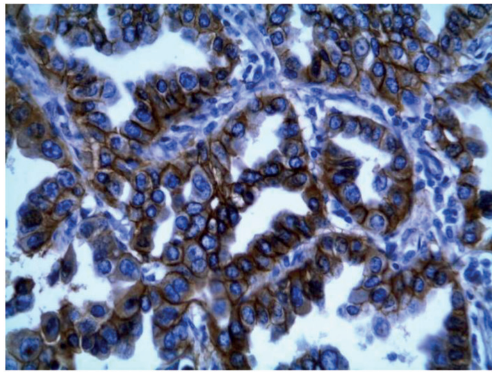
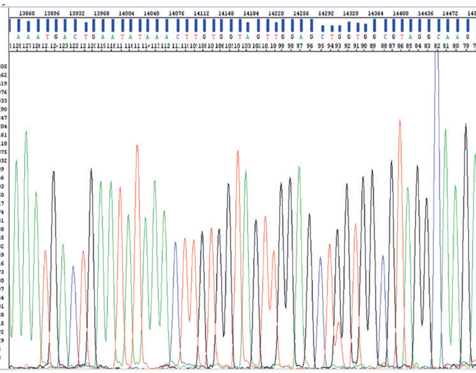
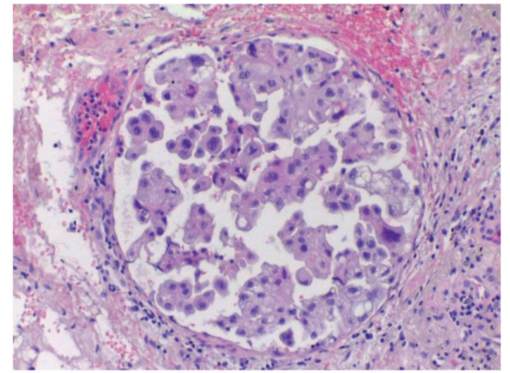
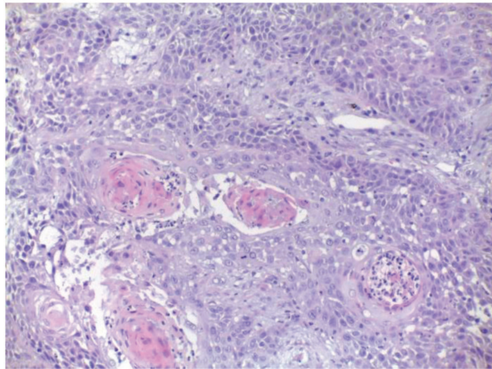
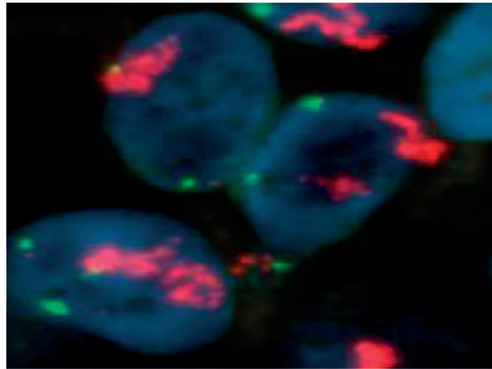
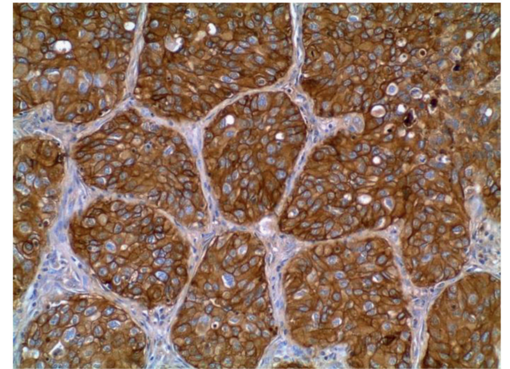

Vitor Manuel Leitão de Sousa

HETEROGENEITY IN BRONCHIAL-PULMONARY CARCINOMAS: IMMUNOHISTOCHEMICAL AND MOLECULAR CHARACTERIZATION

Tese de doutoramento em Ciências da Saúde, ramo de Medicina, especialidade de Patologia (Anatomia Patológica), orientada pela Professora Doutora Lina Maria Rodrigues de Carvalho e apresentada à Faculdade de Medicina da Universidade de Coimbra

Junho 2015

UNIVERSIDADE DE COIMBRA

VITOR MANUEL LEITÃO DE SOUSA

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PREFACE

PREFACE

The present PhD thesis “HETEROGENEITY IN BRONCHIAL-PULMONARY CARCINOMAS: IMMUNOHISTOCHEMICAL AND MOLECULAR CHARACTERIZATION” is submitted in fulfilment of the requirements of the PhD degree at the Faculty of Medicine, University of Coimbra, on the basis of data obtained in the Pathology Department of the Coimbra Hospital and University Centre (SAP – CHUC) and in the Pathology Institute of the Faculty of Medicine, University of Coimbra (IAP – FMUC).

The thesis is divided into a general introduction and six accompanying manuscripts, of which four have already been published, one is accepted for publication and one is undergoing revision. The manuscripts are present in six chapters. They are:

I – **Sousa V**, Espírito Santo J, Silva M, Cabral T, Alarcão AM, Gomes A, Couceiro P, Carvalho L. EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and P53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study. Virchows Arch. 2011 May; 458(5):571-81.

II – Couceiro P, **Sousa V**, Alarcão A, Silva M, Carvalho L. Polysomy and amplification of chromosome 7 defined for EGFR gene in epidermoid carcinoma of the lung together with exons 19 and 21 wild type. Rev Port Pneumol. 2010 May-Jun;16(3):453-62.

III – **Sousa, V**; Bastos, B; Silva, M; Alarcão, A; Carvalho, L. Bronchial-Pulmonary Adenocarcinoma Subtyping Relates With Different Molecular Pathways. In publication in Revista Portuguesa de Pneumologia - Received 30 October 2013, accepted 30 May 2014, available online 5 March 2015 – doi:10.1016/j.rppnen.2014.05.006.

IV – **Sousa, V**; Rodrigues, C; Silva, M; Alarcão, A; Carvalho, L. Lung adenocarcinoma: sustained subtyping with immunohistochemistry and EGFR, HER2 and KRAS mutational status. Rev Port Pneumol. 2015; 21:113-25.

V – **Sousa, V**; Silva, M; Alarcão, A; D’Aguiar, M; Ferreira, T; Carvalho, L. EGFR and KRAS mutations coexistence in lung adenocarcinomas. Diagnostic Pathology 2015, 1:13 - http://www.diagnosticpathology.eu/content/index.php/dpath/article/view/13.

VI – **Sousa, V**; Silva, M; Alarcão, A; Reis, D; Ladeirinha, AF, D’Aguiar, M; Ferreira, T; Carvalho, L. Targeted therapy for FGFR1 may be independent of the histological type of bronchial-

pulmonary carcinomas. Manuscript submitted for publication – Submited / undergoing revision – Virchows Archive.

The manuscripts reflect the Material, Methods and Results and are organized in chapters, followed by twelve posters presented at national and international meetings, with relevance or related to the published abstracts, as follows:

I – "EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 AND P53 EXPRESSION IN PRENEOPLASTIC LESIONS OF BRONCHIAL EPITHELIUM"
The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.
BMC Proceedings 2010, 4(Suppl 2):P64.

II – "*EGFR* UNUSUAL MUTATION STATUS IN LUNG ADENOCARCINOMAS"
The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.
BMC Proceedings 2010, 4(Suppl 2):P61.

III – "*KRAS* AND *EGFR* MUTATIONS COEXISTING IN LUNG ADENOCARCINOMA"

The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.
BMC Proceedings 2010, 4(Suppl 2):P57.

IV – "*EGFR* and *KRAS* mutations in mixed type of Pulmonary Adencocarcinoma"
Intercongress Meeting of the European Society of Pathology, Krakow – Poland, 31st August - 4th September, 2010.
Virchows Arch (2010) 457: 91-281.

V – "*KRAS* Mutations in Lung Adenocarcinomas may be Relevant in Histological Patterns, *EGFR* Mutational Status and Male Smokers"
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.
Virchows Archiv 2011; Supplement 459: SI – S320.

VI – "TTF1 Negative Solid Pattern in Lung Adenocarcinomas May Reflect Morphogenesis"
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.
Virchows Archiv 2011; Supplement 459: SI – S320.

VII – “Lung Adenocarcinomas Subtyping is not Relevant for *EGFR* Mutations, Gene Copy Number and Protein Expression”
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.
Virchows Archiv 2011; Supplement 459: SI – S321.

VIII – “*ALK* Gene Rearrangment in EGFR and KRAS Positive Lung Adenocarcinomas”
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.
Virchows Archiv 2011; Supplement 459: SI – S320.

IX – “ALK expression in Pulmonary Adenocarcinomas”
24thEuropean Congress of Pathology, Prague, September 8 – 12, 2012.
Virchows Arch (2012) 461: S1-S332.

X – “Bronchial-Pulmonary Carcinoma Cases with *EGFR*, *ALK* and *MET* Alterations”
XXIX Congresso de Pneumologia, Albufeira-Portugal, October 25 – 27, 2013.
Rev Port Pneumol. 2013;19(Esp Cong 4): 127-176.

XI – “*EGFR* Mutations, *MET, EGFR* Amplification and *ALK* Rearrangement Simultaneous in Five Bronchial-Pulmonary Adenocarcinomas”
Biennial Pulmonary Pathology Society (PPS) Meeting, Grenoble, June 25-28, 2013.
Pulmonary Pathology Society Biennial Meeting Abstracts. Archives of Pathology & Laboratory Medicine: May 2014, Vol. 138, No. 5, pp. 700-709.

XII – “Pulmonary Squamous Cell Carcinoma can harbor *EGFR* mutations”
5º Congresso de Pneumologia do Centro, June 26-27, 2014.
Rev Port Pneumol. 2014; 20(Esp Cong 3): 1-37.

The posters are organized as a chapter.

Vitor Manuel Leitão de Sousa
Coimbra, June 2015

ABSTRACT

ABSTRACT

Lung cancer is still one of the most frequent cancers in the world, with a high mortality rate. Bronchial-pulmonary carcinomas are still undergoing morphological, immunohistochemical and genetic characterization, with important clinical implications. The characterization of preneoplastic lesions may bring a better understanding of carcinogenesis.

Bronchial-pulmonary carcinomas and preneoplastic lesions of epidermoid carcinoma were studied using immunohistochemical and molecular pathology techniques, on a morphological pattern-based approach.

Basal cell origin was proposed for preneoplastic epidermoid carcinoma lesions, where Ki67, p53 and EGFR constitute biomarkers of agressiveness. *EGFR* amplification is not the main mechanism for overexpression in preneoplastic lesions.

High *EGFR* gene copy number and EGFR overexpression may play a role in epidermoid carcinoma carcinogenesis. *EGFR* mutations are rare. Epidermoid carcinomas with solid, large or clear cell phenotype or with CK7 or Vimentin (EMT phenotype) expression should be submitted to EGFR mutational analysis.

Expression differences between adenocarcinoma patterns reinforce the importance of pattern classification due to their implications for diagnosis, pathogenic understanding and therapeutic outcome. There was higher expression of the gene products studied in adenocarcinomas compared to normal tissue, reinforcing their importance in adenocarcinoma carcinogenesis.

Non-smoking female adenocarcinomas were diagnosed in earlier stages and showed higher ERCC1 expression. Advanced stages (IIA and IIIA) adenocarcinomas were characterized by higher Ki67, APC, ERCC1 expressions and lower TTF1 expression, reflecting more mitotically aggressive adenocarcinomas and a possible non-TRU origin. Solid patterns showed lower expression of nuclear TTF1 and higher expression of Ki67, reflecting aggressive biological behaviour, to be reported to clinicians avoiding poorly differentiated carcinoma reports. TTF1 provides significant guidance for therapy concerning lung adenocarcinoma as *EGFR* mutations were more frequent in TTF1 positive TRU-type adenocarcinomas. On the other hand TTF1 negative adenocarcinomas, some representing non-TRU adenocarcinomas are less prone to harbour *EGFR* mutations. Three clusters of adenocarcinoma patterns were identified: 1 – papillary; 2 – solid; and 3 – lepidic/BA, acinar and micropapillary.

Molecular markers related to therapy resistance were explored and were found to demonstrate higher MRP1 and LRP expression in adenocarcinomas, indicating acquisition of drug-resistance mechanisms during carcinogenesis. Micropapillary, solid and also lepidic patterns showed ERCC1 expression indicating a capacity for Cisplatin-adducts removal.

EGFR mutations were generally present in all the patterns of the same adenocarcinoma reinforcing mutational evaluation in small biopsies. Complex *EGFR* mutations and *KRAS* and *EGFR* mutations coexistence were detected, demonstrating molecular complexity.

FGFR1 protein expression was present in the majority of the bronchial-pulmonary carcinomas, and is higher in adenocarcinomas and pleomorphic carcinomas. Higher expression in pleomorphic carcinomas may reflect the importance of FGFR1 controlling EMT pathways.
FGFR1 amplification is identified in adenocarcinomas (14.7%), epidermoid carcinomas (20.8%), adenosquamous carcinoma (25%) and pleomorphic carcinomas (30%), without significant differences.
Although *FGFR1* amplification has been observed more frequently in epidermoid carcinomas than in adenocarcinomas, these results suggest that the other histological types harbouring *FGFR1* amplification, such as adenosquamous, pleomorphic carcinomas, adenocarcinomas and even small cell lung carcinoma, may benefit from targeted therapy.

Bronchial-pulmonary carcinomas have particular biological, clinical and therapeutic implications. The importance of precise pathological diagnosis, adenocarcinoma patterns recognition, and bronchial-pulmonary carcinogenesis related to biomarker predictive value for targeted therapy is highlighted.

RESUMO

O cancro do pulmão continua a ser um dos cancros mais frequentes no mundo, com elevada taxa de mortalidade. Os carcinomas bronco-pulmonares estão ainda a ser caracterizados morfologicamente, imunohistoquimicamente e geneticamente, com importantes implicações clínicas.

Os carcinomas bronco-pulmonares e as lesões pré-neoplásicas do carcinoma epidermoide foram estudados fazendo uso de técnicas imunohistoquímica e de patologia molecular, numa abordagem baseada no padrão morfológico.

Para as lesões pré-neoplásicas do carcinoma epidermoide foi proposta origem em células basais, constituindo o Ki67, a p53 e o EGFR biomarcadores de agressividade.

O elevado número de cópias do gene *EGFR* e a sobreexpressão de EGFR podem desempenhar um papel na carcinogénese do carcinoma epidermoide. As mutações do *EGFR* são raras. Os carcinomas epidermoides com fenótipo sólido, com células claras ou grandes ou com expressão de CK7 ou Vimentina (fenótipo EMT) devem ser submetidos a análise de mutações do *EGFR*.

As diferenças de expressão entre os padrões de adenocarcinoma reforçam a importância da classificação dos padrões devido às implicações para o diagnóstico, compreensão da patogénese e para o resultado terapêutico. A expressão mais elevada dos produtos de genes estudados em adenocarcinomas comparado com o tecido normal reforça a sua importância na carcinogénese dos adenocarcinomas.

Os adenocarcinomas de mulheres não fumadoras foram diagnosticados em estádios mais precoces e evidenciaram expressão mais elevada de ERCC1. Os adenocarcinomas diagnosticados em estádios mais avançados (IIA e IIIA) caracterizaram-se por expressão mais elevada de Ki67, APC e ERCC1 e por expressão menor de TTF1, refletindo adenocarcinomas mais mitoticamente ativos e agressivos e uma possível origem não-URT (Unidade Respiratória Terminal). Os padrões sólidos evidenciaram uma menor expressão de TTF1 nuclear e uma mais elevada expressão de Ki67, refletindo um comportamento biológico agressivo, que deve ser referenciado aos clínicos, evitando nos relatórios a terminologia carcinoma pouco

diferenciado. A expressão de TTF1 fornece informação significativa na orientação terapêutica para adenocarcinomas do pulmão, tendo sido as mutações do *EGFR* mais frequentes em adenocarcinomas positivos para TTF1, do tipo URT. Por outro lado, os adenocarcinomas TTF1 negativos, alguns representando adenocarcinomas sem origem na URT, revelaram menor propensão a apresentar mutações do *EGFR*. Três grupos de padrões de adenocarcinoma foram identificados: 1 – papilar; 2 – sólido; e 3 – lepídico/BA, acinar e micropapilar.

Foram explorados marcadores moleculares relacionados com a resistência à terapêutica, que demonstraram expressão mais elevada de MRP1 e de LRP em adenocarcinomas, indicando aquisição de mecanismos de resistência a agentes terapêuticos durante a carcinogénese. Os padrões micropapilar, sólido e lepídico demonstraram expressão de ERCC1, indicando capacidade para remoção de aditivos de Cisplatina.

As mutações de *EGFR* estavam presentes, de forma geral, em todos os padrões do mesmo adenocarcinoma, reforçando a avaliação de mutações em pequenas biópsias. Mutações complexas de *EGFR* e a coexistência de mutações *KRAS* e *EGFR* foram identificadas, demonstrando complexidade molecular.

A expressão proteica de FGFR1 estava presente na maioria dos carcinomas bronco-pulmonares, sendo a expressão mais elevada em adenocarcinomas e em carcinomas pleomórficos. A expressão mais elevada em carcinomas pleomórficos pode refletir a importância do FGFR1 no controle das vias EMT.
A amplificação de *FGFR1* foi identificada em adenocarcinomas (14.7%), carcinomas epidermoides (20.8%), carcinomas adenoescamosos (25%) e em carcinomas pleomórficos (30%), sem diferenças significativas. Apesar da amplificação *FGFR1* ter sido observada mais frequentemente em carcinomas epidermoides que em adenocarcinomas, estes resultados sugerem que os outros tipos histológicos, tal como os carcinomas adenoescamosos, carcinomas pleomórficos, adenocarcinomas e mesmo os carcinomas de células pequenas, podem apresentar amplificação de FGR1 e beneficiar de terapêutica dirigida.

Os carcinomas bronco-pulmonares demonstram particularidades com implicações biológicas, clinicas e terapêuticas. A importância do diagnóstico anátomo-patológico preciso, do reconhecimento dos padrões dos adenocarcinomas e da carcinogénese bronco-pulmonar relacionada com biomarcadores com valor preditivo para terapia dirigida é salientada.

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ABBREVIATIONS LIST

ABBREVIATIONS LIST

34βE12 – Cytokeratin 34 beta E12

AAH – Atypical Adenomatoid Hyperplasia

AC – Atypical Carcinoid

ADC – Adenocarcinoma

ADSQC – Adenosquamous Carcinoma

AIS – Adenocarcinoma *in situ*

AKT – v-Akt Murine Thymoma Viral Oncogene Homolog 1

ALK – Anaplastic Lymphoma Kinase

AMP – Amplification

APC – Adenomatous Polyposis Coli Gene

ATM – ATM Serine/Threonine Kinase

ATS/ERS/IASLC – American Thoracic Society / European Respiratory Society /International Association for the Study of Lung Cancer

BA – Bronchioloalveolar

BAC – Bronchioloalveolar Carcinoma

BAX – BCL2-Associated X Protein

BCH – Basal Cell Hyperplasia

BCL XL – B-Cell Lymphoma-Extra Large

BCL2 – B-Cell CLL/Lymphoma 2

BER – Base Excision Repair

BRCA1 – Breast Cancer 1 Protein

CDK – Cyclin-Dependent Kinase

CDKN2A/ p14ARF/ p16INK4/ p21WAF1/ p27- Cyclin-Dependent Kinase Inhibitor 2 A

CEA – Carcinoembryonic Antigen

CHUC – Centro Hospitalar e Universitário de Coimbra

CIS – Carcinoma *in situ*

CK – Cytokeratin

c-KIT – v-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog

c-MET-HGF/SF – MET Proto-Oncogene - Hepatocyte Growth Factor / Scatter Factor

c-MYC – v-Myc Avian Myelocytomatosis Viral Oncogene Homolog

DAPK – Death-Associated Protein Kinase 1

Del – Deletion

DIPNECH – Diffuse Idiopathic Neuroendocrine Cell Hyperplasia

DIS – Disomy

DNA – Deoxyribonucleic acid

E2F – E2F transcription factor

EBV – Epstein - Barr Virus

EGFR/ErbB-1 – Epidermal Growth Factor Receptor / Erythroblastic Leukemia Viral Oncogene Homolog 1

EMA – Epithelial Membrane Antigen

EML4 – Echinoderm Microtubule Associated Protein Like 4

EMT – Epithelial Mesenchymal Transition Characteristics

EQA – European Quality Assurance

ERCC1 – Excision Repair Cross-Complementation Group 1

F – Female Gender

FGFR – Fibroblast Growth Factor Receptor

FFPE – Formalin Fixed Paraffin Embedded

FHIT- Fragile Histidine Triad Gene

FISH – Fluorescence *in situ* Hybridization

FRAB3 – Fragile Region FRAB 3

FTIs – Farnesyl Transferase Inhibitors

FUS1 – FUS RNA Binding Protein

GADD45 – Growth Arrest and DNA-Damage-Inducible

GRB2 – Growth Factor Receptor-Bound Protein 2

HER2/ErbB-2/neu-2 – Heregulin-2 / Erythroblastic Leukemia Viral Oncogene Homolog 2 / Proto-Oncogene 2

HER3/ErbB-3 – Heregulin-3 / Erythroblastic Leukemia Viral Oncogene Homolog 3

HER4/ErbB-4 – Heregulin-4 / Erythroblastic Leukemia Viral Oncogene Homolog 4

HR – Homologous Recombination

hTERT – Human Telomerase Reverse Transcriptase

HP – High Polysomy

HUC-CHUC – Hospitais da Universidade de Coimbra – Centro Hospitalar e Universitário de Coimbra

IAP – Instituto de Anatomia Patológica

IHC – Immunohistochemistry

KRAS – Kirsten Rat Sarcoma Viral Oncogene Homolog

LCC – Large Cell Carcinoma

LCNEC – Large Cell Neuroendocrine Carcinoma

LELC – Lymphoepithelial-Like Carcinoma

LOH – Loss of Heterozigoty

LP – Low Polysomy

LPA – Lepidic Predominant Adenocarcinoma

LRP – Low Density Lipoprotein Receptor

M – Male Gender

MAPK – Mitogen-Activated Protein Kinases

MCC – Mutated in Colorectal Cancers Gene

MDM2 – MDM2 Proto-Oncogene

MEN-1 – Menin 1 Gene

MetMAb – Anti-MET Antibodies

MGMT – O-6-Methylguanine-DNA Methyltransferase

MIA – Minimally Invasive Adenocarcinoma

MLH1 – mutL Homolog 1

MMR – Mismatch Repair

MRP1 – Multidrug Resistance Protein

MSH2 – mutS Homolog 2

mTOR – Mechanistic Target of Rapamycin

NE – Neuroendocrine

NER – Nucleotide Excision Repair

NF1 – Neurofibromin 1

NRG – Neuregulins

NSCLC – Non Small Cell Lung Carcinoma

p53/*TP53* – Tumour Protein p53 / Tumour Protein p53 Gene

p63/*TP63* – Tumour Protein p63 / Tumour Protein p63 Gene

PCR – Polymerase Chain Reaction

PDGF-α – Platelet-Derived Growth Factor α

PDGFR – Platelet Derived Growth Factor Receptor

PI3K – Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

PII – Type II Pneumocytes

PIP3 – Phosphatidylinositol (3,4,5)-Trisphosphate

PLC-γ – Phospholipase C-γ

PLEOMC – Pleomorphic Carcinoma

pRB – Retinoblastoma Protein

PTEN – Phosphatase and Tensin Homolog

RARβ – Retinoic Acid Receptor, beta

RASSF1A – Ras association (RalGDS/AF-6) domain family member 1

RB – Retinoblastoma Gene

RET – Rearranged During Transfection Proto-Oncogene

RTK – Membrane Tyrosine Kinase Receptors

RT – Room Temperature

RT – PCR - Reverse Transcription PCR

SAP – Serviço de Anatomia Patológica

SCC – Small Cell Carcinoma

SCLC – Small Cell Lung Cancer

SD – Squamous Dysplasia

SEMA3B/3F – Sema Domain, Immunoglobulin Domain, Short Basic Domain, Secreted (Semaphorin) 3B/3F

SH2 – Src Homology 2 Domain

SHP2 – Tyrosine Phosphatase Containing Src Homology 2 Domains

SM – Squamous Cell Metaplasia

SRC – Proto-Oncogene Tyrosine-Protein Kinase Src

SQC – Squamous Cell Carcinoma

STAT – Signal Transducer and Activator of Transcription

TC – Typical Carcinoid

TIMP3 – TIMP Metallopeptidase Inhibitor 3

TLS – Translesion DNA Synthesis

Tris – Trisomy

TRU – Terminal Respiratory Unit

TSC – Tuberous Sclerosis

TSC1 – Complex of Tuberous Sclerosis 1

TSGs – Tumour Suppressor Genes

TTF1 – Thyroid Transcription Factor 1

USA – United States of America

VEGF – Vascular Endothelial Growth Factor

WHO – World Health Organization

PART I – INTRODUCTION AND OBJECTIVES

CHAPTER 1 – INTRODUCTION

CHAPTER 1 – INTRODUCTION

Lung bronchial pulmonary carcinomas are considered the most frequent cancer worldwide [1,2], responsible, in the USA, for 14 % of new cancer cases, and 28 and 26% of cancer related death, respectively in men and women. This makes it a frequent type of cancer and the most important cancer related death cause for both genders [3].

Bronchial-pulmonary carcinomas are more frequently diagnosed in Western countries compared to developing countries. However, it is increasing in developing countries and amongst women; while a decline in males incidence rate in most Western countries reflects changes in smoking habits and environmental pollution, as well as the progressive improvement in life expectancy [4-6].

WHO classification for lung cancer recognizes the most important malignant epithelial tumours as: small-cell lung cancer, epidermoid carcinoma, adenocarcinoma, large-cell carcinoma including large-cell neuroendocrine carcinomas, adenosquamous and sarcomatoid carcinomas [6-8]. This classification also recognizes carcinoid tumours and salivary gland-like tumours [6,7].

Three preinvasive lesions are recognized: squamous cell carcinoma *in situ*; atypical adenomatous hyperplasia and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia [6,7,9,10].

There is a strong association with tobacco consumption, describe mainly in small-cell lung carcinomas, epidermoid carcinomas and adenocarcinomas [11]. Relative risk for lung cancer development is 8 to 15 times higher among male smokers and 3 to 10 times higher in women smokers, when compared to non-smokers [12]. It is estimated that about 85% and 45% of bronchial-pulmonary carcinomas diagnosed in men and women respectively are related with tobacco exposure [13,14]. The dose and duration of exposure is also important [11]. The risk of bronchial-pulmonary carcinomas reduces rapidly with tobacco cessation in about 5 years and it nears the risk of non-smokers in about 20 years [11]. Involuntary exposure to tobacco increases the lung cancer risk by about 25% [15-17]. Other factors have been associated with the risk of bronchial-pulmonary carcinomas development such as exposure to asbestos, silica, radon, heavy metal and polycyclic aromatic hydrocarbon [18].

Major Histological Types:

Epidermoid / squamous cell lung carcinoma is defined as a carcinoma originating in the epidermoid bronchial epithelium, and which reveals keratinisation and/or intercellular bridges

[6,7]. Differentiation is defined upon the relative presence or absence of those features. Therefore, these tumours should be classified as well or poorly differentiated. Papillary, clear cell, small cell and basaloid variants are recognized [6,7,19-27]. Survival is usually better when compared to adenocarcinomas [28]. Prognosis is especially related on clinical/pathological staging. Poorly differentiated epidermoid carcinoma are associated with earlier metastatic risk and with a worse behaviour/prognosis when compared to well differentiated tumours [7]. In some countries it is still the most frequent lung carcinoma [29] It has the strongest relationship with tobacco, and its incidence is greater in men, with a ratio of between 6.6 to 15 men / 1 women [30-33]. Squamous carcinoma *in situ*, following squamous dysplasia is considered the precursor lesion [6,7,34,35].The segmental and lobar bronchi are the most frequently affected [36].

Macroscopically it consists of firm (according to the extent of stromal fibrosis) white masses with imprecise limits. Necrosis and cavitation are present in bigger lesions. Central tumours may form masses with endoluminal growth and/or direct invasion of the bronchial wall and lung parenchyma. Some cases (a minority) are diagnosed in the peripheral airways [6,7,36].
Well differentiated epidermoid carcinomas have clear keratinisation features such as keratin pearls, cellular pearls and intercellular bridges. These features are absent or focally present in poorly differentiated epidermoid lung carcinomas.

The papillary variant frequently exhibits endobronchial growth with intra-epithelial dissemination and bronchial wall invasion [19,20].
The clear cell variant has clear cells which constitute the major feature [21,22].

When a carcinoma is made of small cells with focal epidermoid/squamous differentiation, a small cell variant of the epidermoid lung carcinoma is considered in the diagnosis. Differential diagnosis, of this poorly differentiated carcinoma, must be made with small cell lung carcinoma combined with epidermoid carcinoma. Irregular or vesicular chromatin and prominent nucleoli of epidermoid carcinoma are important for the differential diagnosis, as well as large cytoplasm and defined cellular limits present in this variant [6,7,23-25].
The basaloid variant shows nuclear palisading. If squamous differentiation is not identified, these tumours should be diagnosed as the basaloid variant of large cell carcinoma [6,7,26,27].

Epidermoid carcinoma shows high weight cytokeratin positivity, such as CK5, CK6 and 34BE12, and also expresses CEA. Fewer cases may express CK7 and even TTF1 (thyroid transcription factor 1) [21,37-41], which may indicate adenosquamous differentiation at cellular level.

Adenocarcinoma diagnose is made when a malignant epithelial tumour has glandular/acinar differentiation or demonstrate production of mucin [6,7,42]. This type is the most common cancer in non-smokers and its incidence has increased in the past years. There is apparently a relationship between adenocarcinoma and pulmonary scars. Adenocarcinomas are usually localized/developed peripherally from terminal airways and alveolar epithelial cells [6,7,42,43]. Adenocarcinomas are sub-classified according to the following patterns: acinar, papillary, micropapillary, BA (bronchioloalveolar)/lepidic, solid with mucin production and mixed type [6]. Differences between subtypes should be taken into account to improve diagnosis, prognosis and the selection of treatment [7,8,43].
Non-BA/non lepidic adenocarcinoma is usually a peripheral tumour that can have different growth patterns. The mixed sub-type is the most common histological pattern in the WHO classification [6,7], of which there are at least two individual patterns: the solid or acinar type occurs most frequently in the centre, with a BA/lepidic pattern at the periphery [6,7]. When an adenocarcinoma has only on pattern, this is more frequently acinar type [6,7]. Also, acinar pattern is considered the most frequently subtype present in mixed adenocarcinomas [6,7]. Papillary patterns are present in 10% of adenocarcinomas [7]. Sometimes, it is difficult to make a clear distinction between papillary adenocarcinoma and BA/lepidic pattern. The important of this distinction lie on implications for therapeutic strategies [42]. The micropapillary type has small cellular clusters projected and growing from alveolar septa or present/floating within alveolar spaces. The small papillary like cellular structures do not have fibro vascular cores. It is observed in papillary adenocarcinomas but also in mixed type tumours, and metastasizes earlier, with less favourable prognosis [42]. Solid pattern adenocarcinoma accompanied of mucin (solid adenocarcinoma with mucin) production is represented in 15% of mixed-type adenocarcinomas [6,7,42]. BA/lepidic tumoural cells grow alongside alveolar septa with aerial spread through the pulmonary lobules. It is frequently diagnosed in women, young and non-smoking patients [6-8,42,44].
The incidence rate differs according to gender, with roughly 28% occurring in men and 42% in women. A new classification was developed by the International Association for the Study of Lung Cancer, the American Thoracic Society and The European Respiratory Society (ATS/ERS/IASLC) [8]. It recognizes several patterns, such as lepidic /BA, acinar, papillary, solid,

and micropapillary [8]. It also recognizes mucinous adenocarcinomas including the former mucinous BA carcinomas and colloid carcinomas [8].

This ATS/ERS/IASLC recognizes new categories such as AIS (adenocarcinoma *in situ*) and MIA (minimally invasive adenocarcinomas) [8]. According to this proposed classification, preinvasive lesions include AAH and AIS [8]. AIS correspond to the formers ≤3cm non-mucinous BA carcinomas while minimally invasive adenocarcinomas (MIA) are similar (carcinomas ≤3cm) but with an invasion area of ≤5mm; hence, it is an adenocarcinoma with a predominantly lepidic pattern with a small area of invasion (≤5mm) [8].

Some recommendations were made in this newly proposed classification. These included: discontinuation of the term BAC; the use of these new categories; classification according to the predominant pattern with reference to the % of each pattern present in 5% increments; apply the terminology LPA (lepidic predominant adenocarcinoma) for the former mixed-type adenocarcinomas with non-mucinous BA pattern predominance [8]. It also highlighted the need for as assertive diagnosis especially in biopsy material, supported by immunohistochemistry, with clinical, prognostic and therapeutic implications [7,8,45,46]. For personalized treatment, it is important to differentiate between adenocarcinoma and epidermoid carcinoma and to restrict the use of NSCLC terminology [8].

This classification system also recognizes the importance of pattern identification with prognostic implications. Solid and micropapillary patterns are associated with a worse prognosis, while acinar and papillary tend to indicate an intermediate prognosis, and non-mucinous lepidic / BA pattern a good prognosis [8].

The WHO classification also recognizes bronchial-pulmonary carcinomas other than adenocarcinoma or epidermoid carcinoma, such as small cell carcinoma (pure or in combination), large cell carcinoma (including combined, basaloid, lymphoepithelioma-like and clear cell carcinomas as well as large cell carcinoma showing rhabdoid features/phenotype), adenosquamous, and sarcomatoid carcinomas (including spindle and giant cell carcinomas, pleomorphic carcinoma, carcinosarcomas and blastomas), typical and atypical carcinoids and salivary gland type neoplasia such as mucoepidermoid carcinoma, adenoid cystic and epithelial-myoepithelial carcinomas [6,7].

Large cell carcinomas (LCC) are undifferentiated bronchial-pulmonary carcinomas that lack the morphological and cellular features of the small cell carcinoma, glandular and epidermoid/squamous features [6,7]. It is an exclusion diagnosis. Large cell neuroendocrine

carcinomas have NE phenotype with cells organized in trabeculae or as rosettes, defining the so called organoid morphology [6,7], and may be combined with adenocarcinoma, epidermoid/squamous cell carcinoma, or spindle / giant cell carcinomas [6,7,47].

Adenosquamous carcinoma (ADSQC) has compound features of epidermoid/squamous cell carcinoma and of adenocarcinoma [6]. To make this diagnosis, the squamous and adenocarcinoma components must comprise at least 10% of the total tumour [6,7,48-52]. Their frequency varies between 0.4-4% of lung carcinomas. The incidence is said to be increasing alongside the increasing incidence of the adenocarcinoma [6,7,48,50,52-56].

Sarcomatoid carcinomas are bronchial-pulmonary lung carcinomas with a sarcoma or sarcoma-like (spindle and/or giant cell) differentiation. Pleomorphic Carcinoma, Spindle Cell Carcinoma, Giant Cell Carcinoma, Carcinosarcoma and Pulmonary Blastoma are considered as sarcomatoid carcinomas [6,7].
These are rare tumours, representing only 0.3-1.3% of all lung malignancies [6,7,57-61]. The median age at diagnosis is 60 years, and there is a clear predominance of males, with a male/female ratio of approximately 4 to 1) [6,7,19,62-66]. Sarcomatoid lung carcinomas correspond to a poorly differentiated bronchial-pulmonary carcinoma, namely epidermoid carcinoma, adenocarcinoma or large cell carcinoma with areas with spindle cells and/or giant neoplastic cells or, to a carcinoma composed exclusively of spindle and giant cells[6]. The spindle or giant cell component should comprise a minimum of 10% of the tumour. Histological sections demonstrate conventional non-small cell carcinoma, like an adenocarcinoma, epidermoid or large cell subtypes, with at least 10% of spindle cells and/or giant malignant cells [6,7].

Spindle cell carcinoma is defined as a carcinoma composed of only spindle malignant cells[6,7]. Giant cell carcinoma is defined as a carcinoma with highly pleomorphic malignant multinucleated or mononucleated giant cells. It is composed entirely of giant cells [6,7].

Carcinosarcoma diagnose is made when a there is a combination of carcinoma and sarcoma; the last showing differentiated sarcomatous elements, like bone, malignant cartilage, or skeletal muscle malignant elements [6,7]. So, it is considered as a biphasic tumour, which means that there is a mixture of a bronchial-pulmonary carcinoma and a sarcoma component with differentiated elements [6,7]. The most frequently carcinomatous component identified in carcinosarcomas is epidermoid carcinoma (45-70%) [6,7]. The second most frequent

carcinomatous component is adenocarcinoma (20-31%), followed by large cell carcinoma (representing only 10%) [6,7,67].

Neuroendocrine tumours comprise: carcinoid tumours, small cell lung carcinoma and large cell neuroendocrine carcinoma. **Carcinoid tumours** morphologically demonstrate typical pattern growths, such as rosette-like, organoid, insular, trabecular, ribbon formations and palisading [6,7]. These patterns, when present in a tumour, are characteristic or suggestive of neuroendocrine differentiation. Tumour cells are relatively uniform. Cytoplasm is frequently finely granular and eosinophilic [6,7]. The nucleus shows a characteristic finely granular chromatin pattern [6,7]. Carcinoid tumours without necrosis and with less 2 mitoses per 2 mm2 are diagnosed as typical carcinoid (TC) [6,7]. If there are foci of necrosis and if mitotic count is higher (2-10 mitoses per 2 mm2), the carcinoid tumour is classified as atypical [6,7].

Small cell carcinoma (SCC) is a malignant epithelial tumour with small cells with nuclei containing fine granular chromatin [6,7]. Nucleoli are very small or absent. It also has neuroendocrine differentiation, necrosis and are characterized by high mitotic index [6,7]. It is an aggressive neoplasia that can be combined with adenocarcinomas and / or epidermoid carcinomas, large cell carcinomas and less frequently with sarcomatoid carcinomas [6,7,68].

Complementary Histological Types:

Basaloid carcinoma shows a nodular or trabecular pattern, where peripheral cells have nuclear palisading [6,7]. Cells are generally small in size with scant cytoplasm, monomorphic with moderately hyper-chromatic nuclei, finely granular chromatin and small or no nucleoli, with a high mitotic rate [6,7]. Differential diagnosis is established with epidermoid carcinoma [7,27]. Basaloid carcinoma is considered as a LCC variant, with features that resembles basal cell carcinomas of the skin [6,7]. These tumours tend to have an endobronchial growth, and the adjacent mucosa shows epidermoid/squamous dysplasia. Basal or suprabasal bronchial cells are considered as the cells of origin [69,70].

Lymphoepithelial-like carcinoma (LELC) is a rare carcinoma characterized by syncytial growth pattern, large nuclei, prominent nucleoli, and dense lymphocytic infiltration [6,7,71-74]. It often presents with epidermoid differentiation [6,7]. These tumours are more frequently diagnosed in Asians, with association with EBV and less association with tobacco [6,7].

Abundant lymphocytes and plasma cells surrounds the epithelial cells organized in a syncytial pattern [6,7]. Mitosis are frequent but tumour necrosis is rare [72-77].

Clear cell carcinomas have cells with clear cytoplasm with or without glycogen [6,7]. Clear cell carcinoma is a rare, although almost one third of bronchial-pulmonary carcinomas and rare small cell carcinomas show areas with clear cell phenotype [22,78]. So, clear cell variants of adenocarcinoma and of SCC are recognized [6,7,22].

Large cell carcinoma with rhabdoid phenotype is characterized by the presence of rhabdoid cells in lung bronchial-pulmonary carcinomas including small cell carcinoma [79-85]. These cells contain eosinophilic cytoplasmatic globules (intermediary filaments), frequently positive for vimentin and cytokeratins. Pure forms are very rare [6,7,86,87], and bronchial-pulmonary carcinomas composed exclusively of rhabdoid cells are extremely rare. Thus, there need to be more than 10% rhabdoid cells to diagnose a bronchial-pulmonary carcinoma with rhabdoid phenotype [6,7,86,87]. Rhabdoid tumour cells demonstrate immunohistochemical positivity for vimentin, and usually for cytokeratin (often CK7 but not CK20) and EMA [83].

Large cell neuroendocrine carcinoma (LCNEC) corresponds to a large cell carcinoma with neuroendocrine histology/morphology and immunohistochemical or ultrastructure characteristics of neuroendocrine differentiation [6,7]. This type of tumour may arise in combination with other bronchial-pulmonary carcinomas, such as SCC, adenocarcinomas and sarcomatoid carcinomas [6,7].

Pulmonary blastoma is diagnosed when there is an epithelial component similar to a fetal adenocarcinoma (well-differentiated) and a mesenchymal component with primitive features [6,7]. Thus it is considered as a biphasic tumour that sometimes shows small areas of chondrosarcoma, osteosarcoma or rabdomyosarcoma differentiation [6,7,66].

Mucoepidermoid carcinoma is a malignant epithelial tumour that resembles a salivary gland mucoepidermoid carcinoma [6,7]. The diagnosis relies in the identification of squamous cells, intermediate type cells and cells with mucin production [6,7]. These rare tumours represent less than 1% of the bronchial-pulmonary carcinomas [6,7,88-90]. There is equal distribution by sex, with a slight predominance in male gender, with a large age range, frequently diagnosed in younger patients, with less than 50 years [6,7,88-91].

Adenoid cystic carcinoma is also a rare carcinoma (<1% of bronchial pulmonary carcinomas) that resembles its counterpart in salivary glands [6,7,61,92]. There are no differences according to gender and it is more frequently diagnosed in the fourth and fifth decades of life [6,7,61,92,93].

Epithelial-myoepithelial carcinomas are characterized by the presence of an epithelial duct-forming component and of a myoepithelial cells component [6,7]. The last may show a spindle, plasmacytoid or clear phenotype. There could be a variable proportion of the two components [6,7].

Pre-Invasive Lesions:

As has already been said, **pre-invasive** lesions include **squamous cell *in situ* carcinoma** and for adenocarcinomas, **atypical adenomatoid hyperplasia (AAH)** and the newly proposed **adenocarcinomas *in situ*** (**AIS**, formerly pure BA non-mucinous carcinomas) [6-8].

AAH is frequently recognized adjacent to lung adenocarcinomas [94-97]. Epidemiological and morphological features such as clonality, and other molecular changes like *EGFR* and *KRAS* mutations as well as *TP53* methylation expression, have been described in both AAH and adenocarcinomas supporting pre-invasive lesions [6-8,98-102].
AAH is defined as an epithelial proliferation with atypia, and lines the alveoli and respiratory bronchioles for less than 5mm with some gaps between the cells, generally without accompanying fibrosis and/or inflammation [6,7]. It is considered an adenocarcinoma precursor lesion [103].
The incidence varies between 5.7% and 21.4% in patients with lung adenocarcinoma [6,7]. AAH is identified in 19% of women and 9.3% of men with lung bronchial-pulmonary carcinomas and in 30.2% of women and 18.8% of men with adenocarcinoma diagnose [6,7]. It is more frequent in smokers than in patients who have never smoked [94,104,105]. It is frequently an incidental finding, and forms nodules of up to 5mm, sometimes with more than 10mm [10,106]. AAH lesions can be multiple, especially in the peripheral lung parenchyma [6,7]. They are composed by a unique layer of cylindrical or cuboidal epithelial cells with mild to moderate dysplasia with inconspicuous nucleoli and dense chromatin [6,7,96,97].
The differential diagnosis includes the lepidic pattern of lung adenocarcinoma / *in situ* adenocarcinoma of the non-mucinous type, Type II pneumocytes (PII) hyperplasia and

papillary adenoma. The AIS / lepidic pattern of adenocarcinoma should be suspected when it is larger than 5mm, there are no gaps/spaces between the cells or if there are many pleomorphic nuclear features or nuclear stratification [6,7]. PII or Clara cells are believed to be the progenitor cells [6,7].

Different genetic and molecular alterations have been identified in AAH, such as *KRAS*, *TP53*, *FHIT*, *P16INK4* and *TSC* mutations. *KRAS* mutations could be an earlier event [107-109]; 3p and 9p allelic losses have also been diagnosed in AAH involving the *FHIT* (Fragile Histidine Triad) gene and $P16^{INK}$ [109-112]. Loss of heterozigoty (LOH) of the suppressor genes *TSC1* (9q) and *TSC2* (16p) (complex of tuberoses sclerosis) have also been described [113]. Chromosome 7 aneuploidy or polyploidy is increasingly present from AAH till adenocarcinoma [114].

AIS, is defined as a localized adenocarcinoma of ≤ 3cm with non-mucinous lepidic growth, without papillary or micropapillary patterns of growth [6,8]. **MIA** (Minimally Invasive Adenocarcinoma) is defined as a localized adenocarcinoma of ≤ 3cm with lepidic predominant growth, with > 5mm invasion area [6,8].

Squamous dysplasia and squamous cell carcinoma *in situ* are considered to be preinvasive lesions. Basal cell hyperplasia and squamous metaplasia are recognized as non-preinvasive precursor lesions [6,7]. Respiratory epithelium squamous metaplasia is related to a history of smoking as well as to chronic inflammations, as seen in asthma, chronic obstructive pulmonary diseases or chronic irritant exposures [115-118]. Squamous dysplasia and *CIS* may or may not exist in association with epidermoid carcinoma. Generally asymptomatic, they are more frequent in smokers, males and in association with chronic obstructive pulmonary diseases [34,119,120].

Squamous dysplasia should be classified as mild, moderate or severe, taking into account the epithelial layers affected by nuclear changes, cellular loss of orientation, absence of superficial maturation and mitosis level [6,7].

Mild dysplasia shows discrete nuclear size increase, fine granular chromatin, small nucleoli or their absence, vertical nuclear orientation and rare mitosis [6,7]. These alterations are observed in the lower third of the epithelium [6,7]. Moderate dysplasia shows maturation limited to the upper third, higher nuclear pleomorphism and mitosis. Severe dysplasia exhibits marked pleomorphism and prominent nucleoli, with cellular maturation only in the most superficial cells; mitoses are identified in the lower two thirds of the epithelium [6,7]. *CIS* loses nuclear orientation as well as superficial cell maturation; mitoses are identified in all layers [6,7].

Basal cells have been considered as the precursor cells for squamous metaplasia and dysplasia [9].

Frequent cytogenetic changes include aberrations to chromosome 3 and 7 number, 3p, 9p21, 8p21-23, 13q14 (*RB*), 17p13 (*TP53*) LOH, and 5q21 (APC-MCC region) LOH are detected mainly in *CIS* [114,121-125]. 3p LOH was detected in larger regions in *CIS* when compared to earlier lesions, and may involve the entire 3p arm [124,126]. *TP53* mutations can be identified in the different phases of the sequence [124,125,127]. *P16INK4a* hypermethylation was identified, increasing from basal cell hyperplasia to *CIS* [128]. Telomerase higher expression was also found in more severe lesions [129]. Dysplasia frequently shows LOH 8p22-24 and *TP53* inactivation while *CIS* and subsequent invasive epidermoid carcinomas show *KRAS* mutations and 5q22 LOH [7].

Another preneoplastic lesion is **DIPNECH (Diffuse Idiopathic Neuroendocrine Cell Hyperplasia)**. It is considered a neuroendocrine cell proliferation with linear distribution or forming small nodules, defined as neuroendocrine bodies [6,7]. Neuroendocrine cell nodules of 2 to 5mm are classified as tumourlets, while those bigger than 5mm are called carcinoid tumours [6,7]. DIPNECH, tumourlets and carcinoids form a lesion continuum [130-133].

Bronchial-Pulmonary Carcinoma Carcinogenesis:

Improvements in diagnostic molecular techniques and the growing knowledge related to carcinogenesis have contributed to the recognition of diagnostic markers of early lung bronchial-pulmonary carcinomas. Allelic losses (LOH), chromosomal instability, epigenetic gene silencing, proto-oncogenes activation, loss of tumour suppressor and DNA repair genes have been described in bronchial-pulmonary carcinogenesis [110,134,135].

Some of this genetic of molecular changes are considered as early events during bronchial-pulmonary carcinomas development [136].

Lung carcinogenesis is a multicentre, multiphase process with the sequential accumulation of molecular and genetic alterations. It may be caused by smoking, environmental and occupational factors, diet (low ingestion of vitamin A and antioxidant micronutrients), family and genetic factors (several epidemiologic studies indicate, in some cases, a family and individual susceptibility to lung cancer) and lung injury (caused by non-infectious pulmonary and systemic diseases, such as chronic obstructive pulmonary diseases and inflammatory or fibrotic pulmonary diseases) [42].

Uncontrolled growth is the result of an imbalance between oncogene activation (by mutations, amplification, overexpression or rearrangement) and the inactivation of suppressor genes, transcription genes and genes involved in DNA repair (mutations, LOH or by aberrant promoter methylation) that inactivate or activate four or five different signal transduction pathways [137,138].

Studies have improved the knowledge related to specific individual genetic pathways or molecular events, frequently according to histology subtype. However, carcinogenesis is very complex and there is less knowledge related to different pathways or genetic/molecular events interactions and of their importance to cancer development and progression. Identification of molecular events associated with progression and metastasis would be of great relevance. Also, the identification of one or more molecular events altered in early phases of cancer development would be of great importance, as an opportunity for early diagnosis approaches.

Oncogenes and signal transduction

Oncogenes are involved in cellular signalling through overexpression or by activating mutations [137,139].

Growth factors influence cell proliferation, differentiation, survival and apoptosis [137,139-141]. Extracellular ligands bind to specific receptors, activating messengers in the cytoplasm and nuclear transcription factors [140,141]. The activation and inactivation of proteins occurs through reversible phosphorylation of tyrosine, serine, or threonine residues made by tyrosine kinases and serine/threonine kinases. There are several classes of receptors including receptor tyrosine kinases, G-protein–coupled receptors, and cytokine receptors [137,139]. Molecules in the cytoplasm are responsible for signal transduction, acting as "second messengers" [137,139]. There is overlap/cross-talking in cell signalling, resulting in redundancy [137,139]. Adaptor and scaffolding proteins coordinate signalling cascades [137,139].

Membrane Tyrosine Kinase Receptor (RTK) contains different domains, such as an extracellular (for ligand binding), a transmembrane, justamembrane, protein tyrosine kinase, and cytoplasmatic domains [137,139]. Ligand binding is responsible for tyrosine kinase activation, after the formation of dimmer or oligomer [137,139].

RTK alterations are related to cancer development in several ways. Expression of both the receptor and the ligand (growth factor) could explain a autocrine loop, favouring tumour development and progression [137,139]. Growth factors also act in a paracrine mode [137,139]. High gene copy number, by amplification, and overexpression of the receptor are also responsible for cancer development (as for EGFR, ERBB2/HER2, and MET) [137,139].

Gene mutations affecting the tyrosine kinase domain are also implicated, as the receptor is constitutively activated even in the absence of the growth factor [137,139]. The *RET* gene is activated by rearrangement [137,139]. MET is associated with cancer development by protein overexpression and/or by gene activating mutations [137,139]. Genetic rearrangements are also responsible for receptor activation (PDGF-α and ALK), with the resulting fusion proteins with constitutively activated TK domain [137,139].

EGFR

The erbB family includes EGFR/ErbB-1, HER2/ErbB-2/neu-2, HER3/ErbB-3, and HER4/ErbB-4 [137,142]. Activation occurs upon ligation to one of the eight known growth factors, like EGF and transforming growth factor α (TGF-α) [142]. After ligand attachment, receptor dimerization leads to tyrosine auto phosphorylation and activation [137,142]. Heterodimerization is important allowing the activation of different signal pathways[142].

EGFR dysregulation occurs by the overexpression of the ligand, EGFR, or both, and by mutations, these last responsible as already said for the constitutive activation [137,142]. HER2 does not bind to ligands acting mainly as a co-receptor increasing the ligand affinity of the other receptor forming the dimmer [142]. Interactions between EGFR and other different receptors (endothelion-1/thrombin/LPA) may also be implicated in EGFR activation [142].

EGFR is responsible for the activation of different signal transduction cascades. Phosphorylated EGFR activates several molecules, such as SRC, SH2, SHP2, GRB2, phosphatidylinositol 3-kinase (PI3K), AKT, phospholipase C-γ (PLC-γ), and the STAT transcription family [142]. A RAS/RAF/Mitogen-Activated Kinase (MAPK) pathway is activated via GRB2 [142]. EGFR also activates the PI3K pathway, important in cancer development and at controlling the progression on cell-cycle [142]. PI3K/AKT pathway downregulates p27 (a cell-cycle inhibitor) [142]. EGFR activation could be responsible for VEGF upregulation, by activation of PI3K [142]. EGFR has relevance in the control of several important cellular functions with implications on cell proliferation and survival, angiogenesis, invasion capabilities with metastasis development [142].

EGFR mutations occur in approximately 24% of unselected bronchial-pulmonary carcinomas and are more common when there is an adenocarcinoma histology, and amongst never-smokers, East Asians and women [137]. *EGFR* activating mutations are related with tumour sensitivity to TKIs, such as gefitinib (Iressa), erlotinib (Tarceva) [137,142]. Activating mutations occur frequently in exons 18 to 21 and are categorized as deletions, missense point mutations, and insertions [137]. T790M mutation ("second mutation") confers resistance to TKIs [137]. T790M mutation can also be found in patients not yet submitted to TKI therapeutics [137].

Other predictive molecular events have been correlated with TKI response, such as EGFR protein overexpression, *EGFR* gene high copy number by amplification, and activation of Akt [137]. On the other hand, amplification of *c-Met* has been related to TKI resistance [137,143-147]. Some studies demonstrated selective transduction of survival pathways, such as Akt and STATs, by *EGFR* activation mutation [137]. *EGFR* mutated tumours are more sensitive to radiation [137].

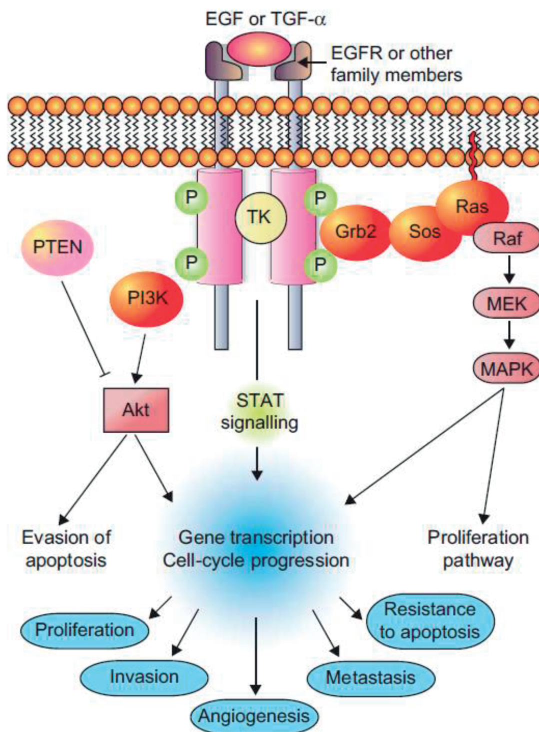

Figure 1: Effects of EGFR signal transduction events. Adapted from: Brambilla, E.; Gazdar, A. Pathogenesis of lung cancer signalling pathways: roadmap for therapies [148].

HER2

Activation of HER2 depends on dimerization with other members of the erbB family [142]. Heterodimers of HER2-HER3 are considered to have potent mitogenic activity [142]. We know that neuregulin (NRG) and heregulin (HRG) are ligands responsible for the activation of HER3 and HER4 [142]. HER2 is responsible for the increase of affinity for the ligand of the other receptor, partner of dimerization, with implications in promotion of increased activation of intracellular molecular pathways [142].

The greatest progress in HER2-targeted therapies have been related to the use of specific monoclonal antibodies (trastuzumab – Herceptin) against the extracellular portion of the HER2 receptor [142]. *HER2* mutations occur in approximately 2% of bronchial-pulmonary carcinomas

as in-frame insertions in exon 20 [137]. *HER2* mutations frequently occur in female gender, non-smokers, Asians patients diagnosed with adenocarcinomas [137].

FGFR1

FGFRs are part of the family of receptor tyrosine kinases and are encoded by 4 genes that are located on chromosome 8p11.23 – *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4* [149-151]. Twenty two Fibroblast growth factors (FGFs) are known until today [149-151].

FGFR1 is one of the most important members of this family of receptors, with tyrosine kynase features [149,152]. Activation of the receptor, by FGFs with subsequent dimerization, is responsible for the activation of different signalling pathways, such as PI3K-AKT, RAS-MEK-MAPK, STAT, SRC and PLC [149,152]. Functions associated with FGFR1 activation include cell growth, differentiation, and survival mechanisms control [149,152]. Epithelial-mesenchymal transition (EMT) could also be induced by FGFR1 upregulation [68,153-156].

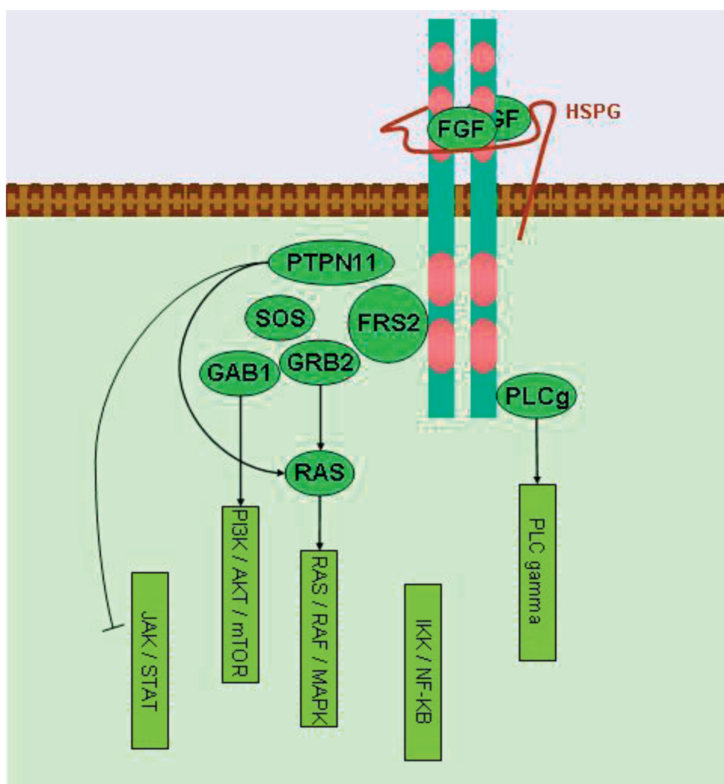

Figure 2: Molecular pathways activated by FGFR1. Adapted from Larsson H, Klint P, Landgren E and Claesson-Welsh. Biol Chem 274: 25726-25734, 1999 [152].

FGFR1 is also associated with the cell cycle control. By RB phosphorylation, promoted by CDK (ciclin dependent kinase), transcription factor E2F is released into the nuclei, where DNA replication is induced, favouring cell cycle progression [157]. It acts also by inhibition of CDKN (ciclin kinase inhibitor), such as p21, p27, p15, p16, p18, and p19 [157].

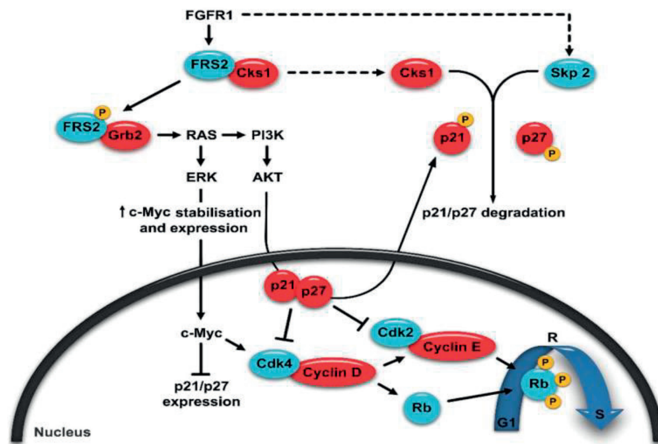

Figure 3: FGFR1 and cell cycle control. Adapted from: Dombrowski C, Helledie T, Ling L, *et al*. (2013). Stem Cells 31: 2724-2736 [157].

By the control of these pathways, these molecules are engaged in the control of physiological processes. Deregulation of these mechanisms could be associated with cancer development [149-151].

Deregulation could be explained by different events, such as high levels of FGFR1 protein expression, which have been diagnosed in lung carcinomas [158,159], co-expression of FGF and FGFR1 [160-163], and amplification of the gene *FGFR*, also described in lung carcinomas [164].

Alterations of the *FGFR* gene have been recognized in many different epithelial malignant tumours, such as in gastric; breast; oral, ovarian, urothelial and lung carcinomas [165,166].

High FGFR1 protein expression, FGF and FGFR co-expression, and *FGFR1* gene amplification has been described in lung bronchial-pulmonary carcinomas, especially on squamous cell lung carcinomas, while mutations are rarely found [158-163,165].

FGFR1 genetic alterations are potentially targetable mutations [167], as FGFR1 can be targeted by tyrosine kinase inhibitors [167]. Therefore, some authors consider that evaluation of *FGFR1* genetic alterations, such as gene amplification, should be considered in the genomic characterization of lung cancers [167].

It has been argued that *FGFR1* amplification is associated with a response to FGFR inhibitors [165,168], and clinical trial investigation is ongoing concerning different FGFR1 inhibitor, such as BGJ 398 (Novartis), AZD 4547 (AstraZeneca), TKI 258 (Novartis - Ponatinib), and BIBF 1120 (Boehringer-Ingelheim), with FGFR1 amplification as an molecular inclusion criteria [168-171].

c-KIT

c-KIT and its ligand (stem cell factor (SCF)) could be overexpressed in SCLC [137]. However *c-KIT* mutations are rarely identified in bronchial-pulmonary carcinomas [137]. There are therapeutic approaches related to the inhibition of c-KIT, such as Imatinib (Gleevec) [137].

RAS

The RAS family is composed of several small proteins with GTPbinding capability, comprising HRAS, NRAS, and KRAS, with relevant functions controlling cellular differentiation, proliferation, apoptosis and survival [142].

Activating mutations, higher protein expression or decreasing in RAS GTPases are associated with RAS function deregulation [142]. RAS overexpression is postulated to be a late event in lung cancers [142]. *RAS* activating mutations are more frequently identified involving the amino acids 12, 13, 59, and 61 [142]. Other mechanisms responsible for RAS activation include the increased stimulation by upstream mediators, related to activating *EGFR* mutations and, c-MET, and platelet-derived growth factor receptor (PDGFR) mediated activation [142].

RAS activating mutations are described bronchial-pulmonary carcinomas (10%–15%), mainly with adenocarcinoma histology, in which the incidence range from 20% to 30%, and are rare in small cell carcinomas [137]. Activating *RAS* mutations are more frequently identified at codons 12, 13, and 61 [137]. Almost 70% of the *KRAS* mutations are G-T transversions, related with tobacco [137]. *KRAS* mutations are associated to EGFR TKIs resistance [172-174]. Farnesyl transferase inhibitors (FTIs) (such as Tipifarnib and lonafarnib) are the most widely studied drugs for targeting *KRAS* mutated tumours.

c-MET and c-MET-HGF/SF

The tyrosine kinase receptor c-MET has the hepatocyte growth factor (HGF)/scatter factor (SF) as its ligand [142]. The c-MET-GF/SF signalling pathway has implications for angiogenesis induction, embryonic and organ development, cellular proliferation, motility, and invasive cellular features [142]. c-MET overexpression has been described in bronchial-pulmonary carcinomas [142]. *c-MET* activating point mutations are associated with the presence of constitutively activated receptors in several tumours [142]. c-*MET* amplification is associated to EGFR TKIs resistance [143,144,175].

SRC Kinase Family

The SRC family include eight members with functions regulating proliferation, apoptosis, angiogenesis and invasiveness, affecting matrix proteases and decreasing cell adhesion by phosphorylation of cadherin with decrease of cadherin-catenin association [142].

Activation of SRC is mediated by c-MET, HER2, EGFR and by *SRC* activating mutations on the catalytic region [142].

c-MYC

MYC family includes c-Myc, b-Myc, l-Myc, n-Myc, and s-Myc [142]. The c-Myc proteins have functions in proliferation, growth, apoptosis, and differentiation [142].

c-Myc may be activated by translocation, as occurs with lymphomas, by increased gene copy number (amplification), by higher protein expression or by deregulation of transcription mechanisms [142]. *MYC* amplification is identified more frequently in SCLCs (ranging from 18% to 31%) compared to other bronchial-pulmonary carcinomas (ranging from 8% to 20%) [137]. Occurrences of *c-MYC* point mutations have been identified [142]. l-Myc high expression is described mainly in small cell lung carcinomas [142].

c-Myc are implicated in cell cycle control, especially controlling transition G1-S phases, by interacting with Cyclins / CDKs and their inhibitors [142]. Myc acts by inducing higher expression of Cyclin A and E with CDK activation [142]. It is also responsible for the downregulation of p27 and p21 (CDKs inhibitors) [142]. Transition G1-S phase is also dependent on the inactivation of RB with E2F release [142]. Myc also target genes implicated in the control of cellular growth [142].

PI3K/Akt/PTEN/mTOR

Activation of phosphatidylinositol–3-kinases (PI3Ks) pathway is associated with stimulation of cellular growth and proliferative activity, apoptosis control and cytoskeletal rearrangement [137]. It acts by the activation of downstream effectors, such as AKT/Protein Kinase B (PKB) [137]. As tumour suppressor gene, *PTEN* controls negatively AKT, loss of PTEN function is associated with PI3K/AKT pathway activation [137]. *PTEN* is infrequently mutated in lung cancer, but reduced or non-existent expression of PTEN protein is common [137].

STATs

There are seven known members of STAT (signal transducers and activators of transcription): STAT1; STAT2; STAT3; STAT4; STAT5A; STAT5B and STAT6 [137]. STAT 3 and STAT5 activation

are associated with induction of a proliferation and with a negative control of apoptosis, mediated by BEL, MYC and Cyclin D1 [137].

Tumour suppressor genes

Tumour suppressor gene products are associated with diverse cellular functions. Some act as a transcription factor, such as p53, other are known to downregulate other transcription factors , such as RB, other act regulating ubiquitin-mediated degradation of molecules by activating ubiquitin ligases, inducing the degradation of oncogene products, such as APC-β-catenin or by inhibiting ubiquitin ligases, reducing the degradation of tumour suppressor gene products [137,138]. Other (GTPases) are responsible by the inhibition of G-coupled proteins and DNA repair, such as MSH2, MLH1, ATM and BRCA1 [137,138]. Other tumour suppressor gene products include cytoskeleton and adhesion molecules (such as E-cadherin, α-catenin, NF2 and RASSF1), and molecules that are responsible for histone changes like Men1 that is a histone methylase associated with transcription silencing [137,138]. Some tumour suppressor gene products act as protein kinases (LKB1) or as kinase inhibitors like PTEN, which is responsible for PIP3 degradation [137,138]. Tumour suppressor genes functions are important in transduction pathways, cell cycle and apoptosis control [137,138]. Mutations, insertions, deletions, and missense mutations, with inactivation of functions, could also be registered in tumour suppressor genes [137,138].

Inactivation of Tumour-Suppressor Genes

Tumour-suppressor pathways, such as p53 and p16-Cyclin D1-CDK4-Rb, are frequently inactivated in lung cancer [137]. It is described an increased expression of p53 upon DNA damage, with cell cycle arrest, followed by DNA repair or by apoptosis if the DNA damage is not repaired [137]. These mechanisms are mediated by p21/WAF/CIP1p53, BAX and GADD45 [137]. p53 is altered in ≈90% of SCLCs and ≈50% of bronchial-pulmonary carcinomas, especially due to missense point mutations (70%–80%), and homozygous deletions [137]. In lung bronchial-pulmonary carcinomas, it is frequently only present the mutated *TP53* allele, due to the deletion of the wild-type *TP53* allele [137]. The loss of p53 wild-type function could be related with the heterodimer formation with mutant p53 forms [137]. Reacquisition of p53 wild-type function is associated with apoptosis, even if tumour cells harbour a mutant *TP53* allele [137]. p53 pathway is regulated by MDM2 and p14ARF [137]. MDM2 induces p53 degradation (by proteosome), reducing p53 levels [137]. It has been described *MDM2* increased gene copy numbers by amplification in approximately 7% of bronchial-pulmonary carcinomas [137]. On the other hand, *p14ARF* gene product binds to MDM2, preventing p53

degradation with p53 stabilization [137]. A decreased expression of p14ARF has been described in approximately 40% of the bronchial-pulmonary carcinomas and over 50% of small cell carcinomas [137].

P16, Cyclin D1, CDK4 and RB have important functions in control of G1-S phases transition [137]. The complex Cyclin D1/CDK4 is responsible for RB phosphorylation, with the consequent release of E2F, and G1-S transition [137]. Decrease of RB protein or *RB* mutations have been described especially in SCLC (90%) and is less frequent in bronchial-pulmonary carcinomas (ranging from 15% to 30%), and are associated with G1-S checkpoint loss [137]. p16INK4 acts by inhibiting RB phosphorylation, mediated by CDK4 [137]. p16INK4 decreased levels or inactivation are frequently observed in bronchial-pulmonary carcinomas (≈70%), and are rare in small cell carcinomas [137]. RB pathway inhibition could be also related to higher CDK4 expression and Cyclin D1 expressions [137]. Cyclin D1 overexpression and *CDK4* amplification have been described in bronchial-pulmonary carcinomas (over 40% and approximately 2% respectively) [137]. Cyclin D1 overexpression is frequently associated with smoking [137].

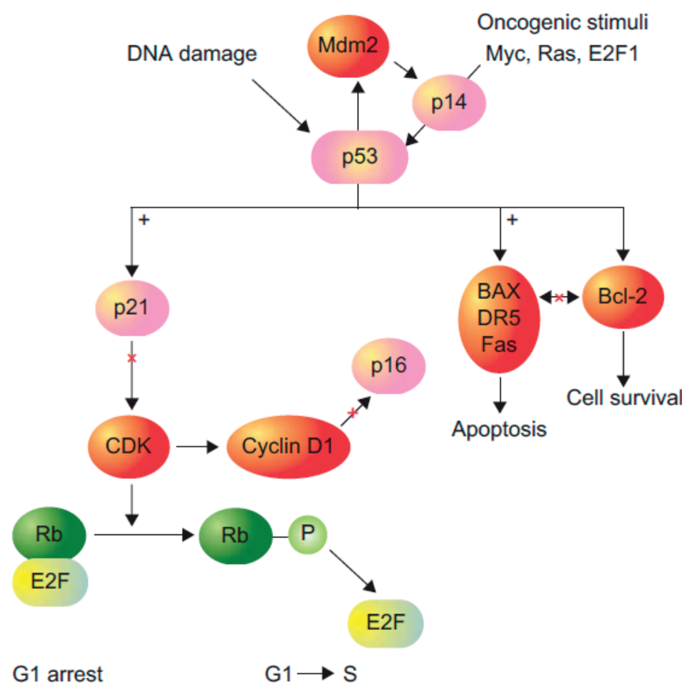

Figure 4: Inactivation of tumour suppressor genes. Adapted from: Brambilla, E.; Gazdar, A. Pathogenesis of lung cancer signalling pathways: roadmap for therapies [148].

The 3p Tumour Suppressor Genes

Genetic alterations involving 3p region are frequently described in bronchial-pulmonary carcinomas (>90%), including deletions and loss of heterozygosity (LOH), considered as an early genetic event in cancer development [137]. Different regions have been identified, including 3p14.2 (*FHIT/FRAB3*), and 3p12 (*ROBO1/DUTT1*) regions [137]. In the 3p21.3 region, 25 genes were identified, including *FUS1*, *RASSF1A*, *TSGs*, *NPRL2*, *SEMA3B*, *SEMA3F* and *101F6* [137]. Alterations in 3p region are described in normal bronchial epithelium, caused by smoking [137]. These genes are implicated in control of the apoptosis, cell cycle, angiogenesis and proliferations control [137].

Cell Cycle control

Cell cycle control deregulation has major importance in cancer development. Five phases are recognized: G0 phase (rest phase), G1 phase (allowing the cell to prepare DNA synthesis), S phase (DNA synthesis engaged phase), G2 phase and M phase (mitosis phase) [140,141]. Cell cycle control is dependent on complexes CDKs (Cyclin Dependent Kinases) with Cyclins [140,141]. Cell cycle checkpoints are important preventing cell division when damaged DNA is present, thus preventing transmission of damaged DNA to daughter cells, by allowing reparation or by the induction of apoptosis [140,141]. There are three important checkpoints: G1–S, S-phase and G2–M checkpoints [140,141]. The cell cycle is initiated by growth factor signalling [140,141]. RB phosphorylation during G1 phase is induced by Cyclin D1 and CDK4 or 6 complexes and by Cyclin E and CDK2 complexes before the S phase [140,141]. CDK inhibitors (p16INK4, p21WAF1 and p27) control Cyclin–CDK complexes [140,141]. Other Cyclins are involved in control of the cell cycle, such as Cyclin A acting in the mitosis phase and in the transition G1-S phase, and Cyclin B associated with the transition to mitosis [140,141].

When DNA damage is present, p53 (as guardian of the genome) is responsible for stopping the cell cycle, allowing DNA repair or inducing apoptosis if the DNA error is not repairable [140,141]. p53 arrests the cell cycle, activating p21WAF1 [140,141]. The cell cycle control depends also of RB [140,141]. Cell cycle control deregulation could be related to loss of p53 or RB function as well by alterations involving other gene products , such as p16, p14, CDKN2A, and MDM2 [140,141].

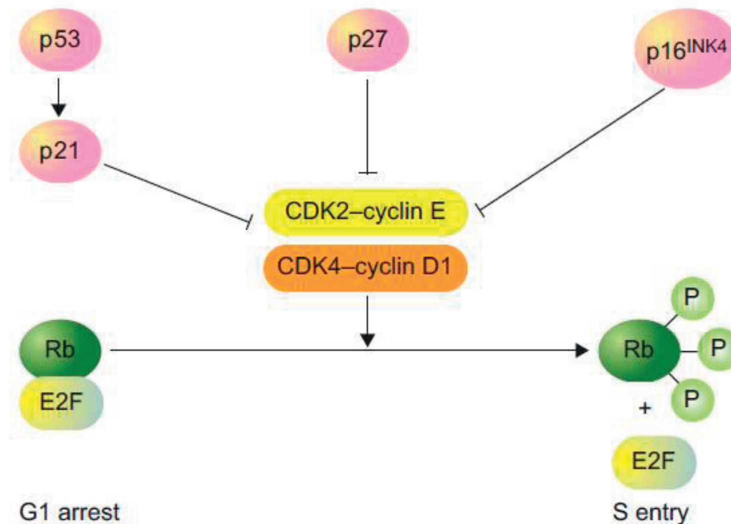

Figure 5: Cell cycle control. Adapted from: Brambilla, E.; Gazdar, A. Pathogenesis of lung cancer signalling pathways: roadmap for therapies [148].

Apoptosis

Apoptosis is defined as the programmed cellular death [176,177]. Cell death types include apoptosis, necrosis, autophagy, paraptosis, and autoschizis [176,177]. There are some interactions between these mechanisms, and the inactivation of one type could lead to the activation of another type of cell death [176,177].

Apoptotic pathways are classified generally as intrinsic (mitochondrial) and extrinsic (receptor pathway). A third pathway is observed in T cell-mediated cytotoxicity [176,177]. The three pathways share a terminal part, that depends on caspase 3 and 7, leading to several cellular events, including protein degradation, DNA fragmentation, apoptotic bodies formation and phagocytosis promotion [176,177].

The BCL protein family has relevant functions in apoptosis control [176,177]. Some members are anti-apoptotic (such as BCL2) and others demonstrated pro-apoptotic proprieties (such as BAX/BAD/BAK and BOK) [176,177]. The balance between proteins with pro-apoptotic and anti-apoptotic functions are related to the response to apoptotic stimuli [176,177].

Cancer cells have the capability to evade apoptosis [137]. As already said, p53 and BCL2 are two most relevant gene products related to apoptosis pathway control [137]. BCL2, responsible for apoptosis inhibition, is overexpressed in bronchial-pulmonary carcinomas (ranging from 10% to 35%) and in the majority of SCLCs (ranging from 75% to 95%) [137]. BCL2 inhibitors are being studied to explore this pathway [137].

DNA repair pathways

DNA repair has important implications in cancer. Deficient DNA repair function allows genomic instability [137,178]. DNA repair impairments could also explain the increased sensitivity of tumour cells to radiation and chemotherapy [137,178]. DNA damage could be related to spontaneous hydrolysis, cytosine deamination, mismatched bases, and secondary to reactive oxygen species (ROS) [137,178]. Also, anticancer agents, such as alkylating agents and bleomycin, are responsible for DNA breaks [137,178]. Six different mechanisms are involved in DNA repair, and when one is impaired another is frequently upregulated [137,178]. The six mechanisms are: mismatch repair (MMR), homologous recombination (HR) and nonhomologous end joining (NHEJ), translesion DNA synthesis (TLS), base excision repair (BER), and nucleotide excision repair (NER) [137,178].

Some DNA repair pathways (BER and NER) have important chemotherapeutic implications, as are responsible for the DNA bases changes repair, induced by alkylant agents [137,178].

Knowledge of the level of DNA repair activation or inhibition could be used to evaluate the potential response to chemotherapy and to define the best chemotherapy combination schemes [137,178]. For example, BRCA1 expression levels are considered as a survival biomarker in patients diagnosed with bronchial-pulmonary carcinomas treated with cisplatin-based chemotherapy agents. Patients with lung cancer with lower ERCC1 levels revealed higher cisplatin sensitivity compared to those with high ERCC1 expression [137,178].

DNA repair biomarkers could be useful to identify tumours resistant to radio or chemotherapy and in the selection of drugs that could selectively inhibit repair pathways/function as chemo sensitizers [137,178].

Genomic instability is frequent in cancer cells and it is associated with at least one DNA repair pathway defect [137,178].

Telomerase activation

Cancer cell immortalization depends on telomere maintenance, regulated by telomerase [137]. hTERT levels are important in control of telomerase activity [137].

Angiogenesis

Angiogenesis, measured by micro-vessel density, is correlated with metastasis risk and with the patient’s survival. VEGF is the most studied angiogenic factor, frequently demonstrating higher expression in bronchial-pulmonary carcinomas [137].

Cancer Stem Cells

The cancer stem cell model hypothesizes that cancer stem cells are less sensitive to radiotherapy and/or chemotherapy, this related to the lower proliferation rate and drug resistance phenotype (by expressing drug transporters) [137]. Thus, cancer stem cells would be able to regenerate tumours [137].The molecular factors related to cancer stem cell phenotype could be explored as potential molecular therapeutic targets [137].

Epigenetic Modifications

Recent evidence suggests that aberrant DNA promoter methylation could be identified early in cancer development [137]. Hypermethylation is frequently identified in several genes, such as *RASSF1A*, *p16INK4a*, *FHIT*, *MGMT*, *ECAD*, *DAPK*, *GSTP1*, *TIMP3*, and *RARβ* [137]. Epigenetic changes, such as promoter hypermethylation, are reversible changes, and so a potential therapeutic target [137].

CHAPTER 2 – OBJECTIVES

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This work aimed to study preneoplastic lung lesions and carcinomas, focusing on: differentiation and diagnostic aspects, prognostic and factors with predictive therapeutic value.

Firstly, adaptive and preneoplastic bronchial lesions were studied, namely respiratory epithelium basal hyperplasia, squamous metaplasia and dysplasia till carcinoma *in situ* (*CIS*).
The aim of this part of the study was to acquire better knowledge of the evolution of preneoplastic bronchial lesions, according to the expression of markers of differentiation and proliferation and EGFR and HER2-neu as a potential biomarker for lung cancer risk assessment. This knowledge would be important to develop new and effective strategies for early diagnosis and insights into chemoprevention strategies and targeted therapy.
Immunohistochemical antibodies (CK5/6, CK7, Chromogranin A, Ki67, p53, HER2 and EGFR) were applied in the study of preneoplastic lesions. The *HER2* and *EGFR* gene copy number were assessed by FISH (fluorescent *in situ* hybridization).

Taking into account the results obtained in the study of preneoplastic epidermoid carcinoma lesions and the increasing importance of targeted therapy, HER2 and EGFR protein expression, *HER2* and *EGFR* gene copy number and *HER2* and *EGFR* mutation status were studied in lung epidermoid carcinoma. The aim was to obtain a better understanding of the molecular pathology of epidermoid carcinoma concerning these genes with clinical and therapeutic implications, and to contribute to knowledge concerning the status of these genes studied by immunohistochemistry, FISH and PCR.

Considering the increasing incidence of lung adenocarcinoma, recent developments in their classification and the growing evidence of the importance of pattern recognition with diagnostic, prognostic, and even therapeutic predictive value, a sample of lung adenocarcinomas was studied according to the expression of differentiation molecular markers, proliferation molecular markers, the expression of different gene products with implications for the signal transduction pathways, cell cycle control, and the apoptosis control, and with potential therapeutic implications, especially concerning targeted therapy. These adenocarcinomas were evaluated using a pattern-based approach, trying to identify differences according to patterns that could reflect different molecular pathways relevance's according to adenocarcinoma patterns. The objectives were to validate a small differentiation

IHC panel, evaluate the protein expression of different genes according to different adenocarcinoma patterns, and correlate this expression with progression and drug resistance cell pathways, in order to understand bronchial-pulmonary adenocarcinoma carcinogenesis. Another objective was to verify possible differences of gene expression in adenocarcinoma taking account of variables such as gender, age, smoking and different tumour stages. The global intention of this project is to acquire knowledge that could be applied in the diagnosis and prognostic evaluation of bronchial-pulmonary adenocarcinoma and its subtypes in order to define individual treatment.

In a fourth stage, *EGFR* and *KRAS* mutational status, *EGFR* and *HER2* gene copy number and immunohistochemical EGFR and HER2 expression were studied in these adenocarcinomas, also taking account of adenocarcinoma patterns. The objectives of this part of the work were 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 better understand the value of pattern sub-typing, supported by an immunohistochemical set routinely used, in adenocarcinoma diagnosis and anti-EGFR / targeted therapy decision.

In a fifth stage, it was studied FGFR1 expression and gene copy number in bronchial pulmonary carcinomas, knowing that *FGFR1* is an oncogene that can potentially be targeted by tyrosine kinase inhibitors. This part of the work aimed to evaluate FGFR1 expression and gene copy number in adenocarcinomas, squamous cell lung carcinomas, pleomorphic carcinomas and adenosquamous carcinomas. EMT pathway was also explored in more complex lung carcinomas such as adenosquamous and pleomorphic carcinomas as well as adenocarcinomas, according to Vimentin and TTF1 expression, and in squamous cell carcinoma, joining CK7 expression. The purpose was to identify differences and/or similarities in between these groups of lung tumours in order to characterize the histological types for targeted therapy.

This work plan gave rise to a work flow with different methodologies reflected in several publications involving specific material and methods, results evaluation and discussion. The results section of this thesis will present several published or forthcoming articles, concerning especially the study of preneoplastic lesions, epidermoid lung carcinomas, and adenocarcinomas. Each publication contains material and methods, results and discussion of the data.

PART II – EXPERIMENTAL WORK

Data obtained during the PhD study period is summarized in the following six manuscripts and twelve published abstracts:

Manuscript I – EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and P53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study.

Virchows Arch (2011) 458:571–581.

Manuscript II – ***Polysomy and amplification of chromosome 7 defined for EGFR gene in squamous cell carcinoma of the lung together with exons 19 and 21 wild type.***

Rev Port Pneumol 2010; XVI (3): 453-462.

Manuscript III – Bronchial-Pulmonary Adenocarcinoma Subtyping Relates With Different Molecular Pathways.

In publication in Revista Portuguesa de Pneumologia - Received 30 October 2013, accepted 30 May 2014, available online 5 March 2015.

doi:10.1016/j.rppnen.2014.05.006

Manuscript IV – Lung adenocarcinoma: sustained subtyping with immunohistochemistry and EGFR, HER2 and KRAS mutational status.

Rev Port Pneumol. 2015;21:113-25.

doi: 10.1016/j.rppnen.2014.09.009. Epub 2015 Mar 11.

Manuscript V – EGFR and KRAS mutations coexistence in lung adenocarcinomas.

Diagnostic Pathology 2015, 1:13.

http://www.diagnosticpathology.eu/content/index.php/dpath/article/view/13

Manuscript VI – Targeted therapy for FGFR1 may be independent of the histological type of bronchial-pulmonary carcinomas.

Submited / undergoing revision – Virchows Archive.

Abstract I – EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 AND P53 EXPRESSION IN PRENEOPLASTIC LESIONS OF BRONCHIAL EPITHELIUM.

BMC Proceedings 2010, 4(Suppl 2):P64.
The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.

Abstract II – *EGFR* UNUSUAL MUTATION STATUS IN LUNG ADENOCARCINOMAS.

BMC Proceedings 2010, 4(Suppl 2):P61.
The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.

Abstract III – *KRAS* AND *EGFR* MUTATIONS COEXISTING IN LUNG ADENOCARCINOMA.

BMC Proceedings 2010, 4(Suppl 2):P57.
The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.

Abstract IV – *EGFR* and *KRAS* mutations in mixed type of Pulmonary Adencocarcinoma.

Virchows Arch (2010) 457: 91-281.
Intercongress Meeting of the European Society of Pathology, Krakow – Poland, 31st August - 4th September, 2010.

Abstract V – *KRAS* Mutations in Lung Adenocarcinomas may be Relevant in Histological Patterns, *EGFR* Mutational Status and Male Smokers.

Virchows Archiv 2011; Supplement 459: SI – S320.
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

Abstract VI – TTF1 Negative Solid Pattern in Lung Adenocarcinomas May Reflect Morphogenesis.

Virchows Archiv 2011; Supplement 459: SI – S320.
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

Abstract VII – Lung Adenocarcinomas Subtyping is not Relevant for *EGFR* Mutations, Gene Copy Number and Protein Expression.

Virchows Archiv 2011; Supplement 459: SI – S321.
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

Abstract VIII – *ALK* Gene Rearrangment in EGFR and KRAS Positive Lung Adenocarcinomas.

Virchows Archiv 2011; Supplement 459: SI – S320.
23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

Abstract IX – ALK EXPRESSION IN PULMONARY ADENOCARCINOMAS.

Virchows Arch (2012) 461: S1-S332.
24thEuropean Congress of Pathology, Prague, September 8 – 12, 2012.

Abstract X – Bronchial-Pulmonary Carcinoma Cases with *EGFR*, *ALK* and *MET* Alterations.

Rev Port Pneumol. 2013; 19(Esp Cong 4): 127-176.
XXIX Congresso de Pneumologia, Albufeira-Portugal, October 25 – 27, 2013.

Abstract XI – *EGFR* Mutations, *MET, EGFR* Amplification and *ALK* Rearrangement Simultaneous in Five Bronchial-Pulmonary Adenocarcinomas.

(*2014*) Pulmonary Pathology Society Biennial Meeting Abstracts. Archives of Pathology & Laboratory Medicine: May 2014, Vol. 138, No. 5, pp. 700-709.
Biennial Pulmonary Pathology Society (PPS) Meeting, Grenoble, June 25-28, 2013.

Abstract XII – Pulmonary Squamous Cell Carcinoma can harbor *EGFR* mutations.

Rev Port Pneumol. 2014; 20(Esp Cong 3): 1-37.
5º Congresso de Pneumologia do Centro, June 26-27, 2014.

Data concerning material and methods, results and discussion will be addressed in the manuscripts and / or abstracts, concerning specifically the studies on preneoplastic bronchial epithelium lesions (Manuscript I and Abstract I), epidermoid carcinoma (Manuscripts II and VI and Abstract XII), and adenocarcinomas (Manuscripts III, IV, V and VI and Abstracts II-XI).

The general discussion following the presentation of papers (Manuscripts and Abstracts) will deal with aspects covering all manuscripts, with reflections and conclusions.

CHAPTER 3 – MANUSCRIPT I

EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and P53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study.

CHAPTER 3 – EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and P53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study.

Virchows Arch (2011) 458:571–581
DOI 10.1007/s00428-011-1062-5

ORIGINAL ARTICLE

EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and P53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study

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Abstract A prognostic interpretation of preneoplastic lesions would have impact in bronchial carcinoma early diagnosis and through the study of Erb-B family receptors as they have an important role in lung carcinogenesis. The existence of drugs as tyrosine kinase inhibitors stressed the importance of studying gene alterations for selected chemoprevention schemes and characterization of carcinogenesis. Bronchial preneoplastic lesions were characterized by immunohistochemistry using the antibodies LP34 (high weigh molecular cytokeratin), CK7, chromogranin A, Ki67, p53, C-erbB-2 and EGFR. *HER2* and *EGFR* gene copy number was also evaluated by fluorescent in situ hybridization in those lesions. The expected results defined the origin cell for basal cell hyperplasia and squamous metaplasia as adaptative lesions and dysplasia. By known experiences and published data, beyond the stem cell, the spectral evolution of bronchial preneoplastic lesions was demonstrated by characterizing basal cells (LP34) and their neoplastic potentiality. Dysplasias showed a higher expression of EGFR, Ki67 and p53 with a stepwise increase with the gravity of the respective grading. C-erbB-2 immunohistochemical overexpression was a rare event in preneoplastic lesions. Polysomy was the main mechanism for *EGFR* and *HER2/neu* higher gene copy number and together with increased proliferation index (Ki67) will account to preview bronchial carcinogenesis.

Keywords Bronchial squamous metaplasia · EGFR, HER2/c-erbB-2 · CK7 · LP34

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Introduction

Lung cancer is the leading cause of death by malignancy in developed countries and throughout the world. Environmental and occupational exposures, such as polyaromatic hydrocarbons, radon, asbestos, nickel, arsenic and chromium, are important determinants of lung cancer risk, but cigarette smoking is the main risk factor, accounting for about 90% of the cases in men, 70% of the cases in women and within today ex-smokers comprising nearly 50% of all new lung cancer cases [1–5].

The overall 5-year survival rate for lung cancer patients remains less than 15% [6], and the death rate for lung cancer exceeds the combined total for breast, prostate and colon cancer in developed countries [5]. The major reasons for the poor prognosis for lung cancer are the lack of effective screening and early diagnosis procedures, the propensity for early metastasis and the inability of systemic therapies to cure patients with widely metastatic disease [7]. Lung cancer is the result of a multi-step accumulation of genetic and/or epigenetic alterations; therefore, a better understanding of the molecular mechanism, by which these alterations affect lung cancer pathogenesis, would provide new and more effective strategies for chemoprevention,

early diagnosis and targeted treatment [8]. The existence of novel drugs against specific molecular targets like EGFR and HER2/neu genes leads to the importance of defining their expression as biomarkers in preneoplastic lesions.

The 1999/2004 WHO lung tumour classification recognizes three types of preinvasive lesions: squamous dysplasia and carcinoma in situ (CIS); atypical adenomatous hyperplasia and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia [9]. Respiratory epithelium adaptative lesions include basal cell hyperplasia, squamous metaplasia, followed by dysplasia and CIS, which precede or accompany microinvasive or invasive carcinoma. Hyperplasia and squamous metaplasia are considered reversible lesions, while dysplasia and CIS are not [10–15].

Squamous metaplasia as a common adaptative lesion of the bronchial mucosae, especially in smokers, represents a precursor step in the development of invasive squamous cell carcinoma, deserving an accurate study to define its evolutionary potential to lung cancer, in order to be determined in bronchial biopsies [10, 14, 16, 17].

Several molecular and genetic abnormalities have been described in lung carcinogenesis, including activation of proto-oncogenes and loss of tumour suppressor genes and DNA repair genes. Proto-oncogenes can be activated by point mutations, amplification, chromosomal rearrangement and expression of a high gene copy number. [18–20]. Those genomic and molecular abnormalities are early events in lung carcinogenesis and can persist in bronchial lesions for months or years and their persistence or regression have been correlated with lesions' evolution [15, 21–30]. Molecular changes are recognized not only in lung cancer, but also in bronchial epithelium and parenchyma of current and former smokers without the development of lung cancer [31, 32].

Epidermal growth factor receptor (EGFR; *ErbB-1* gene) and ErbB-2 (*HER2/neu*) belong to ErbB family, a family of tyrosine kinase receptor proteins that includes also ErbB-3 (*HER-3*) and ErbB-4 (*HER-4*). The ErbB receptors are composed of three major domains: an extracellular ligand binding; a transmembranar segment and a cytoplasmic with tyrosine kinase activity. ErbB receptor function begins upon ligand binding and is followed by receptor homo (e.g. Erb-1/Erb-1) or heterodimerization (e.g. Erb-1/Erb-2), activating the tyrosine kinase domain of the receptor, which becomes phosphorylated and acts as a docking site for downstream signalling molecules and cytoplasmic messenger proteins that then trigger a cascade of cytoplasmic and nuclear pathways [15].

It has been suggested and supported by experimental data that aberrant activation of the kinase activity of ErbB receptors plays an important role in the neoplastic transformation and progression, prognosis, survival and resistance to cytotoxic therapies [6, 7, 15, 33–38]. EGFR's high expression is an early event in preneoplastic lesions, with an increasing expression from normal mucosae to hyperplasia, metaplasia and dysplasia [6, 38–42]. EGFR is overexpressed in 84% of squamous cell carcinoma, 65% of adenocarcinomas and 68% of large cell lung carcinomas [41]. As it seems, that ErbB2 is also highly expressed in bronchial preneoplastic lesions and its overexpression is less common in carcinomas (less than 35% of non-small cell lung carcinomas (NSCLC), mainly adenocarcinomas) [18, 41, 43]. The heterodimer EGFR/HER2-neu has been shown to have a stronger proliferative effect than the corresponding homodimers [38, 44–46]. Amplification and polysomy of EGFR and HER2-neu and overexpressed proteins have been related to survival in patients with NSCLC (non- small cell lung cancer), but contradictory results have been published [47–51]. New therapies have also been developed recently directed to these two molecular targets, some of them being applied in chemoprevention trials, stressing the necessity to understand their expression in adaptative and preneoplastic lesions [5, 52–56]. This study concerned bronchial preneoplastic lesions observed in biopsies as basal cell hyperplasia, squamous metaplasia and dysplasia. Using immunohistochemistry, those lesions were characterized by antibodies applied against LP34, CK7, chromogranin A, Ki67, p53, C-erbB-2 and EGFR. *HER2* and *EGFR* gene copy number was also evaluated by fluorescent in situ hybridization (FISH), using normal controls. The aim of the present study is to achieve a better understanding of the spectral evolution of bronchial adaptative and preneoplastic lesions, by recognizing the expression of *EGFR* and *HER2-neu* as potential biomarkers for lung cancer risk assessment. This knowledge would help to provide new and more effective strategies for chemoprevention, early diagnosis and targeted treatment.

Materials and methods

Material

A number of 67 bronchial biopsies were included in this study, comprising 89 preneoplastic lesions, selected from recent data base, by considering the preservation, number and dimension of the small fragments obtained by bronchial fibroscopy and represented 16 basal cell hyperplasia, 40 squamous metaplasia and 33 epidermoid dysplasia (7 mild dysplasia, 7 moderate dysplasia, 4 severe dysplasia and 15 CIS) (Fig. 1a, b, c, d, e); in 22 cases there was concomitancy of the lesions. The population consisted of 57 men and 10 women, with 66.3 years as mean age (range 38–92).

Hyperplasia was defined when more than four basal cell layers were observed. Squamous metaplasia was considered when there was replacement of ciliated columnar epithelium by pavimentous epithelium without atypia. Dysplasia

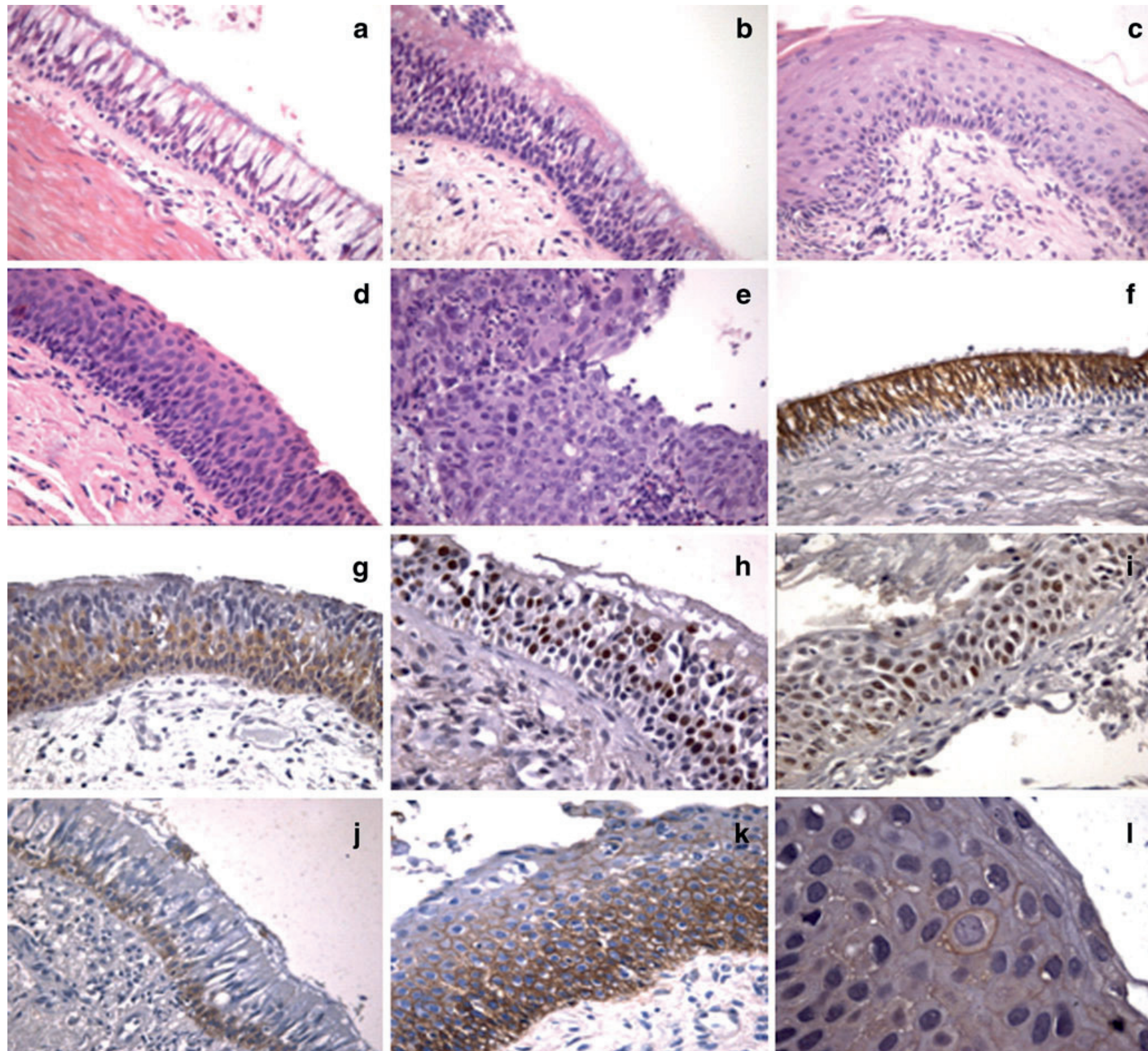

Fig. 1 Morphology and immunostaining: **a** Normal bronchial epithelium, H&E, ×400. **b** Basal cell hyperplasia, H&E, ×400. **c** Squamous metaplasia, H&E, ×400. **d** Moderate dysplasia, H&E, ×400. **e** In situ carcinoma, H&E, ×400. **f** CK7 expression, normal bronchial epithelium, ×400. **g** Basal cell hyperplasia, LP34, ×400. **h** Ki67 3+/70%, ×400. **i** P53 2+/50%, ×400. **j** EGFR negative, normal epithelium, ×400 **k** EGFR, 3+/60%, ×400. **l** C-erbB-2, 2+, ×1,000

was graded as mild, moderate and severe/CIS according to the levels of atypia.

Biopsy products were routinely fixed in 4% neutral buffered formalin immediately after bronchial fibroscopy. They were processed and embedded in paraffin. One paraffin block was chosen from each case, containing several representative fragments. From each paraffin block, several cuts 5-μm thick sections were made and put on glass slides then stained with haematoxylin–eosin and reviewed by two pathologists in order to ensure the optical criteria for preneoplastic bronchial lesions according to the 1999/2004 histological WHO/IASLC classification of preinvasive squamous lesions of the bronchi.

Methods

Immunohistochemistry

The imunnohistochemical analysis included seven different antibodies: CK7 (Monoclonal Mouse Anti-human, OV-TL 12/30, Dako); LP34 (Monoclonal Mouse Anti-human, Clone LP34, Dako); chromogranin A (Monoclonal Mouse

Anti-human, Clone DAK-A3, Dako); Ki67 (Monoclonal Mouse Anti-human, Clone MIB-1, Dako); p53 (Monoclonal Mouse Anti-human, Clone DO-7, Dako); C-erbB-2 (Policlonal Mouse Anti-human, Dako) and EGFR (Monoclonal Mouse Anti-human, Clone 31G7, Zymed Laboratories Inc.). The procedure was performed according to a standard avidin-biotin-peroxidase complex. Three-micrometer tissue sections were placed on coated slides and allowed to dry overnight. After deparaffinization and rehydration, antigen unmasking was performed using Module PT (Lab Vision®) for citrate buffer for 25 min at pH 6, 98°C for chromogranin A, p53, Ki67 and c-erbB-2 antibodies. Antigen unmasking was performed with Pronase E, for 10 min at room temperature for CK7, LP34 and EGFR antibodies. Endogenous peroxidase activity was quenched using 15 min incubation in 3% diluted hydrogen peroxide (H_2O_2). For blocking nonspecific binding, Ultra V Block (Ultra Vision Kit®; TP-015-HL) was applied to the sections and then they were incubated at room temperature, with primary antibodies against CK7 at a dilution of 1/50 for 30 min, LP34 at a dilution of 1/100 for 60 min, chromogranin A at a dilution of 1/300 for 30 min, p53 at a dilution of 1/40 for 30 min, Ki67 at a dilution of 1/50 for 30 min, c-erbB-2 at a dilution of 1/40 for 60 min and EGFR at a dilution of 1/20 for 30 min. After washing with phosphate-buffered saline, slides were incubated with biotin-labelled secondary antibody (Lab Vision®) for 15 min. Primary antibody binding was localized in tissues using peroxidase-conjugated streptavidin (Lab Vision®) and 3,3-diaminobenzidine tetrahydrochloride was used as the chromogen according to manufacturer's instructions. The slides were counterstained with hematoxylin, dehydrated and mounted.

For each biopsy, external and the intrinsic positive control were considered: basal cells for LP34 and columnar ciliated cells for CK7 in normal bronchial epithelium; carcinoid tumour sections for chromogranin A; sections of a colonic tubulo-villous adenoma for p53; female breast ductal carcinoma in situ for C-erbB-2 and sections of lung adenocarcinoma with intense EGFR IHC positivity. Ki67 was validated by a small cell lung carcinoma.

LP34, CK7 and chromogranin A expression were classified as negative if there was no expression or mild focal expression or positive when there was moderate or intense and diffuse expression. The cut off to classify a lesion as positive for proliferative marker Ki67 was 10% in at least two layers of positive nuclei above the epithelial basal membrane, since it is normal to observe some proliferative activity mainly in the basal cell layer.

A score established by other authors was applied to Ki67, p53 and EGFR protein expression: 0-negative; 1-focal positivity; 2-mild positivity; 3-moderate positivity; 4-intense positivity. The intensity was multiplied by the percentage of positive cells, thus defining a 0–200% score as negative or low expression, 201–300% score as intermediate expression and a 301–400% score as high expression [19].

The expression of C-erbB-2 was validated by membrane staining and an identical score to the one used in breast cancer was applied with some alterations because of the limited number of cells observed. Each specimen was scored semi-quantitatively according to the intensity of membrane immunostaining in a 4-point scale: 0/negative - absence of staining; + - weak staining and/or no homogeneous staining; ++ - moderate homogeneous membrane staining; +++ - intense homogeneous membrane staining. For the ++ and +++, we registered the number/percentage of stained cells. Cytoplasmic staining was not considered when present.

Fluorescence in situ hybridization (FISH)

Sections of formalin-fixed paraffin-embedded specimens, 4-μm thick, were incubated at 65°C for 24 h, deparaffinized in two xylene washes for 10 min and dehydrated in ethanol. The slides were then incubated at 120°C for 4 min with 10 mM citrate buffer (ph 6.0), followed by saline sodium citrate buffer (2×SCC pH 7.0) washes. Sections were digested with proteinase K (0.25 mg/ml in 2×SCC, Sigma, pH 7.0) for 5 min at 37°C, followed by new 2×SCC wash and post-fixation with 1% formalin for 10 min at room temperature. The slides were later on dehydrated in ethanol series (70–90–100%) for 2 min. Dual-colour FISH assays for HER2 were performed by using Qbiogene HER2/neu (17q21)/Alphasatellite 17 dual colour probe, and for EGFR, the Vysis, Inc. USA LSI EGFR Spectrum Orange/CEP 7 Spectrum Green probe was used. Ten-microliter probe set was applied to the selected area of the slides and the hybridization areas were covered with a glass cover slip and sealed with rubber cement. The slides were incubated at 90°C for 6 min for co-denaturation and at 37°C for 12–18 h for hybridization in a humidified chamber. Post-hybridization washes were performed with 50% formamide/2×SCC (pH 7.0) at 46°C for 5 min, followed by 2×SSC at 46°C for 2 min. After dehydratation in ethanol series, 4′, 6′-diamidino-2-phenylindole (DAPI; 0.15 mg/ml in Vectashield mounting medium, Vector Laboratories, Bulingame, CA, USA) was applied for chromatin counterstaining.

Microscopic analysis was performed on a Nikon Eclipse 80i bright field and epifluorescent microscope equipped with LUCIA cytogenetics software. Fluorescence signals were scored using single-band filters for DAPI, FITC, Texas Red and triple-band pass filter (DAPI, FITC and Texas Red). Images were recorded with a Nikon digital DMX 1200 F camera in monochromatic layers, which were subsequently merged by Nikon ACT-1 capture software.

The reference slide (stained with haematoxylin–eosin) usually contained normal bronchial epithelium adjacent to the adaptative and preneoplastic lesions and at least 100 non-

overlaping interphase nuclei were scored in each fragment for both *HER2* and chromosome17 centromer signals and for *EGFR* and chromosome7 centromer signals, following scoring guidelines and constant adjustments of microscope focus when signals were located at different levels.

Two independent observers performed analysis in a blinded study and scored results, restricted to the selected lesions and bronchial epithelium in each case, with reproducible results.

Six major FISH patterns were identified: disomy (≤2 gene copies per nucleus in >90% of cells); low trisomy (≤2 gene copies per nucleus in ≥40% of cells, 3 gene copies in 10–40% of cells, ≥4 gene copies in <10% of cells); high trisomy (≤2 gene copies per nucleus in ≥40% of cells, 3 gene copies in ≥40% of cells, ≥4 gene copies in <10% of cells); low polysomy (≥4 gene copies per nucleus in 10–40% of cells); high polysomy (≥4 gene copies per nucleus in ≥40% of cells) and gene amplification (defined by presence of tight *EGFR* gene clusters and a ratio of *EGFR* gene to chromosome of ≥2 or ≥15 copies of *EGFR* per cell in ≥10% of analysed cells), according to a FISH scoring System defined by Cappuzzo et al. [57]. Amplification and high polysomy were considered as FISH-positive results.

Statistical analysis

Comparisons were performed using bilateral chi-squared tests or Fisher exact test when needed, because of the small number of lesions in some categories. Anova test was applied to compare the markers expression's percentage.

Results

Differentiation markers

Basal cell hyperplasia positive LP34 cells in the lower cell layers revealed clear cut to squamous metaplasia staining up to the top cell layer, by loss of CK7 expression (Table 1, Fig. 1f, g). Accordingly, dysplasias showed always LP34 positivity and CK7 negativity.

Chromogranin A expression was negative in all the cases (Table 1).

Caliciform cell hyperplasia, when present, was negative for LP34 (only the basal cell layer remained positive) and maintained the expression for CK7.

Proliferation (Ki67) and apoptotic (p53) markers

Significant differences of Ki67 expression were found between basal cell hyperplasia and squamous metaplasia group and dysplasia group cases ($p=0.001$). Ki67 expression was higher in dysplasia ($p=0.0007$). There was also found a stepwise increment of expression from basal cell hyperplasias, to squamous metaplasia and dysplasia ($p<0.0001$). The intense expression of Ki67 (intense positive cases) was shown to have a statistical significant increase from basal cell hyperplasia, to squamous metaplasia and dysplasia ($p=0.0002$). (Tables 2 and 3, Fig. 1h). Intense positive cases were observed in 19 (57.6%) dysplasias, 9 (22.5%) metaplasias and one basal cell hyperplasia (6.25%). There were no significant differences between the three grades of dysplasia. Despite the intense positive cases observed, moderate expression of Ki67 was the second predominant type of expression in dysplasia (11 cases, 33.3%). Weak expression of Ki67 was mainly observed for basal cell hyperplasia (8 cases, 50%) and squamous metaplasia (13 cases, 32.5%).

An increasing expression for p53 in all the three types of lesions was observed ($p<0.0001$). P53 expression was significantly higher in dysplasia when compared with basal cell hyperplasias and metaplasias ($p=0.0007$). Intense positive cases were also more frequent in dysplasia ($p=0.0001$). In 10 basal cell hyperplasias (62.5%) and 22 squamous metaplasias (55%), it was possible to observe a weak p53 expression. An intense expression of p53 was observed in 20 cases (60.6%) of dysplasia (Tables 2 and 3, Fig. 1i).

EGFR and C-erbB-2/HER2 protein and gene expression

EGFR immunohistochemical expression was significantly higher in dysplasias when compared with other preneo-

Table 1 LP34, CK7 and chromogranin A expression in preneoplastic lesions

	LP34		CK7		Chromogranin A	
	Positive	Negative	Positive	Negative	Positive	Negative
Basal cell hyperplasia $n=16$	16	0	0	16	0	16
	100%	0%	0%	100%	0%	100%
Squamous Metaplasia $n=40$	40	0	0	40	0	40
	100%	0%	0%	100%	0%	100%
Dysplasia $n=33$	33	0	0	33	0	33
	100%	0%	0%	100%	0%	100%

Table 2 Ki67, p53, C-erbB-2 and EGFR intensity of expression in preneoplastic lesions

		Ki 67		p53		C-ErbB-2		EGFR	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
Basal cell hyperplasia (*n*=16)	Negative	1	6.25	1	6.25	15	93.75	5	31.25
	+	9	56.25	10	62.5	1	6.25	9	43.75
	++	5	31.25	5	31.25	0	0	1	6.25
	+++	1	6.25	0	0	0	0	1	6.25
Squamous metaplasia (*n*=40)	Negative	8	20	5	12.5	39	97.5	6	15
	+	18	45	22	55	0	0	13	32.5
	++	9	22.5	12	30	1	2.5	14	35
	+++	5	12.5	1	2.5	0	0	7	17.5
Dysplasia (*n*=33)	Negative	0	0	1	3	27	81.8	4	12.1
	+	3	9.1	8	24.2	5	15.2	7	21.3
	++	17	55.5	9	27.3	1	3	15	45.5
	+++	13	39.4	15	45.5	0	0	4	12.1

plastic lesions (p=0.009) (Tables 2 and 3, Fig. 1j, k, Fig. 2). EGFR IHC expression increased between the three groups of lesions (p=0.0005). The intensity of the expression was higher in the group of dysplasia followed by squamous metaplasia, with 18 (54.5%) dysplasias showing moderate expression and 3 cases (9.1%) intense expression, while 14 (35%) squamous metaplasia presented moderate expression and 1 (2.5%) intense expression (p=0.005). Basal cell hyperplasia showed mainly a low EGFR expression, counting ten (62.5%) cases. Statistical differences between the different grades of dysplasia were not found (Fig. 2). C-erbB-2 protein overexpression was clearly observed in only one severe dysplasia (Tables 2 and 3, Fig. 1l), without statistical differences between the three groups of lesions or according to the severity of the dysplasia (p=0.14). C-erbB-2 protein expression was less frequent than EGFR.

EGFR and *HER2* gene increased copy number was more often due to polysomy than to amplification. Among the four cases with intense EGFR protein expression, three had high polysomy and one showed disomy.

The number of FISH EGFR positive cases were higher in the group of dysplasia (p=0.0002) (Fig. 3). Statistical significant correlation between EGFR IHC expression and FISH *EGFR* results was determined when the intensity of IHQ expression (positive intense vs. non-intense positive cases) was considered (p=0.0092) (Fig. 4).

HER2 FISH positive cases (n=4) were due to high polysomy. In one case (n=1), high trisomy was observed. All high polysomy cases were identified in CIS cases, and the high trisomy case was identified in a basal cell hyperplasia. These cases were IHC negative. The IHC-positive C-erbB-2 case showed low trisomy and was considered FISH *HER2* negative (Fig. 5a, b, c).

Table 3 Ki67, p53, c-erbB-2 and EGFR expression in preneoplastic lesions considering intensity and percentage of cells with expression

		Ki 67		p53		C-ErbB-2		EGFR	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
Basal cell hyperplasia (*n*=16)	Neg	2	12.5	1	6.25	15	93.75	5	31.25
	Low pos	8	50	10	62.5	1	6.25	10	62.5
	Mod pos	5	31.25	2	12.5	0	0	1	6.25
	Pos int	1	6.25	3	18.75	0	0	0	0
Squamous metaplasia (*n*=40)	Neg	13	32.5	5	12.5	39	97.5	6	15
	Low pos	13	32.5	22	55	0	0	19	47.5
	Mod pos	5	12.5	4	10	1	2.5	14	35
	Pos int	9	22.5	9	22.5	0	0	1	2.5
Dysplasia (*n*=33)	Neg	0	0	1	3	27	81.8	4	12.2
	Low pos	3	9.1	8	24.2	5	15.2	8	24.2
	Mod pos	11	33.3	4	12.2	1	3	18	54.5
	Pos int	19	57.6	20	60.6	0	0	3	9.1

Virchows Arch (2011) 458:571–581

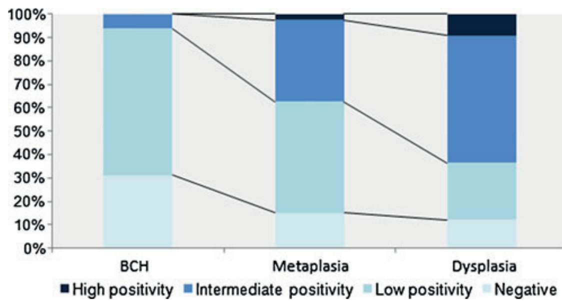

Fig. 2 (MS Excell) Immunohistochemical EGFR expression between the preneoplastic lesions

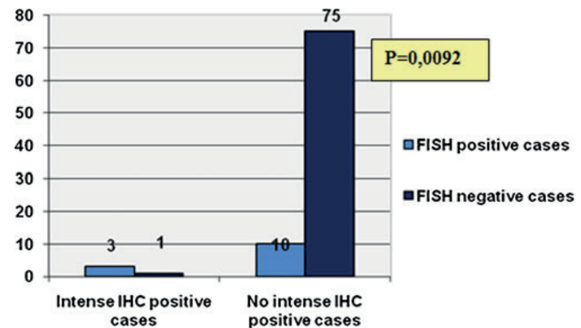

Fig. 4 (MS Excell) Correlation between immunohistochemical EGFR expression and FISH results

Discussion

As lung carcinogenesis is a multistep process, preneoplastic or preinvasive bronchial lesions' evolution to bronchial carcinoma depends upon the molecular and genetic abnormalities accumulated in time. Those abnormalities increase from the normal bronchial epithelium through basal cell hyperplasia, squamous metaplasia and low-grade to high-grade dysplasia. Certain proteins and genes play key roles in lung carcinogenesis and their aberrant expression in that process makes them as potential biomarkers susceptible to discriminate a subpopulation of patients with particularly active mutagenesis, progressive cancerization process and at very high risk to develop lung cancer [58].

LP34, CK7, chromogranin A, Ki67, p53, EGFR and C-erbB2 were the seven biomarkers chosen for this study as their abnormal expression is related with the oncogenic process. The first three were used successfully as differentiation markers of the preneoplastic lesions.

A basal cell origin for basal hyperplasia, squamous metaplasia and dysplasia was clearly defined, based on LP34 and CK7 expression. Immunohistochemical differentiation markers like LP34, CK7 and chromogranin A demonstrated useful to discriminate preneoplastic lesions from other proliferative lesions like caliciform cell hyperplasia or neuroendocrine cell hyperplasia. As expected, basal cell hyperplasia and squamous metaplasia are always LP34-positive and CK7 and chromogranin A-negative, indicating basal cell origin and conditioning squamous cell carcinoma as a basal cell carcinoma. In contrast, caliciform cell hyperplasia maintains cytoplasmic CK7 positivity cells and inconstant LP34 membrane positivity, reflecting an origin in cylindrical cells of the upper cell layer of the respiratory epithelium in bronchial adenocarcinoma carcinogenesis. Chromogranin A, a neuroendocrine marker, was constantly negative in this study, emphasizing that neuroendocrine cell hyperplasia has a different cell commitment.

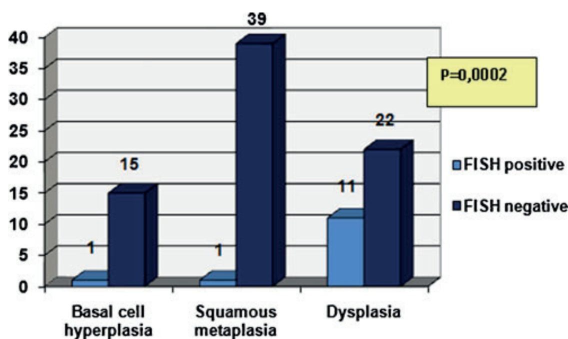

Fig. 3 (MS Excell) *EGFR* FISH results according to preneoplastic lesions

Ki67 expression stepwise increment reflects the increasing proliferative index observed in the spectrum of preneoplastic lesions. The statistical higher expression observed in dysplasia group compared to basal cell hyperplasia and squamous metaplasia defines two groups of lesions according to their proliferative index. As other authors observed, the intensity of Ki67 expression is also higher in dysplasia. This way, we reinforce the utility of Ki67 as a biomarker, namely for dysplasia, as it appears as a group characterized by high proliferative index, subscribing the literature and other authors' study of preneoplastic lesions [22–26, 59].

P53, the “guardian” maintaining the integrity of the genome by participating in the DNA damage checkpoints in the cell cycle, regulating cellular proliferation and apoptosis [20], has been detected in preneoplastic lesions of the lung, suggesting that it occurs early during lung carcinogenesis [14, 18, 20] as the obtained results confirmed. P53 expression was significantly higher in dysplasia when compared with hyperplasia and metaplasia, and the intensity of expression was also higher in the dysplasia group, supporting p53 accumulation involvement in the development of squamous cell lung cancer and fixing p53 as a remarkable biomarker in lung carcinogenesis.

There are many studies concerning EGFR, C-erbB-2, Ki67 and p53 expression in lung cancer. However, there are

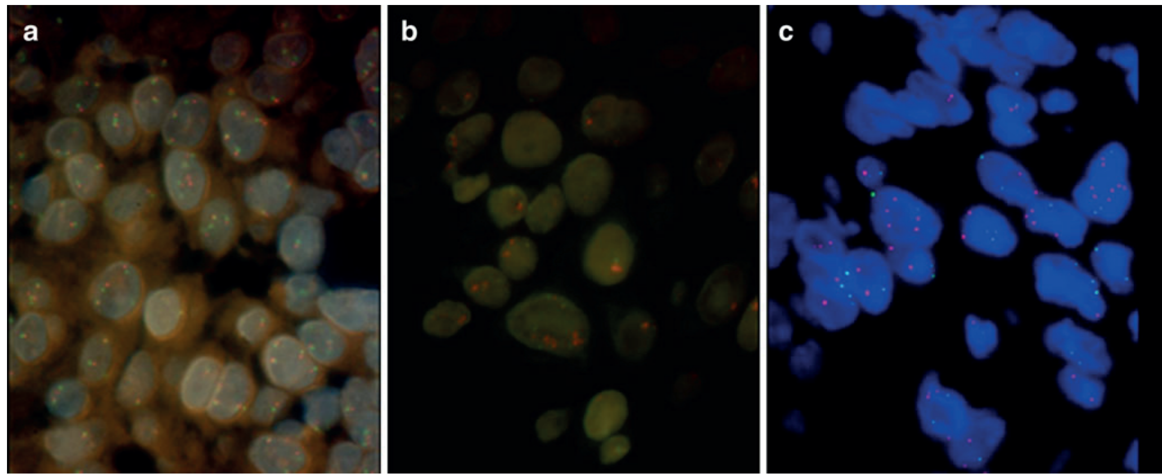

Fig. 5 **a** Dual-colour FISH assay with EGFR (*red*) and chromosome 7 (*green*), balanced polysomy, ×1,000. **b** Dual-colour FISH assay with EGFR (*red*) and chromosome 7 (*green*), amplification, ×1,000. **c** Dual-colour FISH assay with HER2neu (*red*) and chromosome 17 (*green*), amplification, ×1,000

fewer results validating these markers in preneoplastic lesions and most of them used only immunochemistry techniques. By analysing the expression of those biomarkers, using not only immunohistochemistry but also feasible molecular techniques, such as FISH and correlating the results obtained by the two different techniques, bronchial preneoplastic lesions achieve prognostic significance.

EGFR immunohistochemical increased expression in preneoplastic lesions, increasing from basal cell hyperplasia to dysplasia, had been demonstrated [6, 7, 21, 38–42, 55, 60]. Meert et al. [21] also demonstrated not only an increasing EGFR rate from normal bronchial epithelium to CIS and microinvasive tumours, but also a statistically significant cut off between mild dysplasia and severe dysplasia. Although the greater expression has been seen in dysplasia, statistical differences between the different grades of dysplasia were not demonstrated (probably because the number of cases of dysplasia was limited).

EGFR high gene copy number was due more frequently to polysomy than to amplification. FISH-positive *EGFR* cases are present in all types of preneoplastic lesions and were higher in dysplasia group. Polysomy appears as an early event in the sequence of hyperplasia–metaplasia–dysplasia. The higher frequency of polysomy in preneoplastic lesions is reflected in the high frequency of this event in squamous cell carcinomas, constituting the earliest molecular event in lung carcinogenesis [17, 22, 41].

EGFR protein was overexpressed in all lesions with an increased gene copy number. There was statistical significant correlation between EGFR immunohistochemical expression and FISH *EGFR* results, when considering the intensity of IHC expression [17]. Therefore, it was demonstrated that protein expression does reflect high gene copy number, like polysomy and amplification. Although *EGFR* gene amplification is implicated as one mechanism for EGFR overexpression, it does not seem to be the main one [61].

Despite an increasing expression of EGFR and Ki67 along the spectrum of preneoplastic lesions, no correlation between the two markers was found. Meert et al. recently described that low-grade lesions showed no EGFR or Ki67 expression contrasting with high-grade lesions. EGFR overexpression is associated with cell proliferation, making EGFR an early marker of malignant transformation in lung carcinogenesis [21, 38, 59].

C-erbB-2 immunohistochemical overexpression is less frequent than EGFR overexpression, supporting the minor role of C-erbB-2 in squamous cell carcinogenesis when compared with EGFR [17, 62]. In our study, only one case of severe dysplasia showed C-erbB-2-positive immunohistochemical expression and polysomy was observed in 70% of the cells. *C-erB-2* FISH-positive cases were due to high polysomy, showing that amplification is less frequent as a high gene copy number event. No statistical significant correlation was obtained between *C-erbB-2* gene and protein expression.

C-erB-2 does not seem to be involved in the first steps of lung carcinogenesis [16]. For Piyathilake et al., the expression of C-erbB-2 was significantly higher in squamous cell carcinoma and associated precancerous lesions than in normal epithelium and hyperplastic lesions, but no stepwise expression of C-erbB-2 was observed, suggesting its lack of importance in the squamous cell lung carcinogenesis [38]. *HER2/neu* increased copy number is observed in approximately 35% of adenocarcinomas and slightly less

frequently in squamous cell carcinomas [18], assuming more importance in the development of lung adenocarcinomas. The increased copy number of *HER2/neu* in NSCLC is most often attributable to chromosome duplication and polysomy [50], rather than amplification [18].

Although EGFR and Ki67 expression increases from normal epithelium to preinvasive lesions together, this was not seen for C-erbB-2. Therefore, any correlation between EGFR and Ki67 expressions and C-erbB-2 expression was established, like other studies also registered [21, 41, 63–66]. Interestingly, other studies revealed that EGFR/HER2/neu heterodimerization expression is capable to induce a stronger and more sustained proliferative signal than EGFR homodimers [60, 62].

The need to accurate detection of the *EGFR* and *HER2/neu* gene copy number and protein overexpression, reflecting their importance as biomarkers in squamous cell pathogenesis, has become even more important because despite recent advances in lung cancer treatments, improvement in survival has only been modest, showing that effective therapeutic and early detection approaches are still lacking [38]. Selection of patients with preneoplastic lesions expressing selected biomarkers is the way to provide new and more effective strategies for early diagnosis, a posterior closer follow-up and eventually chemoprevention and targeted treatment with better safety profiles. A variety of new approaches that target selected biomarkers in lung carcinogenesis are in clinical development or have already been approved for second and third line lung cancer treatment [5, 52–56, 67].

Our comparison between gene copy number and protein expression contributes to define which are the most reliable methods to be used in patient selection. Since our study includes a large number of preneoplastic lesions in a different population analysed by two different techniques (IHC and FISH), the results obtained reinforce other results published before, some of them centred only in one molecular marker and/or have used a single technique, which measures either the protein level or the gene expression. By identifying dysplasia as a group of preneoplastic lesions, chemoprevention targeted schemes can be delineated to select individual patients, considering protein expression, gene copy number and gene mutational status with repercussions in drug sensitivity.

Conclusions

We conclude that squamous cell carcinoma preneoplastic lesions studied have a basal cell origin, as demonstrated by the unbalance between LP34 and CK7. The differentiation markers, LP34, CK7 and chromogranin A, successfully discriminate preneoplastic lesions origin.

Dysplasias showed a higher expression of EGFR, Ki67 and p53 with a stepwise expression with the gravity of the preneoplastic lesions, reflecting their importance as potential biomarkers of preinvasive lesions of the bronchial epithelium and may be used to identify patients with a higher risk of developing squamous cell lung carcinoma. C-erbB-2 immunohistochemical overexpression is a rare event in bronchial preneoplastic lesions, emphasizing the minor role of C-erbB-2 as a biomarker in squamous cell carcinogenesis.

EGFR and *HER2/neu* high gene copy number was present in preneoplastic lesions, with a higher EGFR gene copy number in dysplasias. *EGFR* and *HER2/neu* high gene copy number was due to polysomy more often than to amplification, reinforcing that amplification is not the main mechanism for protein overexpression. Although *HER2/neu* does not seem to be involved in the early steps of squamous cell lung carcinogenesis, *HER2/neu* gene copy number is important because this gene is a HER family member and an important heterodimerizarion partner for EGFR.

Biological/genetic markers associated with several molecular and genetic abnormalities, such as Ki67, p53, EGFR and HER2/neu, need to be validated as potential and useful preneoplastic bronchial lesion biomarkers. Risk assessment should be based not only on smoking consumption and on histopathology of bronchial lesions, but also on the basis of in situ molecular biomarkers [58]. Therefore, in the future, the recognition of those biomarkers will allow a targeted screening and a posterior closer follow-up to select patients for chemoprevention schemes and will have a positive impact on survival.

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Conflicts of interest We declare that we have no conflict of interest.

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CHAPTER 4 – MANUSCRIPT II

Polysomy and amplification of chromosome 7 defined for EGFR gene in squamous cell carcinoma of the lung together with exons 19 and 21 wild type.

Artigo Original
Original Article

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Carcinoma epidermóide do pulmão: Polissomia e amplificação do cromossoma 7 e do gene EGRF com forma *wild type* nos exões 19 e 21

Polysomy and amplification of chromosome 7 defined for EGFR gene in squamous cell carcinoma of the lung together with exons 19 and 21 wild type

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Resumo

Objectivo: O receptor do factor de crescimento epidérmico (EGFR) está sobreexpresso na maioria dos carcinomas do pulmão de não pequenas células (CPNPC) e é um dos principais alvos específicos dos inibidores da tirosina cinase (TKI) utilizados para o tratamento do CPNPC avançado. Apesar disto, há um considerável número de factores biológicos que também estão associados à resposta dos EGFR-TKIs. Este estudo teve como principal objectivo a pesquisa de mutações somáticas e amplificação do *EGFR* em casos de carcinoma epidermóide do pulmão. **Material e métodos:** Secções

Abstract

Purpose: The epidermal growth factor receptor (EGFR) is overexpressed in the majority of non-small-cell lung cancers (NSCLC) and is a major target specific EGFR tyrosine kinase inhibitors (TKIs) developed and used for the treatment of advanced NSCLC. A number of biological factors are also associated with EGFR-TKIs responsiveness. This study was focused on EGFR somatic mutations and amplifications in squamous cell lung cancer. **Material and methods:** Representative sections of squamous cell carcinoma were selected from 54 surgical specimens

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CARCINOMA EPIDERMÓIDE DO PULMÃO: POLISSOMIA E AMPLIFICAÇÃO DO CROMOSSOMA 7 E DO GENE EGRF COM FORMA *WILD TYPE* NOS EXÕES 19 E 21

Patrícia Couceiro, Vítor Sousa, Ana Alarcão, Maria Silva, Lina Carvalho

representativas de carcinoma epidermóide foram seleccionadas de 54 casos em que o tecido estava fixado em formal e incluído em parafina, sendo depois submetidos à construção de TMA. A determinação da expressão proteica do EGFR foi feita por imunoistoquímica (IHQ) (Zymed, laboratórios). A hibridização *in situ* de fluorescência (FISH) foi realizada com a sonda EGFR LSI / CEP 7 (Vysis; Abbott Molecular, EUA). O ADN genómico foi extraído de 48 casos, amplificado por reacção em cadeia da polimerase (PCR) para pesquisa de mutações nos exões 19 (deleções) e 21 (mutações pontuais). Todos os casos expressaram positividade para a citoqueratina de alto peso molecular e foi observada negatividade para CK7, CD56 e cromogranina. **Resultados:** A sobreexpressão proteica do EGFR foi identificada em 49 casos, pela aplicação do *score* de Hirsh/ Cappuzzo (2005). A pesquisa de alterações génicas no cromossoma 7 e do gene *EGFR* foram analisadas por FISH e de acordo com o método de Cappuzzo (2005), foi identificada alta polissomia em 31 casos e amplificação em 7 casos. Por electroforese capilar, foram detectadas no exão 19 do *EGFR*: deleções em heterozigotia em 3 dos 48 casos estudados e o exão 21 apresentou-se sempre na sua forma *wild-type*, quando estudado por enzimas de restrição. **Conclusões:** A detecção de deleções e mutações pontuais no *EGFR* mostrou ser um evento raro no carcinoma epidermóide do pulmão. Apesar de a presença de mutações no *EGFR* ser um indicador molecular e de sensibilidade eficaz em doentes com CPNPC avançado, submetidos ao tratamento com EGFR-TKIs, é a determinação de amplificações e de polissomias no gene *EGFR* que melhor traduz a eficácia do tratamento nos doentes com carcinoma epidermóide, quando isolado do grupo de CPNPC.

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Palavras-chave: Carcinoma do pulmão, carcinoma epidermóide, tecido incluído em parafina, EGFR, amplificação, polissomia.

from formalin-fixed paraffin-embedded tissues and submitted to TMA construction. Determination of EGFR protein expression was done by immunohistochemistry(IHC) (Zymed, Laboratories). Fluorescence *in situ* hybridization (FISH) was performed with LSI EGFR/CEP 7 (*Vysis*; Abbott Molecular, USA). Genomic DNA was extracted from 48 cases and exon 19 was amplified by polymerase chain reaction (PCR) for search deletions and point mutations for exon 21. All cases expressed high weigh cytokeratin and were observed negativity for CK7, CD56 and chromogranin. **Results:** EGFR protein overexpression was identified in 49 cases, by the application of Hirsh's scoring system. The chromosome 7 and EGFR gene were analyzed by FISH and scored according to Cappuzzo's method that showed high polysomy in 31 cases and amplification in 7 cases. Deletion in exon 19 of EGFR was detected in 3 cases of 48 samples; the exon 21 of EGFR was expressed in its wild type by RFLP in all cases. **Conclusions:** Detection of common EGFR deletion and mutation showed to be a rare event in Squamous cell carcinoma of the lung. While EGFR mutation is the most effective molecular predictor or sensitivity in patients with advanced NSCLC submitted to EGFR-TKIs treatment, amplification and polysomy is the most effective molecular predictor for EGFR-TKIs responsiveness in squamous cell carcinoma, when validated isolated from the group of NSCLC.

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Key-words: Lung cancer, epidermoid carcinoma, paraffin-embedded tissue, EGFR, gene amplification, gene polysomy.

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Introduction

Lung cancer is one of the most common human cancers and the leading cause of cancer death world-wide[1,2].

Non-small cell lung cancer (NSCLC) comprises approximately 85% of all cases divided into squamous-cell carcinoma (SCC), adenocarcinoma (AC), large cells carcinomas and others[3-5].

The treatment of lung cancer is based on the stage of the cancer, and on patients performance status: for patients with early stage disease (stage I or II) surgical resection is considered the primary therapeutic choice. However, that majority of NSCLC cases have reached locally advanced (stage III) or metastatic stages (stage IV) at the time of diagnosis and chemotherapy is usually recommended as first line therapy.

Surgical resection is considered to be a curative treatment during earlier stage disease, but 5-year survival after surgical resection remains less than optimal, ranging from 67% for pT1N0 patients to 23% for patients with ipsilateral mediastinal lymph node involvement[3-6].

Receptor and non-receptor tyrosine and serine/threonine kinases have emerged as promising targets for specific drug development because overexpression or aberrant activation of these kinases often play an important role in the molecular pathogenesis of solid tumours. Further, the structural heterogeneity of these kinases has permitted development of small compounds which is a specific way inhibit their activity.

Epidermal growth factor receptor (EGFR), a 170 kDa tyrosine kinase (TK), is a member of the human epidermal receptor (HER) family that consists of four transmembrane tyrosine kinase receptors, EGFR (HER1, erbB-1), HER2 (erbB-2, HER/neu), HER3 and HER4. Upon ligands binding, the receptors homo or hetero-dimerize in between or either other growth factors. Subsequently, activation of receptor intrinsic tyrosine kinase activity occurs together either downstream signalling cascades, mainly including Ras-Raf-MAP-kinase pathway, PI3K-Akt pathway, and STAT pathway. All these have strong stimulatory effect on cell proliferation, differentiation, survival, angiogenesis and migration[7,8].

EGFR has emerged as a critical tumorigenic factor in the development and progression of NSCLC. EGFR and erbB-2 are expressed in many solid tumours and their overexpression is associated with poor prognosis.

Activating mutations of EGFR are related to increased response rate and survival in patients treated with EGFR tyrosine kinase inhibitors (TKIs)[9]. Specific EGFR tyrosine kinase inhibitors (TKIs) have been developed and used clinically in the treatment of advanced NSCLC. They disrupt EGFR signalling by competing with adenosine triphosphate (ATP) for binding sites at tyrosine kinase domain, and thus inhibiting the phosphorylation and activation of EGFRs and the downstream signalling network[10].

Tyrosine kinase inhibitors after first line chemotherapy are used to complement conventional chemotherapy and several studies are required to define molecular characteristics, different in adenocarcinoma and squamous cell carcinoma, to predict the response to those drugs[11].

Somatic mutation is the mutation that occurs only in somatic cells, which are in contrast to germ cells. A large number of somatic mutations have been identified in the EGFR gene in NSCLC. In general, these

mutations can be included into three major types: in-frame deletion, insertion, and missense mutation. Most of these mutations are located in the tyrosine kinase coding domain (exons 18-21) of the EGFR gene. The amino acids 746-750 encoded by exon 19 and amino acid 858 encoded by exon 21 are two mutation hotspots, which accounts for over 80% of all the detected mutations[12,15].
A considerable number of retrospective studies have reported that two activating mutations, small in-frame deletion in exon 19 and substitution of leucine for arginine at amino acid 858 in exon 21 (L858R), have a striking correlation with EGFR-TKI sensivity.
Both activating mutations are able to enhance kinase activity of EGFR and the activation of its downstream signaling, and play a pivotal role in supporting NSCLC cell survival. When the specific EGFR-TKIs are applied, the excessive survival signals that cancer cells are "addicted to" are counteratcted and a clear apoptosis occurs[14].
The present study intended to clarify the status of EGFR gene by FISH, PCR and its immunohistochemical expression in squamous cell carcinoma of the lung.

Material and methods

Sample selection

A cohort of 54 formalin-fixed paraffin-embedded samples, adequate for analysis were included in this retrospective study, concerning sections of squamous cell carcinoma selected from surgical specimens. In Table I is resumed the age and gender of the patients.
Slides of tumor samples stained with hematoxylin-eosin were independently reviewed by two pathologists and representative areas were marked. Core tissue biopsy speciemens (3mm in diameter) in triplicate were obtained from individual paraffin-embedded samples (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a punch-extractor pen (Histopat, Histopathology, Ltd.). Each tissue array block contain 12 specimens, which allowed all 162 specimens (triplicate speciemens of 54 cases) to be contained in 13 array blocks. This TMA construction was used for FISH and immunohistochemical analysis.

Table I – Patients characteristics

	Female (7)	Male (47)
Age (years) median	65,8	66,9
Age (years) range	54-73	48-84

Immumohistochemistry

Three µm sections of TMA were placed on coated slides and were allowed to dry overnight. After deparaffinization and rehydration, antigen unmasking was performed using pronase E for 10 minutes. Endogenous peroxidase activity was quenched using 15 minutes incubation in 3% diluted hydrogen peroxide (H_2O_2). For blocking nonspecific binding of secondary antibody, Ultra V Block (Ultra Vision Kit; TP-015-HL; LabVision) was applied to the sections and then the sections were incubated at room temperature with primary anitbodies against EGFR (clone 31G7; Zymed Laboratories) at a dilution of 1:20 for 30 minutes. After washing with phosphate-buffered saline (PBS) the slides were incubated with biotin-labeled secondary antibody for 30 minutes. Primary antibody biding was located in tis-

sues using peroxidase-conjugated streptavidin (LabVision). 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen, according to manufacturer's instructions. The slides were counterstained with hematoxylin, dehydrated and mounted. In parallel we used known positive and negative controls.

IHC staining for EGFR antibody was scored according to Hirsch: intensity and percentage of cells. The staining was read as follow:

Grade +	Score	Intensity/positivity
0	<10 +	IHC negative
1 50% cells	10-100	1 – Weak → IHC low positive
2 75% cells	101-200	2 – Moderate → IHC moderate positive
3 >75% cells	201-300	3 – Strong → IHC intense positive

Fluorescence *in situ* hybridization

In situ hybridization is a sensitive method that is generally used to detect specific gene sequences in tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest.

Dual-color FISH analysis was performed on paraffin-embedded tissue blocks on the cases studied, using the Vysis LSI EGFR/CEP 7 probe assay (Vysis; Abbott Molecular, USA). In brief, sections of 4 µm thickness from the tissue microarray blocks were used, they were baked overnight at 56°C, deparaffinized in xylol, rehydrated in 100%, 70% ethanol and bidestilled water. Slides were then submitted to a pre-treatment in a pressure cooker with 10mM citric acid-trisodium salt buffer pH6 for 4 minutes. Washed in 2× SSC salts (sodium chloride and sodium citrate) pH 7 for 5 minutes at room temperature. Slides were immersed for 15 minutes in proteinase K solution at 37°C, they were then rinsed in 2x SSC pH7 for 5 minutes at room temperature. The slides were then dehydrated in 70%, 90% and 100% ethanol then air dried. Ten microliters of probe mixture were applied on the target areas and a 22mmx22mm glass coverslip was placed over the probe.

Coverslips were sealed with rubber cement and codenatured at 83°C for 5 minutes and incubated overnight at 37°C in a humidity chamber. Post-hybridization washes in buffer (50% formamide 2x SSC pH7) at 46°C and washed with 2x SSC pH7. Slides were air-dried in the dark and counterstained with DAPI.

Analysis was performed using Nikon Eclipse 80i and DXM 1200F Workstation equipped with Nikon filter set with single-band exitors for rhodamine, fluorescein isothiocyanate, DAPI.

Overlapping cells were excluded from analysis. Two signals were counted as adjacent or fused only if they were separated by less than one domain.

One hundred spindel cells interphase nuclei with strong and well delineated signals were examined by two different individuals. A split signal in 5% or more of spindle cells was required for a result to be classified as positive.

DNA preparation and molecular analysis of the EGFR gene

Polymerase Chain Reaction (PCR) – is a method that allows logarithmic amplification of short DNA sequences within a lon-

ger DNA molecule. Briefly, genomic DNA was extracted from serial 5-µm archival paraffin-embeded tissue, containing a representative portion of each tumor block, using the QIAmp DNA Mini Kit (Qiagen, IZASA, Portugal). One hundred nanograms (ng) of DNA were amplified in a 50 µl reaction solution containing 5 µl of 10x buffer (Roche, Portugal), 2,5 mM $MgCl_2$, 0,2 µM of each complementary primer, 200 µM deoxynucleoside triphosphate and 1 unit of DNA polymerase (Roche, Portugal). Amplifications were performed using a 5 minutes initial denaturation at 95ºC; follwed by 40 cycles 30 seconds at 95ºC, 1 minute at 60ºC 1 minute at 72ºC and a 10 minutes of final extension at 72ºC. EGFR gene (exons 19 and 21) mutations were determinated using the intron-based primers according to a published method[6]. We performed mutational analysis of exon 19 deletion and L858R point mutation of the EGFR gene, as previously described[7]. Briefly, exon 19 deletion was determined by common fragment analysis using PCR with an FAM-labeled primer set, and the products were electrophoresed on ABI PRISM 3100 (Applied Biosystems) and all electropherograms were reanalysed by visual inspection to check for the mutations. The L858R mutation was performed using also MJ MiniOpticon (Bio-Rad), and its products were then detected by restriction fragment length polymorphism (RFLP) analysis. The restriction enzyme MscI was used to digest the TGGCCA sequence in the amplicon of the wild-type allele. In contrast, mutant type (L858R) was not digested because of the base substitution of T to G at the first base of TGGCCA. On the other hand, a new FauI restriction site, CCCGC, that can be used to distinguish L858R mutant allele from wild-type. The PCR products were digested with the restriction enzymes MscI and FauI and run on 3% agarose gel, and the existence of this mutation was accessed. The Fig. 4 presents the predicted gel-electrophoresis patterns for PCR-RFLP samples.

Statistical analysis

Statistical analyses of categorical variables were performed using bilateral Chi-squared tests or Fisher exact test as appropriate. To compare percentage of markers expression ANOVA test was applied.

Results

Patient characteristics

This study included 47 male and 7 female patients with a median age of 66 years. All of them had a history of smoking. Stages were diagnosed I (52.8%), II (31.6%) and III (15.6%) (Table I).

EGFR expression by immunohistochemistry

Immunohistochemical EGFR protein overexpression was identified in 49 cases, by the application of Hirsh's scoring system (Table II).

Table II – EGFR protein expression detected by IHC in squamous cell carcinoma of the lung

Results	Immunohistochemistry		
	Low	Mod	Int
IHC +	14	14	17
IHC -		9	

Table III – Comparision of detected EGFR copy number using FISH and IHC in Squamous cell carcinoma of the lung

TNM status	Patients		EGFR protein expression IMC		EGFR gene expression FISH Cappuzzo	
	N	%	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)
T1NOMx	14	26%	11 (79%)	3 (21%)	7 (50%)	7 (50%)
T1N1Mx	4	7%	4 (100%)	0	4 (100%)	0
T2N0Mx	19	35%	16 (84%)	3 (16%)	16 (84%)	3 (16%)
T2N1Mx	4	7%	4 (199%)	0	1 (25%)	3 (75%)
T2N2Mx	2	4%	1 (50%)	1 (50%)	1 (50%)	1 (50%)
T3N0Mx	8	15%	7 (100%)	1 (50%)	4 (50%)	4 (50%)
T3N1Mx	2	4%	1 (50%)	1 (50%)	2 (100%)	0
T4N0Mx	1	2%	1 (100%)	0	1 (100%)	0

Table IV – EGFR molecular alterations scored according Cappuzzo *et al*, 2005 criteria

Results	FISH					
	Disomy	Low trisomy	High trisomy	Low polysomy	High Polysomy	Ampli cation EGFR / CEP 7
IHC +	5	1	1	6	25	7
IHC -	1	1	1	–	6	–
TOTAL	6	2	2	6	31	7

EGFR mutations determinated by FISH and PCR

The chromosome 7 and EGFR gene were analyzed by FISH and scored according to Cappuzzo's (2005) method that showed in 31 cases high polysomy and in 7 cases amplification (Table III and Fig. 5).

Through capillary electrophoresis, deletion in exon 19 of EGFR was detected in 3 cases of 48 samples; the exon 21 of *EGFR* was expressed in its wild type by RFLP in all cases (Figs. 1-4).

Correlation between EGFR expression and EGFR mutations

(Table V).

Table V – Relationship between the results obtained in IHC and FISH of 54 screened patients

Results	FISH NEG	FISH POS	TOTAL
IHC NEG	3	6	9
IHC LOW POS	8	7	15
IHC MOD POS	4	10	14
IHC INT POS	3	13	16
TOTAL	18: 3 / 15	36: 6 / 30	54

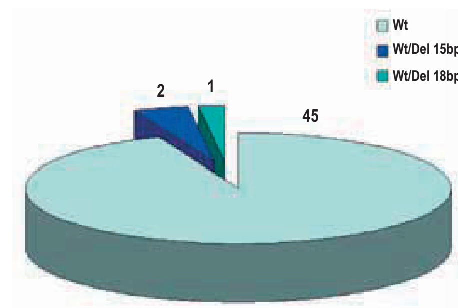

Fig. 1 – Capillary electrophoresis – Exon 19 of EGFR

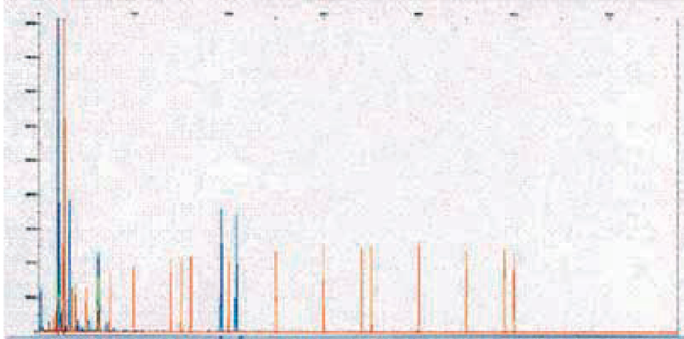

Fig. 2 – In–frame deletion of exon 19 (del E746-A750): Representative nucleotide sequence of the EGFR gene in tumor speciemens, the nucleotide sequence of heterozygous in–frame deletions in exon 19 by direct sequencing (double peaks)

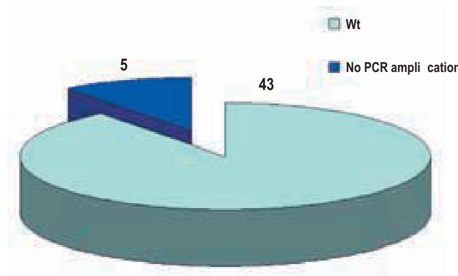

Fig. 3 – RFLP – Exon 21 of EGFR

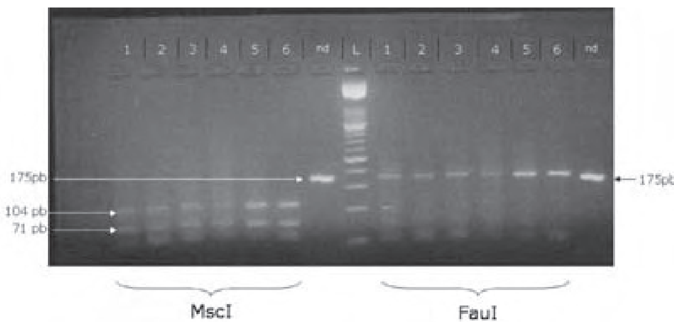

Fig. 4 – Agarose gel – Exon 21 of EGFR

Discussion/Conclusions

EGFR mutation screening could be number one test to provide the most direct and valuable information to help clinicians to make treatment decision when dealing with NSCLC in general[1].

Meanwhile somatic mutations of EGFR gene are found in about 2-8% of USA non-small-cell lung cancer patients because of considering together all histological types of NSCLC. In the current study – all smokers: exon 19 deletion was found in 3/48 (6.25%) of Portuguese derived lung epidermoid carcinomas and in exon 21 – any L858R mutation in the 48 cases studied was identified. In-frame deletions in exon 19 and in exon 21 mutations L858R are the EGFR mutations most commonly identified and most clearly associated with TKI responsiveness.

Low number of EGFR gene copies per cell, including disomic and trisomic patterns, did not influence the level of protein expression, whereas a high gene copy number, clustered amplification or high polysomy, correlated with protein level.

EGFR protein overexpression in all tumours with gene amplification is the mechanism for overexpression. Balanced disomy for chromosome 7 and EGFR gene while expressing high protein expression might be controlled by other mechanisms, the complex interaction between gene and protein levels.

Identifying a panel of predictive markers is very important for selection of advanced NSCLC patients for EGFR-TKI therapy. Although several important demographic and clinical factors are associated with treatment response, EGFR somatic mutations are still the most effective predictor for EGFR-TKI sensitivity in lung cancer histological types.

Our study is the first that concerning only cases of squamous cell carcinoma of the lung in surgical stages, after reviewing the published medical literature which only includes cases of non-small-cell lung Cancer in advanced stages with higher expressions of the parameters here included.
However, the results obtained showed that squamous cell lung carcinoma in surgical stages expressed EGFR protein and high polysomy of chromosome 7 in 46.3% and amplification in 12.9% of the cases. Only in 3 cases we have detected deletions in exon 19.
The presence of high polysomy, amplification and deletions in exon 19 of EGFR gene have implications on tumorigenesis process due to his increased enzymatic activity become a TKIs therapeutic target.
These conclusions have to be correlated with follow-up of these patients to define potential recurrence related with the results obtained.

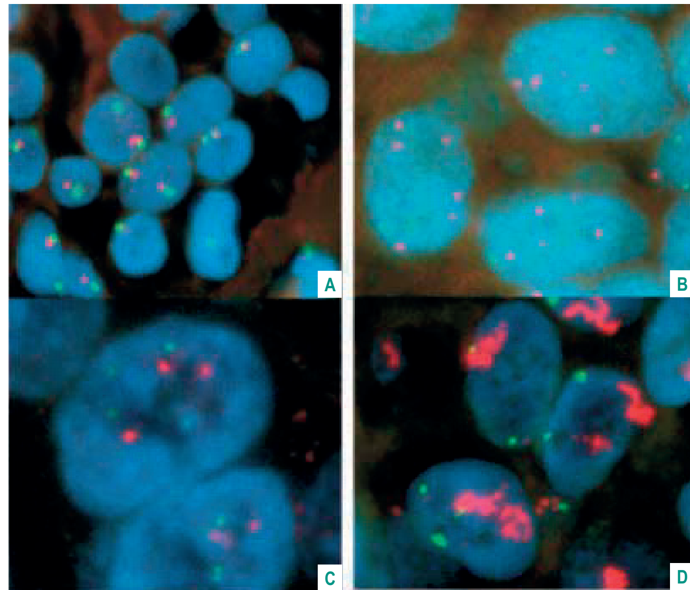

Fig. 5 – FISH analysis for EGFR (7p12/CEP7) for squamous cell carcinoma of the lung (A) – Disomy, (B) – Polysomy, (C) – Trisomy, and (D) - Ampli cation

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CHAPTER 5 – MANUSCRIPT III

Bronchial-Pulmonary Adenocarcinoma Subtyping Relates With Different Molecular Pathways.

CHAPTER 5 – Bronchial-Pulmonary Adenocarcinoma Subtyping Relates With Different Molecular Pathways.

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ORIGINAL ARTICLE

Bronchial-pulmonary adenocarcinoma subtyping relates with different molecular pathways

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LRP;
MRP

Abstract Lung cancer is one of the most common cancers in the world with a high mortality rate. We analyzed 45 surgical samples of the adenocarcinoma, 13 with lymph node metastasis. APC, BCL2, chromogranin A, CK 5/6/18 (LP34), CK20, CK7, cyclin D1, EGFR, ERCC1, HER2, Ki67, LRP, MRP, P53, RB and TTF1 expressions were evaluated by immunohistochemistry (IHC).

Higher Ki67, APC, ERCC1 expressions and lower TTF1 expression were identified in advanced stages (IIA and IIIA) of adenocarcinomas, which reflect a more aggressive, less differentiated, possibly a non-TRU adenocarcinoma.

Acinar, micropapillary and BA/lepidic adenocarcinoma patterns were the most similar patterns and papillary was the most different pattern followed by solid pattern, according to expression of these markers. Different adenocarcinoma patterns are engaged with different molecular pathways for carcinogenesis, based on the differences of expression. Acinar, BA/lepidic and micropapillary showed higher TTF1 expression (type TRU), and papillary and solid patterns revealed less TTF1 expression, exhibiting a non-TRU/bronchial phenotype. Solid pattern revealed lower HER2 and higher EGFR and ERCC1 (this compared to papillary) expression; papillary higher HER2 and lower ERCC1 expressions; micropapillary higher RB expression; and acinar lower ERCC1 and higher EGFR expressions. Ciclin D1 seems to have more importance in acinar and BA/lepidic patterns than in micropapillary. ERCC1 protein expression in micropapillary, solid and BA/lepidic patterns may indicate DNA repair activation. Inhibition of apoptosis could be explained by BCL2 overexpression, present in all adenocarcinoma patterns. MRP-1 and LRP were overexpressed in all patterns, which may have implications for drug resistance.

Further studies are needed to interpret these data regarding to therapy response in advanced staged bronchial-pulmonary carcinomas.
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Introduction

Tobacco, environmental and genetic factors and several lung diseases contribute to lung cancer carcinogenesis. Lung cancer is one of the most common cancer diagnosed, and has also the highest mortality rate due to the advanced stages at time of diagnosis, and when the options for treatment have to be understood as personalized therapy.[1-8]

Bronchial-pulmonary carcinomas consist of small cell lung cancer (SCLC), squamous cell carcinoma (SCC), adenocarcinoma, large cell carcinoma and sarcomatoid/pleomorphic carcinomas.[8] Adenocarcinoma is the most common type in non-smokers and its incidence has increased in recent years, developing after terminal respiratory unit (TRU) but also recognized in respiratory epithelium.[8-10] Adenocarcinoma pure patterns are recognized [acinar, papillary, micropapillary, BA (bronchioloalveolar/lepidic/AIS-adenocarcinoma in situ), solid with mucin production] but mixed type predominates. Differences between the subtype/patterns of adenocarcinoma should be taken into account to improve diagnosis, prognosis and as an enhancement in therapy selection.[8,10]

Cytokeratin 7 (CK7), high weight molecular cytokeratins (HWMC) like CK5/6, CK20, chromogranin A and TTF1 are currently used in lung cancer diagnosis.[11,12] Ki67 is a cell proliferation marker with prognostic value.[13-15] In the apoptosis pathway, p53 induces Bax transcription and reduces bcl2. Bcl2/Bax ratio is negative in lung carcinomas.[16] Inactivation of RB pathway is considered a requirement for lung carcinogenesis.[16] Adenomatous polyposis coli (APC) gene is involved in the canonical Wnt/β-catenin signal transduction. *APC* is mutated in liver, colorectal adenomas and lung tumors.[17] Cyclins D (D1, D2, and D3) are activators of cyclin-dependent kinases cdk4 and cdk6, to translate growth signals into cell cycle progression, leading to proliferation.[18,19] Cyclin D1 overexpression and pRB inactivation indicates a poor prognosis.[20]

Bcl2 as an anti-apoptotic protein has a more important role in neuroendocrine (NE) lung cancer than in other bronchial-pulmonary carcinomas.[21] Excision repair cross-complementing 1 (ERCC1) genetic polymorphisms may affect patient's response to platinum-based chemotherapy.[22,23] However, another study concluded that patients with a bronchial-pulmonary carcinoma with high ERCC1 expression have a better prognosis than those with low expression.[24] Multidrug Resistance Protein 1 (MRP-1) and lung resistance-related protein (LRP) may confer resistance to cytotoxic and antiviral drugs.[25-31]

EGFR and HER2 play a central role in tumorigenesis.[32,33] Targeting those receptors provides a unique approach for treating EGFR/HER2 expressing cancers.[34,35] The most common alteration of HER2 is overexpression/amplification; mutations are less frequent.[33,35,36] The activated receptors trigger Ras-Raf-MEK (mitogen-activated and extracellular signal regulated kinase), ERK1 and ERK2 (extracellular-signal regulated kinase 1 and 2) pathways leading to cell growth, mTOR (mammalian target of rapamycin) pathway leading to protein synthesis, and PI3K-AKT (phosphatidylnositol-2 kinase Akt) pathway sustaining cell survival.[36]

The objectives of this work were to validate a small differentiation IHC panel, evaluate protein expression of different genes according to different adenocarcinoma patterns, and correlate this expression with cell pathways of progression and drug resistance, in order to understand bronchial-pulmonary adenocarcinoma carcinogenesis. Another objective was to verify possible differences of gene expression in adenocarcinoma taking into account variables such as gender, age, smoking and different tumor stages. The global intention of this project is to acquire knowledge which can be applied in the diagnosis and prognosis evaluation of bronchial-pulmonary adenocarcinoma and its subtypes in order to define individual treatment.

Materials and methods

Materials

Surgical samples of bronchial-pulmonary adenocarcinomas ($n = 45$) covering all subtypes recognized by WHO/2004 were randomly selected and staged by the 2010 TNM system; the ERS/ATS proposal was also applied. At least two sections of each tumor and sections of lymph node metastasis were selected (Table 1).[12,37]

Methods

CK7, TTF1, CK20, CK 5/6/18 (LP34), chromogranin A, Ki67, P53, RB, BCL2, cyclin D1, APC, ERCC1, LRP, MRP-1, EGFR, and HER2 expressions were evaluated by immunohistochemistry (IHC). IHC was performed on formalin-fixed, paraffin-embedded tissue samples in automatic immunostainer (Autostainer 360 – LabVision®). Streptavidin biotin protocol was applied according to the manufactures indications for each antibody. Three-micrometer tissue sections were placed on coated slides and allowed to dry overnight. After deparaffinization and rehydration, antigen retrieval was performed according to Table 2. Endogenous peroxidase activity was quenched using 15 min incubation in 3% diluted hydrogen peroxide (H_2O_2). For blocking non-specific binding, Ultra V Block (Ultra Vision Kit®; TP-015-HL) was applied to the sections and then they were incubated at room temperature, with primary antibodies according to Table 2. After washing with phosphate-buffered saline (PBS), slides were incubated with biotin-labeled secondary antibody (LabVision®) for 15 min. Primary antibody binding was localized in tissues using peroxidase-conjugated streptavidin (LabVision®) and 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen, according to manufacturer's instructions. The slides were counterstained with hematoxylin, dehydrated and mounted. In parallel, known positive (Table 2) and negative controls were used.

Three-micrometer tissue sections were placed on coated slides and allowed to dry overnight. IHC scoring was applied independently by two pathologists who registered the intensity of expression (1 – low expression, 2 – intermediate expression and 3 – high expression) and percentage of stained cells. For statistical analyses we grouped the immunohistochemistry results in 4 categories. For each case we multiplied the intensity of expression by the percentage of staining cells and we used this score for the formation of

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Table 1 Gender (M – male; F – female), age, smoking status, TNM classification, stage and patterns present (A – acinar; B – BA/lepidic; C – papillary; D – micropapillary and E – solid).

	Gender	Age	Tobacco	TNM	Stage	Patterns
1	M	73	Non-smoker	pT1aN0Mx	IA	A; B
2	F	71	Non-smoker	pT1aN0Mx	IA	A; B
3	M	69	Smoker	pT4N0Mx	IIIA	A; B; C
4	M	75	Non-smoker	pT1aN2Mx	IIIA	A; E
5	F	53	Non-smoker	pT1aN0Mx	IA	A; B
6	M	76	Ex-smoker	pT2bN0Mx	IIA	A; B; E
7	F	62	Ex-smoker	pT1aN2Mx	IIIA	A; B; E
8	F	74	Non-smoker	pT1aN0Mx	IA	A; B; E
9	F	68	Non-smoker	pT2aN0Mx	IB	A; D; E
10	F	50	Non-smoker	pT1bN2Mx	IIIA	A; E
11	F	57	Smoker	pT2aN0Mx	IB	A; E
12	M	51	Non-smoker	pT1aN1Mx	IIA	A; B
13	F	75	Non-smoker	pT2aN0Mx	IB	A; D; E
14	M	85	Ex-smoker	pT1aN0Mx	IA	A; B
15	F	63	Smoker	pT2aN0Mx	IB	A; B; D
16	F	55	Smoker	pT1aN0Mx	IA	A; E
17	F	67	Smoker	pT1aN0Mx	IA	A; B; D
18	M	48	Smoker	pT1aN0Mx	IA	A; B; E
19	M	71	Non-smoker	pT2aN1Mx	IIA	A; D
20	M	49	Smoker	pT2aN0Mx	IB	A
21	F	80	Non-smoker	pT1bN0Mx	IA	A; D
22	F	68	Non-smoker	pT2aN1Mx	IIA	A; D
23	M	48	Non-smoker	pT2bN0Mx	IIA	A; B
24	F	67	Non-smoker	pT2aN0Mx	IB	A
25	M	76	Ex-smoker	pT1bN0Mx	IA	A; B
26	F	56	Non-smoker	pT1aN0Mx	IA	A; B; C; D
27	M	64	Smoker	pT2bN0Mx	IIA	A; C
28	F	71	Non-smoker	pT2aN0Mx	IB	A; B; E
29	F	50	Smoker	pT2aN0Mx	IB	B; E
30	F	80	Non-smoker	pT1aNxMx	IA	E
31	M	56	Smoker	pT1aN0Mx	IA	A; B
32	F	56	Non-smoker	pT1aN0Mx	IA	A; B
33	F	54	Non-smoker	pT1aN0Mx	IA	B
34	M	67	Smoker	pT2aN1Mx	IIA	A; E
35	F	62	Non-smoker	pT1bN1Mx	IIA	A; C; D
36	F	68	Non-smoker	pT1bN0Mx	IA	A; B
37	F	44	Non-smoker	pT2bN2Mx	IIIA	A; E
38	F	52	Smoker	pT3N1Mx	IIIA	A; B; D
39	M	45	Smoker	pT2aN1Mx	IIA	A; C
40	M	61	Smoker	pT2aN0Mx	IB	A
41	F	74	Non-smoker	pT2aN0Mx	IB	A; D
42	F	74	Non-smoker	pT2aN0Mx	IB	A; B
43	M	73	Non-smoker	pT2aN1Mx	IIA	A; B
44	F	60	Non-smoker	pT1bN0Mx	IA	A; B; D
45	F	79	Non-smoker	pT2aN0Mx	IB	A; B; D

each category (category (−) 0–10; (+) 11–100; (++) 101–200; (+++) 201–300) (Table 3)

The non-parametric Wilcoxon-Mann–Whitney test was used to analyze the differences between categories. Hierarchical clustering and Principal Component Analysis (PCA) was made to validate relationships between patterns. We used the software STASTISTICA 7. *P* values less than 0.05 were considered significant.

Results

Predominantly mixed-type adenocarcinomas,[40] three acinar adenocarcinomas, one solid mucin producing adenocarcinoma and one bronchioloalveolar (BA)/lepidic mucinous adenocarcinoma/mucinous adenocarcinoma in situ were studied to validate the different patterns. Forty-two cases (93.3%) with acinar pattern (being the most prevalent),

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Table 2 Antibodies applied antigen retrieval, dilution and incubation time and staining patterns. RT – room temperature.

Primary antibody	Manufacture	Clone	Positive control	Antigen retrieval	Dilution and incubation time	Staining pattern
APC	Novocastra	EMM43	Colon	PT module, EDTA	1:40, 60′	Membrane and cytoplasmic
Bcl2	Novocastra	3.1	Tonsil	PT module, EDTA	1:150, 60′	Membrane and cytoplasmic
Chromogranin A	DAKO	DAK-A3	Carcinoid tumor	PT module, citrate	1:200, 30′	Cytoplasmic
CK (5/6/18)	Novocastra	LP34	Lung	Pronase E (10′, RT)	1:100, 60′	Cytoplasmic
CK20	DAKO	KS20.8	Colon	Pronase E (10′, RT)	1:50, 30′	Cytoplasmic
CK7	DAKO	KS20.8	Lung	Pronase E (10′, RT)	1:50, 30′	Cytoplasmic
Cyclin D1	Novocastra	P2D11F11	Breast carcinoma	PT module, EDTA	1:30, 60′	Nuclear
EGFR	Zymed	31G7	Respiratory epithelia	Pronase E (10′, RT)	1:20, 30′	Membrane and cytoplasmic
ERCC1	Thermo	8F1	Tonsil	PT module, EDTA	1:100, 60′	Nuclear
HER2	DAKO	Polyclonal	Breast carcinoma	PT module, citrate	1:200, 30′	Membrane
Ki67	DAKO	MIB-1	Small cell carcinoma	PT module, citrate	1:50, 30′	Nuclear except mitotic cells
LRP	Novocastra	9D6	Ileon	PT module, EDTA	1:300, 30′	Cytoplasmic
MRP-1	Novocastra	33A6	Colon	PT module, citrate	1:50, 60′	Cytoplasmic
P53	DAKO	DO-7	Skin	PT module, citrate	1:50, 30′	Nuclear and cytoplasmic
RB	Novocastra	13A10	Tonsil	PT module, EDTA	1:50, 60′	Nuclear
TTF1	DAKO	8G7G3/1	Small cell carcinoma	PT module, EDTA	1:100, 60′	Nuclear

26 (57.8%) with BA/lepidic pattern, 15 (33.3%) with solid pattern, 5 (11.1%) with papillary and 15 (33.3%) with micropapillary pattern were registered.

Thirteen cases (28.9%) showed lymph node metastasis as follows: acinar pattern was present in 13 cases (100%); solid and micropapillary patterns were present in 3 cases each (23%); and papillary pattern in 2 cases (15.4%). Two metastasis with solid pattern belonged to primary adenocarcinomas which harbored, respectively, acinar and BA/lepidic and acinar, BA/lepidic and micropapillary patterns.

Seventeen cases (37.8%) were diagnosed in men and twenty-eight cases (62.2%) in women. Age at diagnosis ranged from 44 to 85 years with a median of 67 years. Fourteen cases (31.1%) were diagnosed in smokers (8 males and 6 females), four (8.9%) in ex-smokers (3 males and 1 female) and twenty-seven (60%) in never smokers (6 males and 21 females). Female patients were more frequently nonsmokers than men ($p = 0.0251$).

Seventeen cases (37.8%) were diagnosed as stage IA (5 males/12 females), twelve (26.7%) as IB (2 males/10 females), ten (22.2%) as IIA (8 males/2 females) and six (13.3%) as stage IIIA (2 males/4 females). Females were diagnosed more frequently in lower stages when compared to men ($p = 0.029$). There were no differences between stages according to smoking habits.

All adenocarcinomas ($n = 45$) expressed CK7. No expression was observed for CK20, HWMC or chromogranin A. TTF1 was expressed in the great majority of lung adenocarcinomas, with statistical significant higher expression in acinar pattern ($p = 0.0094$), micropapillary pattern ($p = 0.012$) and BA/lepidic patterns ($p = 0.00046$). The expression was significantly higher in BA/lepidic pattern when compared to solid pattern ($p = 0.021$).

Nuclear expression of P53 was statistically higher in all patterns when compared to normal tissue ($p < 0.05$), without differences between patterns.

RB expression was higher in micropapillary pattern when compared to normal tissue ($p = 0.011$). The other patterns showed no differences from basal expression in normal tissue.

There were no significant differences between primary adenocarcinomas and their metastasis, except for cyclin D1 whose expression was higher in the metastasis when compared to the primary neoplasia ($p = 0.0409$).

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Table 3 TTF1, KI67, P53, RB, APC, BCL2, Ciclin D1, ERCC1, MRP1, LRP, HER2 and EGFR expression according to adenocarcinoma pattern. A – acinar; B – BA/lepidic; C – papillary; D – micropapillary and E – solid patterns. Expression categories: 0 = (–) = 0–10%; 1 = (+) = 11–100%; 2 = (++) = 101–200%; 3 = (+++) = 201–300%.

—	TTF1					Ki67					P53					RB					APC					BCL2				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
1	3	3				1	1				3	3				0	0				0	0				2	2			
2	0	1				2	2				0	0				3	3				1	2				2	2			
3	0	0	0			1	2	2			0	3	0			1	2	2			1	2	2			2	2	2		
4	0				0	3				3	3				3	0				0	2				2	1				1
5	3	3				1	1				1	1				1	1				1	1				1	1			
6	3	3			1	1	1			1	1	1			1	1	1			1	1	1			1	1	1			1
7	3	3			2	2	2			2	3	3			3	2	2			2	2	2			2	2	1			2
8	3	3			3	2	2			2	1	3			1	0	0			0	1	2			2	2	2			2
9	3			2	1	2			1	2	3			3	3	2			0	0	2			2	2	1			1	1
0	2				2	1				1	1				1	2				2	1				1	2				2
11	0				0	3				2	0				0	3				2	2				2	0				0
12	0	0				2	2				2	3				1	2				0	0				1	1			
13	3			3	3	2			1	1	1			1	1	1			0	0	2			2	1	2			2	2
14	2	2				2	3				3	3				1	1				1	2				1	1			
15	3	3		3		2	3		3		3	3		3		1	1		1		1	1		1		2	2		2	
16	2				2	1				1	1				1	1				1	1				1	2				2
17	3	3		3		1	1		1		0	0		0		0	0		0		2	2		2		2	2		2	
18	3	3			3	1	1			1	3	3			3	1	1			1	1	1			1	2	2			2
19	3			3		0			0		1			1		0			0		2			2		3			2	
20	3					0					1					0					2					3				
21	3			3		1			1		1			1		0			0		1			1		2			2	
22	3			3		0			0		1			1		1			1		2			2		3			3	
23	0	1				0	0				0	0				3	1				2	2				2	2			
24	2					1					0					1					1					2				
25	3	3				1	1				0	0				1	1				1	1				3	3			
26	3	3	3	3		0	0	0	0		0	0	0	0		0	0	0	0		2	2	2	2		2	3	3	3	
27	1		1			2		1			2		3			2		2			2		2			2		2		
28	2	3			2	0	0			0	0	0			0	1	1			1	2	1			2	2	2			1
29		3			2		3			3		3			3		3			2		1			1		1			0
30					2					0					1					2					0					2
31	3	3				0	1				0	0				2	3				1	2				2	3			
32	3	3				1	1				0	0				1	3				2	2				2	2			
33		3					1					1					1					1					1			
34	3				3	0				1	0				0	0				0	2				2	3				2
35	3		3	3		1		1	1		3		3	3		1		1	1		2		2	2		2		2	2	
36	3	3				0	0				0	0				2	2				1	1				2	2			
37	3				2	0				1	3				3	2				2	2				2	2				2
38	3	3		3		1	1		1		1	1		1		0	0		0		2	2		2		1	2		2	
39	3		3			2		2			3		3			1		1			2		2			2		2		
40	2					2					2					1					1					0				
41	3			1		1			1		1			1		3			3		1		1	1		2			2	
42	3	3				0	0				0	1				1	1				1	2				2	2			
43	2	3				0	0				1	2				0	0				2	2				1	2			
44	1	3		1		0	0		0		0	0		0		0	0		0		2	2	2	2		1	1		1	
45	3	3		3		1	1		1		3	3		3		3	3		3		1	1		2		1	2		2	

—	Ciclin D1					ERCC1					MRP-1					LRP					HER2					EGFR				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
1	0	0				0	1				2	2				3	3				3	3				1	1			
2	0	1				2	2				2	2				2	3				1	1				0	0			
3	0	1	1			0	0	0			2	2	2			2	3	3			2	2	2			0	0	0		
4	0				1	2				2	2				2	3				3	1				1	0				0
5	0	1				1	1				2	2				2	2				2	2				0	0			
6	1	1			1	1	1			1	1	1			1	1	1			1	1	1			1	1	1			1
7	3	3			1	2	2			1	2	2			2	3	3			3	1	1			1	0	0			0
8	0	0			0	1	1			1	2	2			2	3	3				2	3			1	0	0			0
9	0			0	0	1			1	1	3			1	3	3			3	3	3			3	2	2			2	3
0	1				1	1				1	2				2	3				3	1				1	1				1
11	0				2	1				1	1				2	3				3	2				2	0				0
12	0	0				0	1				1	1				3	3				1	2				1	1			
13	2			2	2	1				1	1			1	1	3			3	3	2			2	1	3			3	3
14	1	1				0	1				1	1				3	3				1	2				0	0			
15	0	0		0		0	1		1		2	2		2		3	3		3		2	2		2		2	2		1	
16	1				1	0				2	0				0	3				3	1				1	1				0
17	1	2		2		1	1		1		2	2		2		3	3		3		2	2		2		0	0		0	
18	1	1			1	0	0			0	1	2			1	3	3			3	1	1			1	0	0			1
19	1			1		0			0		1			1		3			3		2			2		0			0	
20	0					0					2					3					1					1				
21	0			2		1			0		1			1		3			3		1			1		1			0	
22	0			0		0			0		2			3		3			3		3			3		0			1	
23	0	0				0	0				1	1				3	3				2	2				0	0			
24	0					0					1					3					1					0				
25	0	0				0	0				2	2				2	3				2	2				1	0			
26	0	0	0	0		0	0	0	0		2	2	2	2		3	3	3	3		2	2	2	2		0	0	0	0	
27	1		1			0		0			1		1			2		3			2		2			0		0		
28	1	1			1	0	0			0	1	1			1	3	3			1	2	2			1	0	0			
29		1			1		2			2		2			2		3			1		2			1		0			0
30					0					1					1					2					1					1
31	0	0				0	0				2	2				3	3				2	3				0	0			
32	0	1				1	1				2	2				3	3				2	2				0	0			
33		1					2					2					2					2					0			
34	0				0	0				0	2				2	3				3	3				2	0				0
35	0		0	0		0		0	0		2		2	2		2		2	2		3		3	3		0		0	0	
36	1	2				1	1				1	2				2	2				3	3				0	0			
37	0				0	0				0	1				1	1				1	3				3	0				1
38	0	0		0		1	1		1		1	1		1		2	2		2		3	3		3		1	0		0	
39	0		0			1		1			1		2			2		2			2		2			0		0		
40	0					1					1					3					2					0				
41	0			0		1			1		2			2		3			3		2			2		0			0	
42	0	0				1	1				2	2				3	3				2	2				0	0			
43	0	0				0	1				1	1				3	3				2	2				0	0			
44	0	1		0		0	1		1		1	2		1		3	3		3		3	3		3		0	0		0	
45	0	0		0		1	1		1		2	2		2		2	2		3		3	3		3		0	0		0	

APC expression was higher in all patterns when compared to normal tissues ($p < 0.05$), with no differences between patterns.

BCL2 expression was higher in all patterns studied than normal adjacent tissue ($p < 0.05$), without differences between patterns.

Cyclin D1 expression differed between normal tissue and solid ($p = 0.0002$) and BA/lepidic ($p = 0.002$) patterns, being higher in these patterns than in the normal tissue.

EGFR protein expression was higher in acinar ($p = 0.033$) and solid ($p = 0.012$) patterns when compared to normal tissues, concerning membrane expression only. If we take into account cytoplasmic expression the overall expression was significantly higher in all patterns without differences between them.

For HER2 we observed significantly higher expression in all patterns compared to normal tissue ($p < 0.05$). Expression is also higher in acinar, papillary, micropapillary and BA/lepidic patterns compared to solid pattern ($p < 0.05$).

ERCC1 expression was lower in acinar ($p = 0.0016$) and papillary ($p = 0.019$) patterns compared to normal tissue. Expression in the other patterns revealed no differences to basal expression in normal tissue. Papillary pattern has significant less expression than solid ($p < 0.05$) and BA/lepidic patterns ($p < 0.05$).

Ki67 expression was higher in all adenocarcinoma patterns when compared to normal lung tissue ($p < 0.05$). There were no differences between the patterns.

LRP expression was different (higher) between normal and the different patterns of the adenocarcinomas ($p < 0.05$).

MRP-1 expression was higher in all patterns compared to normal lung tissue, without differences between patterns ($p < 0.05$).

Some immunohistochemical results are illustrated in Fig. 1.

The analysis of the dendrogram clearly showed that adenocarcinoma is genetically different from normal tissue (Fig. 2). Concerning the patterns, BA/lepidic and acinar are the more similar and papillary the most distinct or genetically separated pattern. By PCA analysis (Fig. 3) we were able to identify three clusters: the first composed of papillary pattern adenocarcinomas; the second corresponding to solid; and the third to the group of acinar, BA/lepidic and micropapillary patterns.

Looking at gender, we have identified higher expression of ERCC1 in female patients when compared to males ($p = 0.0008$). We did not find any differences according to age. HER2 expression was higher in adenocarcinomas of non-smokers or ex-smokers ($p = 0.0384$). Ki67 expression

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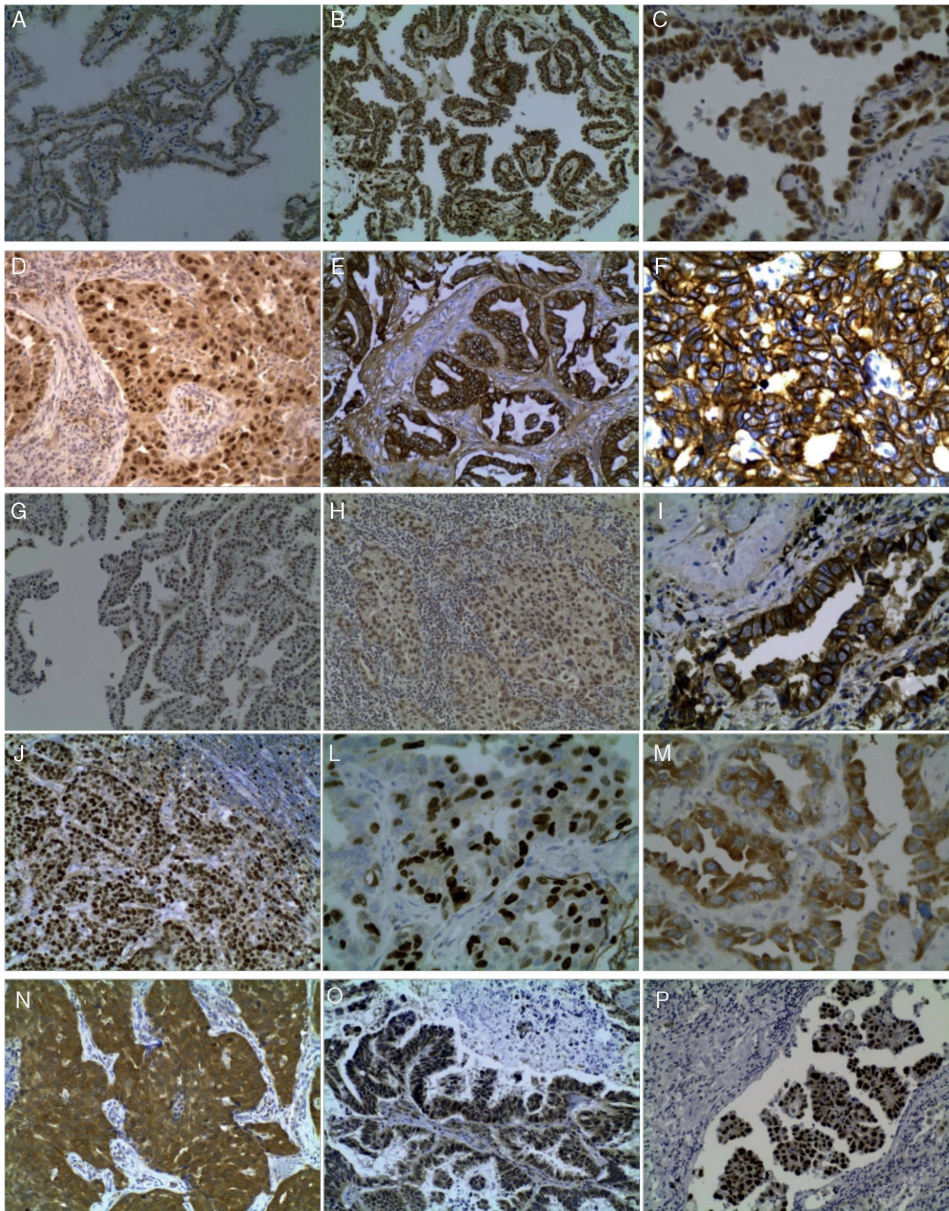

Figure 1 (A) Expression (++) of APC in BA pattern, 200×; (B) Expression (+++) of BCL2 in papillary pattern, 200×; (C) BA pattern with *Cyclin D1* expression, 400×; (D) *Cyclin D1* expression in solid pattern, 200×; (E) *EGFR* expression (+++) in acinar pattern, 200×; (F) *EGFR* expression (+++) in solid pattern, 200×; (G) *ERCC1* expression (++) in BA pattern, 200×; (H) *ERCC1* expression (++) in solid pattern, 200×; (I) *HER2* expression (++) in acinar pattern, 400×; (J) *Ki67* expression (+++) in lymph node metastasis, 200×; (L) *Ki67* expression (+++) in acinar pattern, 400×; (M) *LRP* expression (+++) in acinar pattern, 400×; (N) *MRP-1* expression (+++) in solid pattern, 200x; (O) *RB* expression (++) in papillary pattern, 200×; and (P) *P53* expression (+++) in micropapillary pattern, 200×.

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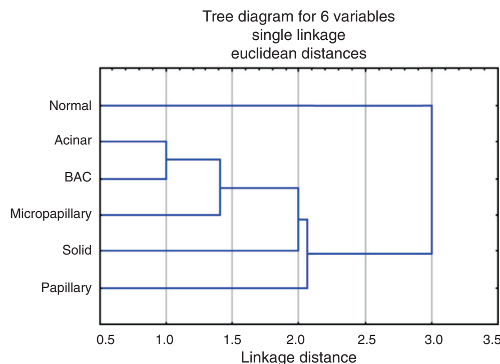

Figure 2 Dendrogram for the hierarchical clustering analysis of transcripts according to their expression values, using Single linkage algorithm, Euclidean distances, targeting the various patterns and normal tissue.

was higher in adenocarcinomas of smokers and ex-smoker patients ($p = 0.0034$ and $p = 0.0064$).

TTF1, Ki67, APC, RB, P53, BCL2, ERCC1 and EGFR showed significantly different expressions according to the stage of the tumor. TTF1 expression showed differences between stage IA and IIIA, being higher in stage IA ($p = 0.02$). Ki67 was higher in IIIA compared to IA and IIA ($p = 0.02$ and $p = 0.02$). APC expression was higher in IIA and IIIA compared to IA ($p = 0.065$ and $p = 0.025$). RB expression was higher in stage IB tumors compared to stage IA ($p = 0.04$). P53 expression was higher in IIA, IB and IIIA compared to IA ($p = 0.01$; $p = 0.03$; $p = 0.01$). BCL2 expression was higher for IB compared to IA ($p = 0.039$). ERCC1 was higher in stage IA compared to IIA ($p = 0.02$) and in IIIA compared to IIA ($p = 0.01$). EGFR expression was lower in stage IB compared to IA ($p = 0.032$).

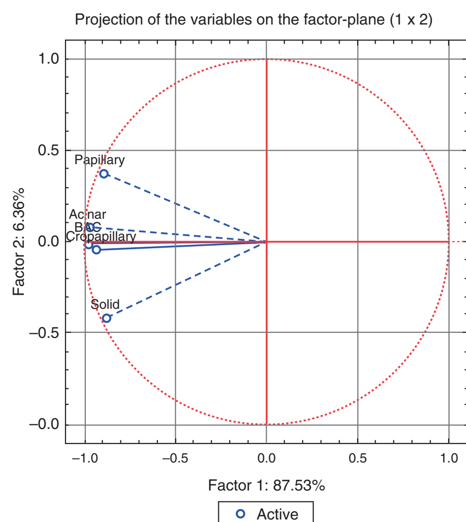

Figure 3 The correlation of the patterns with the advanced PCA factor analysis.

To sum up, almost all patterns showed differences of expression in relation to normal tissues with some exceptions for cyclin D1 (papillary and micropapillary patterns), for EGFR membrane staining (papillary and micropapillary patterns), for ERCC1 (in micropapillary, solid and BA/lepidic patterns), for RB (in acinar, papillary and solid patterns) and for TTF1 (in papillary and solid patterns).

Solid pattern demonstrated lower HER2 expression compared to acinar, papillary, micropapillary and BA/lepidic patterns. Papillary pattern had lower ERCC1 expression than solid and BA/lepidic patterns. Solid pattern showed lower TTF1 expression than BA/lepidic pattern.

Discussion

Non-BA/lepidic adenocarcinomas are most often peripheral tumors with different growth patterns considered mixed type according to WHO classification, with at least two individual patterns; solid or acinar patterns are the most frequent in the central tumoral bulk and BA/lepidic at the periphery. Acinar has been described as the most common pure subtype and the most common in the mixed type. Papillary pattern was present in 10% of the adenocarcinomas, often difficult to differentiate from BA/lepidic, which has important therapeutic strategies' implications.[9] Micropapillary pattern is characterized by small papillary tufts growing from alveolar septa or floating within alveolar spaces lacking fibrovascular cores and is usually observed with papillary and mixed patterns; they are more aggressive due to their increased capacity for metastasize, indicating unfavorable prognosis.[9] Solid pattern with mucin production has been reported in 15% of mixed-type adenocarcinomas.[9,10] BA/lepidic pattern shows tumor cell growth along alveolar septa and aerogeneous spread through the lung. It is frequently diagnosed in former and non-smoking women. BA/lepidic is classified as non-mucinous, mucinous or mixed.[9,10,38]

Markers of differentiation

CK20, HWMC and chromogranin A were negative in all our samples and CK7 and TTF1 were positive, which means morphology almost always sustains bronchial-pulmonary adenocarcinomas' diagnosis when mucin is not present. These results corroborated those that have been described in the literature. CK7 is positive in 90–100% and CK20 is positive in only 7–10% of primary lung adenocarcinomas.[39] Chromogranin A is not expressed in an ordinary adenocarcinoma.[40] TTF1 is a useful marker for lung adenocarcinoma and is also expressed in thyroid normal tissue and tumors.[22] The use of TTF1, as a differentiation marker, is useful not only for diagnostic purpose but especially to differentiate between different histological types of bronchial and TRU adenocarcinomas.

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Genetic expression in each pattern of bronchial-pulmonary adenocarcinoma

Acinar pattern

Starting with membrane proteins that play a central role in the control of cellular proliferation, differentiation, apoptosis and angiogenesis, EGFR and HER2 had a higher expression in acinar pattern than in normal epithelium/lung cells, highlighting the importance of AKT/mTOR, PI3K and MAPK pathways, essential for oncogenic transformation and tumor maintenance.[41] Blockage of both results in apoptosis and tumor shrinkage.[42,43] AKT, by inhibiting GSK-3, changes canonical Wnt pathway, releasing β-catenin, and leading to angiogenesis and proliferation.[44]

TTF1 positivity was statistically higher in the adenocarcinomas (with acinar pattern) compared to normal tissue. While the majority of the adenocarcinomas expresses TTF1, this marker is misunderstood, because its positivity is associated inversely with proliferation but it is correlated with P53 expression and HER2/neu involved in lung cancer.[45] TTF1, NKX2-8 and PAX9 are very important in initiation and progression of lung cancer. It is said that MAPKinase, JAK/STAT and Wnt are important in activation of TTF1 and NKX8, connected with EGFR and Her2.[46]

The expression of cyclin D1 was higher in tumoral than in normal tissue and RB expression had no statistical difference when compared to normal tissue. This fact may indicate a tendency to proliferate, because once cyclin D1 is in high concentration, it could phosphorylate more RB protein and release E2F, involved in the regulation of genes with implications in cell cycle progression (G1 to S phase).[43,47] One of the genes that correlate with E2F is Ki67, still a good marker for proliferative index.[48] We obtained statistical significant higher expression of Ki67 in all patterns of adenocarcinoma, indicating active proliferative activity.[49] Another overexpressed gene in this pattern was P53 and some studies indicate that ''a low level of p53 leads to transient growth arrest and cell survival, whereas a high level promotes irreversible apoptosis'', and *P53* levels may have oscillations, and it has to be explored as a target for therapy.[50] ERCC1 is downregulated in acinar pattern, may be indicating that DNA repair pathway is not activated in the acinar pattern. ERCC1 is associated with radio resistance, so it is important for treatment choice.[51] High BCL2 expression may favor cell cycle arrest.

LRP and MRP1 were also overexpressed in our samples, as has been described in lung cancer.[52,53] As mentioned these genes can lead to chemotherapy resistance. This fact should be taken into consideration when assessing prognosis and treatment issues.

APC was also overexpressed in the acinar pattern, and being involved in β-catenin degradation is important in progression, with GSK-*3* and *Axin* downregulating β-catenin (leading to β-catenin degradation).[54] Our findings also suggest that β-catenin may be downregulated, already described in lung cancer, which is related to cell invasion and metastasis.[55] β-Catenin is connected to cytoskeleton and cadherins and regulates E- or N-cadherin-mediated cell adhesion. Notch activation (that inactivates β-catenin) may repress E-cadherin, leading to tumor growth and metastasis.[56] So, overexpression of APC leads to β-catenin degradation that weakens cell adhesion promoting tumor growth and metastasis.

In relation to the acinar pattern the genes analyzed expressions could lead to cell proliferation and survival, angiogenesis and metastasis.

Papillary pattern

This pattern showed some differences compared to acinar pattern. There were no differences of expression for cyclin D1 and TTF1, compared with normal tissue. Since cyclin D1 level is not upregulated as well as RB gene, the concentration of active E2F must be regular. However Ki67 was overexpressed, in contrast to previous findings.[21] So other pathways could explain cellular proliferation. There was higher expression of EGFR but just in cytosol compartment; membrane EGFR had the same level of expression as normal tissue, so we can conclude that activity of EGFR is normal. Thus, all pathways that this gene regulates are functioning normally or if activated, this activation is not induced by EGFR.[41,57,58]

Papillary pattern proliferation properties could in part be explained by APC upregulation when compared to normal tissue.

Micropapillary pattern

Micropapillary pattern is characterized by small papillary tufts and is considered an aggressive adenocarcinoma, more than papillary pattern.[59–61] There were three differences with papillary patterns concerning expression of ERCC1, RB and TTF1. RB expression was downregulated in micropapillary, being almost non-existent. Some authors said that downregulation of RB leads to the progression of cell cycle.[62] Micropapillary pattern had a basal level of ERCC1, this indicating that DNA repair may occur with NER activation. TTF1 was overexpressed in this pattern and, according with what has been said before, this fact indicates lung cancer progression and development after the terminal respiratory unit. These considerations are consistent with our results because downregulation of RB and TTF1 overexpression are responsible for cell cycle progression and basal level of ERCC1 allows DNA repair. This last consideration is very important to keep in mind in treating lung cancer.

Solid pattern

Genetic expression in this pattern is identical to acinar pattern with only two differences for ERCC1 and TTF1 expressions. These showed no differences compared to normal tissue in solid pattern, whereas in acinar pattern there was a higher expression for TTF1 and downregulation for ERCC1. A lower TTF1 expression reflects a lower differentiation of this pattern and eventually means a more aggressive behavior since in general less differentiated tumors are more aggressive. Higher EGFR expression could be the reflex of EGFR mutations and/or overexpression activating several pathways involved in cell cycle control, proliferation and even metastization. EGFR overexpression is also associated with poor prognosis.[33,63] Solid pattern has more ERCC1 expression than acinar and this aspect is very important because ERCC1 repairs DNA and confers resistance to some drugs.[51]

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BA/lepidic pattern

BA pattern, called lepidic in ATS/ERS proposed classification can be difficult to distinguish morphologically from papillary pattern.[12] Cyclin D1 had higher expression in BA/lepidic pattern and a basal level in papillary pattern. This fact and knowing that RB was not overexpressed, enhances the idea that this pathway leads to cell progression. As TTF1 is related with tumor progression and is overexpressed, unlike in papillary pattern, and ERCC1 has a basal level in BA/lepidic pattern and is downregulated in papillary pattern, this may explain chemical resistance.

By evaluating the expression of these 13 genes, BA/lepidic pattern has more cell progression pathways activated than papillary pattern. This finding proves that they are biologically different. The differences could be explained by the origin in TRU with the different activated pathways, with probable implication in the prognosis and treatment, knowing that different pathways are targets for therapeutic approach.

Metastasis analysis

Metastasis formation is a complex process with several pathways activated leading to loss of cellular adhesion, increased motility and invasiveness.[55] Acinar pattern metastasis had cyclin D1 over-expression, statistically different between primary and metastatic tumor. This gene is involved in cell progression that might be helping cell survival in circulation and colonization.[55]

Gender, age, smoking and tumor staging

ERCC1 expression was higher in females, correlating in some studies to a better prognosis.[64] However ERCC1 may confer resistance to cisplatin.[65]

Lung adenocarcinoma is more frequently diagnosed in patients over 60 years and it is infrequent before age 40 and generally more aggressive.[66] All our samples were taken from patients older than the age of 40. There were no difference in the expression of the markers studied between the group under and over 60 years.

Concerning smoking history, HER2 expression was higher in non-smokers than in ex-smokers. Ki67 expression was higher in tumors of smokers and ex-smoker patients than in non-smokers, indicating a higher proliferative rate and more aggressive/mitotically active tumors.

Women diagnosed with adenocarcinoma were more frequently non-smokers, reflecting the cultural tobacco habits and some of those adenocarcinomas belonging to the group of adenocarcinomas diagnosed in women, non-smokers and young that could harbor *EGFR* mutations.[67] Adenocarcinomas diagnosed in women patients were more frequently diagnosed at stages IA and IB reflecting somehow a less aggressive behavior demonstrated also by a lower proliferative index in these stages. Tobacco habits could also explain the activation of different pathways and genetic alterations in these cases. Our results also demonstrated a relation between higher Ki67 expression and smoking habits.

Many gene products have their expression altered when correlating with stage, such as APC, BCL2, EGFR, ERCC1, Ki67, P53, RB and TTF1, showing that gene expression is a dynamic and unforeseen process. From stage IA to IB we observed lower Bcl2 and EGFR expressions and RB higher expression. From stage IB to IIA we observed higher Bcl2 and lower ERCC1 expressions. IIIA stage showed higher ERCC1 and Ki67 expressions. From IA and IB to IIA we observed also higher APC and Bcl2 expressions. In advanced stages we observed especially higher Ki67, APC and ERCC1 expressions and lower TTF1 expression, the last reflecting a lower differentiation and even a non-TRU adenocarcinoma with a more aggressive behavior. Ki67 higher expression in higher stages was an expected result as it reflects higher proliferation index and thus aggressive behavior. APC mutations were described in several tumors.[17] Taking into account our results, it seems that APC genetic and pathways changes are important to consider in advanced stages of adenocarcinomas.

One of the most important molecule in this study was P53 because it was higher expressed in all patterns and is involved in several pathways and mechanisms in normal cell cycle, carcinogenesis and DNA repair with obvious therapeutic implications. Some authors relate P53 with MRP1, because their parallel increase is frequent. It was demonstrated that P53 wild-type suppressed MRP1 promoter. These hypotheses are corroborated by our data, because we always obtained high expression of MRP1 and P53. According to these authors P53 was mutated in all patterns.[68]

RB expression was lower in micropapillary pattern, which also explained a more aggressive phenotype because free E2F activates transcription of genes related with tumor progression.

TTF1 was connected with P53 and HER2 in lung cancer and some authors relate this protein with initiation and progression in lung carcinogenesis.[19,23] The relation between TTF1 and HER2 was observed between solid and BA/lepidic patterns, because both gene expression was higher in BA/lepidic pattern than in solid.

Conclusions

Female adenocarcinoma patients were more frequently non-smokers and diagnosed in earlier stages, with higher ERCC1 expression involved in DNA repair.

Advanced stages (IIA and IIIA) of adenocarcinomas showed higher Ki67, APC, ERCC1 expressions and lower TTF1 expression reflecting a more aggressive, mitotically active and less differentiated adenocarcinoma and eventually a non-TRU adenocarcinoma.

There was generally higher expression of the products of genes studied in the adenocarcinomas compared to normal adjacent cells reinforcing their importance in lung adenocarcinoma carcinogenesis.

There were two specific gene expressions with differences between patterns, HER2 and TTF1 that interfere with gene transcription. Papillary and solid patterns revealed less TTF1 expression, identical to normal tissue exhibiting a non-TRU/bronchial phenotype. At the other end acinar, BA/lepidic and micropapillary showed higher TTF1 expression. Solid pattern revealed also lower HER2 and higher EGFR and ERCC1 (this compared to papillary) expression. Papillary showed higher HER2 and lower ERCC1 expressions. Adenocarcinomas showed higher TTF1 expression (type TRU) in

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acinar, BA/lepidic and micropapillary patterns, micropapillary with higher RB expression, acinar with lower ERCC1 and higher EGFR. Ciclin D1 seems to have more importance in acinar and BA/lepidic patterns than in micropapillary.

ERCC1 protein expression in micropapillary, solid and BA/lepidic patterns indicates DNA repair and in acinar and papillary patterns there was lower expression. BCL2 was overexpressed in all patterns, suggesting that there is inhibition of apoptosis. MRP-1and LRP were overexpressed in all patterns and it is important to further analyze these proteins for a better understanding of the response to therapy.

By hierarchical clustering test we highlight papillary pattern as the most different pattern followed by solid pattern; acinar and BA/lepidic patterns are most alike. Using PCA analysis we realized that acinar, micropapillary and BA/lepidic patterns are the most alike, so we made three clusters of patterns: papillary, solid and micropapillary/acinar/lepidic (BA).

Furthers studies are needed in order to interpret these results regarding therapeutic response in advanced staged bronchial-pulmonary carcinomas.

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 center on the publication of patient data.

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

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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CHAPTER 6 – MANUSCRIPT IV

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

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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-erB-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 semiquantitatively 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 μm 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× 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 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× SCC pH 7) at 46 °C and were also washed with 2× 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 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 solution 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 eletropherograms 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, Papillay, 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

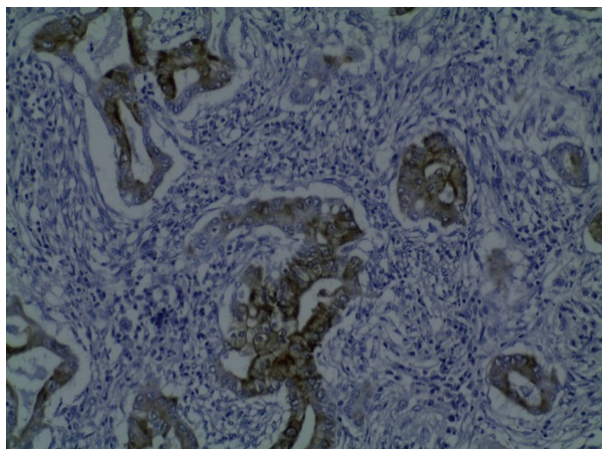

Figure 1 Case 21: 2+ CK20 expression; acinar pattern. 200×.

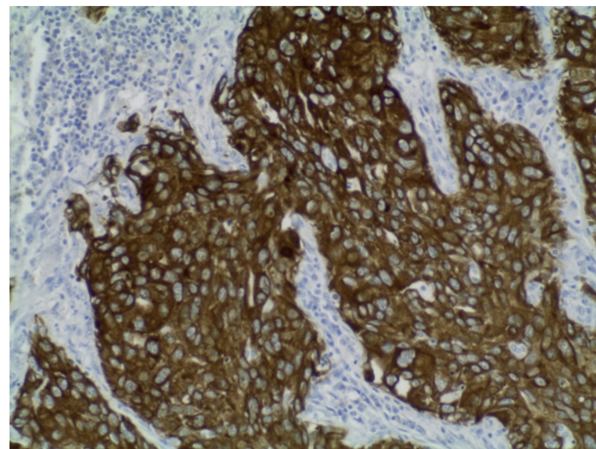

Figure 2 Case 6: CK7 intense expression (3+) in solid pattern. 200×.

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 ($p = 0.0073$).

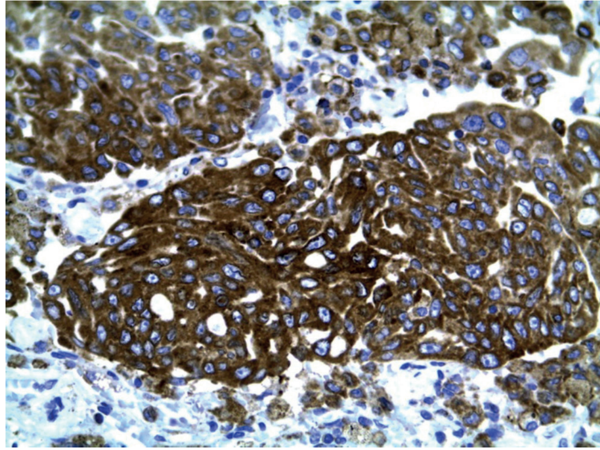

Figure 3 Case 7: ErbB-2 3+ cytoplasmatic expression; solid pattern. ErbB-2, 400×.

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

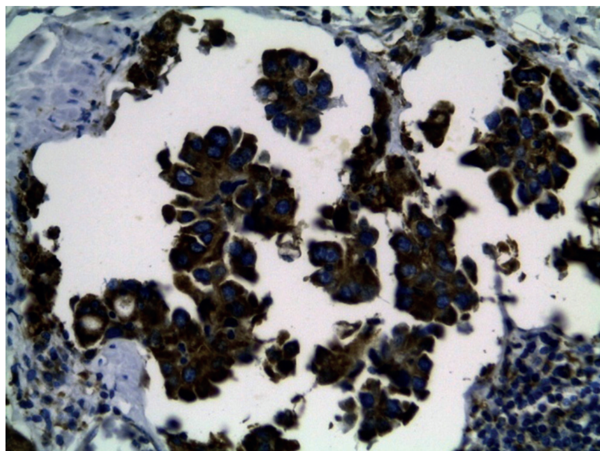

Figure 4 Case 8: ErbB-2 3+ cytoplasmatic expression; micropapillary pattern. ErbB-2, 400×.

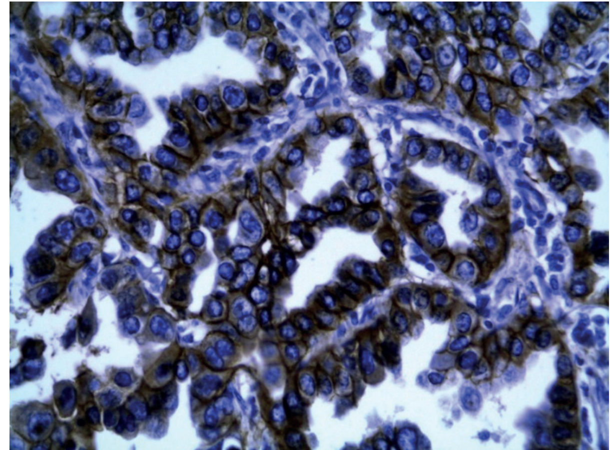

Figure 5 Case 12: Intense (3+) EGFR expression; acinar pattern. EGFR, 200×.

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 (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

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 (*EGFR* L858R)	G12V (*EGFR* L858R)	n.p.	n.p.	n.d.
29	G12C	G12C	n.p.	n.p.	n.p.

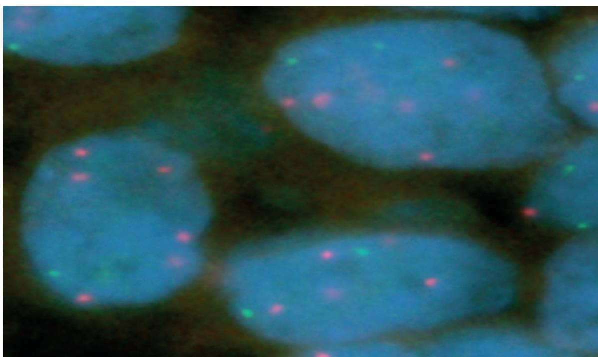

Figure 6 Case 7: FISH positive for EGFR/chromosome 7 centromere. Gene high polysomy; *EGFR* – red signal, chromosome 7-green. 1000×.

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

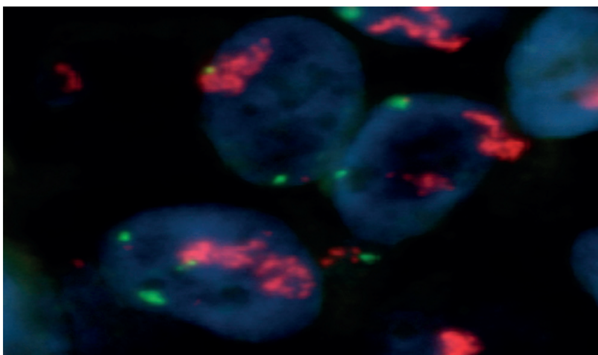

Figure 7 Case 12: EGFR/chromosome 7. Gene amplification; FISH positive. EGFR/chromosome 7 centromere. *EGFR* – red signal, chromosome 7-green. 1000×.

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 weights, 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 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 TTF1[35] 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 αC-helix) in exon 20 just as *EGFR* in-frame duplications/insertions, present in a very small fraction of adenocarcinomas of non-smokers and females, (the same as *EGFR* mutations) [10,19]; 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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CHAPTER 7 – MANUCRIPT V

EGFR and KRAS mutations coexistence in lung adenocarcinomas.

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EGFR and KRAS mutation coexistence in lung adenocarcinomas

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Lung cancer is one of the most common causes of cancer deaths. The development of EGFR targeted therapies, including monoclonal antibodies and tyrosine kinase inhibitors have generated an interest in the molecular characterization of these tumors. KRAS mutations are associated with resistance to EGFR TKIs. EGFR and KRAS mutations have been considered as mutually exclusive.

This paper presents three bronchial-pulmonary carcinomas, two adenocarcinomas and one pleomorphic sarcomatoid carcinoma, harboring EGFR and KRAS mutations.

Case 1 corresponded to an adenocarcinoma with EGFR exon 21 mutation (L858R) and KRAS codon 12 point mutation (G12V); case 2, a mucinous adenocarcinoma expressed coexistence of EGFR exon 21 mutation (L858R) and KRAS codon 12 point mutation (G12V); and case 3 a sarcomatoid carcinoma with EGFR exon 19 deletion – del 9bp and KRAS codon 12 point mutation (G12C - cysteine).

Based on our experience and on the literature, we conclude that EGFR and KRAS mutations can indeed coexist in the same bronchial-pulmonary carcinoma, either in the same histological type or in different patterns. The biological implications of this coexistence are still poorly understood mainly because these cases are not frequent or currently searched. It is therefore necessary to study larger series of cases with the two mutations to better understand the biological, clinical and therapeutic implications.

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Introduction

Lung cancer is one of the most common causes of cancer deaths [1,2]. Despite improvements in diagnostic and surgical techniques and chemotherapy protocols, overall survival is still low [3,4]. The NSCLC designation includes different carcinoma types such as squamous cell carcinoma, adenocarcinoma, large cell carcinoma and pleomorphic carcinoma [5,6]. Lung adenocarcinomas account for about 28% and 42% of the bronchial-pulmonary carcinomas diagnosed in women and men respectively [6]. The newly proposed lung adenocarcinoma classification recognizes a lepidic pattern instead of bronchiole-alveolar pattern, acinar, solid, papillary, micropapillary patterns as well as mucinous adenocarcinomas. This classification reinforces the importance of pattern recognition in lung adenocarcinoma diagnosis [6].

The development of EGFR-targeted therapies, including monoclonal antibodies and tyrosine kinase inhibitors, has generated interest in the molecular characterization of these tumors. Patients with lung cancer who benefit from EGFR tyrosine kynase inhibitors (EGRF TKI) may show dramatic responses with gefitinib or erlotinib therapy [7-11].

Activating EGFR mutations have been identified to predict EGRF TKI response [12-16].

KRAS mutations have been associated with EGFR TKIs resistance [17,18].

EGFR and KRAS mutations have been considered as mutually exclusive, as the KRAS-MAPKinase signaling pathway is also one of the signaling pathways for EGFR [18-20].

The objectives of this paper are to present three cases of bronchial-pulmonary carcinoma with coexisting EGFR and KRAS mutations and to discuss the clinical-pathological and therapeutic implications.

Material and Methods:

Material

This paper presents two adenocarcinomas and one pleomorphic carcinoma, harboring EGFR and KRAS mutations. Adenocarcinoma patterns present were registered.

The tissue for the analysis was obtained from 4% formalin-fixed paraffin embedded sections of lung surgical specimens and transthoracic biopsy (third case). All patterns present were manually dissected for selection after identification on hematoxylin – eosin stained slides.

Methods

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EGFR exons 19 and 21 and KRAS codon 12 and 13 mutations were evaluated after DNA extraction and polymerase chain reaction (PCR) amplification; EGFR exon 19 was studied by fragment analysis and EGFR exon 21 and KRAS codons 12 and 13 were studied by Sanger direct sequencing. Tissue representative of the adenocarcinoma patterns was selected after identification on HE stained slides and manual dissection of the tissue. The percentage of neoplastic cells was registered (in the three cases it was over 50%).

Genomic DNA was extracted from 5 µm section of paraffin-embedded tissue. For that, the QIAmp DNA Mini Kit (Qiagen, IZAZA, Germany) was used. One hundred nanograms (ng) of DNA was amplified in a 50 µl reaction solution containing 5 µl of 10x buffer (Roche, Germany), 2.5 mM MgCl2, 0.2 µM of each complementary primer, 200 µM deoxynucleoside triphosphate and one unit of DNA polymerase (Roche, Germany). A 5-minute initial denaturation at 95ºC was used to perform the amplifications; this was followed by 40 cycles of 30 seconds at 95ºC, 1 minute at 60ºC (for exon 19) or 57ºC (for exon 21), 1 minute at 72ºC and 10 minutes of final extension at 72ºC. The EGFR gene mutations located at exons 19 and 21 were determined using the intron-based primers according to the published method [21]. EGFR mutations were analyzed / detected according to the published method [22]. Exon 19 deletion was determined by common fragment analysis using PCR with an FAM-labeled primer set, and the products were submitted to electrophoresis on ABI PRISM 3100 (Applied Biosystems®). All electropherograms were reanalyzed by visual inspection in order to check for mutations. To evaluate the L858R mutation, MyCcycler (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 was performed using 5-minute initial denaturation at 95ºC; followed by 40 cycles of 30 seconds at 95ºC, 1 minute at 53ºC, 1 minute at 72ºC and a 10 minutes of final extension at 72ºC.

The same DNA samples were used for EGFR and KRAS mutational testing. The results were confirmed by double checking after another PCR reaction. Rules to avoid contamination were applied. Positive and negative (WT) controls were used in every test. Before sequencing an electrophoresis in agarose gel was performed to check the blank. Since 2012 our laboratory has participated in the European Society of Pathology (ESP) Lung External Quality Assessment Scheme (EQA) to ensure optimal accuracy and proficiency in lung cancer biomarker testing. Our score has been consistently ≥90%, considered as a successful participation.

Results

Case 1

A 77-year-old man had a 3 cm central tumor in the left lower lobe, corresponding to an adenocarcinoma with acinar, lepidic, solid and mucinous patterns, pT1bN0.

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EGFR exon 21 mutation (L858R) was present in the acinar and lepidic patterns (Figure 1).

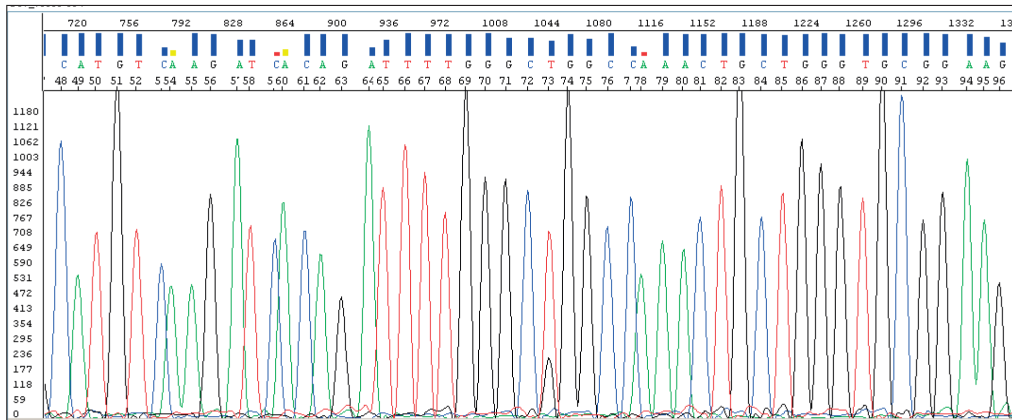

Figure 1. Exon 21 (L858R) EGFR mutation.

EGFR exon 19 was Wild Type (WT) in all patterns.

KRAS codon 12 point mutation (G12V) was also present in the acinar and lepidic patterns (Figure 2) (Table 1).

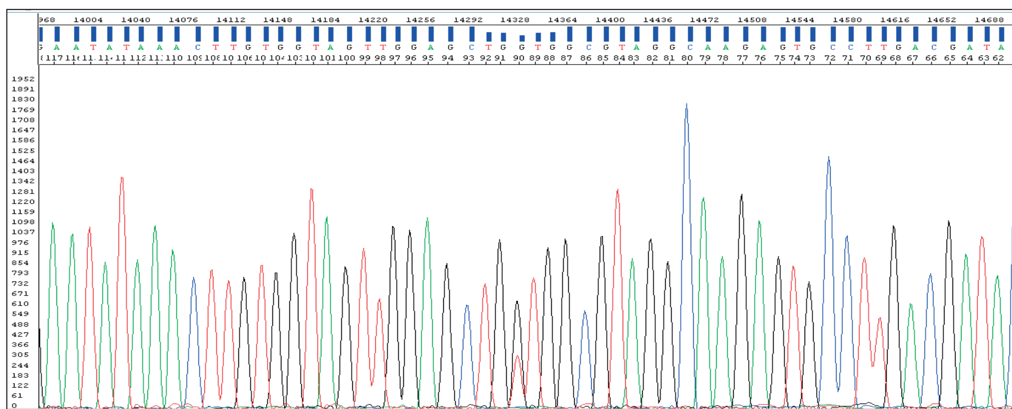

Figure 2. Codon 12 KRAS (G12V) mutation.

Case 2

A 77-year-old woman had a 6cm right upper lobe mucinous adenocarcinoma (with mucinous lepidic and mucinous acinar patterns), pT2b N0.

EGFR exon 21 mutation (L858R) was present in both patterns, while EGFR exon 19 was WT.

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KRAS codon 12 point mutation (G12V) was also concomitant (Table 1).

Case 3

A 76-year-old man had an 8 cm nodule in the right lower lobe with nodular pleural invasion as well as malignant pleural effusion, staged as pT3N0M1a. A transthoracic biopsy revealed a pleomorphic carcinoma, with acinar and giant/large cells.

EGFR exon 21 was WT and exon 19 deletion – del 9bp was present in both patterns (Figure 3) (Table 1).

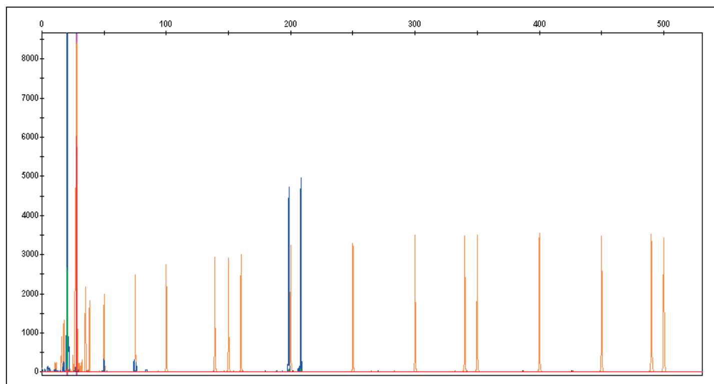

Figure 3. Exon 19 (Del 9bp) EGFR mutation.

KRAS codon 12 point mutation (G12C - cysteine) was also present in both patterns (Figure 4) (Table 1).

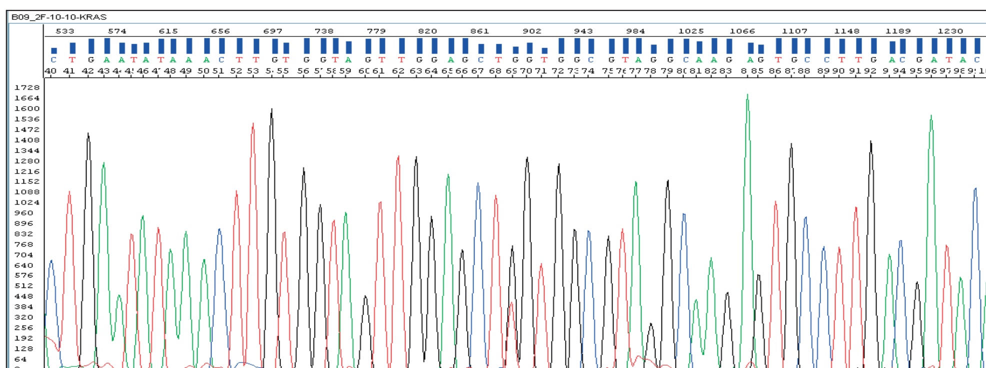

Figure 4. Codon 12 KRAS (G12C - cysteine) mutation.

Table 1: EGFR and KRAS mutations identified according to histologic patterns. WT – wild type; L858R - exon 21 EGFR point mutation; G12V – codon 12 KRAS mutation G12V; G12C – Codon 12 KRAS mutation G12C.

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Case 1	Mutations /patterns	Acinar	Lepidic mucinous	Solid	Mucinous
	Exon 21 EGFR	L858R	L858R	WT	WT
	Exon 19 EGFR	WT	WT	WT	WT
	KRAS	G12V	G12V	WT	WT
Case 2	Mutations /patterns	Acinar mucinous	Lepidic mucinous		
	Exon 21 EGFR	L858R	L858R		
	Exon 19 EGFR	WT	WT		
	KRAS	G12V	G12V		
Case 3	Mutations /patterns	Acinar	Giant cells		
	Exon 21 EGFR	WT	WT		
	Exon 19 EGFR	del 9bp	Del 9bp		
	KRAS	G12C	G12C		

Discussion

EGFR and KRAS mutations are considered as driver mutations because they are responsible for the initiation, progression and maintenance of lung cancers. Since 2004 the scientific community has known that pulmonary carcinomas harboring EGFR mutations are highly sensitive to EGFR TKIs [15].

Several studies have shown that the classical brochioloalveolar carcinomas and mixed-type adenocarcinomas have more frequently EGFR activating mutations [23-25]. Other studies have demonstrated that papillary, micropapillary and hobnail patterns are correlated with EGFR mutations [26, 27].

Dacic et al. demonstrated that EGFR mutated mixed-type adenocarcinomas showed the following predominance of primary histological patterns: acinar (20%), BA/lepidic (12.5%), mucinous (9%), papillary (3%), micropapillary (3%) and solid (0%); and KRAS mutated mixed-type adenocarcinomas showed acinar (32%), solid (18%), BA/lepidic (16%), mucinosous (16%), papillary (10%) and micropapillary (4%) [28]. Dacic et al. also demonstrated that tumors with a lymphocytic prominent response are less prone to have EGFR mutations and more likely to harbor KRAS mutations [28].

Intra - tumors heterogeneity for EGFR mutations has also been demonstrated [29,30]. KRAS mutations have been more frequently associated with mucinous differentiation, including mucinous BA/lepidic, acinar patterns, goblet cell morphology and poorly differentiated adenocarcinomas, especially in adenocarcinomas with solid patterns [28,31-37]. KRAS mutations are rare in squamous cell carcinoma and significantly more frequent in proximal lung adenocarcinomas (the bronchial type TTF1 negative) than EGFR mutations [38,39].

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Our two adenocarcinoma cases showed one lepidic pattern (second case) and the other (first case) mixed patterns where EGFR mutations are supposed to be more frequent. However the lepidic case had mucinous differentiation. KRAS mutation was present in the same area/patterns of the mixed adenocarcinoma (first case), namely in the acinar and lepidic patterns but not in the solid or mucinous patterns. In the second case it was present in the mucinous lepidic pattern, in accordance with the most frequent patterns harboring KRAS mutations in the literature.

In our laboratory, taking these three cases into account, we have a rate of 2.1% EGFR and KRAS coexistence. In our laboratory, the overall mutation rate for EGFR is 20% and for KRAS is 11%.

None of our patients underwent EGFR TKI therapy, so we cannot discuss the therapeutic consequences of EGFR and KRAS mutation coexistence in our cases.

EGFR mutations define a group of lung cancer which are dependent on EGFR signaling pathways and are more responsive to EGFR TKIs. EGFR mutations may also be a positive prognostic factor for advanced tumors treated with erlotinib [40].

Recent works have also demonstrated that EGFR mutational status is associated with sensitivity to first-line EGFR TKI in patients with advanced staged tumors [41].

Iressa Pan-Asia Study provided evidence that EGFR mutations are predictive of longer progression-free survival when treated with gefitinib compared with conventional chemotherapy protocols [42, 43].

The major signaling pathways for EGFR are RAS/MAPK and PI3K/AKT, with implications for cell proliferation, differentiation and survival [44]. Mutations on the downstream effectors of those pathways could be responsible to EGFR TKIs resistance [45,46]. KRAS is an important downstream effector in the MAPK pathway. Concomitancy of KRAS and EGFR mutations could seem redundant in their functional results. Mutant EGFR selectively activates AKT and STAT signaling pathways, which promotes cell survival [8].

KRAS mutations are associated with an unfavorable response to EGFR TKIs and resistance to conventional adjuvant chemotherapy with cisplatin/vinorelbine [18,47-50]. The TRIBUTE study also demonstrated that patients with KRAS mutation treated with erlotinib failed to benefit from erlotinib plus chemotherapy [40].

Evidence suggests that a tumor can harbor EGFR and KRAS mutations, which means that upstream inhibition of EGFR will have no therapeutic effect in these cases [51].

Recently Jackman et al. found no impact of KRAS mutations on the overall survival in patients without EGFR mutations treated with EGFR TKIs [41]. The role of KRAS mutation as a negative predictor of response of lung cancer treated with cetuximab is not clear either [52, 53]. Because EGFR mutation is a predictor of EGFR TKIs response and EGFR and KRAS mutations are generally mutually exclusive, it is not clear whether the response to TKIs differs between tumors with KRAS mutations and those without KRAS and EGFR mutations [54].

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There is a report of two cases of patients with colonic adenocarcinoma metastatic to lung EGFR WT and with KRAS mutation with durable responses to erlotinib [30]. There are other rare cases of TKI minor or transient response in patients with KRAS mutation [41,55]. Choughule et al described in their series three patients with KRAS and EGFR mutations with partial response to EGFR TKI [56]. Benesova et al also found response to gefitinib in three of five patients with this coexistence [57]. These facts raise the idea that KRAS mutation is not always associated with a lack of efficacy of EGFR TKI. The authors hypothesize that EGFR activation is not only mediated through KRAS signaling but also involves other pathways like PI3K/AKT/mTOR, phospholipase C or STAT. Negative feedback loops could also down regulate RAS signaling pathways [30]. Another possible explanation includes eventual concomitant molecular abnormalities that could activate EGFR [30]. Epigenetic variations, alternative splicing or posttranslational modifications could explain non active KRAS isoforms [30]. These authors therefore conclude that a small portion of patients with KRAS mutations might paradoxically benefit from EGFR TKIs.

One patient with KRAS mutations in the Southwest Oncology Group S0126 trial study responded to gefitinib, but none of four patients with both EGFR and KRAS mutations responded [58].

Zhu et al. also demonstrated that one patient with KRAS mutation responded to EGFR TKI [55]. The tumor had EGFR amplification but not mutation. It is argued that tumors with KRAS mutation are unlikely to respond to EGFR TKIs unless they have EGFR amplification [55].

Not all lung cancers with KRAS mutations are addicted to KRAS, demonstrated by in vitro studies [59]. Dependency was correlated with KRAS overexpression while the well-differentiated epithelial phenotype is correlated with RAS dependency [59].

Pao et al. first suggested that EGFR and KRAS mutations are mutually exclusive [18]. Other studies have also shown mutual exclusivity. Nevertheless combined EGFR and KRAS mutations do exist, though they are rare [40, 55, 56, 60, 61]. Li et al showed that when there was EGFR and KRAS mutation coexistence, EGFR mutations were more frequently identified in exon19 [61].

In our study, the EGFR and KRAS mutations present were always activating mutations, two identified in exon 21 and one in exon 19. These cases demonstrate that EGFR and KRAS mutations can coexist even in the same patterns of adenocarcinoma, although they have been discerned as mutually exclusive.

Lung adenocarcinoma comprises a group of tumors that are heterogeneous as regard histopathology typing, patterns and genetic alterations; hence, it is important to review a large number of histological sections to characterize the patterns present and even the whole tumor. In future, a more accurate molecular profile needs to be defined to overtake tumor morphological and genetic heterogeneity in order to explain the coexistence of KRAS and EGFR mutations.

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In the second case reported here, the EGFR mutation occurred in the lepidic pattern, although with mucinous differentiation. Nevertheless, there are EGFR mutations described in mucinous patterns. KRAS mutation is described as frequent in this pattern.

In the first case, the mutations were present in acinar and lepidic patterns and not in solid and mucinous patterns. Thus, tumor heterogeneity does not explain all the cases of EGFR and KRAS mutation coexistence. In these cases, genetic instability could explain the simultaneous or possibly sequential occurrence of the two mutations. Different clones of cells present in the same patterns could also explain the coexistence. Nor should we exclude the possibility of coexistence in the same cells that could overcome the downstream KRAS mutations by alternative signaling pathways or other regulatory mechanisms.

In the third case, EGFR and KRAS mutations were present in a pleomorphic sarcomatoid carcinoma. We know that KRAS mutations are more frequent in less differentiated lung cancers. These tumors are biologically aggressive and more prone to accumulate genetic instability and alterations.

Pleomorphic and sarcomatoid carcinomas are rare. One study revealed no EGFR mutations and a frequency of 38% KRAS mutations in primary sarcomatoid lung carcinomas. However, high EGFR polysomy (23%) and EGFR protein overexpression were identified in all the cases. These authors hypothesized that EGFR overexpression and KRAS mutation could explain aggressive biological behavior and worse prognosis of these subset of NSCLC [62].

Conclusions

Our findings strongly support the idea that EGFR and KRAS mutations can coexist in the same lung cancer, even in the same cell type of an adenocarcinoma. The biological implications of this coexistence are still poorly understood, mainly because these cases are not frequent, and bronchial-pulmonary carcinomas are not routinely studied in this extensive manner. In the future, studies of larger case series are required to better understand the biological, clinical and therapeutic implications of the discussed molecular alterations, based on uniformly designed studies.

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Authors' contributions

VS was responsible for drafting the manuscript, diagnosis, data collection and interpretation. LC was responsible for diagnosis, orientation and corrections. MS, AMA, TF and AL carried out technical issues and mutational status evaluation.

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CHAPTER 8 – MANUSCRIPT VI

Targeted therapy for FGFR1 may be independent of the histological type of bronchial-pulmonary carcinomas.

CHAPTER 8 – Targeted therapy for FGFR1 may be independent of the histological type of bronchial-pulmonary carcinomas.

Targeted therapy for FGFR1 may be independent of the histological type of bronchial-pulmonary carcinomas

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INTRODUCTION

Although bronchial-pulmonary carcinomas are still the most important cause of cancer-related death, personalized therapy and accurate diagnosis is believed to raise survival, after the recent advances in molecular targeted therapies [1-5].

Targetable and predictive oncogenic mutations have been mainly identified in lung adenocarcinomas (ADC) and in never smokers' tumours. Recently, several genomic and molecular alterations have been demonstrated in squamous cell/epidermoid carcinoma (SQC), such as fibroblast growth factor receptor 1 (FGFR1), PTEN and PIK3CA/AKT1 alterations [6].

FGFRs belong to the family of receptor tyrosine kinases (including FGFR1, FGFR2, FGFR3 and FGFR4), encoded by 4 genes, *FGFR1* located on chromosome 8p11.23-p11.22; *FGFR2* on 10q26; *FGFR3* on 4p16.3; and *FGFR4* on 5q35.2. They are responsible for the control of several physiological processes, whose deregulation may lead to cancer development [7-9].

FGFR1, as a transmembrane tyrosine kynase receptor, promotes cell growth, differentiation, and survival by downstream signalling of PI3K-AKT, RAS-MEK-MAPK, STAT, SRC and PLC genetic cascades [7,10].

FGFR1 promotes epithelial-mesenchymal transition (EMT) by activating the same pathways and favouring tissue remodelling, while upregulation leads to cell transformation and carcinogenesis [11-15].

To the growing number of targeted therapies routinely applied for adenocarcinomas therapy, it may be added recent comprehensive molecular/genomic characterization of squamous cell lung carcinomas for new potential targetable mutations [16].

FGFR1 is considered as a new potential target for specific tyrosin kinase inhibitors. Our study aimed to evaluate FGFR1 expression and gene copy number in adenocarcinomas, squamous cell lung carcinomas, pleomorphic carcinomas and adenosquamous carcinomas. EMT pathway was also explored in more complex lung carcinomas such as adenosquamous and pleomorphic carcinomas as well as adenocarcinomas, according to Vimentin and TTF1 expression, and in squamous cell carcinoma, joining CK7 expression. The purpose was to identify differences and/or similarities in between these groups of lung tumours in order to characterize the histological types for targeted therapy.

MATERIAL AND METHODS

Material

Tumour samples were selected from 76 bronchial pulmonary carcinomas submitted for surgical resection (34 adenocarcinomas, 24 squamous cell lung carcinomas, 10 pleomorphic carcinomas and 8 adenosquamous carcinomas).

Clinical and pathological features are summarized in Table 1. The median age at diagnosis was 65 years (range 40-87 years). 56 patients were male and 20 were female. 23 were non-smokers, 29 ex-smokers and 24 current smokers. Male gender and smokers were more prevalent in squamous cell lung cancer. Adenocarcinomas also demonstrated male predominance, but in this case were more non-smokers and ex-smokers. A predominance of males and smokers was registered for pleomorphic and adenosquamous carcinomas. Stages I and II were more prevalent. Metastases were more frequently diagnosed in lymph nodes.

Adenocarcinomas were classified in accordance to 2015 WHO criteria (concerning the main patterns and all represented patterns in decreasing percentage) and the following immunohistochemical combinations were considered as study sub-groups with positivity (pos) taken as over 75% tumoural cells: 1 – CK7 pos / TTF1 pos / Vimentine (Vim) pos: 14; 2 – CK7 pos / TTF1 pos / Vim negative (neg): 9; 3 – CK7 pos / TTF1 neg / Vim neg: 6; 4 – CK7 pos / TTF1neg / Vim pos: 5. The squamous cell carcinomas were separated into two groups according to CK7 expression: 1 – CK7 neg: 17; 2 – CK7 pos: 7, with CK5.6 expression in all cases. Pleomorphic carcinomas and adenosquamous carcinomas were diagnosed according to the 2015 WHO criteria [17].

Methods

At least two sections of each tumour were evaluated and the results were validated by two pathologists. Differentiation markers, such as CK7, TTF1, CK5.6 and Vim, were applied according to defined protocols (see Table 2).

FGFR1 protein expression was evaluated by immunohistochemistry applying the FGFR1 antibody. Endogenous peroxidase activity was quenched using 15 minutes of 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 FGFR1 (Polyclonal; Thermo Scientific) at a dilution of 1/75 for 30 minutes, 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 minutes, the slides were incubated with the 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 the manufacturer's instructions. Haematoxylin was used to counterstain the slides which were then dehydrated and mounted. In parallel, known positive (squamous cell carcinoma with keratin pearls) and negative controls were used.

The immunohistochemistry evaluation was undertaken by two experienced thoracic pathologists. The intensity of expression was registered in four grades: 0; 1+; 2+; and 3+. The percentage of positive cells was also registered, allowing a global score to be obtained by multiplying the intensity by the percentage of positivity. The results were then stratified into 4 categories: 0-10% - negative/without expression; 11-100% - low expression; 101-200% moderate expression; 201-300% - high expression.

FISH was carried out in FFPE using *FGFR1* (8p11) / SE 8 (D8Z1) (Kreatech, Leica Biosystems). Sections were deparaffinized in xylene 15 min and dehydrated in ethanol (2 x for 5 min). Sections were pre-treated using a Citrate buffer in a pressure cooker for 4 min, followed by 5 min in 2 X sodium citrate (SSC), 10 min in proteinase K solution at 37°C, washing in 2 X SSC for 5 min and dehydrating for 2 min in increasing concentrations of alcohol (70, 90 and 100%) after which they were air dried. Ten microliters were applied to the slides, overlaid with a coverslip, which was sealed with rubber cement. Slides were denatured for 8 min at 78°C and hybridized for at least 16 h at 37°C in a humidity chamber. They were then washed post – hybridization in 50% formamide and in PBS buffer at 46°C for 4 min and 2XSSC for 2 min and air-dried, and the counterstain DAPI 4',6-diamidino-2-phenylindole was applied.

The samples were analyzed using x100 oil immersion objective using a Nikon 80i fluorescence microscope with the appropriate filters. One hundred tumour cells were evaluated. A specimen was considered *FGFR1* positive ('amplified') if: (1) the *FGFR1*/CEN8 ratio was ≥2.0; (2) the average number of *FGFR1* signals per tumour cell nucleus was ≥6; (3) the percentage (%) of tumour cells with ≥15 *FGFR1* signals or large clusters was ≥10%; (4) the % of tumour cells with ≥5 *FGFR1* signals was ≥50%, with classifications 1 to 3 considered as a high-level amplification and classification 4 as a low-level amplification [18]. Two independent technicians and a pathologist applied the score.

RESULTS

In non-tumour tissue, FGFR1 expression was seen in respiratory epithelium; pneumocytes and stromal cells showed no or rare expression (figure 1).

We found significantly higher FGFR1 protein expression in all groups of tumours compared to non-tumour tissue (Table 3). Significantly higher expression was seen in ADC compared to SQC (p=0.0232). Significantly higher expression was also observed in pleomorphic carcinomas compared to SQC (p=0.0265). No significant differences in expression were found between adenocarcinomas and adenosquamous carcinomas (p= 0.158) and pleomorphic carcinomas (p=0.3190), between squamous cell carcinoma and adenosquamous carcinoma (p=0.9651), or between adenosquamous and pleomorphic carcinomas (p=0.1189) (Table 3).

When comparing FGFR1 expression according to squamous cell carcinomas with or without CK7 expression, we found no significant differences (p=0.2502). There were also no significant differences between adenocarcinomas with or without TTF1 expression (p=0.123) or according to Vimentin expression (p=0.301) (Table 3)

When immunohistochemical results were stratified according to two expression categories: 1+ and 2+ / 3+ cases, reflecting the intensity of expression, we found a tendency to higher expression in adenocarcinomas compared to squamous cell carcinomas (p=0.061), and significantly higher expression in pleomorphic carcinomas compared to adenocarcinomas (p=0.0212), squamous cell carcinomas (p=0.0004) and adenosquamous carcinomas (p=0.0015). No differences were observed between adenosquamous and adenocarcinomas (p=0.1122) and squamous cell carcinomas (p=1). No differences were also observed in squamous cell carcinomas according to CK7 expression (p=0.167), and in adenocarcinomas according to TTF1 (p=0.4653) and Vimentin (p=1) expressions (Table 4).

FGFR1 gene copy number, evaluated by FISH, identified 15 (19.7%) FISH-positive cases: 5 (14.7%) adenocarcinomas; 5 (20.8%) squamous cell carcinomas; 3 (30%) pleomorphic carcinomas; and 2 (25%) adenosquamous carcinomas (Table 5).

FGFR1 FISH-positive cases (amplification) were more frequent in squamous cell carcinoma compared to adenocarcinomas, but without significant differences. *FGFR1* FISH-positive cases were also identified in adenosquamous carcinomas (2/8 - 25%) and pleomorphic carcinomas (3/10 - 30%). Although amplification was more frequent in squamous cell carcinoma than in adenocarcinoma, no significant differences were identified according to histology (Table 6). There were no significant differences in *FGFR1* FISH results according to gender or smoking status, either for adenocarcinoma and squamous cell carcinoma (Table 7), though there was a

tendency for a higher frequency of *FGFR1* amplification in the adenocarcinomas of smoking patients.
No correlations were found between FGFR1 immunohistochemical expression and FISH results (Spearman Rank Correlation p=0.3323 and Linear Correlation p=0.681).

DISCUSSION

Cancer is driven by different types of genetic mutations, which need to be identified in order to understand tumour growth (oncogenic drivers), so that they can be targeted, bringing improvements in the survival rate [3-5]. Genetic driver events are infrequent and limited mostly to ADC and never smokers [3-5]. Squamous cell lung carcinoma, primarily a smoker's disease, still lacks genetic targets and more effective targeted therapies [6,19]. *FGFR1* and FGF have recently emerged as driving oncogenes, sufficient to drive tumour growth [20]. Alterations of the *FGFR* gene have been recognized in many epithelial malignancies (10%): oral squamous cell carcinoma, breast, gastric, bladder and ovarian carcinomas and in squamous cell carcinoma of the lung [6,21,22].
Several reports have shown high levels of FGFR1 protein expression in lung carcinomas [23,24], co-expression of FGF and FGFR1 [25-28], and *FGFR1* was found to be amplified in lung carcinomas [29]; *FGFR1* amplification and overexpression are a frequent event in lung squamous cell carcinoma [6]. It is described by some authors as an early event, even identified in squamous metaplasia and dysplasia [19,27]. However, there are conflicting results as some authors did not identify *FGFR1* amplification in T1 NSCLC [30]. While *FGFR1* is frequently amplified in NSCLC, mutations are absent or extremely rare in NSCLC (both SQC and ADC) [6,19]. So, the copy number increase appears to be responsible for the activations of the FGFR1 pathway [6,19]. We consider that the use of NSCLC terminology should be avoided because it is not the most appropriate way of classifying lung carcinomas in order to apply targeted therapies.
FGFR1 amplification is associated with a response to FGFR inhibitors [6,31]. Amplified cells showed robust phosphorylation of FGFR1 and their effectors molecules [6,31], where treatment with inhibitors resulted in less phosphorylation, less growth and apoptosis [6,31,32]. Inhibition of FGFR signalling pathway can be achieved by: dominant negative FGFR1; anti-FGF2 Antibodies; FGFR TKIs; and antisense DNA [23,24,33]. There are several phase I and II clinical trials evaluating FGFR1 inhibitors, such as BIBF 1120 (Boehringer-Ingelheim), TKI 258 (Novartis - Ponatinib), AZD 4547 (AstraZeneca) and BGJ 398 (Novartis). One of the inclusion criteria for

most of these trials was *FGFR1* amplification [31,34-36]. Therefore, evaluation of the *FGFR1* gene will gain a growing importance especially in squamous cell lung carcinoma patients.

MYC expression has been described as a predictive biomarker for FGFR1 TKIs in squamous cell lung carcinomas probably due to pro-apoptotic functions of MYC that facilitate FGFR-inhibitor-mediated cell death [37]. Tumour cells co-expressing MYC and *FGFR1* amplification were more sensitive to FGFR inhibition, suggesting that patients with both *FGFR1* and MYC alterations may benefit from FGFR inhibitor therapy [38].

We know that about 80% of patients harbouring *EGFR* mutations initially shows response to EGFR TKIs, and they will eventually relapse [39-41]. Acquired resistance is associated with mortality and morbidity [39-41]. The FGF-FGFR pathway is one of the important mechanisms of “intrinsic” EGFR-TKI resistance [33]. This could be explained by activation of PI3K –AKT and MEK-ERK pathways [42]. Some authors have investigated the presence of *FGFR1* amplification and *EGFR* mutations, which were considered as mutually exclusive [43].

In our study, higher FGFR1 protein expression was seen in tumours compared to non-tumour adjacent parenchyma / bronchial epithelium, demonstrating that, even in the absence of *FGFR1* gene copy number increase, the FGFR1 pathway could be implicated in lung carcinogenesis and tumour growth. Other authors have also found higher protein expression in lung carcinomas compared to non-tumour tissue [27]. Our results suggest that protein overexpression is frequent in lung carcinomas, with higher expression observed in adenocarcinomas and pleomorphic carcinomas compared to squamous cell lung carcinomas. No differences were found between adenocarcinomas, pleomorphic and adenosquamous carcinomas. Hence, in our results, squamous carcinomas showed lower immunohistochemical protein expression. However, Behrens *et al* did not find differences of FGFR1 protein expression between the carcinomas studied. Higher immunohistochemical expression in pleomorphic carcinomas compared to squamous cell carcinomas suggests that overexpression is important in this group of tumours, probably reflecting activation of the EMT pathway. As some authors have identified FGFR1 therapy efficacy in 3+ cases, we have also stratified our results according to this end-point [44]. Intense (3+) immunohistochemical expression was observed more frequently in adenocarcinomas compared to squamous carcinomas and in pleomorphic carcinomas compared to all the other types of carcinomas. These last results could reinforce the importance of FGFR1 in the activation of the EMT pathway. No differences were found between adenocarcinomas and adenosquamous carcinomas, reflecting the compound morphology of the latter and the carcinogenesis of both. Some squamous carcinomas also expressed CK7, reflecting a group of less differentiated tumours with some features overlapping with adenocarcinomas, without significant differences of FGFR1

expression and gene copy number, compared to the other squamous cell carcinomas studied. Between adenocarcinomas, no significant differences were found, with regard to the expression of TTT1 (TTF1 positive/TTF1 negative cases). Thus, our results suggest that TTF1 expression, reflecting cell origin, has probably no implications on FGFR1 pathway activation. Also, adenocarcinomas expressing Vimentin, associated with EMT phenotype in carcinomas where biological behaviour is not yet clarified, had no significant differences of either FGFR1 expression or gene copy number compared to Vimentin negative cases, suggesting that in adenocarcinomas FGFR1 pathway activation precedes or is independent of EMT activation.

In the future, it will be necessary to clearly define the predictive biomarkers for anti-FGFR1 treatment, as some authors have identified a response in tumours with *FGFR1* amplification and EGFR 3+ [44]. It is important to understand the predictive value of protein expression to be used as a biomarker, after clear cut-offs, in order to select the patients will benefit more.

We found *FGFR1* amplification in 19.7% (15) of the studied cases: 5 adenocarcinomas (14.7%), 5 squamous cell carcinomas (20.8%), 3 pleomorphic carcinomas (30%) and 2 adenosquamous carcinomas (25%). FISH positive cases were more frequent in squamous cell carcinomas, though without significant differences to adenocarcinomas. An Wa Seo *et al* have reported a lower rate in NSCLC (8.7%) and a higher rate in squamous cell carcinoma (18%) than in adenocarcinomas (3%) [45]. Sasaki *et al* showed that the *FGFR1* gene copy number was increased in 32 (32%) lung bronchial-pulmonary carcinoma patients, representing higher frequencies than in our results. There were also higher in squamous cell carcinomas (41.3%) than in the other histological types (14.3%) [43]. Cihoric *et al* have reported a global rate of 12.5%, significantly higher in squamous cell carcinomas (20.7%) compared to adenocarcinomas (2.2%) and large cell carcinomas (13%) [30]. Tran *et al* demonstrated squamous cell carcinomas as having an higher frequency of *FGFR1* amplification and large cell carcinomas and adenocarcinomas with lower rates [46]. Other authors have shown *FGFR1* amplification in squamous carcinomas varying according to the respective series: 10.5% (Kohler *et al*); 16% (Goke *et al*); 20% (Shildhaus *et al*); and 22% (Weiss *et al*) [6,18,47,48]. For adenocarcinomas, *FGFR1* amplification rates also varied according to the series: 4.7% (Kohler *et al*); 0 % (Shildhaus *et al*); rare cases (Weiss *et al*) [6,18,47]. In a review of 6 papers, *FGFR1* amplification ranged from 9 to 16% in squamous carcinoma and was found in a maximum of 32% for NSCLC [49].

As *FGFR1* amplification was also identified in adenosquamous and pleomorphic carcinomas, with no differences compared to the other histological sub-types, it seems that the FGFR pathway is also important in these tumours; hence, *FGFR1* amplification and protein expression should be performed in these tumours in order to select patients for targeted

therapy. Cihoric *et al* also have found *FGFR1* amplification in 16% of large cell carcinomas [30], as did Tran *et al* at lower rate [46]. In our work, we also found amplification in adenosquamous and pleomorphic carcinomas. Adenosquamous carcinomas showed pathological characteristics common to adenocarcinomas and squamous carcinomas [50,51]. Pleomorphic carcinomas were characterized when the squamous pattern, adenocarcinoma or large cell carcinoma also exhibited a spindle and/or giant cells in at least 10% of the tumour area [50]. These tumours share some characteristics of the EMT pathway. Behrens *et al* identified protein overexpression in bronchial-pulmonary carcinomas, without significant differences between histological subtypes [27]. In our results, we observed a tendency to higher expression in pleomorphic carcinomas in adenocarcinomas, when comparing to squamous cell carcinomas. Schultheis *et al* have also identified *FGFR1* amplification in 5.6% of small cell lung cancers small, without age, gender, staging, tobacco or overall survival associations [52]. According to these authors, *FGFR1* amplification testing should be considered in patients diagnosed with small-cell carcinomas [52].

We did not find any associations between FGFR1 expression / gene copy number and clinical parameters, such as gender and smoking habits. Like us, Tran *et al*, and Kohler *et al* found no correlations with clinical parameters [46,47]. Zhang *et al* found associations of *FGFR1* amplification with male gender and smoker status [44], while Cihoric *et al* reported a higher *FGFR1* amplification frequency in males (14.8%) and on smokers or ex-smokers [30]. Sasaki *et al* have demonstrated increased *FGFR1* copy number correlations with gender (higher frequency in males) and smoking status (higher frequency in smokers), in lung bronchial pulmonary carcinomas [43]. However, no correlations (with smoking status, gender, or tumour stage) were identified when considering only squamous cell carcinomas [43]. An Na Seo *et al* have identified *FGFR1* amplification significantly higher in male smokers [45]. We have, however, found a tendency to higher frequencies of *FGFR1* amplification in adenocarcinomas of smoking patients.

No correlations between FISH results (*FGFR1* amplification) and FGFR1 protein expression were found in our series. Similarly, An Na Seo *et al* found no correlation between *FGFR1* gene copy number and FGFR1 protein expression [45].

A meta-analysis (6 studies) revealed no association of *FGFR1* amplification with poorer survival [49]. Further studies will be needed to evaluate survival implications, as there are some conflicting studies: Volm *et al* demonstrated that high FGFR1 expression is associated with worse prognosis; Cihoric *et al* identified lower overall survival associated with *FGFR1* amplification in NSCLC; Tran *et al* demonstrated, on the other hand, a tendency for longer survival while Seo *et al* showed that *FGFR1* amplification was associated with lower overall

survival and disease-free survival in patients diagnosed with squamous cell carcinoma, though not significant in multivariate analysis [28,30,45,46,49].

As already mentioned, *FGFR1* amplification is an inclusion criteria for phase I and II clinical trials. However, according to our data and those of other series, amplification may not reflect overexpression. Thus, it seems reasonable that Clinical Trials should also evaluate the predictive value of both *FGFR1* gene copy number and protein expression, in order to definitively clarify which is the best predictive testing, knowing that some authors identified a response in tumours with *FGFR1* amplification and EGFR 3+ [44].

Some authors have also investigated *EGFR* mutations in their series, and *EGFR* mutations and *FGFR1* increased copy number appeared to be as mutually exclusive [43].

CONCLUSIONS

FGFR1 protein expression is present in the majority of the bronchial-pulmonary carcinomas, and is higher in ADC and Pleomorphic carcinomas. Higher expression in pleomorphic carcinomas may reflect the importance of FGFR1 controlling EMT pathways, as Vimentin expression is found in an indeterminate percentage of all histological types of lung carcinomas.

FGFR1 amplification is identified in ADC (14.7%), SQC (20.8%), adenosquamous carcinoma (25%) and pleomorphic carcinomas (30%), without significant differences.

Although *FGFR1* amplification has been observed more frequently in squamous cell carcinomas, these results and literature suggest that the other histological types harbouring *FGFR1* amplification, such as adenosquamous, pleomorphic carcinomas, adenocarcinomas and even small cell lung carcinoma, may benefit from targeted therapy.

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Table 1: Clinicopathologic characteristics distribution according to lung carcinomas histological subtype – WHO 2015.

CLINICOPATHOLOGIC CHARATERISTICS					
CHARACTHERISTIC	ALL PATIENTS No./ %	SQUAMOUS CARC.	ADENOCARCINOMA	ADENOSQUAMOUS	PLEOMORPHIC
NUMBER PATIENTS	**76**	**24 (31.6%)**	**34 (44.7%)**	**8 (10.6%)**	**10 (13.1%)**
AGE (YEARS)					
MEDIAN	**65**	64	63	67	**72**
RANGE	**40-87**	40-87	47-82	53-81	56-82
SEX					
MALE	**56 (73.7%)**	**22 (91.7%)**	**22 (64.7%)**	**5 (62.5%)**	**7 (70%)**
FEMALE	**20 (26.3%)**	2 (8.3%)	12 (35.3%)	3 (37.5%)	3 (30%)
SMOKING HISTORY					
NON-SMOKER	**23 (30.3%)**	3 (12.5%)	**14 (41.2%)**	**2 (25%)**	**4 (40%)**
EX-SMOKER	**29 (38.1%)**	8 (33.4%)	**11 (32.3%)**	**6 (75%)**	**4 (40%)**
SMOKER	**24 (31.6%)**	**13 (54.1%)**	9 (26.5%)	0 (0%)	2 (20%)
pT STAGE					
T1	**22 (28.9%)**	**8 (33.3%)**	**9 (26.5%)**	**4 (50%)**	1 (10%)
T2	**43 (56.6%)**	**12 (50%)**	**20 (58.8%)**	**4 (50%)**	**7 (70%)**
T3	6 (7.9%)	3 (12.5%)	1 (2.9%)	0 (0%)	**2 (20%)**
T4	1 (1.3%)	1 (4.1%)	0 (0%)	0(0%)	0 (0%)
pTNM STAGE					
IA	20 (26.3%)	7 (29.2%)	8 (23.5%)	4 (50%)	1 (10%)
IB	18 (23.7%)	4 (16.7%)	8 (23.5%)	2 (25%)	4 (40%)
IIA	13 (17.1%)	7 (29.2%)	3 (8.8%)	1 (12.5%)	2 (20%)
IIB	5 (6.6%)	1 (4.1%)	3 (8.8%)	0 (0%)	1 (10%)
IIIA	11 (14.5%)	2 (8.3%)	7 (20.6%)	0 (0%)	2 (20%)
IV	1 (1.3%)	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)
METASTASIS					
LYMPH NODE	**27 (35.5%)**	**11 (45.8%)**	**13 (38.2%)**	0 (0%)	**3 (30%)**
DISTANT	**3 (3.9%)**	0 (0%)	**1 (2.9%)**	**1 (12.5%)**	**1 (10%)**

Table 2: Immunohistochemistry method and antibodies applied.

Primary antibody	**Manufacture**	**Clone**	**Method**	**Positive control**	**Antigen retrieval**	**Dilution and incubation time**	**Staining Pattern**
CK7	DAKO	OV-TL12/30	LSAB	Lung	Pronase E (10', RT)	1:50, 30'	Cytoplasmic
TTF1	DAKO	8G7G3/1	LSAB	Small cell carcinoma	EDTA (Mw,40')	1:100, 60'	Nuclear
CK (5/6/18)	Novocastra	LP34	LSAB	Skin	Pronase E (10', RT)	1:100, 60'	Cytoplasmic
Vimentin	DAKO	Vim3B4	LSAB	Colon	Citrato (Mw,20')	1:200, 30'	Cytoplasmatic

LSAB - Labeled Streptavidin Biotin Method; Mw – microwave.

Table 3: Comparisions of FGFR1 protein expression between bronchial-pulmonary carcinomas and non-tumoural tissue. ANOVA results. ADC – Adenocarcinoma: SQC – Squamous cell carcinoma; ADSQC – Adenosquamous cell carcinoma; PLEOMC – Pleomorphic carcinoma

STATISTICS / Differences	**p VALUE**	**RESULTS**
ADC / Non tumour tissue	p<0.0001	Higher expression in ADC
SQC / Non tumour tissue	p<0.0001	Higher expression in SQC
ADSQC / Non tumour tissue	p=0.0078	Higher expression in ADSQC
PLEOMC / Non tumour tissue	p=0.002	Higher expression in PLEOMC
ADC / SQC	p=0.0232	Higher in ADC
ADC / ADSQC	p= 0.158	Not significant
ADC / PLEOMC	p=0.3190	Not significant
SQC / ADSQC	p=0.9651	Not significant
SQC / PLEOMC	p=0.0265	Higher in PLEOMC
ADSQC/ PLEOMC	p=0.1189	Not significant
ADC TTF1+ / ADC TTF1-	p=0.123	Not significant
ADC VIM+ / ADC VIM-	p=0.301	Not significant
SQC CK7-/SQC CK7+	p=0.2502	Not significant

ANOVA results. ADC – Adenocarcinoma: SQC – Squamous cell carcinoma; ADSQC – Adenosquamous cell carcinoma; PLEOMC – Pleomorphic carcinoma.

Table 4: Differences of FGFR1 protein expression between bronchial-pulmonary carcinomas, according to intensity of expression (1+ and 2+ / 3+ cases).

STATISTICS / Differences	VALUE p	RESULTS
ADC / SQC	p=0.061	Not quite significant
ADC / ADSQC	p=0.1122	Not significant
ADC / PLEOMC	p=0.0212	Significant higher in PLEOMC*
SQC / ADSQC	p=1	Not significant
SQC / PLEOMC	p=0.0004	Significant higher in PLEOMC*
ADSQC/ PLEOMC	p=0.0015	Significant higher in PLEOMC*
ADC TTF1+ / TTF1-	p=0.4653	Not significant
ADC VIM- / VIM+	p=1	Not significant
SQC CK7- / CK7+	p=0.167	Not significant

ADC – Adenocarcinoma: SQC – Squamous cell carcinoma; ADSQC – Adenosquamous cell carcinoma; PLEOMC – Pleomorphic carcinoma.

Table 5: FISH *FGFR1* results.

FISH *FGFR1* RESULTS					
CHARACTHERISTIC	All patients No. (%)	SQC.	ADC	ADSQC	PLEOMC
NUMBER OF CASES	76 (100%)	24 (31.6%)	34 (44.7%)	8 (10.6%)	10 (13.1%)
FISH RESULTS					
NEGATIVE	61 (80.3%)	19 (79.2%)	29 (85.3%)	6 (75%)	7 (70%)
POSITIVE	**15 (19.7%)**	**5 (20.8%)**	**5 (14.7%)**	**2 (25%)**	**3 (30%)**

ADC – Adenocarcinoma: SQC – Squamous cell carcinoma; ADSQC – Adenosquamous cell carcinoma; PLEOMC – Pleomorphic carcinoma.

Table 6: FISH *FGFR1* results according to histology.

FGFR1 FISH RESULTS		
STATISTICS / Differences	VALUE p	RESULTS
ADC / SQC	p=0.227	Not significant
ADC / ADSQC	p=0.289	Not significant
ADC / PLEOMC	p=0.188	Not significant
SQC / ADSQC	p=0.6464	Not significant
SQC / PLEOMC	p=0.281	Not significant
ADSQC/ PLEOMC	p=0.392	Not significant

Fischer exact test. ADC – Adenocarcinoma: SQC – Squamous cell carcinoma; ADSQC – Adenosquamous cell carcinoma; PLEOMC – Pleomorphic carcinoma.

Table 7: FISH *FGFR1* results according to gender and smoking status.

	FISH Negative	FISH Positive	Statistics
SQC			
Male	17 (70.8%)	5 (8.3%)	p=0.620
Female	2 (20.8%)	0 (0%)	
ADC			
Male	17 (50%)	5 (14.7%)	p=0.137
Female	12 (35.3%)	0 (0%)	
SQC			
Smoker (1)	10(41.7%)	3(12.5%)	1/2 - p= 1
Ex-smoker (2)	6(25%)	2(8.3%)	1/3 - p= 1
Non-smoker (3)	3(12.5%)	0(0%)	
ADC			
Smoker (1)	6(17.6%)	3(8.8%)	1/2 - p=0.074
Ex-smoker (2)	11 (32.3%)	0 (0%)	1/3 - p=0.343
Non-smoker (3)	12(35.3%)	2(5.9%)	

Fischer exact test. ADC – Adenocarcinoma: SQC – Squamous cell carcinoma.

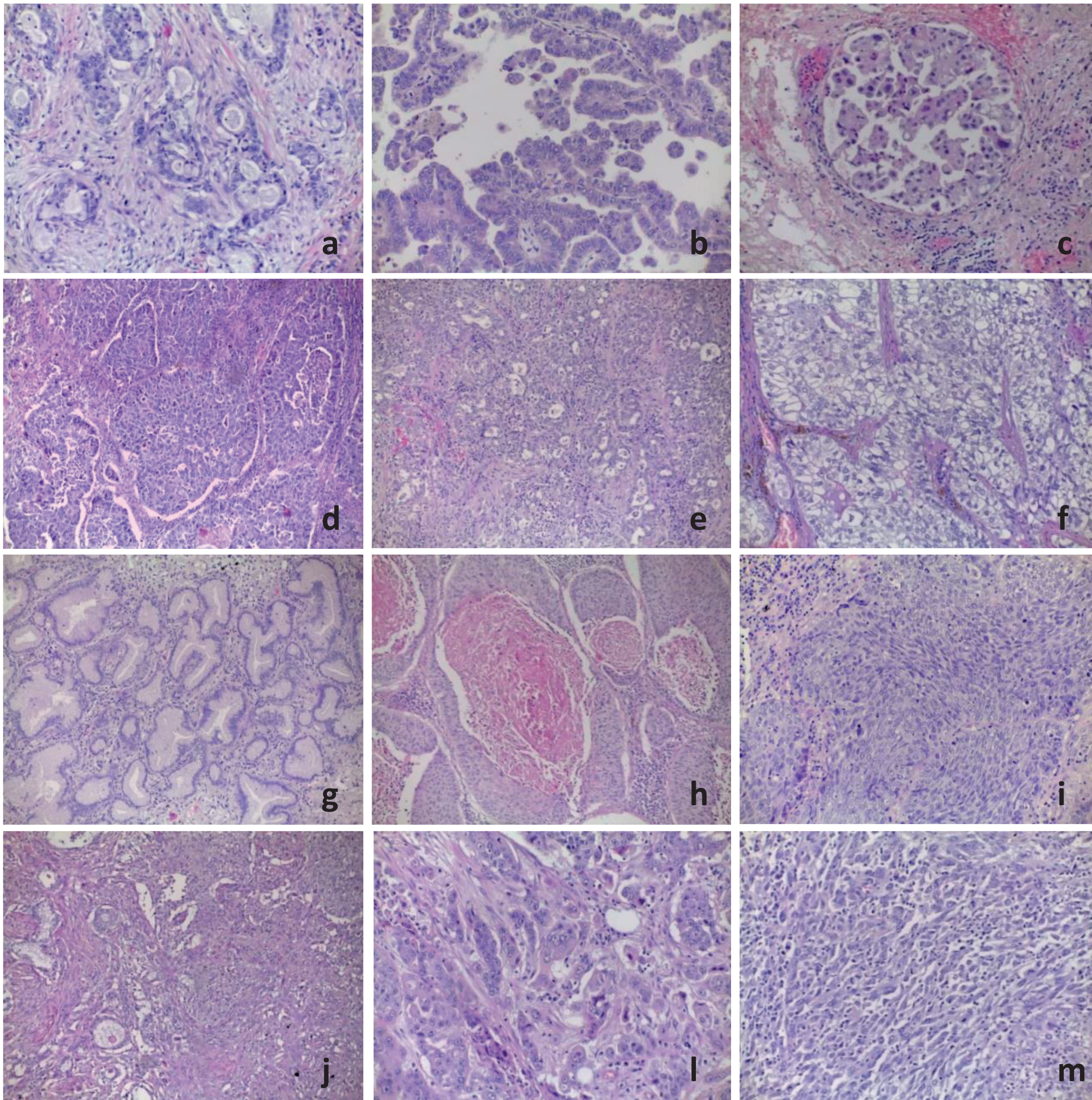

Figure 1: a - Adenocarcinoma (ADC) Vim positive (+) TTF1 negative (-), acinar pattern, HE, 200x; b - ADC Vim- TTF1-, papillary and micropapillary patterns, HE, 200x; c - ADC Vim+ TTF1+, micropapillary pattern, HE, 400x; d - ADC Vim- TTF1+, solid pattern, HE, 100x; e - ADC Vim- TTF1+ , cribriform pattern, HE, 100x; f - ADC Vim- TTF1-, solid with clear cells, HE, 200x; g - ADC Vim- TTF1-, mucinous pattern, HE, 100x; h – squamous cell carcinoma (SQC), CK7 +, HE, 100x; i – SQC, CK7-, HE, 200x; j – adenosquamous carcinoma, HE, 100x; l – pleomorphic carcinoma (PLEOMC) with giant cells, HE, 200x; m –PLEOMC with fusiform cells, HE, 200x.

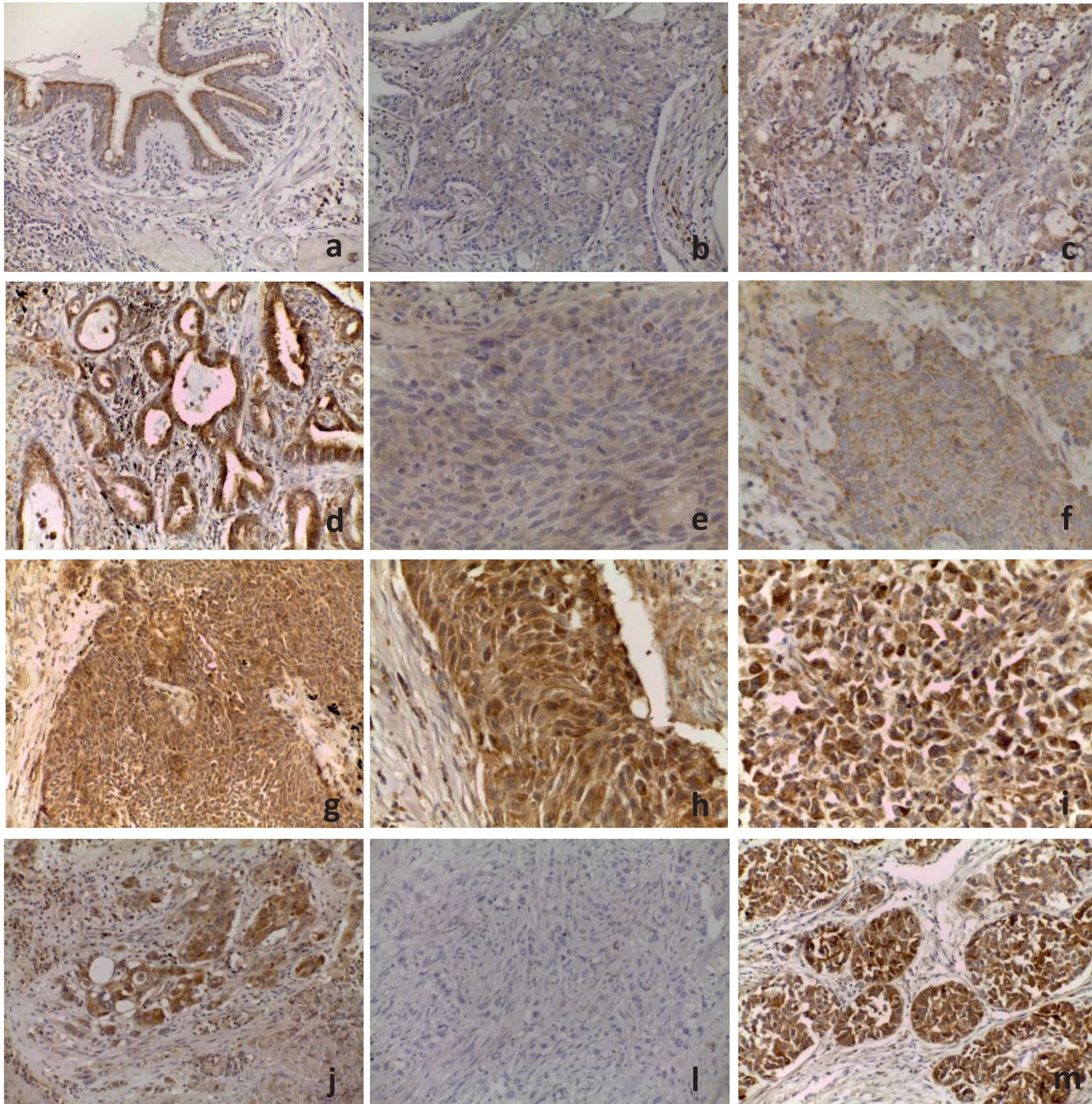

Figure 2: a – normal respiratory epithelium, FGFR1, 100x; b – adenocarcinoma (ADC) Vim- (negative) TTF1+ (positive), FGFR1 1+, 200x; c - ADC Vim+ TTF1-, FGFR1 2+, 200x; d - ADC TTF1+ Vim+, FGFR1 3+, 200x; e – squamous cell carcinoma (SQC) CK7 -, FGFR1 1+, 400x; f - SQC CK7 -, FGFR1 2+, 400x; g - SQC CK7 -, FGFR1 3+, 200x; h - SQC CK7 +, FGFR1 3+, 400x; i – pleomorphic carcinoma (PLEOMC), FGFR1 3+, 400x; j - PLEOMC, FGFR1 3+, 200x; l – adenosquamous carcinoma (ADSQC), FGFR1 -, 200x; m - ADSQC, FGFR1 3+, 200x.

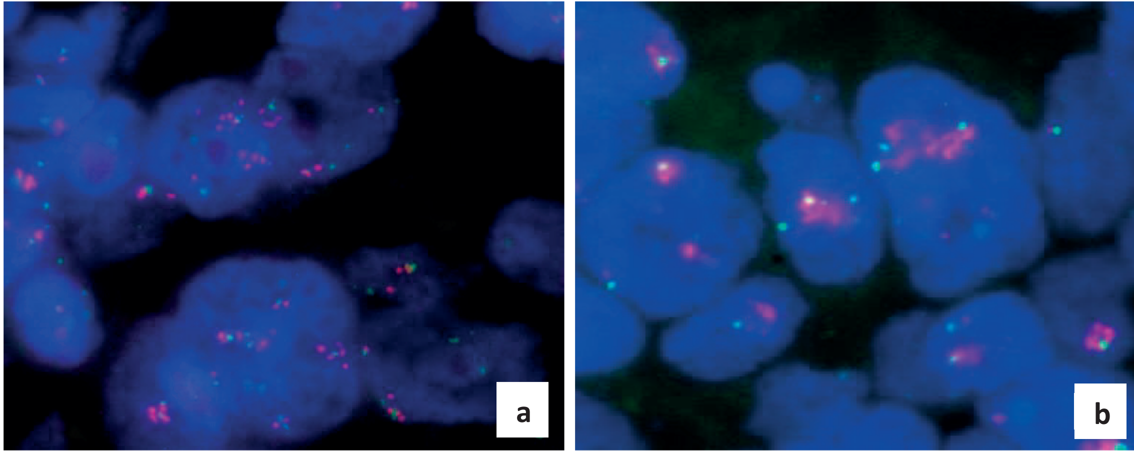

Figure 3: a - *FGFR1* amplification, *FGFR1* (red) / CEP 8 (green), adenocarcinoma, 1000x; b - *FGFR1* amplification, *FGFR1* (red) / CEP 8 (green), squamous cell carcinoma, 1000x.

CHAPTER 9 – ABSTRACTS PUBLICATIONS – Indexed Journals

CHAPTER 9 – Abstracts Publications – Indexed Journals

Abstract I (2010):

The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.

EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 AND P53 EXPRESSION IN PRENEOPLASTIC LESIONS OF BRONCHIAL EPITHELIUM

Lina Carvalho,[1-4]* Joana E. Santo,[1] Ana Alarcão,[1-2] Patricia Couceiro, [1-2] Maria R. Silva, [1-3] Ana Gomes,[2,4] Maria J. d'Aguiar,[1] Lia. Teixeira,[1] Vitor Sousa[1-4]

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ABSTRACT

A prognostic interpretation of preneoplastic lesions would have impact in bronchial carcinoma early diagnosis and through the study of Erb-B family receptors as they have an important role in lung carcinogenesis. The existence of drugs as tirosine kinase inhibitors (TKis) stressed the importance of studying gene alterations for selected chemoprevention schemes and characterization of carcinogenesis.

Bronchial preneoplastic lesions were characterized by immunohistochemistry using the antibodies LP34 (high weigh molecular cytokeratin), CK7, Chromogranin A, Ki67, p53, C-erbB-2 and EGFR. *HER2* and *EGFR* gene copy number was also evaluated by fluorescent *in situ* hybridization (FISH) in those lesions.

The expected results defined the origin cell for basal cell hyperplasia and squamous metaplasia as adaptative lesions and dysplasia. By known experiences and published data, beyond the

stem cell, the spectral evolution of bronchial preneoplastic lesions was demonstrated by characterizing basal cells (LP34) and their neoplastic potentiality.

Dysplasias showed a higher expression of EGFR, Ki67 and p53 with a stepwise increase with the gravity of the respective grading. C-erbB-2 immunohistochemical overexpression was a rare event in preneoplastic lesions. Polysomy was the main mechanism for *EGFR* and *HER2/neu* higher gene copy number and together with increased proliferation index (Ki67) will account to preview bronchial carcinogenesis.

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EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 AND P53 EXPRESSION IN PRENEOPLASTIC LESIONS OF BRONCHIAL EPITHELIUM

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INTRODUCTION

Lung cancer is the leading cause of death by malignancy in developed countries and throughout the world. Environmental and occupational exposures are important determinants of lung cancer risk, but cigarette smoking is the main risk factor, accounting for about 90% of the cases in men, 70% of the cases in women and with in today ex-smokers comprising nearly 50% of all new lung cancer cases. The overall 5-year survival rate for lung cancer patients remains less than 15% and the death rate for lung cancer exceeds the combined total for breast, prostate and colon cancer in developed countries. The major reasons for the poor prognosis for lung cancer are the lack of effective screening and early diagnosis procedures, the propensity for early metastasis and the inability of systemic therapies to cure patients with widely metastatic disease. Lung cancer is the result of a multi-step accumulation of genetic and/or epigenetic alterations, therefore a better understanding of the molecular mechanism by which these alterations affect lung cancer pathogenesis would provide new and more effective strategies for chemoprevention, early diagnosis and targeted treatment. The existence of novel drugs against specific molecular targets like EGFR and HER2/neu genes leads the importance of defining their expression as biomarkers in preneoplastic lesions.

A prognostic interpretation of preneoplastic lesions would have impact in bronchial carcinoma early diagnosis and through the study of Erb-B family receptors as they have an important role in lung carcinogenesis. The existence of drugs as tirosine kinase inhibitors (TKis) stressed the importance of studying gene alterations for selected chemoprevention schemes and characterization of carcinogenesis.

MATERIAL & METHODS

Material

67 bronchial biopsies were included in this study, comprising 89 preneoplastic lesions, 16 basal cell hyperplasia (BCH), 40 squamous cell metaplasia (SM) and 33 epidermoid dysplasia (7 mild dysplasia, 7 moderate dysplasia and 19 severe dysplasia/CIS). From each paraffin block, several cuts 5µm thick sections were made and then stained with H-E and reviewed by 2 Pathologists in order to ensure the optical criteria for preneoplastic bronchial lesions, according to the 1999/2004 histological WHO/IASLC classification of preinvasive squamous lesions of the bronchi .

Methods

Immunohistochemistry

The imunnohistochemical analysis included seven different antibodies: CK7 (Dako); LP34 (Dako); Chromogranin A (Dako); Ki67 (Dako); p53 (Dako); C-erbB-2 (Dako) and EGFR (Zymed Laboratories Inc.). The procedure was performed according to a standard avidin-biotin-peroxidase complex.

Fluorescence in situ hybridization (FISH)

Dual-color FISH assays for HER2 were performed by using Qbiogene HER2/neu (17q21)/Alphasatellite 17 dual color probe and for EGFR it was used the Vysis, Inc. USA LSI EGFR Spectrum Orange/CEP 7 Spectrum Green probe. Microscopic analysis was performed on a Nikon Eclipse 80i bright field and epifluorescent microscope equipped with LUCIA cytogenetics software. Six major FISH patterns were identified: disomy (≤ 2 gene copies per nucleus in > 90% of cells); low trisomy (≤ 2 gene copies per nucleus in ≥ 40% of cells, 3 gene copies in 10-40% of cells, ≥ 4 gene copies in < 10% of cells); high trisomy (≤ 2 gene copies per nucleus in ≥ 40% of cells, 3 gene copies in ≥ 40% of cells, ≥4 gene copies in < 10% of cells); low polysomy (≥4 gene copies per nucleus in 10-40% of cells); high polysomy (≥ 4 gene copies per nucleus in ≥ 40% of cells) and gene amplification (defined by presence of tight *EGFR* gene clusters and a ratio of *EGFR* gene to chromosome of ≥ 2 or ≥ 15 copies of *EGFR* per cell in ≥ 10 % of analyzed cells), according to a FISH scoring System defined by Cappuzzo *et al.* Amplification and high polysomy were considered as FISH positive results.

Statistical analysis

Comparisons were performed using bilateral Chi-squared tests or Fisher exact test when needed, because of the small number of lesions in some categories. Anova test was applied to compare the markers expression's percentage.

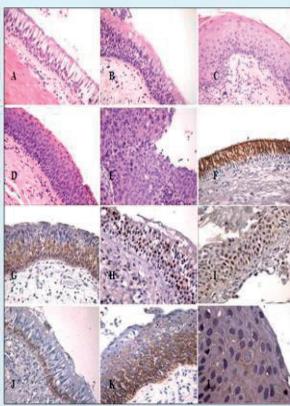


Figure 1: Morphology and immunostaining: A) Normal bronchial epithelium, H&E, 400x. B) Basal cell hyperplasia, H&E, 400x. C) Squamous metaplasia, H&E, 400x. D) Moderate dysplasia, H&E, 400x. E) *In situ* carcinoma, H&E, 400x. F) CK7 expression, normal bronchial epithelium, 400x. G) Basal cell hyperplasia, LP34, 400x. H) Ki67 3+ / 70%, 400x. I) P53 2+ / 50%, 400x. J) C-erbB-1 negative, normal epithelium, 400x C-erbB-1, 3+ / 60%, 400x. K) C-erbB-1, 3+ / 60%, 400x. L) C-erbB-2, 2+, 1000x .

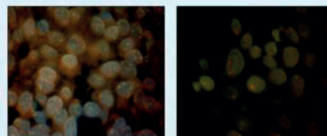


Figure 2: *EGFR* FISH positive (high polissomy) case in a severe dysplasia (1000x)

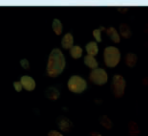


Figure 3: *EGFR* FISH positive (amplification) case in a severe dysplasia (1000x)

RESULTS

16 basal cell hyperplasia (BCH), 40 squamous cell metaplasia (SM) and 33 epidermoid dysplasia (7 mild dysplasia, 7 moderate dysplasia and 19 severe dysplasia/CIS) were diagnosed. In 22 cases there was concomitancy of the lesions. The population consisted in 57 men and 10 women, with 66,3 years as mean age (range 38-92).

The lesions showed LP34 positivity and CK7 negativity. (Table 1)

Significant differences of Ki67 expression were found between basal cell hyperplasia and squamous metaplasia group and dysplasia group cases ($p=0,001$). Ki67 expression was higher in dysplasia ($p=0,0007$). There was also found a stepwise increment of expression from basal cell hyperplasias, to squamous metaplasia and dysplasia ($p<0,0001$). The intense expression of Ki67 (intense positive cases) was shown to have a statistical significant increase from basal cell hyperplasia, to squamous metaplasia and dysplasia ($p= 0,0002$). There were no significant differences between the three grades of dysplasia.(Graphic 1) An increasing expression for p53 in all the three types of lesions was observed ($p<0,0001$). P53 expression was significantly higher in dysplasia when compared with basal cell hyperplasias and metaplasias ($p=0,0007$). Positive intense cases were also more frequent in dysplasia ($p=0,0001$).(Graphic 2)

EGFR expression was higher in dysplasias when compared with other preneoplastic lesions ($p= 0,009$). EGFR expression increased between the three groups of lesions ($p=0,0005$). The intensity of the expression was higher in the group of dysplasia. Statistical differences between the different grades of dysplasia were not found.

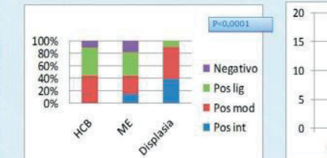
C-erbB-2 protein overexpression was clearly observed in only one severe dysplasia, without statistical differences between the three groups of lesions or according to the severity of the dysplasia ($p=0,14$). C-erbB-2 protein expression was less frequent than C-erB-1.

EGFR and *HER2* gene increased copy number was more often due to polisomy than to amplification. Among the 4 cases with intense EGFR protein expression, 3 had high polisomy and 1 showed disomy. The number of FISH EGFR positive cases were higher in the group of dysplasia ($p=0,0002$). Statistical significant correlation between EGFR IHC expression and FISH *EGFR* results was determined when the intensity of IHQ expression (positive intense vs non intense positive cases) was considered ($p=0,0092$). (Graphic 3-5)

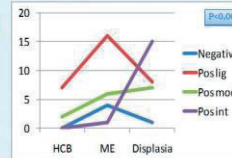
HER2 FISH positive cases (n=4) were due to high polisomy. In one case (n=1) was observed high trisomy. All high polisomy cases were identified in CIS cases and the high trisomy case was identified in a basal cell hyperplasia. These cases were IHC negative. The IHC positive C-erbB-2 case showed low trisomy and was considered FISH *HER2* negative.

Table 1 – LP34, CK7 and Chromogranin A expression in preneoplastic lesions

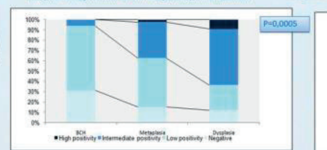
	LP34		CK7		CHROMOGRANIN A	
	Pos	Neg	Pos	Neg	Pos	Neg
Basal cell hyperplasia n=16	16 100%	0 0%	0 0%	16 100%	0 0%	16 100%
Squamous Metaplasia n=40	40 100%	0 0%	0 0%	40 100%	0 0%	40 100%
Dysplasia n=33	33 100%	0 0%	0 0%	33 100%	0 0%	33 100%



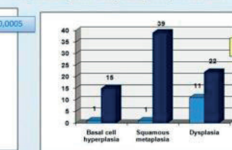
Graphic 1: Expression of Ki67 in preneoplastic lesions.



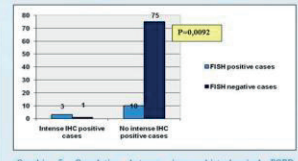
Graphic 2: Expression of p53 in preneoplastic lesions.



Graphic 3: Immunohistochemical EGFR expression between the preneoplastic lesions.



Graphic 4: *EGFR* FISH results according to preneoplastic lesions.



Graphic 5: Correlation between immunohistochemical EGFR expression and FISH results.

DISCUSSION / CONCLUSIONS

We conclude that squamous cell carcinoma preneoplastic lesions studied have a basal cell origin, as demonstrated by the unbalance between LP34 and CK7. The differentiation markers LP34, CK7 and Chromogranin A successfully discriminate preneoplastic lesions origin.

Dysplasias showed a higher expression of EGFR, Ki67 and p53 with a stepwise expression with the gravity of the preneoplastic lesions, reflecting their importance as potential biomarkers of preinvasive lesions of the bronchial epithelium and may be used to identify patients with an higher risk of developing squamous cell lung carcinoma.

C-erbB-2 immunohistochemical overexpression is a rare event in bronchial preneoplastic lesions, emphasizing the minor role of C-erbB-2 as a biomarker in squamous cell carcinogenesis.

EGFR and *HER2/neu* high gene copy number was present in preneoplastic lesions, with a higher *EGFR* gene copy number in dysplasias. *EGFR* and *HER2/neu* high gene copy number was due to polysomy more often than to amplification, reinforcing that amplification is not the main mechanism for protein overexpression. Although *HER2/neu* does not seem to be involved in the early steps of squamous cell lung carcinogenesis, *HER2/neu* gene copy number is important because this gene is a HER family member and an important heterodimerizarion partner for *EGFR*.

The need to accurate detection of the *EGFR* and *HER2/neu* gene copy number and protein overexpression, reflecting their importance as biomarkers in squamous cell pathogenesis, has become even more important because despite recent advances in lung cancer treatments, improvement in survival has only been modest, showing that effective therapeutic and early detection approaches are still lacking. Selection of patients with preneoplastic lesions expressing selected biomarkers is the way to provide new and more effective strategies for chemoprevention, early diagnosis and targeted treatment with better safety profiles. A variety of new approaches that target selected biomarkers in lung carcinogenesis are in clinical development or have already been approved for second and third line lung cancer treatment. Among those approaches are therapeutic agents such as small molecule tyrosine kinase inhibitors (TKis) and monoclonal antibodies (mAbs) specifically targeted against C-ErbB-1 and C-ErbB-2.Biological/genetic markers associated with several molecular and genetic abnormalities, such as Ki67, p53, EGFR and HER2/neu, need to be validated as potential and useful preneoplastic bronchial lesion biomarkers. Risk assessment should be based not only on smoking consumption and on histopathology of bronchial lesions, but also on the basis of in situ molecular biomarkers. Therefore, in future the recognition of those biomarkers will allow a targeted screening and a posterior closer follow-up to select patients for chemoprevention schemes and will have a positive impact on survival.

Figure 1: Poster I: EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 AND P53 EXPRESSION IN PRENEOPLASTIC LESIONS OF BRONCHIAL EPITHELIUM.

Abstract II (2010):

The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.

EGFR UNUSUAL MUTATION STATUS IN LUNG ADENOCARCINOMAS

Ana Alarcão,[1-2]* Vitor Sousa,[1-4] Patrícia Couceiro, [1-2] Maria Silva, [1-3] Maria J. d'Aguiar,[1] Lia Teixeira,[1] Lina Carvalho[1-4]

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* *Presenting author.*

ABSTRACT

Lung cancer is the most common cause of cancer deaths in both men and women. Adenocarcinoma represents about 28% of the NSCLC cases in men and 42% in women. EGFR is a member of the ERBB family of tyrosine kinases (TK). *EGFR* mutations are more frequently observed in female, non-smokers, East-Asian and in patients with adenocarcinomas, and predict response to TK Inhibitors (TKIs).

Sections of adenocarcinomas of the lung, formalin-fixed paraffin-embedded tissues (FFPE), were selected to analyze mutations in *EGFR* exons 19 and 21 by DNA extraction for polymerase chain reaction (PCR). Exon 19 was studied by fragment analysis and exon 21 was studied by direct sequencing. The analysis of FISH results was done by Cappuzzo's score to *EGFR* gene. Determination of EGFR protein expression was done by immunohistochemistry (IHC) (*Zymed*, Laboratories)

The author's present two cases of lung adenocarcinoma that harbours coexisting *EGFR* exon 19 and 21 mutations and one case of *EGFR* multiple in frame-deletions. The patients were female (n=3), with mixed type adenocarcinoma overexpressing EGFR by IHC.

Most reports demonstrate one *EGFR* mutation per adenocarcinoma. We demonstrated that a single adenocarcinoma can harbour more than one *EGFR* activating mutations.

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EGFR UNSUAL MUTATIONS STATUS IN LUNG ADENOCARCINOMA

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INTRODUCTION

Lung cancer is the most common cause of cancer deaths in both men and women. Adenocarcinoma represents about 28% of the NSCLC cases in men and 42% in women. EGFR is a member of the ERBB family of tyrosine kinases (TK). EGFR mutations are more frequently observed in female, non-smokers, East-Asian and in patients with adenocarcinomas, and predict response to TK Inhibitors (TKIs). Two types of mutations account for ~90% of mutated cases: short in-frame deletions in exon 19 and a point mutation, L858R, in exon 21.

MATERIAL & METHODS

Sections of adenocarcinomas of the lung, formalin-fixed paraffin-embedded tissues (FFPE), were selected to analyze mutations in EGFR exons 19 and 21 by DNA extraction for polymerase chain reaction (PCR). Exon 19 was studied by fragment analysis and exon 21 was studied by direct sequencing. The analysis of FISH results was done by Cappuzzo's score to EGFR gene. Determination of EGFR protein expression was done by immunohistochemistry (IHC) (Zymed, Laboratories).

RESULTS

Table 1 – EGFR analysis by IHC and FISH; Mutational status of EGFR and KRAS in Lung Adenocarcinoma

#	PATTERN	IHC		*EGFR*		*KRAS*	FISH-*EGFR*
		MEMBRANE	CYTOPLASM	EXON 19	EXON 21	EXON 2	
10635/08 1	ACINAR	-	+1/25%	WT/DEL	MUT	WT	HIGH POLYSSOMY
7450/07 1A	ACINAR	-	+1/25%	WT/DEL	WT	WT	-
	BA	-	+1/50%	WT/DEL	MUT	WT	-
	MICROPAP	-	+1/25%	WT/DEL	WT	WT	-
11008/08 1A	ACINAR	3+/50%	3+/50%	WT/DEL	WT	WT	HIGH POLYSSOMY
	SOLID	3+/75%	3+/75%	WT/DEL	WT	WT	HIGH POLYSSOMY
	MICROPAP	3+/50%	3+/50%	WT/DEL	WT	WT	HIGH POLYSSOMY

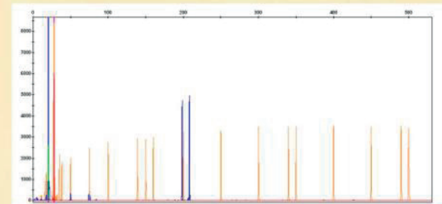

Fig. 1 – *EGFR* – Exon 19: In-frame deletions – 9bp by fragment analysis

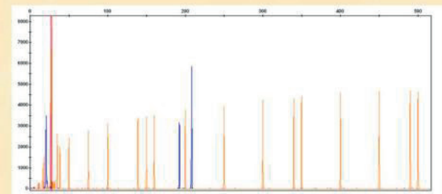

Fig. 2 – *EGFR* – Exon 19: In-frame deletions – 15bp by fragment analysis

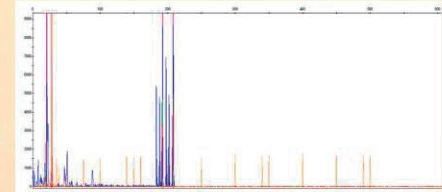

Fig. 3 – *EGFR* – Exon 19: Multiple in-frame deletions in acinar pattern of mixed type adenocarcinoma by fragment analysis

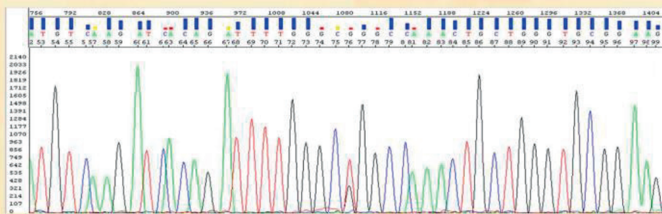

Fig. 4 – *EGFR* – Exon 21: Mutation L858R by direct sequencing

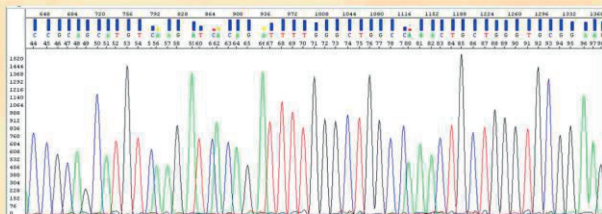

Fig. 5 – *EGFR* – Exon 21: Wild-type by direct sequencing

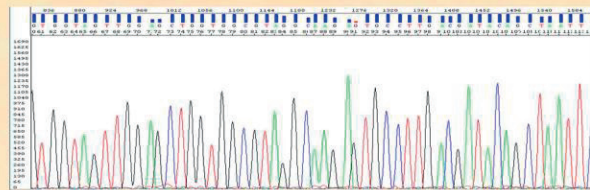

Fig. 6 – *KRAS* – Exon 2: Wild-type by direct sequencing

DISCUSSION / CONCLUSIONS

The authors present two cases of lung adenocarcinoma that harbours coexisting *EGFR* exon 19 and 21 mutations and one case of *EGFR* multiple in frame-deletions due probably to differences between clone cancer cell lines. The patients were female (n=3), with mixed type adenocarcinoma overexpressing EGFR by IHC. Most reports demonstrate one EGFR mutation per adenocarcinoma. We demonstrated that a single adenocarcinoma can harbour more than one *EGFR* activating mutations. The authors didn't found any published article making reference to synchronous *EGFR* mutations in more than these studied exons.

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Qiulu Pan, William Pao, and Marc Ladanyi (2005) Rapid Polymerase Chain Reaction-Based Detection of Epidermal Growth Factor Receptor Gene Mutations in Lung Adenocarcinomas. Journal of Molecular Diagnostics, 7:3, 396-403.

Figure 2: Poster II: *EGFR* UNUSUAL MUTATION STATUS IN LUNG ADENOCARCINOMAS.

Abstract III (2010):

The 16th International Charles Heidelberger Symposium on Cancer Research, Coimbra, Portugal, September 26-28, 2010.

***KRAS* AND *EGFR* MUTATIONS COEXISTING IN LUNG ADENOCARCINOMA**

Vitor Sousa,[1-4]* Ana Alarcão,[1-2] Patricia Couceiro, [1-2] Maria R. Silva, [1-3] Maria J. d'Aguiar,[1] Lia Teixeira,[1] Lina Carvalho[1-4]

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ABSTRACT

Lung adenocarcinoma represents about 42% and 28% of NSCLC in women and men. Adenocarcinomas incidence is still rising being the most frequent type of NSCLC diagnosed in USA. Both *EGFR* and *KRAS* gene mutations can contribute to the development of NSCLC, namely adenocarcinomas. *EGFR* and *KRAS* mutations are considered by some authors as mutually exclusive explained by the fact that KRAS-MAPK pathway is one of the downstream signalling pathways of EGFR. Lung cancers with *KRAS* mutations are resistant to EGFR tyrosine kinase inhibitors.

Sections of the adenocarcinoma of the lung, formalin-fixed paraffin-embedded tissues (FFPE), were selected to analyze mutations in *EGFR* exons 19 and 21 and *KRAS* - codons 12 and 13 by DNA extraction for polymerase chain reaction (PCR). Exon 19 was studied by fragment analysis and exon 21, codons 12 and 13 were studied by direct sequencing. The analysis of FISH results was done by Cappuzzo's score to *EGFR* gene. Determination of EGFR protein expression was done by immunohistochemistry (IHC) (*Zymed*, Laboratories)

The authors present a rare case with synchronous *EGFR* and *KRAS* mutations. The patient is a 77 years old, male with a central 3cm mixed adenocarcinoma. The tumour showed EGFR protein overexpression identified by IHC and chromosome 7 high polyssomy by FISH.The authors call attention to the fact that although *EGFR* and *KRAS* mutations are almost always mutually exclusive in some cases they may coexist in the same neoplasia.

BMC Proceedings 2010, 4(Suppl 2):P57

KRAS AND *EGFR* MUTATIONS COEXISTING IN LUNG ADENOCARCINOMA

Vitor Sousa,[1-4] Ana Alarcão,[1-2] Patrícia Couceiro,[1-2] Maria R. Silva,[1-3] Maria J. d'Aguiar,[1] Lia Teixeira,[1] Lina Carvalho[1-4]

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INTRODUCTION

Lung adenocarcinoma represents about 42% and 28% of Non-Small-Cell Lung Cancer (NSCLC) in women and men, respectively. Adenocarcinomas incidence is still rising being the most frequent type of NSCLC diagnosed in USA. Both *EGFR* and *KRAS* gene mutations can contribute to the development of NSCLC, namely adenocarcinomas. *EGFR* and *KRAS* mutations are considered by some authors as mutually exclusive explained by the fact that KRAS-MAPK pathway is one of the downstream signalling pathways of EGFR. Lung cancers with *KRAS* mutations are resistant to EGFR tyrosine kinase inhibitors.

Two types of mutations account for ~90% of mutated *EGFR* cases: short in-frame deletions in exon 19 and a point mutation, L858R, in exon 21. About 15-30% of NSCLCs harbour activating mutations in codons 12 and 13 of the *KRAS* gene and 15-20% of all NSCLCs, with a *KRAS* mutation at codon 12 being most frequent.

MATERIAL & METHODS

Sections of the adenocarcinoma of the lung, formalin-fixed paraffin-embedded tissues (FFPE), were selected to analyze mutations in *EGFR* exons 19 and 21 and *KRAS* - codons 12 and 13 by DNA extraction for polymerase chain reaction (PCR). Exon 19 was studied by fragment analysis and exon 21, codons 12 and 13 were studied by direct sequencing. The analysis of FISH results was done by Cappuzzo's score to *EGFR* gene. Determination of EGFR protein expression was done by immunohistochemistry (IHC) (Zymed, Laboratories).

RESULTS

The authors present a rare case with synchronous EGFR and KRAS mutations. The patient is a 77 years old, male with a central 3cm mixed adenocarcinoma. The tumor showed EGFR protein overexpression identified by IHC and chromosome 7 high polyssomy by FISH. Polyssomy is a frequent event in lung carcinomas, although the majority of studies indicate that EGFR and KRAS mutations do not coexist, there are some articles that reports cases with this rare event, as we do in this case.

Table I- EGFR analysis by IHC and FISH; Mutational status of *EGFR* and *KRAS* in Lung Adenocarcinoma

#	Pattern	IHC		*EGFR*		*KRAS*	FISH-*EGFR*
		Membrane	Cytoplasm	Exon 19	Exon 21	Exon 2	
5858/07	BA	-	2+/75%	Wt	Mut	G12V	High polyssomy
5858/07	Acinar	2+/10%	2+/75%	Wt	Mut	G12V	High polyssomy

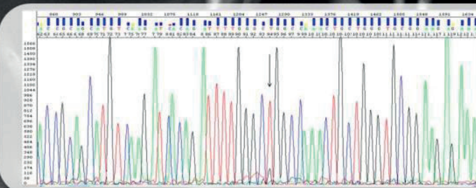

Fig. 1 – *EGFR* – Exon 21: Mutation L858R by direct sequencing

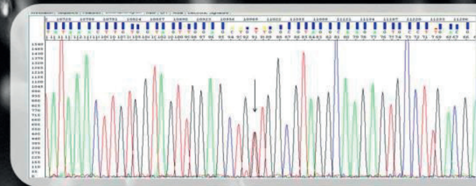

Fig. 2 – *KRAS* – Exon 2: Mutation G12V by direct sequencing

CONCLUSION

The authors call attention to the fact that although EGFR and KRAS mutations are almost always mutually exclusive in some cases they may coexist in the same neoplasia.

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- Yoshitaka Sekido, Kwun M. Fong, and John D. Minna (2003). MOLECULAR GENETICS OF LUNG CANCER. Annu. Rev. Med. 54:73–87. doi: 10.1146/annurev.med.54.101601.152202

Figure 3: Poster III: *KRAS* AND *EGFR* MUTATIONS COEXISTING IN LUNG ADENOCARCINOMA.

Abstract IV (2010):

Intercongress Meeting of the European Society of Pathology, Krakow – Poland, 31st August - 4th September, 2010.

EGFR and *KRAS* mutations in mixed type of Pulmonary Adencocarcinoma

Author's: Ana Alarcão[1;2], Patrícia Couceiro[1;2], Maria Silva[1;2], Maria João d'Aguiar[1], Lia Teixeira[1], Vitor Sousa[1-3], Lina Carvalho[1-3].

Institution: [1] Instituto de Anatomia Patológica – Faculdade de Medicina da Universidade de Coimbra, Coimbra, Portugal; [2] Centro de Investigação em Meio Ambiente, Genética e Oncobiologia, Coimbra, Portugal; [3] Serviço de Anatomia Patológica dos Hospitais da Universidade de Coimbra, Coimbra, Portugal.

Background:

The *EGFR* and *KRAS* genes act sequentially in the MAPK signaling pathway. The *KRAS* gene is located downstream in the transduction of signals transmitted from the transmembrane receptor EGFR to the nucleus through a series of intermediate genes. Patients with non-small-cell lung cancer (NSCLC) with *EGFR* mutations are more sensitive to treatment with tyrosine kinase inhibitors (TKIs) without *KRAS* mutations. The different patterns recognized in adenocarcinomas were microdissected to identify mutations in both *EGFR* and *KRAS* in order to clarify their significance when selecting patients either to TKIs.

Methods:

Material and methods: Histological sections of 31 adenocarcinomas of the lung, FFPE, were selected to analyze *EGFR* exons 19 and 21 mutations and *KRAS* - codons 12 and 13, DNA was extracted for polymerase chain reaction (PCR). Exon 19 was studied by fragment analysis, exon 21 and codons 12 and 13 were studied by direct sequencing.

Results:

From the 31 samples studied, 10/31 showed in-frame deletions, 4/20 L858R substitution in the *EGFR*, and 5/31 point mutations in codon 12 of *KRAS*. In all cases mutations were exclusive for *EGFR* and *KRAS* in alternative. Only one mixed adenocarcinoma showed *EGFR* in-frame deletions in bronchiolo-alveolar pattern and *Wt* in acinar pattern (*KRAS Wt*).

Conclusion:

In this set of mixed type adenocarcinomas, the different histological patterns revealed to be inconsequent for *KRAS* and *EGFR* determination of mutations, reinforcing the technical feasibility and reliability of small biopsies of lung cancer to determine personalized therapy.

Virchows Arch (2010) 457: 91-281

EGFR AND *KRAS* MUTATIONS IN MIXED TYPE OF PULMONARY ADENOCARCINOMA

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INTRODUCTION

The *EGFR* and *KRAS* genes act sequentially in the MAPK signaling pathway. The *KRAS* gene is located downstream in the transduction of signals transmitted from the transmembrane receptor *EGFR* to the nucleus through a series of intermediate genes. Patients with non-small-cell lung cancer (NSCLC) with *EGFR* mutations are more sensitive to treatment with tyrosine kinase inhibitors (TKIs) without *KRAS* mutations. The different patterns recognized in adenocarcinomas were microdissected to identify mutations in both *EGFR* and *KRAS* genes in order to clarify their significance when selecting patients either to TKIs.

MATERIAL AND METHODS

Histological sections of 31 adenocarcinomas of the lung, FFPE, were selected to analyze *EGFR* exons 19 and 21 mutations and *KRAS* - codons 12 and 13. DNA was extracted for polymerase chain reaction (PCR). Exon 19 was studied by fragment analysis, exon 21 and codons 12 and 13 were studied by direct sequencing.

RESULTS

From the 31 samples studied, 10/31 showed in-frame deletions, 4/20 L858R substitution in the *EGFR*, and 5/31 point mutations in codon 12 of *KRAS*. In all cases mutations were exclusive for *EGFR* and *KRAS* in alternative. Only one mixed adenocarcinoma showed *EGFR* in-frame deletions in bronchiolo-alveolar pattern and wild-type in acinar pattern (*KRAS* wild-type).

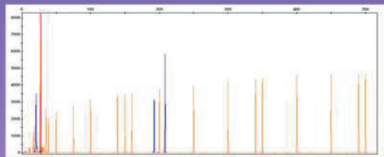
Fig. 1 – *EGFR* – Exon 19: in-frame deletions: Del 15bp/Wt by fragment analysis

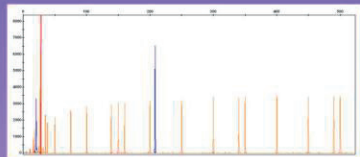
Fig. 2 – *EGFR* – Exon 19: Wt by fragment analysis

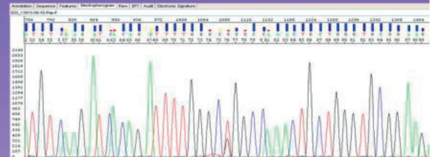
Fig. 3 – *EGFR* – Exon 21: Mutation L858R by direct sequencing

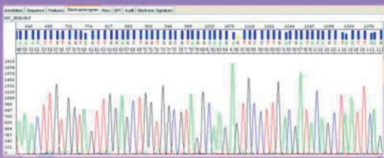
Fig. 4 – *KRAS* Wt by direct sequencing

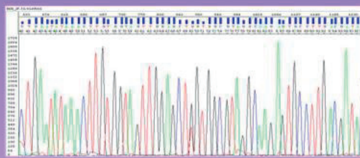
Fig. 5 – *KRAS* Mutation G12C by direct sequencing

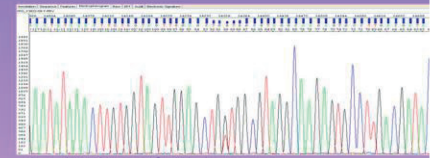
Fig. 6 – *KRAS* Mutation G12V by direct sequencing

DISCUSSION

Mixed type Adenocarcinoma	Acinar & BAC	Acinar & Solid	Acinar & Pappillary	Acinar & Pappillary & Solid	Acinar & BAC & Pappillary	Acinar & BAC & Clear Cell	Acinar & BAC & Micropappillary	Acinar & Micropappillary & Solid	Acinar & Micropappilary	Acinar
EGFR – Exon 19	3/8[A]	1/7		-	-	1/1[C]	1/1[D]	1/1	1/1	2/6[E]
EGFR – Exon 21	3/8[A&B]		2/3	-	-	1/1[C]	1/1[D]	-	-	1/6[E]
KRAS	4/8[B]	1/7	1/3	-	-	-	-	-	-	-

Table 1 – *EGFR* and *KRAS* mutations in mixed type adenocarcinoma
A – BAC: *EGFR* – Exon 19 & Acinar: *EGFR* – Exon 21
B – BAC: *EGFR* – Exon 21 + *KRAS* & Acinar: KRAS
C – BAC: *EGFR* – Exon 19 & Acinar: *EGFR* – Exon 21
D – Acinar & Micropaqppillary – *EGFR* Exon 19 & BAC – *EGFR* – Exons 19 & 21
E – 1 sample with mutations on *EGFR* – Exons 19 & 21 + 1 sample with mutation s on *EGFR* – Exon 19

CONCLUSION

In this set of mixed type adenocarcinomas, the different histological patterns revealed to be inconsequent for *KRAS* and *EGFR* determination of mutations, reinforcing the technical feasibility and reliability of small biopsies of lung cancer to determine personalized therapy.

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- A Kalikaki, A Koutsopoulos, M Trypaki, J Souglakos, E Stathopoulos, V Georgoulias, D Mavroudis and A Voutsina (2008). Comparison of EGFR and K-RAS gene status between primary tumours and corresponding metastases in NSCLC. British Journal of Cancer 99, 923–929;
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Figure 4: Poster IV: *EGFR* and *KRAS* mutations in mixed type of Pulmonary Adenocarcinoma.

Abstract V (2011):

23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

KRAS Mutations in Lung Adenocarcinomas may be Relevant in Histological Patterns, *EGFR* Mutational Status and Male Smokers

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Lung adenocarcinomas may show different patterns/subtypes and as *EGFR* and *KRAS* mutations rationalize personalized therapy, mutations were searched in all different patterns: acinar, papillary, micropapillary, solid and mucin producing, after immunohistochemistry characterization.

KRAS and *EGFR* mutations in 31 lung resected adenocarcinomas were studied by polymerase chain reaction (PCR), fragment analysis and direct sequencing.

There were 5/31 cases with *KRAS* point mutations, 3 of them G12V and 2 of them G12C; 26 cases were Wt. In *KRAS* mutated cases, 4 had no *EGFR* mutations and one case exhibited exon 21 *EGFR* point mutation in two patterns (BAC and Acinar) of the same mixed type adenocarcinoma.

No statistical relevance was found between exon 2 *KRAS* status and age (for WT and mutated) (p=0,401). *KRAS* mutation was more frequent in male (p=0,000) and in smokers (p=0.014).

EGFR mutations and *KRAS* mutations have been described as mutually exclusive but in this small series one case had *KRAS* and *EGFR* mutations simultaneously. Although *EGFR* and *KRAS* mutations are generally mutually exclusive, they can coexist and clinical, therapeutic and prognostic issues concerning male smoking patients become relevant as may direct personalized therapy.

Virchows Archiv 2011; Supplement 459: SI – S320. **IF: 2.491**

KRAS Mutations in Lung Adenocarcinomas may be Relevant in Histological Patterns, *EGFR* Mutational Status and Male Smokers

Lina Carvalho (1-4), Ana Alarcão (1-3), Patrícia Couceiro (1-2), Maria R Silva (1-3), M João d'Aguiar (1), Lia Teixeira (1), Teresa Ferreira (1), Vitor Sousa (1-4)

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Introduction

Lung adenocarcinoma represents about 42% and 28% of NSCLC in women and men. The incidence of lung adenocarcinomas is still rising, being in some countries the most frequent NSCLC.
EGFR and *KRAS* mutation have been described in lung adenocarcinomas and considered by some authors as mutually exclusive mutations. KRAS mutations may become relevant in bronchial-pulmonary adenocarcinomas in order to refine personalized therapy with tyrosine-kynase inhibitors. KRAS mutated adenocarcinomas are associated with poor response to anti-EGFR therapies. KRAS-MAPK pathway is one of the downstream signalling pathways of EGFR.
Two types of mutations account for ≈90% of mutated *EGFR* cases: short in-frame deletions in exon 19 and a point mutation, L858R, in exon 21. About 15-30% of NSCLCs harbour activating mutations in codons 12 and 13 of the *KRAS* gene and 15-20% of all NSCLCs, with a *KRAS* mutation at codon 12 being most frequent.
The authors intend to evaluate *EGFR* and *KRAS* mutations in lung adenocarcinomas patterns/subtypes (acinar, bronchiolo-alveolar, papillary, micropapillary, solid and mucinous patterns).

Objective

The authors intend to evaluate *EGFR* and *KRAS* mutations in lung adenocarcinomas patterns/subtypes (acinar, bronchiolo-alveolar, papillary, micropapillary, solid and mucinous patterns).

Material & Methods

Thirty one formalin-fixed paraffin-embedded (FFPE) lung adenocarcinomas were reviewed by Pathologists after clinical demanding, to tumour fragment selection in haematoxylin-eosin stains, as representative areas for analyze mutations in *EGFR* exons 19 and 21 and *KRAS* - codons 12 and 13. DNA extraction was performed for polymerase chain reaction (PCR) and exon 19 was studied by fragment analysis and exon 21, codons 12 and 13 were studied by direct sequencing.

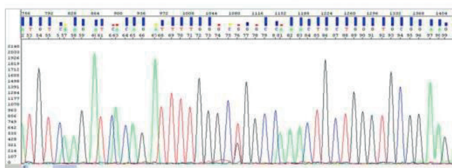

Fig. 1 – *EGFR* – Exon 21: Mutation L858R by direct sequencing

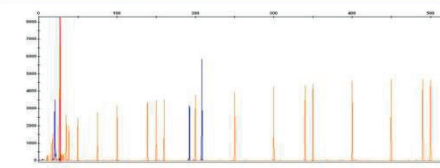

Fig. 3 – *EGFR* – Exon 19: in-frame deletion: Del 15bp/Wt by fragment analysis

Results

The authors identified 8/31 cases with L858R *EGFR* exon 21 point mutation; 10/31 cases with exon 19 in-frame deletion (9bp (3/31), 12bp (1/31), 15bp (4/31), 18pb (1/31)).
There were 5/31 cases with *KRAS* codon 12 point mutation, 3 of them G12V and 2 of them G12C; 26/31 cases were Wt. In *KRAS* mutated cases, 4/31 had no *EGFR* mutations and 1/31 case exhibited L858R *EGFR* exon 21 point mutation in two patterns (BAC and Acinar) of the same mixed type adenocarcinoma. No statistical relevance was found between exon 2 KRAS status and age (for wild-type and mutated gene) (p=0,401). KRAS mutation was more frequent in male (p=0,000) and in smokers (p=0.014).

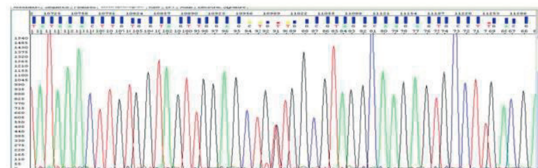

Fig. 2 – *KRAS*– Exon 2: Mutation G12V by direct sequencing

Conclusion

EGFR mutations and KRAS mutations have been described as mutually exclusive but in this small series one case had KRAS and EGFR mutations simultaneously. Although EGFR and KRAS mutations are generally mutually exclusive, they can coexist and clinical, therapeutic and prognostic issues concerning male smoking patients become relevant as may direct personalized therapy.

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Figure 5: Poster V: *KRAS* Mutations in Lung Adenocarcinomas may be Relevant in Histological Patterns, *EGFR* Mutational Status and Male Smokers.

Abstract VI (2011):

23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

TTF1 Negative Solid Pattern in Lung Adenocarcinomas May Reflect Morphogenesis

Lina Carvalho (1) (2) (3) (4), Ana Alarcão (1) (2), Patrícia Couceiro (1) (2), Maria R. Silva (1) (2) (3), M João d'Aguiar (1), Lia Teixeira (1), Teresa Ferreira (1), Vitor Sousa (1) (2) (3) (4)

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(4) Serviço de Anatomia Patológica dos Hospitais da Universidade de Coimbra, Coimbra, Portugal.

Lung adenocarcinomas are classified according with histological subtypes/patterns. Thyroid transcription factor-1 (TTF-1) is a tissue-specific transcriptional factor important in differentiation and morphogenesis of lung/thyroid/brain and was applied to classify pulmonary adenocarcinomas and also to evaluate its expression in the reported patterns.

In a series of 44 lung adenocarcinoma there were: 41 acinar, 5 papillary, 13 micropapillary, 14 solid, 25 bronchiolo-alveolar (BAC) and 3 mucinous patterns. Applyed immunohistochemistry panel was: TTF1, CK7 and Ki67.

TTF1 expression was higher in adenocarcinomas / normal lung (p=0.0009). Expression was greater in acinar (p=0.0014), BAC (p<0.0001) and micropapillary patterns (p=0,0019) than in normal parenchime. Mucinous pattern had lower expression / normal lung (p=0.02). There were no differences between normal lung and papillary (p=0.5074) and solid patterns (p=0.5659); 6/14 cases of solid pattern were TTF1 negative and expressed CK7 while Ki67 expression was higher in this TTF1 negative group when compared to the TTF1 positive (p=0.0015).

Decreased expression of TTF1 in some patterns identifies a small number of cases developing from pure CK7 positive cells (bronchial?). Solid pattern show lower nuclear expression of TTF1 and combined with higher expression of Ki67 in TTF1 negative cases may reflect different morphogenesis and more aggressive biological behaviour.

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TTF1 Negative Solid Pattern in Lung Adenocarcinomas May Reflect Morphogenesis

Lina Carvalho (1-4), A na Alarcão (1-3), Patrícia Couceiro (1-2), Maria R Silva (1-3), M João d'Aguiar (1), Lia Teixeira (1), Teresa Ferreira (1), Vitor Sousa (1-4)

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(4) Serviço de Anatomia Patológica dos Hospitais da Universidade de Coimbra, Coimbra, Portugal

Introduction

The more recent studies of pulmonary carcinogenesis give relevance to embryological pathways to understand lung carcinoma morphology and prognosis. Thyroid transcription factor-1 (TTF-1) is a tissue-specific transcriptional factor important in differentiation and morphogenesis of lung/thyroid/brain and was applied to classify pulmonary adenocarcinomas and also to evaluate its expression in the reported patterns of mixed type adenocarcinomas.

Objective

Authors aims to evaluate differentiation markers TTF1 and CK/ in lung adenocarcinomas according to the patterns and compare with the proliferative index (Ki67).

Material and methods

In a series of 44 lung adenocarcinoma there were: 41 acinar, 5 papillary, 13 micropapillary, 14 solid, 25 bronchiolo-alveolar (BAC) and 3 mucinous patterns. The applied immunohistochemistry panel was: TTF1, CK7 and Ki67. Statistical analysis was performed using the software GraphPad Instat 3.

Results

41 (93,18%) cases with acinar pattern, 5 (11,36%) papillary pattern, 13 (29,55%) micropapillary pattern, 14 (31,8%) solid pattern, 25 (56,81%) with non-mucinous bronchiolo-alveolar pattern (BAC) and 3 (6,8%) with mucinous pattern were identified. (Figure 1)

TTF1 expression was higher in adenocarcinomas when compared with normal lung (*p*=0.0005). (Figure 2)

Expression was greater in acinar (*p*=0.0014), BAC (*p*<0.0001) and micropapillary patterns (*p*=0,0019) than in normal parenchyma. (Figure 3)

Mucinous pattern had lower expression again when normal lung (*p*=0.02) was considered. (Figure 4)

There were no differences between normal lung and papillary (*p*=0.5074) and solid patterns (*p*=0.5659) expression of TTF1.

6/14 cases of solid pattern were TTF1 negative and expressed CK7 while Ki67 expression was higher in this TTF1 negative group when compared to the TTF1 positive (p=0.0015). (Figure 1-11)

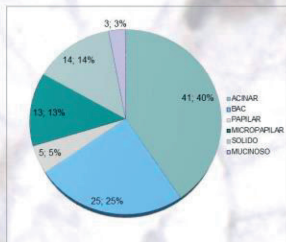

Figure 1 – Patterns observed in the adenocarcinomas .

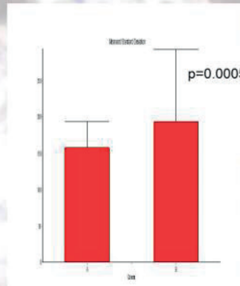

Figure 2 – TTF1 expression: Normal (A) versus Adenocarcinomas (B). Anova test – p=0.0005

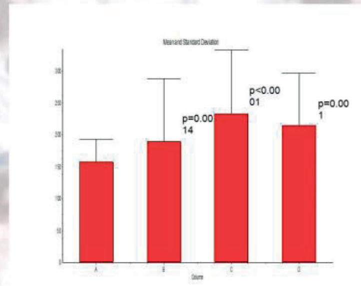

Figure 3 – TTF1 expression: Normal (A) versus acinar (B), BAC (C) and micropapillary patterns (D). Anova test – p=0.0014(A/B); p<0.0001(A/C); p=0.001(A/D)

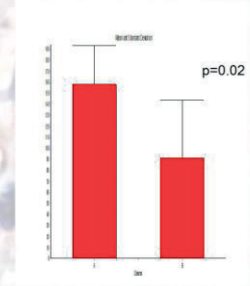

Figure 4 – TTF1 expression: Normal (A) versus Mucinous patterns (B). Anova test – p=0.02

Discussion and Conclusion

Decreased expression of TTF1 in some patterns identifies a small number of cases developing from pure CK7 positive cells. Solid pattern, showing lower nuclear expression of TTF1 and combined with higher expression of Ki67 in TTF1 negative cases, may reflect different morphogenesis and more aggressive biological behavior, possibly due to development after bronchial cylindrical cells that are TTF1 negative.

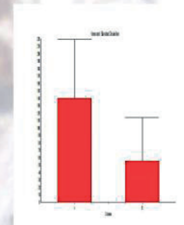

Figure 11 – Comparision of Ki67 expression between TTF1 negative and TTF1 positive adenocarcinomas. TTF1 negative (A) versus TTF1 positive (B). Mann-Whitney Test – p=0.0015

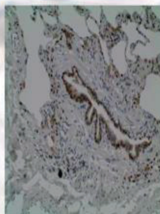

Figure 5 – TTF1 expression in normal lung tissues. TTF1, 100x

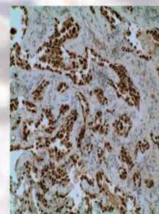

Figure 6 – TTF1 expression in acinar pattern. TTF1, 200x

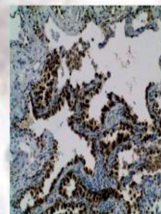

Figure 7 – TTF1 expression in BAC pattern. TTF1, 200x

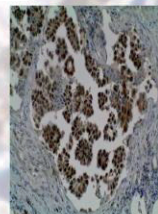

Figure 8 – TTF1 expression in micropapillary pattern. TTF1, 200x

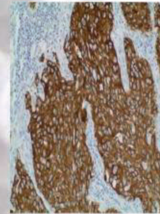

Figure 9 – CK7 expression in solid pattern. CK7, 200x

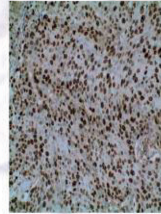

Figure 10 – Ki67 expression in solid pattern. Ki67, 200x

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Figure 6: Poster VI: TTF1 Negative Solid Pattern in Lung Adenocarcinomas May Reflect Morphogenesis.

Abstract VII (2011):

23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

Lung Adenocarcinomas Subtyping is not Relevant for *EGFR* Mutations, Gene Copy Number and Protein Expression

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Lung adenocarcinomas simple/mixed type show various (till six) patterns whose clinical relevance must be tested. A series of 44 resected adenocarcinomas were studied with c-erbB1 (clone 31G7), *EGFR* gene copy number by FISH and *EGFR* exons 19 and 21 sequenced.

Being mixed type Adenocarcinomas, the following patterns were characterized in at least two sections: 41 acinar, 5 papillary, 13 micropapillary, 14 solid, 25 with non-mucinous bronchiolo-alveolar (BAC) and 3 mucinous.

EGFR higher expression in solid pattern was relevant and lower in BAC pattern. *EGFR* mutational status was present in 8/31 cases with exon 21 point mutations and in 10/31 cases exon 19 deletions; 4 cases had coexisting exon 21 and exon 19 mutations. Concordance between FISH results ad each mutation type (exon 21 and 19) was considered fair (k=0.3702 and k=0.3841).

The concordance between mutations and immunohistochemistry expression and between FISH and IHC was not relevant (k=0.2556 and k=0.2635).

EGFR mutations are frequent and reflect their importance in lung adenocarcinomas pathogenesis. There is rough concordance with high gene copy number and in less extent with protein expression. Mutations were generally present in all the patterns of the same adenocarcinoma meaning that biopsies are prone to evaluation of *EGFR* mutational status.

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Lung Adenocarcinomas Subtyping is not Relevant for *EGFR* Mutations, Gene Copy Number and Protein Expression

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Introduction

Mixed type adenocarcinoma of the lung show various (till six) patterns whose clinical relevance has not been tested and their significance in small biopsies of patients in advanced stages determinates prognosis as the available tissue to define therapy.

Objective

A series of 44 resected adenocarcinomas were studied by immunohistochemistry (IHC) with c-erbB1 (clone 31G7), *EGFR* gene copy number by fluorescence in situ hybridization (FISH) and *EGFR* mutations - exons 19 and 21 by fragment analysis and direct sequencing, in order to verify different molecular behaviors of the present patterns.

Material and methods

Being mixed type Adenocarcinomas, the following patterns were characterized in at least two sections: 41 acinar, 5 papillary, 13 micropapillary, 14 solid, 25 with non-mucinous bronchiolo-alveolar (BAC) and 3 mucinous.

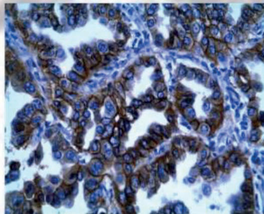

Figure 1 – Intense (3+) EGFR expression; acinar pattern. EGFR, 200x

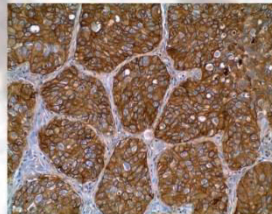

Figure 2 – Intense (3+) EGFR expression; solid pattern. EGFR, 200x.

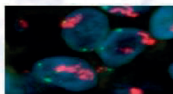

Figure 3 – Gene amplification; FISH positive result. EGFR/chromosome 7 centromer, FISH, 1000x

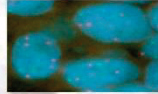

Figure 2 – Gene high polisomy; FISH positive result. EGFR/chromosome 7 centromer, FISH, 1000x

	EXON 19 EGFR MUTATION	EXON 21 EGFR MUTATION	FISH RESULT
Case 1	WT (acinar and BAC)	Point mutation (acinar and BAC)	Positive – HP
Case 8	Del9pb (acinar)	Point mutation (acinar)	Positive – HP
Case 9	Del9, 15, 18pb (acinar, solid, micropapillary)	WT (acinar, solid, micropapillary)	Positive – HP
Case 10	Del 9pb (acinar)	WT (acinar)	Negative – DIS
Case 13	Del 15pb (acinar and solid)	WT (acinar and solid)	Positive – AMP
Case 15	WT (acinar and papillary)	Point mutation (acinar and papillary)	Positive – AMP
Case 17	Del12pb (acinar, BAC and micropapillary)	WT (acinar ,BAC and micropapillary)	Negative – DIS
Case 19	WT (acinar and papillary)	Point mutation (acinar and papillary)	Negative – DIS
Case 20	Del15 pb (acinar and micropapillary)	WT (acinar and micropapillary)	Positive – HP
Case 21	Del 15 pb (acinar and BAC)	WT (acinar and BAC)	Positive – HP
Case 24	WT (acinar and BAC)	Point mutation (acinar and BAC)	Positive – HP
Case 25	Del 9 pb (acinar, BAC and micropapillary)	Point mutation (acinar, BAC and micropapillary)	Positive – HP
Case 27	Del 18 pb (BAC) (acinar not determined.)	Point mutation (acinar) (BAC WT)	Negative – DIS
Case 31	Del 9pb (BAC) (acinar WT)	Point mutation (acinar) (BAC WT)	Positive – HP
Case 3	WT	WT	Positive – HP
Case 7	WT	WT	Positive – HP
Case 29	WT	WT	Positive – HP

Cases with EGFR mutations and / or FISH positive. WT – Wild type; HP – High polisomy; AMP – amplification; DIS – Disomy.

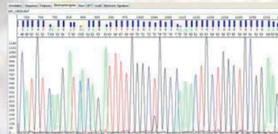

Figura 5: Exon 21 *EGFR* mutation (L858R) do gene *EGFR*.

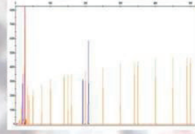

Figure 6 – *EGFR* (exon 19) – Del 15pb/Wt, fragment analysis.

Results

41 (93,18%) cases with acinar pattern, 5 (11,36%) papillary pattern, 13 (29,55%) micropapillary pattern, 14 (31,8%) solid pattern, 25 (56,81%) with non-mucinous bronchiolo-alveolar pattern (BAC) and 3 (6,8%) with mucinous pattern were identified.

EGFR membranar expression is higher in adenocarcinomas when compared to normal tissue ($p=0{,}0018$). Solid patterns showed higher expression when compared to BA pattern. We did not found any difference between the other patterns.

We have identified 8 cases of the 31 adenocarcinomas with exon 21 point mutations, in 6 cases, the mutation was present in all the patterns of the adenocarcinoma, in 2 cases the mutation was present in the acinar pattern and the other patterns were WT (BAC and solid and BAC patterns respectively).

10 cases showed exon 19 deletions, 7 present in all patterns of the neoplasic, in one case was present in BAC pattern being acinar pattern WT. In 3 cases the tumor had only one pattern (2 acinar; 1 BA).

Deletion of 9pb (del 9pb) was present in 3 cases, del 15pb (del 15pb) in 4 cases, del 18pb in 1 cases and del 12pb in 1case.

One singular case showed 3 types of deletion, namely del 9pb, 15 and 18pb in the acinar pattern and del of 15pb in solid and micropapillary patterns.

In 4 cases we identified coexisting exon 21 point mutations and exon 19 deletion. In two of these cases the mutation were present in the same patterns (acinar, BAC and solid). In two cases the mutations were present in different patterns (del 9pb in BAC pattern and exon 21 mutation in the acinar pattern).

We have identified 13 FISH positive cases and 18 FISH negative cases. The 13 FISH positive cases, 3 were EGFR WT and 10 had EGFR mutation. On the other hand of the 18 FISH negative cases, 14 were EGFR WT and 4 had EGFR mutations with concordance K Test of $k=0{,}5412$ (moderate agreement).

When considering the K Test for concordance between FISH results ad each mutation type (Exon 21 and 19) the concordance was only considered as fair ($k=0{,}3702$ and $k=0{,}3841$ respectively).

The concordance (K Test) between mutational status and immunohistochemistry (IHC) expression (positive/negative) was considered fair ($k=0{,}2556$). The concordance (K Test) between FISH status and IHC results was also considered as fair ($k=0{,}2635$).

There was moderate agreement/concordance between FISH and mutational status.

Only fair agreement was identified between protein expression and FISH results and mutational status.

In general, when a mutation is identified, it is present in all patterns of the same adenocarcinoma.

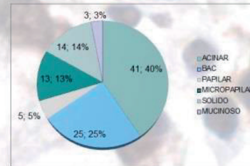

Figure 3 – Patterns observed in the adenocarcinomas .

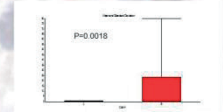

Figure 4 – Comparison of EGFR expression between normal (A) and adenocarcinomas tissues (B). Mann-Whitney ANOVA test. p=0.0018

Discussion and Conclusion

EGFR mutations are frequent and reflect their importance in lung adenocarcinomas pathogenesis. There is rough concordance with high gene copy number and in less extent with protein expression. Mutations were generally present in all the patterns of the same adenocarcinoma meaning that biopsies are prone to evaluation of *EGFR* mutational status.

	EGFR WT	*EGFR* MUT	TOTAL
FISH -	14	4	18
FISH +	3	10	13
TOTAL	17	14	31

K=0.5412

Concordance between FISH and mutational status. Moderate concordance (K=0.5412)

	IHC -	IHC +	TOTAL
FISH -	13	5	18
FISH +	6	7	13
TOTAL	19	12	31

K=0.2635

Concordance between FISH and IHC. Fair concordance (K=0.2635)

	EGFR WT	*EGFR* MUT	TOTAL
IHC -	11	6	17
IHC +	6	8	14
TOTAL	17	14	31

K=0.2556

Concordance between IHC and mutational status. Fair concordance (K=0.2635)

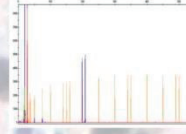

Figure 7 – *EGFR* (exon 19) – Del 9pb/Wt, fragment analysis.

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Figure 7: Poster VII: Lung Adenocarcinomas Subtyping is not Relevant for *EGFR* Mutations, Gene Copy Number and Protein Expression.

Abstract VIII (2011):

23rd European Congress of Pathology, Helsinki August 27– September 01 – 2011.

ALK Gene Rearrangment in EGFR and KRAS Positive Lung Adenocarcinomas

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Lung cancer is still the leading cause-related of death worldwide and adenocarcinomas harbour genetic changes to be further classified into clinical relevant molecular subsets. Tumours sensitive to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors often contain somatic mutations in the tyrosine domain of EGFR. While other tumours exhibit somatic mutations in KRAS which display primary resistance to those drugs. Another mutation is anaplastic large cell kinase gene (ALK) that is rearranged in approximately 5% of lung adenocarcinomas, indicates ALK inhibitors to be prescribed when EGFR and KRAS are wildtype.

We tested 19 lung adenocarcinomas for ALK rearrangments by fluorescent in-situ hybridization (FISH) and immunohistochemistry (IHC), 14 cases harboured EGFR mutations and 5 KRAS mutations.

Both techniques independently identified negative cases 17/19 (89%), except for one positive FISH and IHC case 1/19 (5%), with ALK rearranged gene (50 year old women). One FISH case was inconclusive.

Our findings in this subset population of EGFR and KRAS mutation positive adenocarcinomas of 5% ALK rearrangement suggests that with the emergence of moleculary-targeted therapies, it is reasonable to assume that all adenocarcinomas should be screened for ALK rearrangments, by dual testing IHC and FISH, to ensure accurate diagnosis.

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ALK Gene Rearrangment in EGFR and KRAS Positive Lung Adenocarcinomas

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Introduction

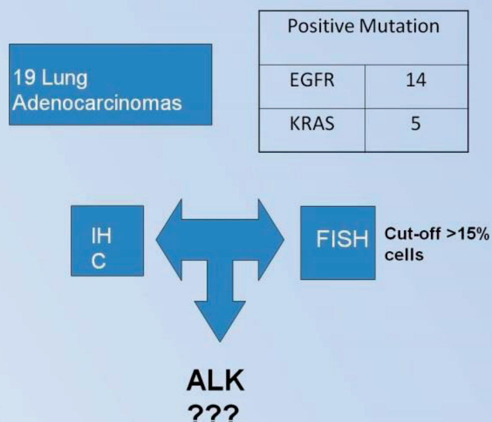
Adenocarcinomas of the lung are under personalized therapy targets because of genetic changes clinically relevant to clarify molecular subsets. Activation of the mutation in EGFR defines a group of patients with sensitivity to the chemical inhibitor for the kinase activity of EGFR, accounting for about 10% of primary lung cancers. While other tumors exhibit somatic mutations in KRAS which display primary resistance to those drugs. In 2007, Soda et al identified the fusion oncogene joining the echinoderm microtubule-associated protein-like-4 (EML4) and the anaplastic lymphoma kinase gene (ALK) in non-small-cell lung cancer (NSCLC).[1] The detection of EML4-ALK-positive lung cancer is needed to identify lung cancer patients for molecular target therapy.

The frequency of EML4-ALK translocation ranges from 3% to 7% in unselected. Similar to EGFR mutations, the frequency of EML4-ALK fusions is increased in people with adenocarcinomas, in young adult patients, and in people who have never smoked (<100 cigarettes in a lifetime) or who are light smokers (≤15 pack-years).

A series of pulmonary adenocarcinomas was submitted to a selected study in order to isolate the different patterns by macrodissection and then determine EGFR and KRAS mutation status and ALK gene rearrangement by FISH, in each section with different morphology.

Materials and Methods

Nineteen cases of lung adenocarcinomas from University Hospital of Coimbra were selected: 14 cases were EGFR mutation positive (exon 19, 21) , by PCR - direct sequencing, and fragment analysis by capillary electrophoresis and five cases had KRAS mutation (exon 2) positive by PCR-direct sequencing.



Results

Both techniques independently identified negative cases 17/19 (89%), except for one positive FISH and IHC case 1/19 (5%), with ALK rearraged gene , an 50 year old women, histology pattern acinar, with a gene copy number increase together with classic rearrangement. One FISH case was inconclusive, but negative for IHC.

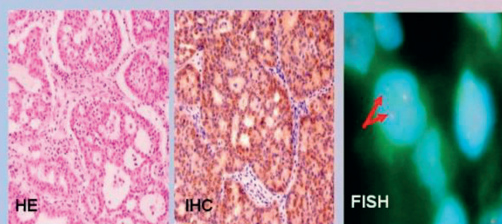
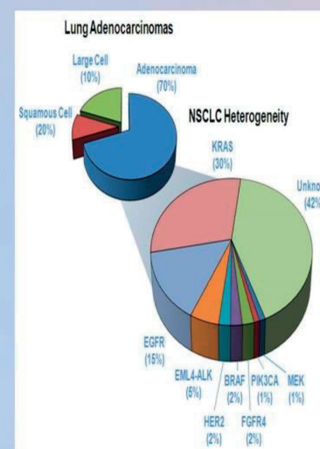


Figure 1. Representative images of , acinar/microacinar adenorcarcinoma: HE X200 , ALK IHC , ALK FISH.

	ALK - FISH POSITIVE	ALK -FISH NEGATIVE	ALK -IHC POSITIVE	ALK -IHC NEGATIVE
EGFR MUTATION POSITIVE (14)	1	13	1	13
KRAS MUTATION POSITIVE (5)	0	5	0	5

Conclusions

We performed this study to evaluate ALK+ in mutated EGFR and KRAS adenocarcinomas. Only one case was ALK+ (5%). With the emergence of molecularly-targeted therapies anti-ALK it is reasonable to assume that all adenocarcinomas should be screened for ALK rearrangements, by FISH as a possible gold standard after IHC, to ensure accurate diagnosis.



References

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(2)Tavazoie SF, Alarcón C, Oskarsson T *et al.* (2008) *Endogenous human microRNAs that suppress breast cancer metastasis.* Nature, 451: 147-152.

[2] Baranwal S and Alahari SK (2010) *miRNA control of tumor cell invasion and metastasis.* Int. J. Cancer: 126, 1283–1290.

[3] Meister J and Schmidt MHH (2010) *miR-126 and miR-126*: New players in Cancer.* TheScientificWorldJOURNAL: *0, 2090–2100.*

Figure 8: Poster VIII: *ALK* Gene Rearrangment in EGFR and KRAS Positive Lung Adenocarcinomas.

Abstract IX (2012):

24thEuropean Congress of Pathology, Prague, September 8 – 12, 2012

ALK EXPRESSION IN PULMONARY ADENOCARCINOMAS

Lina Carvalho (1) (2) (3) (4), Ana Alarcão (1) (2) (3), Ana Filipa Ladeirinha (1), Maria Reis Silva (1) (2) (3), M João d'Aguiar (1), Teresa Ferreira (1), Vitor Sousa (1) (2) (3) (4)

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EML4-ALK crizotinib therapy needs validation at lower cost and rapid answer in Pathology routine.

Histological/WHO 2004 and CK7, TTF1, CK5.6, CD56/chromogranin and vimentin panel classifications with ALK (clone 5A4, Novocastra Laboratories Ltd, Newcastle, United Kingdom) were applied to paraffin sections of 35 bronchial-pulmonary carcinomas: 20 adenocarcinomas, 6 epidermoid carcinomas, 4 pleomorphic carcinomas (mixed type adenocarcinomas with large/giant/fusiform cells), 4 neuroendocrine carcinomas (NEC) (1 combined large cell NEC with adenocarcinoma and 2 with epidermoid carcinomas; 1 SCLC chromogranin positive combined with adenocarcinoma) and 1 adenosquamous carcinoma.

The applied antibodies specified bronchial pulmonary carcinomas subtypes clearly. In 3 over 60 years old non-smoking females mixed type adenocarcinomas ALK expression was over 50%: acinar, solid, micropapillary and microacinar patterns; one glandular mucinous pattern (mucinous BA pattern) and one BA pattern, all expressing TTF-1.

In this study, 3/20 adenocarcinomas of older women had ALK protein expression, only one with a mucinous pattern. As protein positivity cases comprise a lower number, FISH described by S. Lantuejoul seems to be the most appropriate method. It is now necessary to decide whether *KRAS* and *EGFR* mutations have to be determined together and/or select TTF-1 positive adenocarcinomas (from terminal respiratory unit) raised by this approach.

Virchows Arch (2012) 461 : S1-S332. IF: 2.676

ALK EXPRESSION IN PULMONARY ADENOCARCINOMAS

LINA CARVALHO (1) (2) (3) (4), ANA ALARCÃO (1) (2) (3), ANA FILIPA LADEIRINHA (1), MARIA REIS SILVA (1) (2) (3), M JOÃO D'AGUIAR (1), TERESA FERREIRA (1), VITOR SOUSA (1) (2) (3) (4)

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(4) SERVICE OF PATHOLOGY, UNIVERSITY HOSPITAL OF COIMBRA, COIMBRA, PORTUGAL

INTRODUCTION

EML4-ALK crizotinib therapy needs validation at lower cost and rapid answer in Pathology routine.
Histological/WHO 2004 and CK7, TTF1, CK5.6.18, CD56/chromogranin and vimentin panel classifications with ALK (clone 5A4, Novocastra Laboratories Ltd, Newcastle, United Kingdom) were applied to paraffin sections of 35 bronchial-pulmonary carcinomas: 20 adenocarcinomas, 6 epidermoid carcinomas, 4 pleomorphic carcinomas (mixed type adenocarcinomas with large/giant/fusiform cells), 4 neuroendocrine carcinomas (NEC) (1 combined large cell NEC with adenocarcinoma and 2 with epidermoid carcinomas; 1 SCLC chromogranin positive combined with adenocarcinoma) and 1 adenosquamous carcinoma.

MATERIAL & METHODS

Epi. Car.	Age	Sex	CK 5.6	CK7	TTF1	CD56 / chromo	VMT	ALK IHC	ALK FISH
1	79	M	+++	-	-	-/-	+	-	-
2	61	M	++	+	-	-/-	++	-	-
3	84	M	+++	+	-	-/-	+	-	-
4	87	M	+++	++	-	-/-	+	-	-
5	60	M	+++	-	-	-/-	-	-	-
6	58	M	+++	-	-	-/-	-	-	-

NEC	Age	Sex	CK 5.6	CK7	TTF1	CD56 / chromo	VMT	ALK IHC	ALK FISH
1	70	M	++	+++	-	++/-	-	-	-
2	70	M	-	+++	-	+/+	-	-	-
3	60	M	++	+++	-	++/+	-	-	-
4	42	M	-	-	-	++/++	+	-	-

PLM Car	Age	Sex	CK 5.6	CK7	TTF1	CD56 / chromo	VMT	ALK IHC	ALK FISH
1	57	M	-	+++	-	-/-	++	-	-
2	68	M	+	+++	-	-/-	+	-	-
3	68	M	+	+++	-	-/-	++	-	-
4	75	M	+	+++	+	-/-	+	-	-

ADSQC	Age	Sex	CK 5.6	CK7	TTF1	CD56 / chromo	VMT	ALK IHC	ALK FISH
1	70	F	++	-	+	-/-	-	-	-

ADC	Age	Sex	CK 5.6	CK7	TTF1	CD56/chromo	VMT	ALK IHC	ALK FISH
1	83	F	-	+++	+++	-/-	++	-	-
2	64	M	-	+++	++	-/-	-	-	-
3	64	F	-	+++	-	-/-	-	-	-
4	60	M	-	+++	+++	-/-	-	-	-
5	55	M	++	+++	+++	-/-	++	+++ faint	+
6	74	M	-	+++	++	-/-	-	-	-
7	76	M	+	+++	++	-/-	+	-	-
8	51	M	-	+++	-	-/-	++	++ faint	+
9	72	M	-	+++	++	-/-	+	-	-
10	63	F	-	+++	+	-/-	+	-	-
11	79	F	-	+++	+++	-/-	++	-	-
12	69	M	-	+++	+++	-	+	-	-
13	61	F	-	+++	++	-/-	-	-	-
14	59	F	-	+++	++	-/-	-	-	-
15	71	F	-	+++	-	-/-	-	-	-
16	75	M	-	+++	+++	-/-	-	-	-
17	62	F	-	+++	+++	-/-	+	+++	+
18	68	F	-	+++	+++	-/-	-	-	-
19	44	M	-	+++	++	-/-	+	-	-
20	73	M	-	+++	+++	-/-	+	-	-

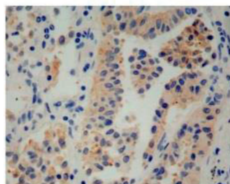

Figure 1 – Intense (3+) ALK expression; acinar and solid patterns. ALK, 400x.

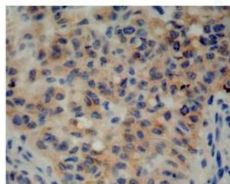

Figure 2 – Moderate (2+) ALK expression; acinar pattern. ALK, 400x.

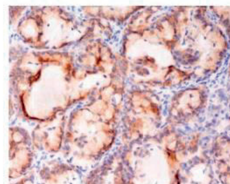

Figure 3 – Intense (3+) ALK expression; acinar pattern with mucinous cells. ALK, 200x.

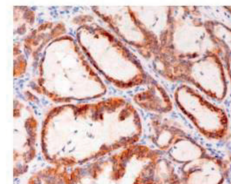

Figure 4 – Intense (3+) ALK expression; mucinous pattern. ALK, 200x.

RESULTS

The applied antibodies specified bronchial pulmonary carcinomas subtypes clearly. In 3 over 60 years old non-smoking females mixed type adenocarcinomas ALK expression was over 50%: acinar, solid, micropapillary and microacinar patterns; one glandular mucinous pattern (mucinous BA pattern) and one BA pattern, all expressing TTF-1.

CONCLUSION

In this study, 3/20 adenocarcinomas of older women had ALK protein expression, only one with a mucinous pattern. As protein positivity cases comprise a lower number, FISH described by S. Lantuejoul seems to be the most appropriate method. It is now necessary to decide whether KRAS and EGFR mutations have to be determined together and/or select TTF-1 positive adenocarcinomas (from terminal respiratory unit) raised by this approach.

Figure 9: Poster IX: ALK expression in Pulmonary Adenocarcinomas.

Abstract X (2013):

XXIX Congresso de Pneumologia, Albufeira-Portugal, October 25 – 27, 2013

Bronchial-Pulmonary Carcinoma Cases with *EGFR*, *ALK* and *MET* Alterations

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Introduction

ALK (encodes a tyrosine kinase receptor) rearrangements are present in 2-7% of lung carcinomas and are responsible for a constitutively active oncogenic protein reported as exclusive of *EGFR* mutations and associated with *EGFR* inhibitors resistance but highly sensitive to treatment with ALK–inhibitor crizotinib. MET amplification is also associated with EGFR inhibitors resistance.

Material and Methods

ALK rearrangement was studied by FISH with break-apart dual color probe (Abbott) and immunohistochemistry (IHC) - monoclonal antibody clone 5A4 (Leica) was applied to sections of a bronchial-pulmonary carcinomas in surgical specimens of all histological types. Simultaneously *EGFR* mutations were determined by DNA direct sequencing and *EGFR* and *MET* genes amplification, by FISH (Abbott).

Results

The authors present two cases showing MET, ALK and EGFR molecular alterations.

One case diagnosed as pleomorphic carcinoma with *ALK* translocation, *MET* amplification and *EGFR* amplification.

Another case diagnosed as an solid and acinar lung adenocarcinoma showed *MET* amplification, *ALK* translocation and exon 21 *EGFR* mutation (L858R)

Conclusion

Concomitant *EGFR* and *MET* alterations were observed with *ALK* rearrangement. This could be the reflex of the biological complexity of cancer cells and of the carcinoma. Although rare this events raises questions concerning the clinical and therapeutical relevance of them.

ALK status must be tested in advanced bronchial-pulmonary adenocarcinoma, as it could be responsible for TKI-resistence of *EGFR* mutated tumours that benefit from ALK-targeted agents.

Key-words: *ALK* rearrangement, EGFR, MET.

Rev Port Pneumol. 2013;19(Esp Cong 4): 127-176

Casos de Carcinoma Bronco-Pulmonar com Alterações do *EGFR*, *ALK* e *MET*.

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INTRODUÇÃO

Os rearranjos do gene *ALK* (que codifica um receptor tirosina-cinase) estão presentes em 2-7% dos carcinomas do pulmão e são responsáveis pela activação constitutiva de uma proteína oncogénica aparentemente sem sobreposição de mutações dos genes *EGFR* e *KRAS* e associada a resistência aos anti-EGFR mas altamente sensível ao tratamento com os inibidores do ALK como o crizotinib. A amplificação do *MET* está também associada a resistência a inibidores de EGFR.

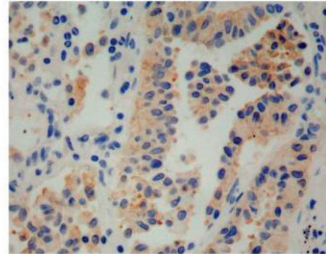

Figura 1 – Expressão de ALK (3+); padrões acinar e sólido. ALK, 400x.

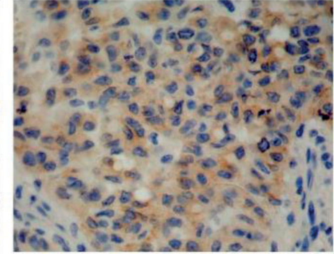

Figura 2 – Expressão de ALK (2+); padrão sólido. ALK, 400x.

MATERIAL E MÉTODOS

Os rearranjos do *ALK* foram estudados por FISH a sonda de dupla cor de separação (Abbott) e por imunohistoquímica (IHQ), o anticorpo monoclonal 5A4 (Leica). Simultaneamente, as mutações dos genes *EGFR* foram determinadas por sequenciação do DNA e a amplificação dos genes *EGFR* e *MET* por FISH (Abbott).

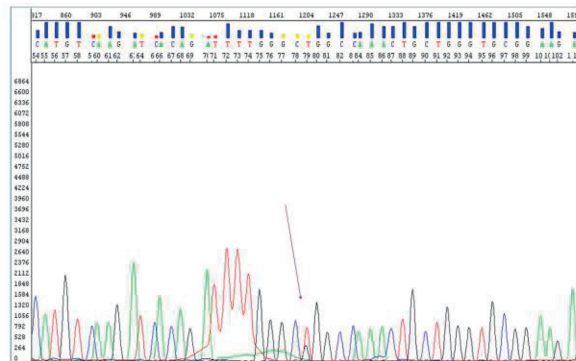

Figura 3 – Mutação pontual do *EGFR* (L858R) – exão 21.

RESULTADOS

Os autores apresentam dois casos com alterações do MET, ALK e EGFR.

CASO 1 – Doente do sexo masculino com 56 anos de idade, com o diagnóstico de carcinoma pleomórfico em biópsia endobrônquica. Mostrava translocação *ALK*, amplificação do *MET* e amplificação do *EGFR*.

CASO 2 – Doente do sexo feminino com o diagnóstico de adenocarcinoma com padrões sólido e acinar do pulmão, com amplificação do *MET,* translocação do *ALK* e mutação do exão 21 do *EGFR* (L858R).

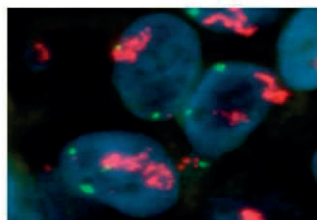

Figura 4 – Amplificação do gene *EGFR*. *EGFR*, FISH, 1000x.

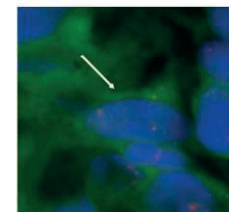

Figura 5: Translocação *ALK*, FISH 1000x.

DISCUSSÃO/CONCLUSÕES

Observaram-se alterações concomitantes do *EGFR* e *MET* com o rearranjo do *ALK*.

Embora raros, estes eventos levantam questões quanto às implicações clinicas e terapêuticas dos mesmos.

Os rearranjos do *ALK* devem ser avaliados nos adenocarcinomas bronco-pulmonares, pela sua responsabilidade na resistência aos inibidores tirosina-cinase dos tumores com mutação no gene *EGFR* e que podem beneficiar da terapia alvo do *ALK*.

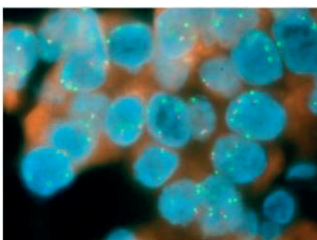

Figura 6: Amplificação *MET*, FISH 1000x.

Figure 10: Poster X: Bronchial-Pulmonary Carcinoma Cases with *EGFR*, *ALK* and *MET* Alterations.

Abstract XI (2013):

Biennial Pulmonary Pathology Society (PPS) Meeting, Grenoble, June 25-28, 2013

***EGFR* Mutations, *MET, EGFR* Amplification and *ALK* Rearrangement Simultaneous in Five Bronchial-Pulmonary Adenocarcinomas**

Silva MR (1) (2) (3), Alarcão A (1) (2) (3), Ladeirinha AF (1), D'Aguiar MJ (1), Ferreira T (1), Sousa V(1) (2) (3) (4), Carvalho L (1) (2) (3) (4),

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(2) CIMAGO – Research Center for Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
(3) Centre of Pulmonology, Faculty of Medicine of the University of Coimbra, Portugal
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Context

ALK rearrangement was found in 2-7% of lung carcinomas resulting a constitutively active and oncogenic protein reported as exclusive of *EGFR* and *KRAS* mutations and associated with resistance to *EGFR* inhibitors but with sensitivity crizotinib.

Design

Sections of 126 bronchial-pulmonary carcinomas of all histological types, obtained from surgical specimens were screened for *ALK* positivity by FISH with break-apart dual color probe (Abbott) and immunohistochemistry (IHC) - monoclonal antibody clone 5A4 (Leica); *EGFR* and *KRAS* mutations were determined by DNA direct sequencing and *EGFR* and *MET* genes amplification, were explored by FISH (Abbott).

Results

IHC was applied in FISH *ALK*+ cases and of the 126 screened tumours, 9 adenocarcinomas (7%) were FISH *ALK +*, 3+ or 2+ score in IHQ, corresponding to 42-79 aged patients. Among these 9 FISH-ALK+ cases, 3 had *EGFR* mutations and 2 had both *EGFR* and *MET* gene amplifications in FISH; *KRAS* was wild type. IHQ correlates with FISH for *ALK* gene rearrangement.

Conclusion

EGFR and *MET* alterations concomitant with *ALK* rearrangement have to be tested in advanced bronchial-pulmonary adenocarcinoma, to validate TKI-resistance of *EGFR* mutated tumours that benefit from ALK-targeted agents. *EGFR* mutations search continue again to be necessary in this context of targeted therapy.

(*2014*) Pulmonary Pathology Society Biennial Meeting Abstracts. Archives of Pathology & Laboratory Medicine: May 2014, Vol. 138, No. 5, pp. 700-709.

EGFR Mutations, *MET*, *EGFR* Amplification and ALK Rearrangement Simultaneous in Five Bronchial-Pulmonary Adenocarcinomas

Silva MR [1][2][3], Alarcão A [1][2][3], Ladeirinha AF [1], d'Aguiar MJ [1], Ferreira T [1], Sousa V [1][2][3][4], Carvalho L [1][2][3][4]

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(2) CIMAGO – Research Center for Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
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1. Context

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2. Design

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3. Results

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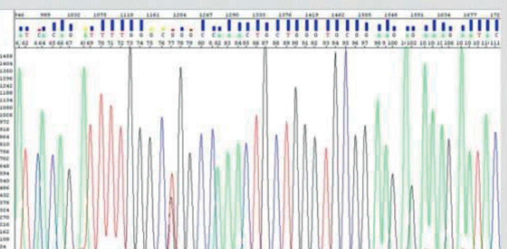
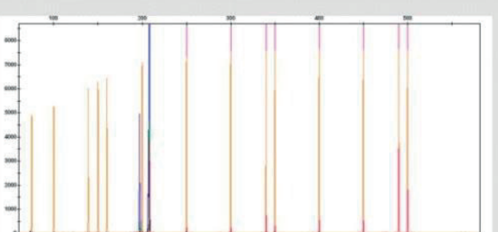


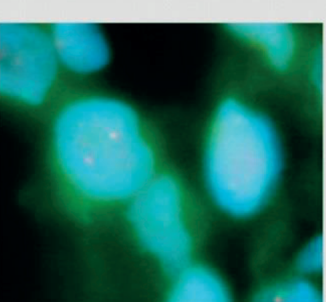
EGFR – Exon 21: mutation L858R by direct sequencing



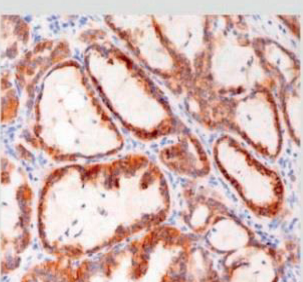
EGFR – Exon 19: *in-frame* deletion – 9 bp by fragment analysis

4. Conclusion

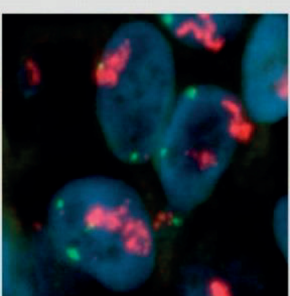
EGFR and *MET* alterations concomitant with *ALK* rearrangement have to be tested in advanced bronchial-pulmonary adenocarcinoma, to validate TKI-resistance of *EGFR* mutated tumors that benefit from ALK-targeted agents. *EGFR* mutations search continue again to be necessary in this context of targeted therapy.



ALK rearrangement by FISH



ALK 3+ by IHC



EGFR amplification by FISH

Figure 11: Poster XI: *EGFR* Mutations, *MET*, *EGFR* Amplification and *ALK* Rearrangement Simultaneous in Five Bronchial-Pulmonary Adenocarcinomas.

Abstract XII (2014):

5º Congresso de Pneumologia do Centro, June 26-27, 2014

Pulmonary Squamous Cell Carcinoma can harbor *EGFR* mutations

L Carvalho [1-3], MR Silva [1-3], A Alarcão [1-3], T Ferreira [1,2], MJ d´Aguiar [1,2], V Sousa[1-3]

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3. Centre of Pulmonology, Faculty of Medicine of the University of Coimbra, Coimbra, Portugal

Introduction

Lung Squamous Cell Carcinomas (SQCC) are tumours that arise from bronchial epithelial cells through squamous metaplasia/dysplasia and are characterized by keratinization and/or intercellular bridges. The presence of at least 10% of the tumour bulk exhibiting these differentiation features is required for the diagnosis. *EGFR* mutations are more frequently described in lung adenocarcinomas.

Objective

Activating mutations in *EGFR* are typically not present in SQCC. We examined the diagnostic accuracy of SQCC to relate with *EGFR* mutations analysis, supported in a small immunohistochemistry panel.

Materials and Methods

Fourty-seven surgical specimens with SQCC were studied. Immunohistochemical markers (TTF1, P63 and CK5/6, CD56, VIM) were used to confirm the diagnosis, to evaluate proliferative index and EMT differentiation. EGFR was studied by IHC (protein expression), FISH (copy number) and direct sequencing (mutational analysis).

Results

EGFR mutation (in-frame deletion in exon 19) was present in three SQCC, one diagnosed as clear cell variant; all TTF1 negative and with high weight cytokeratin expression. Proliferative index varied between 10 and 50% in these cases. EGFR protein expression was present as well as *EGFR* high copy number, by amplification (1 case) or by high polysomy (2 cases).

Conclusion

Authors present three cases of Lung Squamous Cell Carcinoma in never-smokers. *EGFR* mutations occur rarely in SCC with reported frequency of less than 5%; thus routine molecular testing is not usually recommended for SCC histology. Little is known about SQC harboring *EGFR* mutations. Although EGFR-TKIs seem to be generally less effective in *EGFR*-mutated

SQCC than in *EGFR*-mutated ADC patients, some *EGFR*-mutated SQCC patients can obtain clinical benefit from EGFR-TKIs. To better identify these patients, pathological parameters should be taken into consideration: absence of cellular pearl /keratin formation and large/clear cells, where CK7 and Vim are relevant; p63 as a unique marker leads to myoepithelial carcinoma interpretation.

Key words: Squamous Cell Carcinoma, EGFR

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Pulmonary Squamous Cell Carcinoma can harbor *EGFR* mutations

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Introduction

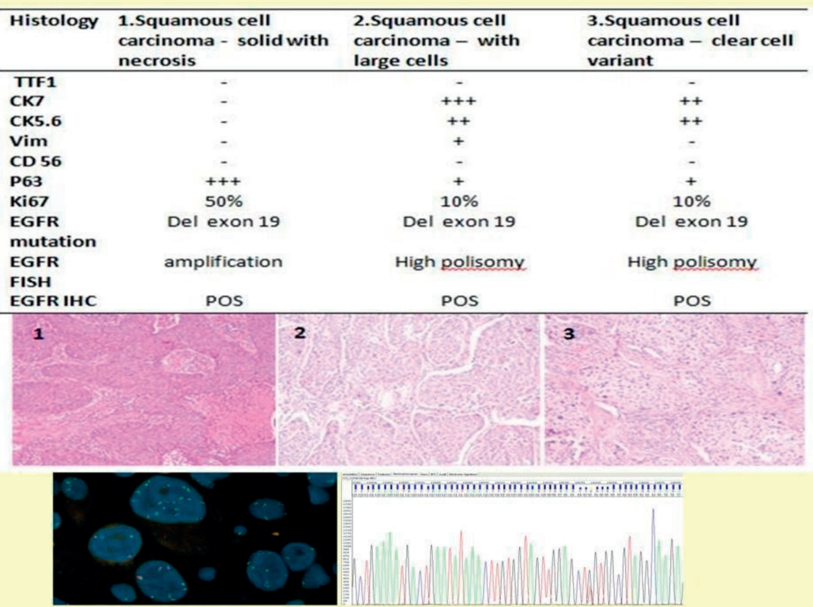
Lung Squamous Cell Carcinomas (SQCC) are tumors that arise from bronchial epithelial cells through squamous metaplasia/dysplasia and are characterized by keratinization and/or intercellular bridges. The presence of at least 10% of the tumor bulk exhibiting these differentiation features is required for the diagnosis. *EGFR* mutations are more frequently described in lung adenocarcinomas.

Objective: Activating mutations in *EGFR* are typically not present in SQCC. We examined the diagnostic accuracy of SQCC to relate with *EGFR* mutations analysis, supported in a small immunohistochemistry panel.

Materials and Methods

Fourty-seven surgical specimens with SQCC were studied. Immunohistochemical markers (TTF1, P63 and CK5/6, CD56, VIM) were used to confirm the diagnosis, to evaluate proliferative index and EMT differentiation. EGFR was studied by IHC (protein expression), FISH (copy number) and direct sequencing (mutational analysis).

Histology	1.Squamous cell carcinoma - solid with necrosis	2.Squamous cell carcinoma – with large cells	3.Squamous cell carcinoma – clear cell variant
TTF1	-	-	-
CK7	-	+++	++
CK5.6	-	++	++
Vim	-	+	-
CD 56	-	-	-
P63	+++	+	+
Ki67	50%	10%	10%
EGFR mutation	Del exon 19	Del exon 19	Del exon 19
EGFR FISH	amplification	High polisomy	High polisomy
EGFR IHC	POS	POS	POS



Results

EGFR mutation (in-frame deletion in exon 19) was present in three SQCC, one diagnosed as clear cell variant; all TTF1 negative and with high weight cytokeratin expression. Proliferative index varied between 10 and 50% in these cases. EGFR protein expression was present as well as *EGFR* high copy number, by amplification (1 case) or by high polisomy (2 cases).

Conclusion: Authors present three cases of Lung Squamous Cell Carcinoma in never-smokers. *EGFR* mutations occur rarely in SCC with reported frequency of less than 5%; thus routine molecular testing is not usually recommended for SCC histology. Little is known about SQC harboring *EGFR* mutations. Although EGFR-TKIs seem to be generally less effective in *EGFR*-mutated SQCC than in *EGFR*-mutated ADC patients, some *EGFR*-mutated SQCC patients can obtain clinical benefit from EGFR-TKIs. To better identify these patients, pathological parameters should be taken into consideration: absence of cellular pearl /keratin formation and large/clear cells, where CK7 and Vim are relevant; p63 as a unique marker leads to myoepithelial carcinoma interpretation.

Figure 12: Poster XII: Pulmonary Squamous Cell Carcinoma can harbor EGFR mutations.

CHAPTER 10 – DISCUSSION AND CONCLUSIONS

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DISCUSSION

This scientific content reflects the candidate's research in the Institute of Anatomic and Molecular Pathology of the Faculty of Medicine, University of Coimbra, as well as the diagnosis work carried out at the Pathology Department of HUC-CHUC, where most of the cases were collected.

The sequence is as follows: basal cell hyperplasia (BCH) – squamous cell metaplasia (SM) – squamous dysplasia (SD). Bronchial-pulmonary carcinomas are discussed with emphasis on epidermoid carcinomas, secondly on adenocarcinomas, followed by adenosquamous and pleomorphic carcinomas.

Epidermoid carcinoma preneoplastic lesions

Knowledge accumulation related to molecular events occurring in preneoplastic lesions may allow a better understanding of carcinogenesis and the molecular pathways implicated. It may also help to define lesions at risk of evolving into invasive carcinomas, and help in the selection of patients for future chemoprevention schemes, always based on the expression of molecular or genetic tumours biomarkers.

Preneoplastic lesions studied in Manuscript I ("EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and p53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study") included BCH, SM, and SD including carcinoma *in situ* (*CIS*); these lesions are considered as a model for epidermoid carcinoma carcinogenesis. One of the reasons for studying these lesions was the frequency of their diagnosis in biopsies and surgical specimens, and their frequent identification alongside invasive epidermoid carcinomas. On the other hand, other recognized preneoplastic lung lesions, such as diffuse idiopathic neuroendocrine cell hyperplasia (DIPNECH) and atypical adenomatous hyperplasia (AAH), are less frequently identified in routine practice. The BCH – SM – SD sequence was also well established, as molecular events reflect the different biological phases of evolution. This sequence therefore constitutes a good model for investigating new molecular markers or genes, such as *EGFR* and *HER2*.

The study of preneoplastic lesions allows a better comprehension of carcinogenesis, including the molecular mechanisms involved. Early diagnosis is essential to reduce mortality rates. We

also have to consider the relevance of molecular biomarkers in the context of preneoplastic lesions, with predictive value for invasiveness or with potential relevance in the context of the development of chemoprevention schemes and actual treatment. Lung carcinogenesis is a complex multistep process that originates in "normal" epithelium and preneoplastic lesions; hence, more information is needed concerning this type of lesions. We also know that some proteins and genes may constitute good biomarkers of the biological risk of progressing to invasive lesions. For all these reasons it is very important to study preneoplastic lesions [179].
Based on a small IHC panel (CK7, CK5.6) our results suggest that preneoplastic lesions of epidermoid carcinoma originate in basal cells of the respiratory epithelium. All lesions tested positive for CK5.6 and negative for CK7, the same pattern observed in the respiratory epithelium basal cells. In this work, Ki67 and p53 also constitute potentially biologically aggressive biomarkers, as we observed an increasing expression along the spectrum of lesions from BCH to *CIS*. As stated before, lung carcinogenesis is a complex multistep process where, based on our results, Ki67 and p53 participate in proliferation with implications for cell cycle and apoptosis control.

Given the current importance of targeted therapy depending on biomarker expression, where EGFR and the signal pathway determine personalised therapy, we studied EGFR expression in these preneoplastic lesions, demonstrating its increasing expression in lesions on this spectrum; therefore, EGFR and the respective pathways may play a role in early steps of epidermoid carcinoma development, reflecting the importance of EGFR signalling transduction pathways in preneoplastic lesions. Other studies also corroborate these results [136,180-186]. The increment in *EGFR* gene copy number (FISH positive cases) was mainly by polysomy than by amplification; so amplification is not the major mechanism related to protein overexpression. Polysomy is considered an early genetic event that could also be used to identify more biologically aggressive lesions [34,134,180,187-189]. On the other hand, it seems that *HER2* might not be involved in the first steps of epidermoid cancer development; other studies have found identical results [185,189,190]. Piyathilake *et al* did not find any stepwise increment of C-erbB-2 along the preneoplastic lesions, suggesting its lack of importance in lung epidermoid carcinoma carcinogenesis [185]. Several other reports also suggest that HER2 has less importance than EGFR in epidermoid lung carcinoma carcinogenesis [135,189,191].

EGFR, Ki67 and p53 might play a role in the identification of epidermoid carcinoma preneoplastic lesions at higher risk of developing epidermoid carcinoma. An increasing expression of these markers was observed. The study reinforced the importance of Ki67 as a

biomarker for dysplasia, a group of preneoplastic lesions characterized by higher proliferative index, also identified by other authors [187,188,192-195]. The “guardian” of genome integrity, which also regulates cellular proliferation and apoptosis, had a higher expression along the preneoplastic lesion sequence indicating that *TP53* mutation and p53 accumulation are early molecular events, a finding corroborated by other published studies [110,135,196,197].

Significant correlation was found between the intense expression of EGFR protein and *EGFR* gene copy number; in the preneoplastic lesions studied, EGFR protein expression reflected gene copy number. Hirsch *et al* also identified a correlation between EGFR protein and *EGFR* gene copy number [198]. Other mechanisms could explain protein overexpression without amplification, such as lower internalization and degradation of EGFR protein or alterations in molecular control mechanisms [199,200]. No correlation was found between EGFR and Ki67 protein expression, although there was an increased expression of both molecules along the spectrum of the studied lesions. Meert *el al* also identified a higher expression of Ki67 and EGFR in high-grade lesions compared to low-grade lesions, associated with proliferation, as a marker of malignant transformation [184,185,194,201].

EGFR protein overexpression as well as high gene copy number in the studied lesions raises the hypothesis of chemoprevention, whose development depends partially on the recognition of the importance of these biomarkers in carcinogenesis, and their ability to select patients that could benefit from targeted therapies. Reliable biomarkers must be identified in the carcinogenesis process so that modulation of their expression correlates with the evolution of the disease and could be easily reproducible in routine practice. In this context, routine biomarkers could comprise histological alterations, molecular patterns and cytogenetic alterations, validated in larger series, to also enable the microenvironment and patients’ habits to be understood [202]. At present there are no chemoprevention schemes with demonstrated benefits [202-207]. However, given that preneoplastic lesions are frequently asymptomatic, and cancer is characterized by a great genetic complexity through multiple molecular pathways alterations, it seems that if an alteration in a single gene or a small group of genes is not identified as associated to epidermoid carcinoma carcinogenesis, a scheme of chemoprevention must be capable of targeting multiple pathways to block epidermoid lung carcinoma carcinogenesis [206-208].

Epidermoid carcinoma

The results of the epidermoid carcinoma study are presented in Manuscript II "Polysomy and amplification of chromosome 7 defined for *EGFR* gene in epidermoid carcinoma of the lung together with exons 19 and 21 wild type". To clearly define epidermoid differentiation, an IHC panel (including CK7, CD56, Chromogranin A and CK5/6) was used, which allowed us to confirm epidermoid differentiation in all our selected cases. Taking account of the results concerning EGFR and HER2 expression in the preneoplastic lesions, we aimed to explore their expression in the invasive counterpart of this spectrum, although we now know that *EGFR* mutations are more frequent in adenocarcinomas [180,189,198,209-212].

EGFR-FISH positive cases, by high polysomy or amplification, were mostly diagnosed in cases with EGFR protein overexpression, with a tendency to significance. EGFR protein expression in FISH-positive cases could be explained by other molecular mechanisms such as lower internalization and degradation. Until our study, most of the studies concerning EGFR (IHC, FISH or mutational) were done in Non-Small Cell Lung Cancer (NSCLC) without histological typing. Our work added value by selecting only epidermoid carcinomas, based on histological and IHC characteristics. Cases of *EGFR* mutations in epidermoid carcinomas should be cautiously interpreted [209,211-215]. In our selected surgical cases, we found 3 cases out of 48 (6.25%) with *EGFR* mutation, namely 3 exon 19 deletion and no exon 21 point mutation.

Epidermoid carcinomas with *EGFR* mutations could be explained by the biological / genetic complexity of lung cancer, including adenocarcinoma differentiation, lower differentiation, or cases with epidermoid and sarcomatoid differentiation (EMT - epithelial mesenchymal transition characteristics) [209,211-224].

Fang *et al* identified a lower *EGFR* mutation rate (2%) in epidermoid carcinoma compared to adenocarcinomas, and a lower TKIs response rate [225]. Fiala *et al* identified 7.2% rate of *EGFR* mutation in epidermoid carcinoma, without significant EGFR TKIs benefit [211]. Other studies demonstrated variable response rates to EGFR TKIs [212,215,221,226].

Recently, Recktman and Travis *et al* evaluated a group of 95 epidermoid carcinomas p63+/TTF1- all without *EGFR* or *KRAS* mutations [220]. They also revised a group of formerly diagnosed epidermoid carcinomas with *EGFR* mutations, and concluded that those cases corresponded to adenosquamous or poorly differentiated adenocarcinomas with a "squamous" morphology, after IHC [220]. Thus, these authors claim that there are no *EGFR* mutations in epidermoid carcinomas.

Several other reports indicate the presence of *EGFR* mutations in adenosquamous carcinomas and pleomorphic carcinomas [213,214,216-218,224].

Jia *et al* observed that adenosquamous carcinomas had an *EGFR* mutation rate of 38%, identifying 5 cases with more than one mutation type. The *KRAS* mutation rate was 3.6% [213].

Kang *et al* identified an *EGFR* mutational rate of 44% in adenosquamous carcinoma, present in both patterns (adeno and squamous), and more frequent in young women and never-smokers [214].They found no relations with the histological pattern of the adenocarcinoma counterpart [214].

Tochigi *et al* found an *EGFR* mutational rate of 13% in adenosquamous carcinoma, also present in both patterns [218]. They also found a 13% mutational rate for *KRAS*, in a western population [218]. *EGFR* mutational rates were similar to the mutational rates for adenocarcinomas and *KRAS* mutation rates were lower [218]. Exon 19 del was more frequent; hence, they argue that adenosquamous carcinoma has a worse prognosis, due probably to exon 19 del associated with the prognosis [218]. Those mutations were present in both components of the adenosquamous carcinomas, suggesting that these are not collision carcinomas based on monoclonal theory [218]. This hypothesis is also raised by Kang *et al*, supported by studies with identical results [214].

During tumour progression, the morphological transformation of a carcinoma from epithelial phenotype to mesenchymal phenotype has been associated with a more aggressive behaviour and metastasis. Yauch *et al* explored the EMT concept and identified the loss of E-cadherin and higher Vimentin expression associated with the EMT pathway in EGFR TKIs insensitive cellular lineages; on the other hand, E-cadherin was upregulated in sensitive cellular lineages [227]. Cross-talk between E-cadherin and EGFR was suggested [227].

Italiano *et al* identified no *EGFR* mutations in pleomorphic carcinomas, where the *KRAS* mutation rate was 38%, suggesting that sarcomatoid carcinomas do not benefit from EGFR TKIs [224]. Lee *et al* instead, identified mutational rates of 14.8% for *EGFR* and 9% for *KRAS* in pleomorphic carcinomas [216].

Epidermoid carcinomas have a basal cell origin, as already stated. We know that there are poorly differentiated epidermoid carcinomas, some with an EMT phenotype and others co expressing CK7. These cases represent more complex carcinomas, which present characteristics that possibly indicate different molecular/genetic events or hybrid characteristics, where *EGFR* mutations could be found. Tumours with poorly differentiated

epidermoid lung carcinoma, especially with CK7 and/or Vimentin expression, may be submitted to *EGFR* mutation testing. In our routine molecular pathology practice, we also identified pleomorphic carcinoma with coexistence of *EGFR* and *KRAS* mutations, again morphology reflecting genetic/molecular complexity and events accumulation. We have also recently (Abstract XII – “Pulmonary Squamous Cell Carcinoma can harbour *EGFR* mutations”) presented three “epidermoid” carcinomas with *EGFR* mutations (all exon 19 deletions), without TTF1 expression. One had a solid pattern and necrosis, with p63 expression and TTF1, CK7, CK5.6 and Vimentin negativity, a second case had large cell phenotype and CK5.6, CK7 and Vimentin positivity, while the third (clear cell variant) showed CK7 and CK5.6 positivity. Amplification was present in the first case and high polysomy in the remaining two cases. CK7 was generally expressed in cylindrical epithelial bronchial cells and pneumocytes. Thus, these three cases expressed characteristics common to bronchial adenocarcinomas. Epidermoid carcinomas with vimentin expression are also more complex carcinomas where EMT pathways are important, which explains their lesser morphological differentiation together with fusocellular phenotype or irregular infiltrative borders with large isolated cells. Consequently, epidermoid carcinomas with these phenotypes should be reported to clinicians, who should be made aware that these patients may benefit from *EGFR* mutation testing. Hence, epidermoid carcinomas with solid, large cells or clear cell phenotype or with CK7 or even Vimentin expression (EMT phenotype) are submitted to *EGFR* mutational analysis in our Hospital.

In small biopsies, solid pattern adenocarcinomas could be difficult to interpret, as we found in these cases lower TTF1 expression. Some could be interpreted as NSCLC, a designation we definitely avoid. These TTF1 negative solid pattern adenocarcinomas have higher Ki67 expression, indicating a more biologically aggressive behaviour.

When considering adenosquamous carcinoma, a pattern-based approach is important as we know that *EGFR* mutations are more frequent in lepidic non-mucinous and acinar patterns [228-230].

Hence, a comprehensive pathological diagnosis is essential, incorporating immunohistochemical markers, such as p63 or CK5/6, CK7 and TTF1, as well as mucine detection by histochemical techniques. This is especially necessary in small biopsies representing poorly differentiated carcinomas with some “squamous” features or solid patterns, where it could be difficult to differentiate between a poorly differentiated epidermoid carcinoma and a solid pattern adenocarcinoma. In these small biopsies we must also take account of incomplete sampling; thus, some cases diagnosed as epidermoid

carcinoma in biopsies may be diagnosed as adenosquamous carcinoma in surgical specimens, after extensive sampling. We could argue for the possibility of *EGFR* mutation testing in biopsies with poorly differentiated epidermoid carcinomas, especially in cases diagnosed in never-smokers or with CK7 / Vimentin expression / TTF1 negative.

We must take in consideration that our cases presented in Manuscript II, "Polysomy and amplification of chromosome 7 defined for *EGFR* gene in squamous carcinoma of the lung together with exons 19 and 21 wild type", were selected by two pathologists, making use of an IHC panel in order to exclude adenosquamous or large cell lung carcinoma cases. These cases were selected from the SAP – HUC/CHUC archive, where total inclusion of lung carcinomas is mandatory; this practice allows us to better classify lung cancers, namely by allowing the identification of glandular or sarcomatoid areas.

Although *EGFR* mutations are infrequent, the high gene copy number is a frequent event in epidermoid carcinoma (with polysomy present in 46.3% and amplification in 12.9% of the cases). It seems that *EGFR* gene copy may play a role in epidermoid lung cancer carcinogenesis, with mutations being a rare event.

Some authors identified a response to TKIs (tyrosine kinase inhibitors) in NSCLC FISH *EGFR* positive cases, especially with amplification and others with EGFR protein overexpression [198,231]. In our work, the percentage of FISH-positive cases was high (46.3% with high polysomy and 12.9% with amplification).

We can infer that EGFR TKI therapy is less prone to be effective in these epidermoid carcinomas. However, tumours that have areas with other morphological or immunohistochemical differentiation may benefit from the mutational search [211,215,226,232].

Adenocarcinoma

Bronchial-pulmonary adenocarcinomas were studied, also following the strategy of EGFR and HER2 analysis, adding a new degree of complexity, namely evaluating expression according to histological subtypes / patterns. Knowing that there are several patterns / histological subtypes recognized, we studied the adenocarcinomas using a pattern-based approach, aiming to identify differences concerning the expression of differentiation markers and several gene

products related with different molecular pathways, by IHC methods (methods that could easily be used in routine diagnosis).

In a first study, several molecular markers were evaluated: CK7, TTF1, CK20, CK 5/6, chromogranin A, Ki67, p53, RB, BCL2, Cyclin D1, APC, ERCC1, LRP, MRP1, EGFR and HER2 in an attempt to differentiate / identify the molecular pathways involved in cancer development according to the present patterns, and to establish expression differences for molecular markers for targeted therapy. Comparisons were made with adjacent normal lung tissue (bronchial epithelium and the epithelium of the terminal respiratory unit (TRU)).

In the manuscript "Bronchial-Pulmonary Adenocarcinoma Subtyping Relates with Different Molecular Pathways", the importance of pattern identification and classification of lung adenocarcinomas was recognized and emphasized by the newly proposed classification of lung adenocarcinomas [233].

Differences between patterns should be taken to account to improve diagnosis and prognosis and enhance therapy selection strategies. We studied differences in the expression of differentiation and proliferation markers, gene products related to apoptosis like p53 and BCL2, products of oncogenes, proteins involved in cell cycle control such as molecules related to chemotherapy resistance, and growth factor receptors like EGFR and HER2.

In this work, we clearly identified differences in expression between all patterns studied and normal tissue. Together with the differences observed between patterns, this suggested, based on a dendogram analysis, that adenocarcinomas are molecularly different from normal adjacent tissue, and that acinar and BA/lepidic patterns are the most alike and papillary the most different. Cluster analysis revealed three clusters: papillary; solid; and a group composed of acinar, BA/lepidic and micropapillary.

There were also clear differences concerning gender (females were more frequently non-smokers, tumours were in earlier stages and with higher ERCC1 expression, involved in DNA repair).

Staging correlated with higher TTF1 expression in stage IA, higher Ki67 expression in IIIA, higher APC expression in IIA and IIIA, higher RB expression in IB compared to IA, higher p53 expression in IB, IIA and IIIA compared to IA, higher ERCC1 expression in IIIA than IIA and lower EGFR expression in stage IB compared to stage IA). Ki67, APC, and ERCC1 had higher expression in the higher stages and TTF1 lower expression in the higher stages, reflecting more aggressive and proliferative adenocrcinomas with less differentiation or even a non-TRU

bronchial adenocarcinoma. Higher ERCC1 expression is also associated with platin chemoresistance. Higher-stage adenocarcinomas are more prone to chemoresistance.

There were two specific gene expression differences concerning patterns and HER2 and TTF1 alterations. Papillary and solid patterns revealed lower TTF1 expression (identical to normal tissue), exhibiting a non-TRU/bronchial phenotype. Acinar, BA/lepidic and micropapillary patterns showed higher TTF1 expression corresponding to TRU origin. These patterns, especially lepidic and acinar, TTF1 positive, are those where *EGFR* mutations are said to be more frequent. TTF1 expression, identifying possible TRU origin, defines a subgroup of adenocarcinomas with molecular and biological particularities.

The solid pattern also revealed lower HER2 and higher EGFR and ERCC1 expression (compared to papillary). In solid patterns, EGFR pathway activation was related with EGFR overexpression. There are some reports indicating that EGFR overexpression is related with poor prognosis or survival [224,234-238]. Based on these facts, we stated that solid pattern adenocarcinomas were less differentiated adenocarcinomas, with a worse prognosis. Papillary patterns showed higher HER2 and lower ERCC1 expressions.

It seems that impaired DNA repair mechanisms are implicated in carcinogenesis when the papillary pattern is dominant. Adenocarcinomas showed higher TTF1 expression in acinar, BA/lepidic and micropapillary patterns corresponding to TRU adenocarcinomas that express TTF1 [239]; probably related with better prognosis when compared to non-TRU type adenocarcinomas [240]. This is also highlighted by some authors that identified an inverse correlation between TTF1 and Ki67, a marker of proliferation and biological aggressiveness [241]. Hence, TRU-type adenocarcinomas represent a biologically less aggressive group, based on TTF1 higher expression and in the absence of higher EGFR expression compared to normal tissue.

In our results, there was no difference in Ki67 expression between patterns. We identified higher TTF1 expression in acinar and lepidic patterns, in TRU- type adenocarcinomas, corroborating the published results. TTF1, expressed in TRU type adenocarcinomas, has been associated with a good prognosis [241-246]. However Pelosi *et al* did not find a correlation between TTF1 expression and prognosis [247]. Several studies identified a correlation between TTF1 expression, like TRU type adenocarcinomas, and *EGFR* mutations [248,249]. TRU-type adenocarcinomas have also been associated with *EGFR* mutations [248-252]. It is also known that *EGFR* mutations are more frequent in lepidic and acinar patterns. EGFR protein expression

has been described as more frequent in TRU-type adenocarcinomas and also in epidermoid lung carcinomas [253].

TTF1 has importance defining adenocarcinomas differentiation as well as providing crucial information for therapy planning in patients diagnosed with advanced lung adenocarcinoma, taking into consideration that *EGFR* mutations are more frequent in TTF1 positive TRU-type adenocarcinomas. On the other hand TTF1 negative adenocarcinomas, some representing non-TRU adenocarcinomas, are less prone to harbour *EGFR* mutations.

The micropapillary pattern had higher RB expression, and the acinar pattern lower ERCC1 and higher EGFR expression when compared with normal tissue. Cyclin D1 seemed to be relevant in acinar and BA/lepidic patterns and not related with micropapillary pattern.

Increased Cyclin D1 expression is associated with G1 to S phase progression, inducing cellular proliferation [254]. The G1-S transition mediated by Cyclin D and CDK4 complexes occurs through retinoblastoma inactivation. Some reports have corroborated the bad prognosis of Cyclin D1 overexpression and pRb inactivation, and the importance of G1/S transition in lung cancer [255]. Thus, the cell cycle progression in acinar and lepidic patterns may be related to higher Cyclin D1 expression together with pRb inactivation. The loss of the *RB* gene is associated with cell proliferation and apoptosis [138]. In lung cancer, the frequency of the inactivation of the RB pathway is so high that it is considered a requirement for carcinogenesis [256].

ERCC1 protein expression in micropapillary, solid and BA/lepidic patterns indicated DNA repair preservation, while in acinar and papillary patterns, there was lower expression. Lung cancer with higher ERCC1 expression was associated with Cisplatin-based chemotherapy resistance as ERCC1 acts by removing DNA adducts, which relates to poor prognosis [257-262]. In our cases, micropapilary, solid and also lepidic patterns had ERCC1 expression, indicating their ability to remove Cisplatin adducts.

BCL2 was overexpressed in all patterns, suggesting that there was inhibition of apoptosis. Higher BCL2 indicates that prevention of apoptosis is a ubiquitous phenomenon transversal to all the patterns of the adenocarcinomas [263].

MRP1 and LRP were overexpressed in all patterns and need further analysis to better understand the role of those proteins in the response to therapy.

MRP1 is a transporter of the cytokine cysteinyl leukotriene C4, releasing it in response to inflammatory stimulus, in order to confer cellular protection against toxic agents [264]. MRP molecules are associated with resistance to cytotoxic agents / therapies [265]. Functional active MRP1 expressed bronchial-pulmonary carcinoma cells correlate inversely with chemosensitivity [266]. In some studies, researchers showed that there were frequently high levels of MRP1 in NSCLC specimens; however, there is no consensus about the prognostic value of NSCLC with MRP1 expression [267]. Lung resistance-related protein forms part of the phenotype MRD (multidrug resistance), expressed in bronchial-pulmonary cell lines and correlated with cisplatin resistance [268]. In lung tissue, some researchers demonstrated that LRP is similarly expressed in normal bronchial cells and in NSCLC cells. The respiratory epithelial cells have detoxification mechanisms, and the LRP can deregulate this mechanism and confer resistance to chemotherapy [266,269-271]. In our selected cases there was higher MRP1 and LRP expression in adenocarcinoma patterns compared to normal tissue, indicating that during carcinogenesis there is an acquisition of drug resistance mechanisms, without correlation with any specific pattern.

Adenocarcinoma subtyping / pattern recognition was considered to have great importance, and one of the most original aspects of our work is to compare the results obtained according to adenocarcinoma patterns. Pattern recognition/classification has important clinical and therapeutic implications as it has been demonstrated to have biological significance. Knowledge of molecular target expression according to the pattern is of great clinical utility and enables us to better recognize the probability of an adenocarcinoma harbouring a specific molecular target for personalized therapy, according to the patterns present in the diagnosed adenocarcinoma, where molecular alterations start to be clearly known.

Molecular characterization of lung adenocarcinomas supports targeted therapies, especially for EGFR. Knowing the importance of targeted therapies and pattern recognition, we evaluated EGFR, HER2 expression, gene copy number and mutational status as *KRAS* mutational status, while also trying to correlate it with adenocarcinoma sub-typing, to better understand the value of pattern sub-typing in lung adenocarcinoma and anticipate molecular alterations.

There are numerous references in the IHC and genetic studies literature to lung adenocarcinoma or NSCLC (under the old nomenclature). One important objective of this work was to look for *EGFR* and *KRAS* mutations together with FISH and IHC assays, always according to adenocarcinoma patterns.

We found a higher *EGFR* mutational rate (46%) than advocated in the literature, especially in western countries [45,172,173,180,198,219,272-278]. This could be explained by the selection of surgical stage adenocarcinomas, not including biopsy cases, the extensive selection of different areas of the same tumour for the assays, and the high number of female patients, where the risk for *EGFR* mutations is higher. Quality control is also a standard in the laboratory, according to the EQA program for testing biomarker mutations in non-small cell lung carcinoma, promoted by the European Society of Pathology. The laboratory has participated with a score of ≥ 90%, considered as a successful participation. It must be emphasized that other studies have shown *EGFR* mutational rates of 49% in females and 19% in males [277]. This may also reflect population features. Advanced stage tumours could explain genetic events accumulation, tumour heterogeneity and genetic instability. Tumour heterogeneity and genetic instability could also explain cases identified with more than one *EGFR* mutation type.

Although *EGFR* mutations were more frequent in women, there were clear significant differences concerning exon 19 mutations but not exon 21 mutations. Like other publications, *EGFR* mutations were associated with female never-smokers [45,277,279]. Smoking cannot prevent *EGFR* mutations as there have been cases diagnosed in smokers, as also stated by other authors [279]. *EGFR* mutations were more frequent in acinar and lepidic patterns compared to the other patterns [228-231]. *EGFR* activating mutations have been described as more frequent in the classical bronchiole-alveolar pattern and in mixed-type adenocarcinomas [228-230]. Others have demonstrated that papillary, micropapillary and hobnail patterns were correlated with *EGFR* mutations [280,281]. Dacic *et al* demonstrated different *EGFR* mutation rate according to primary histological patterns: acinar (20%), BA/lepidic (12.5%), mucinous (9%), papillary (3%), micropapillary (3%) and solid (0%) [273].

KRAS mutations were more frequent in male adenocarcinoma cases and in smokers. These results were corroborated by other studies [173,223,273,279,282,283]. *KRAS* mutations were identified in acinar, lepidic and solid patterns. In the literature, *KRAS* mutations are associated with mucinous differentiation, goblet cells, and solid patterns and in non-TRU adenocarcinomas [8,273,284-290]. Dacic *et al* demonstrated different *KRAS* mutation rates in mixed-type adenocarcinomas that showed acinar (32%), solid (18%), BA/lepidic (16%), mucinous (16%), papillary (10%) and micropapillary (4%) patterns [273]. These differences could be explained by the lower number of mucinous and solid patterns in the studied sample and also by the absence of poorly differentiated adenocarcinoma cases. As in the literature a

correlation was found between *EGFR* gene copy number and mutational status. Amann *et al*, Hirsch *et al* and Takano *et al* also found correlations and some sensitivity for EGFR TKIs[198,291,292]. Although some studies demonstrated that *EGFR* mutations are usually related with amplification in *EGFR locus*, the relationship between *EGFR* mutations, gene copy number, and IHC expression is still unclear [213,215-217,231]. We found no correlation with EGFR expression, perhaps because gene amplification was not the prevalent event but gene polysomy, reflecting aneuploidy.

One important aspect identified in this study was the fact that when a mutation was present, it was generally present in all the patterns of the same adenocarcinoma. These results support a monoclonal origin for the different patterns. Other molecular mechanisms must be implicated in different morphological differentiation, some already explored. This has important clinical and diagnostic implications; biopsies of an adenocarcinoma are reliable to determine *EGFR* and *KRAS* mutational status.

KRAS and *EGFR* mutations coexistence was identified: there was one adenocarcinoma with *KRAS* and *EGFR* mutation coexistence (*EGFR* exon 21 L858R mutation in acinar and lepidic patterns and *KRAS* codon 12 point mutation G12V in acinar and lepidic patterns). In our daily diagnostic routine, we identified two more cases of bronchial-pulmonary carcinoma with this mutational coexistence status: one mucinous lung adenocarcinoma (*EGFR* exon 21 point mutation L858R and *KRAS* codon 12 point mutation G12V in acinar and lepidic mucinous patterns), and one pleomorphic giant-cell carcinoma (*EGFR* exon 19 deletion – 19 bp and *KRAS* codon 12 point mutation G12C). *KRAS* and *EGFR* mutations have generally been considered as mutually exclusive [274,275,277,279,283,293]. However, there are reports indicating their coexistence [173,274,294].

A literature review was conducted into the predictive value of *KRAS* mutations and *KRAS* and *EGFR* mutation coexistence concerning EGFR TKIs. *KRAS* mutations are associated with EGFR TKIs resistance [274,283,295-298]. *EGFR* and *KRAS* mutation coexistence means that upstream inhibition of EGFR will have no therapeutic blockage [299]. Jackman *et al* found no survival impact of *KRAS* mutations in patients without *EGFR* mutations treated with EGFR TKIs [300]. Rare cases of TKI minor or transient response in patients with *KRAS* mutation are described [173,300]. Because *EGFR* mutation is considered a good predictor of EGFR TKIs response and *EGFR* and *KRAS* mutations are generally mutually exclusive, it is not clear whether the response to TKIs differs between tumours with *KRAS* mutations and those without *KRAS* and *EGFR* mutations [301]. This fact raises the idea that *KRAS* mutation is not always associated

with EGFR TKI resistance as EGFR activation was not only mediated through KRAS signalling, and negative feedback loops could downregulate RAS signalling [302]. Non-active KRAS isoforms could also be explained by epigenetic changes, alternative splicing or posttranslational modifications [302].

In our study, the *EGFR* and *KRAS* mutations present were always activating mutations. Other studies have identified rare NSCLC cases with *EGFR* and *KRAS* mutations [173,274,294].
To better understand the biological and therapeutic implications, it is necessary to evaluate a larger number of cases of lung carcinomas harbouring both mutations, knowing that most published articles make reference to sporadic rare case reports. Coexistence of *KRAS* and *EGFR* mutations in the same tumour raises again the question of tumour heterogeneity.
Although when an *EGFR* mutation is identified, it was generally present in all patterns of the same adenocarcinoma, there are cases where it was possible to find more than one type of *EGFR* mutation. One case of lung adenocarcinoma was reported with exon 19 *EGFR* del 9bp and 18bp present in acinar pattern and del 15bp in solid and micropapillary patterns. Two cases showed coexistence of exon 21 *EGFR* point mutation (L858R) and exon 19 *EGFR* del (of 19, 18 1nd 9bp) present in different patterns of the same adenocarcinoma. Complex *EGFR* mutations patterns, like the presence of more than one mutation type, have been being described; for instance Huang *et al* identified 7 cases with two *EGFR* mutations out of a total of 39 mutated cases [303].
These are rare cases that clearly demonstrate tumour heterogeneity in lung adenocarcinomas, reflecting some pattern heterogeneity; they may be the result of different neoplastic cell clones or more probably genomic instability and mutational acquisition in the later phases of carcinogenesis. Neoplastic cells might accumulate multiple molecular events in the EGFR gene in these adenocarcinoma cases. In our study, all *EGFR* mutations were activating mutations, so it could be argued that these adenocarcinoma patients would still benefit from EGFR TKIs.

Several poster presentations with abstract published in different medical journals completed the investigation that sustains the actual discussion.

In "TTF1 Negative Solid Pattern in Lung Adenocarcinomas May Reflect Morphogenesis", some of the results presented in Manuscript IV – "Lung adenocarcinoma: sustained subtyping with immunohistochemistry and EGFR, HER2 and KRAS mutational status" – were illustrated, with incidences in accordance with mutational status and FISH *EGFR* results, a pattern-based approach that show that biopsies are prone to be used in the evaluation of *EGFR* mutational status. Till now the results and discussion have been centred particularly on *EGFR* and *KRAS*

mutational status using an adenocarcinoma pattern-based approach. Work carried out in the laboratory was also essential to improve technical and diagnostic skills that enabled the development of molecular diagnosis for clinical purposes at the Institute of Pathology. The knowledge acquired made it possible to advance to the diagnosis/evaluation of other genes, such as *ALK* and *MET*, all with therapeutic implications. In the last group of abstracts (VIII, IX, X and XI and XII), we searched for the results of the evaluation of these genes in lung bronchial carcinomas, especially adenocarcinomas. Abstract VIII – "*ALK* Gene Rearrangment in EGFR and KRAS Positive Lung Adenocarcinomas" reported one case with *ALK* rearrangement out of a total of 19 carcinomas, with either *EGFR* (14) or *KRAS* (5) mutations. Different *EML4* exons can be fused with the *ALK* gene, and more than nine variants have been identified [304,305]. FISH is the actual method of choice for *ALK* rearrangement detection, although Reverse Trancription PCR (RT-PCR), immunohistochemistry and chromogenic *in situ* hybridization have been also proposed by different authors [305-307]. Results demonstrated that *EGFR* mutation and *ALK* rearrangement coexistence, although rare, can be diagnosed. Patients with both genetic alterations may show different sensitivities to targeted EGFR inhibition [308-311]. *ALK* and *EGFR* signalling are closely related, and the differential sensitivity in these cases may indicate that tumours were dependent on different oncogenes or that they had a dominant driver mutation [312,313]. Further studies, inolving more cases are necessary to clarify the interaction between EGFR and ALK signalling and the biological and clinical implications of *EGFR* and *ALK* genetic alteration coexistence.

Abstract X – "Bronchial-Pulmonary Carcinoma Cases with *EGFR*, *ALK* and *MET* Alterations" presents a pleomorphic carcinoma with *ALK* translocation, *MET* amplification and *EGFR* amplification and an adenocarcinoma with *ALK* translocation, *MET* amplification and exon 21 *EGFR* point mutation (L858R). Abstract XI – "*EGFR* Mutations, *MET, EGFR* Amplification and ALK Rearrangement Simultaneous in Five Bronchial-Pulmonary Adenocarcinomas" concerns a series of 126 cases of bronchial-pulmonary carcinomas including 9 FISH *ALK* positive cases with *EGFR* mutations and *MET* amplification. A correlation was found between ALK IHC and FISH results. These cases may reflect tumour heterogeneity and molecular complexity. *MET* amplification, like *ALK* translocation is responsible for EGFR TKI resistance, associated with poor prognosis [314-316]. There are trials ongoing with anti-MET antibodies (MetMAb) [147]. *MET* amplification may be detected by FISH or RT-PCR [143,145,317]. *MET* amplification is supposed to be unusual in untreated bronchial-pulmonary lung carcinomas, and the incidence varies between 2 to 4% [143,146,175,317,318]. *MET* amplification plays a role in acquired

resistance to EGFR inhibitors in patients with *EGFR* mutated tumours, and is identified in 5% to 20% of these cases [144,175,314,319,320].

Bronchial-pulmonary carcinomas and FGFR1

As oncogenic driver mutations have been mainly described for adenocarcinomas we considered to explore new molecular targets, especially for squamous cell lung carcinomas and other lung cancers, such as adenosquamous and pleomorphic lung carcinomas. *FGFR1* was considered as a good candidate because amplification was recently described in lung carcinomas, more frequently in squamous cell carcinoma, with the recent development of molecular targeted therapies. So it was intended to evaluate FGFR1 protein expression and *FGFR1* copy number in lung bronchial-pulmonary carcinomas, comparing the results between the histological types. Also, EMT pathway was explored in more complex lung carcinomas, such as adenosquamous and pleomorphic carcinomas as well as in adenocarcinomas, according to Vimentin and TTF1 expression. For squamous cell carcinoma, FGFR1 alterations were also explored according to CK7 expression. The purpose was to identify differences and/or similarities in between these groups of lung tumours in order to characterize the histological types for targeted therapy.

Potential targetable molecular events are more frequently diagnosed in lung adenocarcinomas, especially of non smoker's patients [321-323]. In squamous cell carcinoma, more prevalent in smokers, these molecular events / genetic driver mutations are less frequently identified [2,165]. For this reason, targeted therapies are not a reality in a daily routine for squamous cell lung cancer treatment [2,165]. So, new research in this field is crucial for the growing knowledge and to the development of new therapies strategies, we hope with increment in life expectancy and quality. In this context, *FGFR1* and *FGF* emerged recently as a driver potentially targetable oncogenes [324]. There are several ongoing clinical trials with FGFR inhibitors, and in most of these clinical trials one of the inclusion criteria is *FGFR1* amplification [168-171]. Therefore, the assessment of *FGFR1* gene status might become increasingly important in the future, especially for patients with squamous cell lung carcinomas. It is also important to clearly define the criteria to select patients for targeted therapies. Protein overexpression, gene copy number increases (by amplification) and mutations have been described in lung bronchial-pulmonary carcinomas [158-163,165].

Amplification of the *FGFR1* gene is actually considered the best criteria for patient's selection for targeted therapy, as *FGFR1* mutations are rare [2,165]. Therefore, *FGFR1* amplification seems to be the most frequent responsible for the activation of FGFR1 pathways [2,165]. Also, it has been demonstrated response to FGFR inhibitors, in those patients with *FGFR1* amplification [165,168].

FGFR1 amplification as well as FGFR1 overexpression are frequently identified, mainly in squamous cell lung carcinomas [165]. These alterations have been considered as an early event, as they could be identified in squamous metaplasia and dysplasia, with some conflicting results [2,162,325].

Another important aspect to take in account is that patients with *FGFR1* amplification may develop EGFR-TKI resistance [326], being the FGFR pathway a mechanism of "intrinsic" resistance. So it is important to identify genetic *FGFR* alterations in order to better understand therapy responses, and explain resistant cases. *EGFR* mutations and FGFR1 increased copy number have been considered as mutually exclusive [327]. However, there are cases reported of patients with *FGFR1* amplification and high EGFR protein expression (overexpression) [328].

We have demonstrated higher FGFR1 protein expression in tumours compared to non-tumour adjacent parenchyma / bronchial epithelium. This aspect may reinforce the importance of the FGFR1 pathway in lung carcinogenesis, even in the absence of *FGFR1* gene copy number increase. Higher expression was seen in adenocarcinomas and pleomorphic carcinomas compared to squamous cell carcinomas. No differences were found between adenocarcinomas, pleomorphic and adenosquamous carcinomas.
Higher expression in pleomorphic carcinomas suggests that overexpression may also be implicated in the activation of the EMT pathway. Overexpression could also be responsible for tumour growth and proliferation and invasiveness, related with a more aggressive behaviour.
Protein FGFR1 expression showed no differences between adenocarcinomas and adenosquamous carcinomas, may be reflecting the compound morphology of the latter and the carcinogenesis of both.

No differences of FGFR1 protein expression and gene copy number were found in the group of squamous cell carcinomas, according to CK7 expression (CK7 expression reflecting a group of less differentiated tumours with some features overlapping with adenocarcinomas).

Therefore, it seems that the grade of differentiation in squamous cell lung carcinoma has no relevance considering FGFR1 protein expression.
For adenocarcinomas, our results suggest that TTF1 expression, reflecting cell origin, has probably no implications on FGFR1 pathway activation, as no significant differences were found related to the expression of TTT1.

In contrast to the results obtained in the group of pleomorphic carcinomas, adenocarcinomas expressing Vimentin, associated with EMT phenotype in carcinomas, had no significant differences of either FGFR1 expression or gene copy number compared to Vimentin negative cases. This may indicate that in adenocarcinomas FGFR1 pathway activation precedes or is independent of EMT activation or even that activation of EMT pathways occur later than the activation of pathways related to tumour growth. Also, other pathways than FGFR1 activated, might be related with EMT phenotype in lung adenocarcinomas expressing Vimentin.

FGFR1 amplification was identified in 19.7% (15) of the studied cases: 5 adenocarcinomas (14.7%), 5 squamous cell carcinomas (20.8%), 3 pleomorphic carcinomas (30%) and 2 adenosquamous carcinomas (25%). Although FISH positive cases were more frequent in squamous cell carcinomas, no significant differences to adenocarcinomas were registered.
FGFR1 amplification rates vary according to the different series published, ranging from 0% to 4.7% for adenocarcinomas, from 9% to 41.3% for squamous cell lung carcinomas [165,325,327,329-334].

As *FGFR1* amplification was identified in adenosquamous and pleomorphic carcinomas, it seems that the FGFR pathway is also important in these tumours. FGFR1 molecular profile was studied in a subset of 10 pleomorphic carcinomas. No differences of FGFR1 protein expression were identified according to the presence of a component of squamous cell carcinoma or adenocarcinoma, as all showed intense expression. However, *FGFR1* amplification was identified in 2/2 (100%) of the pleomorphic carcinomas with a squamous cell carcinoma component and in 1/8 (12.5%) of the pleomorphic carcinomas with a component of adenocarcinoma.

FGFR1 protein expression is higher and frequent in pleomorphic carcinomas compared to adenocarcinomas and squamous cell carcinomas. Higher FGFR1 expression may reflect a more aggressive behaviour, related with the activation of pathways related to tumour growth and also reflect the importance of FGFR1 in the EMT pathway in this subtype of lung carcinomas.

As these molecular events are frequent in pleomorphic carcinomas, it seems prudent to evaluate FGFR1 protein expression and gene copy number in this subtype of lung carcinomas. FGFR1 molecular testing should also be done in adenosquamous carcinomas, hence FGFR1 protein expression and gene amplification was also identified in adenosquamous lung carcinomas.

We did not found any associations between FGFR1 expression / gene copy number and clinical parameters, such as gender and smoking habits. Therefore, according to our results, it seems that clinical parameters should not be used to select patients for targeted therapy. Other investigators also found no correlation with clinical parameters [330,333]. We have, however, found a tendency to higher frequencies of *FGFR1* amplification in adenocarcinomas of smoking patients and other authors identified correlations with male gender and smoking habits, raising the question of the importance of clinical parameters in the patient selection [325,327,328,332]. Therefore, in the future, more and larger studies must be done to address this question.

As already mentioned, *FGFR1* amplification is an inclusion criteria for clinical trials. However, according to our data and those of other series, amplification may not reflect overexpression. Therefore, in the future, Clinical Trials should also determine the predictive value of both *FGFR1* gene copy number and protein expression, in order to definitively clarify which is the best predictive testing.

Some take home messages related to FGFR1 molecular testing in lung bronchial-pulmonary carcinomas emerge from our study and the literature review. FGFR1 protein expression was identified in all subtypes of lung bronchial-pulmonary carcinomas, especially in pleomorphic carcinomas. *FGFR1* amplification, although more frequent in squamous cell carcinoma, was also identified in adenocarcinomas, adenosquamous and pleomorphic carcinomas. Therefore, it is important to do not exclude from molecular testing patients with lung bronchial-pulmonary carcinomas, other than squamous cell carcinomas. It is also important to clear define, in future studies, the predictive value of both *FGFR1* amplification and FGFR1 protein expression by evaluating these parameters in clinical trials.

Conclusions

Epidermoid carcinomas and adenocarcinomas are distinct entities with different pathological, biological and molecular characteristics.

Epidermoid carcinoma:

Epidermoid carcinoma preneoplastic lesions have a basal cell origin.

Stepwise higher Ki67, p53 and EGFR expression from basal cell hyperplasia to squamous dysplasia reinforce their importance as potentially significant gravity biomarkers for preneoplastic lesions.

EGFR and *HER2* high gene copy number was mainly due to high polysomy, rather than to amplification; amplification is not the main mechanism for protein overexpression in epidermoid carcinoma preneoplastic lesions.

Recognition of preneoplastic lesion biomarkers may allow targeted screening and follow-up, especially if chemoprevention schemes are available.

Epidermoid carcinomas had EGFR protein overexpression, together with high gene copy number (by high polysomy and amplification). *EGFR* mutations were infrequent.

High gene copy number is a frequent event in lung epidermoid carcinoma, suggesting that *EGFR* gene copy may play a role in epidermoid lung cancer carcinogenesis.

In small biopsies, we must also take account of incomplete sampling, and some cases diagnosed as epidermoid carcinoma could be diagnosed as adenosquamous carcinoma in surgical specimens with extensive sampling.

Epidermoid carcinomas with solid, large cells or clear cell phenotype or with CK7 or even Vimentin (EMT phenotype) expression should be submitted to EGFR mutational analysis.

Epidermoid carcinomas with vimentin expression are more complex carcinomas where EMT pathways are important, which explain why they are less morphologically differentiated and have fusocelular phenotype or irregular infiltrative borders with large isolated cells. Thus, epidermoid carcinomas with these phenotypes should be reported to clinicians in order to make them aware that these patients may benefit from *EGFR* mutation testing. In fact, epidermoid carcinomas with solid, large cells or clear cell phenotype or with CK7 or even

Vimentin expression (EMT phenotype) are routinely submitted to *EGFR* mutational analysis in our hospital.

We could argue that *EGFR* mutational testing should also be used in biopsies with poorly differentiated epidermoid carcinomas, especially in cases diagnosed in never-smokers or with CK7/Vimentin expression.

Bronchial-pulmonary adenocarcinoma:

A comprehensive pathological diagnosis incorporating immunohistochemical markers such as p63 or CK5/6, CK7 and TTF1, as well as mucine detection by histochemical techniques, is essential, especially in small biopsies representing poorly differentiated carcinomas or those with some “squamous” features or solid patterns, in which it is difficult to differentiate between a poorly differentiated epidermoid carcinoma and a solid pattern adenocarcinoma.

Adenocarcinomas are molecularly different from normal adjacent lung tissue.

There was generally higher expression of the products of genes studied in the adenocarcinomas compared to normal adjacent cells reinforcing their importance in lung adenocarcinoma carcinogenesis.

Adenocarcinomas in non-smoking females were diagnosed in earlier stages and had higher ERCC1 expression.

Advanced-stages (IIA and IIIA) adenocarcinomas had higher Ki67, APC, ERCC1 expressions and lower TTF1 expression reflecting more mitotically aggressive adenocarcinomas and a possible non-TRU origin.

Differences of expression were identified between adenocarcinoma patterns. Papillary and solid patterns revealed less TTF1 expression, identical to normal tissue exhibiting a non-TRU/bronchial phenotype. Acinar, BA/lepidic and micropapillary patterns showed higher TTF1 expression (TRU type).

Solid patterns showed lower expression of nuclear TTF1 and higher expression of Ki67, reflecting aggressive biological behaviour, to be reported to clinicians in order to avoid poorly differentiated carcinoma reports.

TTF1 provides significant guidance for therapy concerning advanced lung adenocarcinoma, as *EGFR* mutations are more frequent in TTF1 positive TRU-type adenocarcinomas. On the other hand, TTF1 negative adenocarcinomas, some representing non-TRU adenocarcinomas, are less prone to harbor *EGFR* mutations.

The solid pattern revealed lower HER2 expression and higher EGFR and ERCC1 expression compared to the papillary pattern. The papillary pattern showed higher HER2 and lower ERCC1 expressions. The micropapillary pattern showed higher RB expression. Acinar showed lower ERCC1 and higher EGFR expressions. Cyclin D1 seems to have more importance in the acinar and BA/lepidic patterns than in the micropapillary. ERCC1 protein expression in micropapillary, solid and BA/lepidic patterns indicate DNA repair and in acinar and papillary patterns there was lower expression. BCL2 was overexpressed in all patterns, suggesting that there is inhibition of apoptosis.

Chemotherapy resistance might be explained in part by MRP1 and LRP overexpression in adenocarcinomas. Micropapillary, solid and lepidic patterns are also the patterns where ERCC1 expression was relevant, indicating an ability to remove Cisplatin adducts.

Higher MRP1 and LRP expression in adenocarcinoma indicate acquisition of drug resistance mechanisms during carcinogenesis.

The differences identified between the adenocarcinoma patterns reinforce the need to carefully specify the patterns due to their implications for diagnosis, pathogenic understanding and therapeutic outcome.

Cluster analysis yielded three clusters: 1 – papillary; 2 – solid; and 3 – lepidic/BA, acinar and micropapillary.

In adenocarcinomas, a correlation was found between *EGFR* FISH results and mutational status.

In general, *EGFR* mutations were present in all the patterns of the same adenocarcinoma, reinforcing the possibility of mutational status determination in biopsies.

Although rare, *EGFR* and *KRAS* mutation can coexist. The coexistence of *KRAS* and *EGFR* mutations in the same tumour again raises the question of tumour complexity.

Complex *EGFR* mutations (coexistence of more than one type) were detected in adenocarcinomas, clearly demonstrating a molecular complexity that might be related to different cell clones or genomic instability, responsible for the accumulation of multiple molecular events in the *EGFR* gene.

Bronchial-pulmonary carcinomas and FGFR1

FGFR1 protein expression was identified in all subtypes of lung bronchial-pulmonary carcinomas, especially in pleomorphic carcinomas. FGFR1 amplification, although more frequent in squamous cell carcinoma, was also identified in adenocarcinomas, adenosquamous and pleomorphic carcinomas. Therefore it is important to do not exclude from molecular testing patients with lung bronchial-pulmonary carcinomas, other than squamous cell carcinomas.

Bronchial-pulmonary carcinomas have particular biological, clinical and therapeutic implications. The importance of precise pathological diagnosis, avoiding NSCLC terminology, and adenocarcinoma pattern recognition is highlighted, as bronchial-pulmonary carcinogenesis particularly related to biomarker predictive value for targeted therapy, concerning *EGFR*, *KRAS*, *ALK* and *MET*.

Lung cancer is a complex biological and genetic entity that has proved challenging for research.

LIST OF PUBLICATIONS

List of publications

I – **Sousa V**, Espírito Santo J, Silva M, Cabral T, Alarcão AM, Gomes A, Couceiro P, Carvalho L. EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and P53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study. Virchows Arch. 2011 May;458(5):571-81

II – Couceiro P, **Sousa V**, Alarcão A, Silva M, Carvalho L. Polysomy and amplification of chromosome 7 defined for EGFR gene in epidermoid carcinoma of the lung together with exons 19 and 21 wild type. Rev Port Pneumol. 2010 May-Jun;16(3):453-62.

III – **Sousa, V**; Bastos, B; Silva, M; Alarcão, A; Carvalho, L. Bronchial-Pulmonary Adenocarcinoma Subtyping Relates With Different Molecular Pathways. In publication in Revista Portuguesa de Pneumologia - Received 30 October 2013, accepted 30 May 2014, available online 5 March 2015 – doi:10.1016/j.rppnen.2014.05.006

IV – **Sousa, V**; Rodrigues, C; Silva, M; Alarcão, A; Carvalho, L. Lung adenocarcinoma: sustained subtyping with immunohistochemistry and EGFR, HER2 and KRAS mutational status. Rev Port Pneumol. 2015;21:113-25

V – **Sousa, V**; Silva, M; Alarcão, A; D'Aguiar, M; Ferreira, T; Carvalho, L. EGFR and KRAS mutations coexistence in lung adenocarcinomas. Diagnostic Pathology 2015, 1:13 - http://www.diagnosticpathology.eu/content/index.php/dpath/article/view/13

VI – **Sousa, V**; Silva, M; Alarcão, A; Reis, D; Ladeirinha, AF, D'Aguiar, M; Ferreira, T; Carvalho, L. Targeted therapy for FGFR1 may be independent of the histological type of bronchial-pulmonary carcinomas. Manuscript submitted for publication – Submited / undergoing revision – Virchows Archive

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