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Analysis of the molecular expression profile of non small cell lung carcinoma associated to chronic obstructive pulmonary disease

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Summary. Chronic obstructive pulmonary disease (COPD) is an independent risk factor to develop lung cancer but there are no different functional clusters of biomarkers between patients with non-small cell lung cancer (NSCLC) with or without COPD. To analyse protein expression, in order to find out whether samples of resected NSCLC from patients with COPD present a different molecular expression. Observational, cohort, concurrent study with sampling since treatment of disease in patients with NSCLC in initial stages (pIA-pIIB) treated surgically in our hospital between October 1993 and September 1997. The study consisted of the elaboration of tissue arrays with samples from resected tumor, using immunohistochemistry as a study method. Univariate analysis and logistic regression analysis were performed in order to determine molecular markers that showed a differential expression in NSCLC of the patients with COPD. We studied thirty-two proteins in 146 patients. 30% of the patients had COPD. Univariate analysis in patients with COPD showed one molecular marker to be overexpressed and five molecular markers to be underexpressed. Multivariate analysis in patients with COPD identified membranous ß-Catenin as a differential biomarker, which displayed an underexpression, with an Odds Ratio (95% Confidence Interval) of 0.26 (0.07-1.01). A significant lowest expression of membranous ß-catenin was detected in NSCLC of the patients with COPD.

Key words: COPD, Immunohistochemistry, Molecular Biology, Non Small Cell Lung Cancer, Protein expression.

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Introduction

Lung Cancer (LC) is the most frequently occurring tumour accounting for the highest mortality rate in males due to cancer, both in Spain and worldwide (Coleman et al., 1993). Since 1990, cancer has overtaken cardiovascular disease as the leading cause of death in Spain (Alonso et al., 1996) due mainly to an increase in smoking (Doll and Peto, 1981). The consumption of tobacco accounts for 4.8 million deaths each year worldwide and it is estimated that by year 2030 this figure may have reached 10 million people (Ezzati and Lopez, 2003).

The association of LC with chronic obstructive pulmonary disease (COPD) is frequent, as tobacco is the principal causal agent in both diseases. However, a large number of studies have long been known to defend that the presence of COPD is a risk factor to develop LC independent from tobacco (Skillrud et al., 1986; Wasswa-Kintu et al., 2005), further suggesting that these could be two manifestations of the same disease (Petty, 2006). There is a higher incidence of LC in patients with COPD, and the worse COPD severity the greater the incidence, as measured in terms of FEV1 in percentage over the theoretical value (FEV1%) (Tockman et al., 1987).

COPD plays an adverse role in the operative morbimortality of LC (Harpole et al., 1999), with some papers claiming that COPD is also a negative prognostic factor in the long-term (Lopez Encuentra et al., 2005; Birim et al., 2006). FEV1 is the main prognostic factor in COPD (Anthonisen et al., 1986) and it is closely related to operative morbimortality in LC (Kearney et al., 1994; Win et al., 2005).

In the past years, great contributions have been made to further the knowledge in the identification of cancer-related genetic alterations, and especially in what relates

to the development of LC (Salgia and Skarin, 1998; Sekido et al., 1998, 2003; Sanchez-Cespedes et al., 2001). These molecular alterations have been linked to some clinical data, particularly with tobacco exposure (Marchetti et al., 1998; Ahrendt et al., 2001; Miura et al., 2002; Pao et al., 2004; Shigematsu et al., 2005), however, there is no literature demonstrating a different molecular expression of non-small cell lung cancer (NSCLC) of the patients with COPD so there are no different functional clusters of biomarkers between patients with NSCLC with or without COPD.

The principle aim of our study was to analyse whether samples of resected NSCLC from patients with or without COPD present a different molecular expression.

Materials and methods

Study design

Observational, cohort, concurrent study with sampling since treatment of disease. All patients included in the study had initial stages of NSCLC (pIA, pIB, pIIA, pIIB), met resecability and operability criteria and underwent thoracotomy with intent to cure. Criteria of functional operability and oncological resecability are as described by the Task Force of Bronchogenic Carcinoma of the Spanish Society of Pneumology and Thoracic Surgery (Work Group of Spanish Society of Pneumology and Thoracic Surgery, 1998).

Population and definitions

All patients who had been treated surgically in our centre between 1st October 1993 and 30th September 1997 were prospectively included. Exclusion criteria included: induction therapy, incomplete surgery, exploratory thoracotomy or operative mortality, defined as any death directly related to the surgical procedure, regardless of the time and location where death occurred. COPD is defined as FEV1/FVC values <70% from the theoretical value.

Methods

Proteins

Proteins to be studied were selected based on a thorough review of the literature to be potentially implicated in the development of LC, on the availability of specific antibodies and on their indication for use in paraffined material. Thirty-two molecular markers (Table 1) of different paths of NSCLC development were selected: cell cycle, p53 pathway, apoptosis, adhesion molecules, signal transduction receptors and other pathways.

Tissue selection

Anonymized tissue sections were obtained with informed patient consent and full local Medical Research Ethics Committee approval. Tumor samples were collected from 146 patients who underwent resection at the Hospital Universitario 12 de Octubre between 1993 and 1997. All tumors were classified by 2 pathologists (ECG and FLR) according to the 2004 World Health Organization classification. We do not include normal samples for the same patients.

Tissue microarrays of lung tumor tissues

A Tissue Arrayer (Beecher Instruments, Silver Spring, MD) was used to construct the tissue microarrays (TMAs). Formalin-fixed paraffin-embedded tissue blocks from 146 lung tumor tissues were used for the construction of the TMAs. One section of each TMA was stained with hematoxylin-eosin to confirm adequacy.

Immunohistochemistry

TMA blocks were sectioned at a thickness of 3 μm and dried for 16 hours at 56°C before being dewaxed in xylene and rehydrated through a graded ethanol series and washed with phosphate-buffered saline. Citrates tampon 10 mM pH6,5 was a general rule for the

Table 1. Molecular markers included, according to cell pathway.

Cell cycle	Cyclin A Cyclin B1 Cyclin D1 Cyclin E Kinase-dependent cyclin (KDC)2 KDC 6 P16 P21 P27 Retinoblastoma (RB) protein Ki-67 Cdc6
P53 pathway	P53 MDM2
Apoptosis	BCL-2 Caspase-3 Survivin NF-KBc (p65) FAS ligand (CD95)
Adhesion molecules	Membranous Beta-catenin E-cadherin
Receptor/ Transduction signal	AKTp Mammalian Target of Rapamicin (mTORp) Epidermic Growth FactorReceptor (EGFR) c-erbB2/Neu / Herceptest p-Acetyl-CoA Carboxylase (ACC) S6 Protein (PS6) LKB1
Others	Cyclooxigenase 2 (COX2) TTF-1 P63 Alpha-CP4

majority of antibodies except for cyclin D1 (pH 6.5+PK), cdc6 (EDTA 1 mM pH8), EGFR (Citratus tampon 10mM pH6.5, 16h, 4°C), Herceptest (Citratus tampon 10mM pH6), ACC (Citratus tampon AR: Trilogy) and LKB1 (Citratus tampon 10mM pH6.5 + PK). These sections were immunohistochemically stained using monoclonal antibody Ley 37D/G6 (1:100) summarized in Table 2. After incubation, immunodetection was performed by means of the LSAB (Labelled streptavidin-biotin) visualisation technique and the DAKO EnVision Visualization Method (DAKO, Glostrup, Denmark), using diaminobenzidine chromogen as the substrate. Two pathologists (ECG and FLR) reviewed independently the immunostaining score for each protein and performed a semi quantitative evaluation of the percentage of positive cells in each cylinder and the intensity of protein expression. The cut-off values are summarized in Table 2. Two possible categories were ultimately considered: "positive" and "negative". The evaluation was performed using uniform criteria and without prior knowledge of the clinical and pathologic characteristics of the patients. We provide a figure of samples of the immunohistochemical expression (positive and negative results) of Mb B catenin (Fig. 1).

The protein pattern expression in the resected tumour was homogeneous enough to allow the use of TMA technology for the evaluation of their expression.

Statistical analysis

For associative analysis, univariate analysis was initially performed to determine the frequency distribution of expression / non expression for each marker and the presence or absence of COPD, considering a $p<0.2$ as evaluable in this first analysis as a tendency but not for its statistical significance.

These proteins were subsequently included in a multivariate analysis, in which logistic regression was used to identify correlations and independent associations. The efficacy of the regression model was evaluated through the log Likelihood Ratio, used as a ratio of the model's performance and as a test of the model's statistical significance (independence X^2), by means of variance proportion as explained by the model, or adjusted generalized coefficient of determination ($R2_{adj}$) and the C value as a ratio to measure the relative concordance or degree of accuracy of the classification. The C index indicates the accordance level between predicted probabilities and observed responses, being equivalent to the area below the ROC curve (Brunelli et al., 1999).

Table 2. Antibodies, clones, commercial industries, dilution and cutt off value.

PROTEIN	CLON	INDUSTRY	DILUTION	CUTOFF VALUE
Cyclin A	6 E6	Novocastra	1:50	5% cells
Cyclin B1	7A9	Novocastra	1:25	15% cells
Cyclin D1	Sp4	Neomarkers	1:10	5% cells
Cyclin E	13A3	Novocastra	1:10	5% cells
CDK2	8D4	NeoMarkers	1:200	5% cells
CDK6	Poli-rabbit	BD PharMingen	1:350	5% cells
p16 (F-12)	F-12	Santa Cruz	1:25	10% cells
p21 (WAF1)	EA10	Oncogen	1:25	10% cells
p27	57	BD Transduction Lab	1:1000	5% cells
Rb	G3-245	BD PharMingen	1:100	10% cells
Ki-67	MIB-1	DAKO	1:50	20% cells
cdc6	180.2	Santa Cruz	1:25	Staining intensity 2+/3+
p53	DO-7	Novocastra	1:50	10% cells
MDM2	IF2	Oncogen	1:25	10% cells
Bcl-2	124	DAKO	1:25	10% cells
Caspase 3 act	C92-605	BD PharMingen	1:25	75% cells
Survivin	Poli-rabbit	RD System	1:1000	10% cells
NF-kb	F-6	Santa Cruz	1:350	>75%
FAS ligand	GM30	Novocastra	1:25	50% cells
B catenin	14	BD Transduction Lab	1:500	Staining intensity 2+/3+
E-cadherin	4A2C7	Zymed	1:50	75% cells
AKTp	Poli-rabbit	Cell Signaling	1:25	Staining intensity >1+
m-TORp	Poli-rabbit	Cell Signaling	1:10	Staining intensity >1+
EGFR	EGFR.113	Novocastra	1:10	10% cells
Herceptest	Policlonal	DAKO	1:25	25% cells
ACC	Policlonal (rabbit)	Cell Signaling	1:25	5% cells
p-S6	Poli-rabbit	Cell Signaling	1:50	Staining intensity >1+
LKB1	LEY37		1:10	5% cells
COX-2	SP21	NeoMarkers	1:25	Staining intensity 2+/3+
TTF-1	8G7G3/1	DAKO	1:25	50% cells
p63	4A4	DAKO	1:50	25% cells
Alpha-CP4		Zymed	1:1000	

The dependent variable in the multivariate analysis was the presence of COPD, and the independent variables were proteins with differences in expression ($p<0.2$) at univariate analysis. Upon multivariate analysis, molecular markers that expressed differently with a $p<0.05$ were considered to be significant.

Results

Study population

The study included 146 patients corresponding to all the cases with NSCLC in initial stages in which a thoracotomy with intent to cure had been performed during this period.

Table 3 shows the results of the variables relating to the patient, the tumor and the surgery. Over 90% of cases were men; with a high percentage of active smokers (59%), and a 30% of COPD patients. Median value of FEV1% in the study was 83.1%. Most cases were squamous cell carcinoma (68%) in pathological stage IA-IB (84%). 5 years survival was 55%. There are no significant differences in the percentage of squamous cell lung cancer between COPD group (71%) and non COPD group (66%). The percentage of active smoking patients was higher in COPD patients (66%) than in non COPD patients (53%) but there were no statistical differences (p=0.3).

Table 3. General characteristics of study patients (n=146).

Males	134 (91.8)
Age (years)	67; 61-73
Active smoker	85 (58.2%)
Years/package	50; 33-80
Years since beginning	40; 35-50
COPD	43 (29.5)
FEV 1%	83.1; 71.4-97.7
Casual finding	51 (34.9)
Type	
Squamous	99 (67.8)
Adenocarcinoma	33 (22.6)
Large cells	10 (6.8)
Sarcomatoid	4 (2.7)
Pathological tumour size (cm)	4; 3-5.8
Pathological stage	
IA	28 (19.2)
IB	94 (64.4)
IIA	6 (4.1)
IIB	18 (12.3)
Type of surgery	
Pneumonectomy	47 (32.2)
Bilobectomy	11 (7.5)
Lobectomy	85 (58.2)
Others	3 (2.1)
Survival state	
Alive	55 (37.7)
Dead	90 (62.3)

Qualitative variables are expressed in absolute and relative frequencies (n; %) and quantitative variables in median and interquartile range.

Univariate analysis

Table 4 shows the proteins that express differently for a level of significance of $p<0.2$ in NSCLC of patients with COPD. One protein was found to be overexpressed (COX2) and five proteins were found to be underexpressed in NSCL of patients with COPD (Cdc6, Caspase 3, AKTp, membranous expression of B-catenin and FAS ligand).

Multivariate analysis

Results from the COPD patient group are described in table 5. Mb B-catenin was identified as the only differential protein: it expresses more frequently in resected NSCLC of patients without COPD, with an Odds Ratio (95% Confidence Interval) of 0.26 (0.07-

Table 4. Percentage of positive protein expression. Univariate analysis according to COPD.

	TUMOUR TISSUE COPD (-) PATIENTS n=91	TUMOUR TISSUE COPD (+) PATIENTS n=43	p
COX2	77.4	88.4	0.18
Cdc6	23.5	10.1	0.14
AKTp	16.5	4.7	0.12
Caspase 3	28.2	16.3	0.11
FAS ligand	8.7	2.3	0.14
Mb B catenin	20.4	9.5	0.04

Table 5. Multivariate analysis of associated proteins in the patient population with resected NSCLC and COPD.

Likelihood Ratio Analysis				
Protein	Estimator	ErrorC	Chi-Square	Pr > ChiSq
Cdc6	-0.7329	0.4847	2.2859	0.1306
Caspase 3	-0.6015	0.5247	1.3138	0.2517
FAS	-12.5711	247.8	0.0026	0.9595
Mb B-catenin	-1.3397	0.6885	3.7859	0.0497
AKTp	-1.1491	0.8183	1.9717	0.1603
COX2	12.7576	320.1	0.0016	0.9682

Odds Ratio Analysis			
Protein	Estimator	Confidence Intervals 95%	
Cdc6	0.481	0.186	1.243
Caspase 3	0.548	0.196	1.533
FAS	<0.001	<0.001	>999.999
Mb B-catenin	0.262	0.068	1.010
AKTp	0.317	0.064	1.576
COX-2	>999.999	<0.001	>999.999

C Index (ROC area)		
	c	0.749

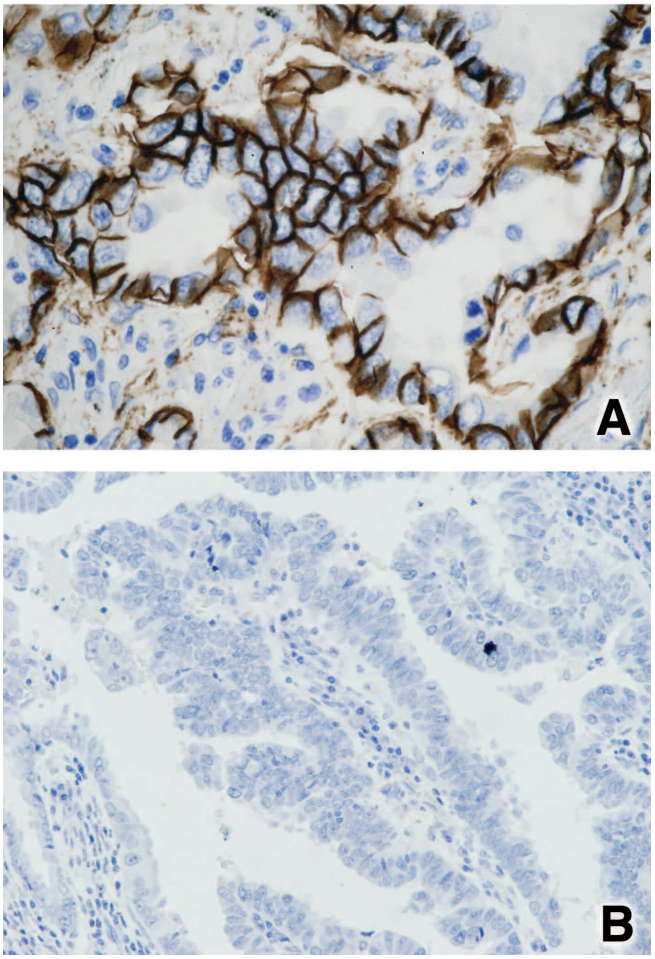

Fig. 1. A. Representative example of positive for the Membranous (Mb) B catenin immunostaining. **B.** Representative example of negative for the Membranous (Mb) B catenin immunostaining. x 400

1.01) and a concordance ratio of 0.75.

Discussion

This prospective study was conducted on a series of consecutive patients with resected NSCLC in initial stages and measured the expression of 32 proteins involved in the neoplastic process by means of tissue arrays.

Univariate analysis found several markers, either directly or inversely associated with the presence of COPD. Nonetheless, upon multivariate analysis, we were only able to find one biomarker that significantly underexpressed in NSCLC patients with COPD. In multivariate analysis we did not find any molecular marker which significantly overexpressed in NSCLC patients with COPD.

This study was based on NSCLC cases in stages pI-II that had been resected between 1993 and 1997. Most patients were men (92%), a figure that does not show an increase in the incidence of LC in women in Spain in these past years (Regidor et al., 1995). Mean age was 67 years, in line with other data available nationwide (Lopez Encuentra et al., 2002). The percentage of active smokers were 58%, similar to other series (Sanchez de Cos et al., 2000).

Squamous cell carcinoma was the most frequently found histological type in the surgical sample (68%). These data are similar to those described in other national series (Agudo et al., 1994; Sanchez de Cos et al., 2000), but different to series from other countries, in which the most prevalent histological type is adenocarcinoma (Rezola et al., 1996; Travis et al., 1995; Janssen-Heijnen and Coebergh, 2001). Although patients with COPD have a higher frequency of squamous cell carcinoma (Papi et al., 2004) in our study there are no statistical differences in percentage of squamous carcinomas between COPD and non COPD patients.

Regarding cigarette smoking, there are studies showing a different gene expression in resected NSCLC between smokers and non-smokers (Miura et al., 2002). K-RAS Oncogen (Marchetti et al., 1998; Ahrendt et al., 2001) and P53 tumour suppressor gene (Sanchez-Cespedes et al., 2001; Pfeifer et al., 2002; Fong et al., 1999) mutations occur more frequently in smokers, and epidermal growth factor receptor gene (EGFR) mutations occur more frequently in non-smokers (Pao et al., 2004; Shigematsu et al., 2005).

In COPD there are some studies claiming the existence of molecular mechanisms as a means to justify the association between LC and COPD, such as common chromosomal areas in chromosome 6q and in chromosome 12 (Schwartz et al., 2007), EGFR overexpression in the normal epithelium of patients with LC and in the epithelium of patients with COPD (Tang et al., 2005; De Boer et al., 2006), or increased expression of NFKB in bronchial biopsies from smoker patients and COPD (Di Stefano et al., 2002). Nonetheless, not much data has been published showing differences in the expression frequency of molecular markers in NSCLC patients with or without COPD.

Upon multivariate analysis, our study found only one protein that expressed differently in NSCLC of COPD population. Mb B-catenin is underexpressed more frequently in cases of resected NSCLC patients with COPD. Multivariate analysis did not find any molecular markers showing an overexpression in this population.

ß-catenin is an adhesion molecule which is involved in the intercellular adhesion of epithelial cells and tumor cells. ß-Catenin participates in signal transduction as a component of the Wnt/Wg signal pathway. The Wnt and epidermal growth factor receptor (EGFR) signaling pathways play crucial roles in the pathogenesis of a variety of malignant tumors, including lung carcinomas. Although the details of each cascade are understood, very little is known about their collective effects in

NSCLC, but is known that an aberrant methylation of Wnt antagonists is common in NSCLCs (Suzuky et al., 2007).

There are studies that demonstrate that EGFR modulation regulates the E-cadherin/ß-catenin complex and cell motility in human lung epithelial carcinoma cells. These results may have important therapeutic implications for the treatment of invasive human lung carcinomas via the restoration of the E-cadherin/ß-catenin complex using inhibitors of EGFR (Al Moustafa et al., 2002).

The Wnt pathway is activated by smoke in bronchial epithelium and treatment with Sulindac (a Wnt pathway specific inhibitor) resulted in decreased tumor mass so this could be an opportunity for inhibition of early stages of tumourogenesis in lung cancer (Emami et al., 2004).

Some studies indicate that Mb ß-catenin expression in NSCLC has a positive prognostic significance (Hommura et al., 2002) and it has been clearly demonstrated that COPD appears to be a negative prognostic factor in the long term in LC (Lopez Encuentra et al., 2005). A possible implication is that the worse survival of NSCLC seen in patients with COPD could partially be explained by the lower molecular expression of Mb B-Catenin in these patients.

The study presents some limitations. First of all, most immunohistochemical biomarkers are far from being homogeneously distributed in tumor bulk and false negative in TMA of tumor samples is an important reproducibility problem. Moreover, the selection of the proteins to be studied was based on a thorough review of the literature to be potentially implicated in the development of LC, availability of specific antibodies, and their indication for use in paraffined material. This is where some of the limitations of the study arise from, since there is no consensus regarding which protein ought to be studied in cases of resected NSCLC (this study did not include members of alpha7 nicotinic receptor, beta adrenergic pathways or COPD related inflammatory mediators, such as TNF alpha and interleukins that may modulate signal transduction pathways in cancer cells) or which study techniques of tissue arrays in LC would be employed, although in the majority of cases immunohistochemistry is used.

We were only able find one biomarker (Mb B-catenin) that underexpressed in NSCLC of the patients with COPD, but we have no data to conclude that this protein could explain the susceptibility of COPD patients to develop lung cancer. May be in future other studies could explain the molecular mechanisms to justify the association between NSCLC and COPD

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