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Aberrant gene methylation and bronchial dysplasia in high risk lung cancer patients☆

Rosell A.[a,j,l,*], Rodríguez N.[c,l], Monsó E.[d,k,l], Taron M.[e], Millares L.[f], Ramírez J.L.[e], López-Lisbona R.[a,l], Cubero N.[a,l], Andreo F.[g,l], Sanz J.[g,l], Llatjós M.[h], Llatjós R.[b], Fernández-Figueras M.T.[h,k], Mate J.L.[h], Català I.[b], Setó L.[a], Roset M.[i], Díez-Ferrer M.[a,l], Dorca J.[a,j,l]

[a] Department of Respiratory Medicine, Hospital Universitari de Bellvitge-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain
[b] Department of Pathology, Hospital Universitari de Bellvitge-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain
[c] Department of Respiratory Medicine, Hospital Comarcal de l'Alt Penedès, Vilafranca, Barcelona, Spain
[d] Deparment of Respiratory Medicine, Fundació Parc Taulí, Sabadell, Barcelona, Spain
[e] Laboratory of Molecular Biology, Institut Català d'Oncologia, Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain
[f] Fundació Parc Taulí, Sabadell, Barcelona, Spain
[g] Department of Respiratory Medicine, Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain
[h] Department of Pathology, Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain
[i] IMS Health, Barcelona, Spain
[j] Universitat de Barcelona, Barcelona, Spain
[k] Universitat Autònoma de Barcelona, Barcelona, Spain
[l] CIBER de Enfermedades Respiratorias, CIBERES, Bunyola, Mallorca, Spain

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ABSTRACT

Introduction: The risk for lung cancer is incremented in high degree dysplasia (HGD) and in subjects with hypermethylation of multiple genes. We sought to establish the association between them, as well as to analyze the DNA aberrant methylation in sputum and in bronchial washings (BW).
Methods: Cross sectional study of high risk patients for lung cancer in whom induced sputum and autofluorescence bronchoscopy were performed. The molecular analysis was determined on DAPK1, RASSF1A and p16 genes using Methylation-specific PCR.
Results: A total of 128 patients were enrolled in the study. Dysplasia lesions were found in 79 patients (61.7%) and high grade dysplasia in 20 (15.6%). Ninety eight patients out of 128 underwent molecular analysis. Methylation was observed in bronchial secretions (sputum or BW) in 60 patients (61.2%), 51 of them (52%) for DAPK1, in 20 (20.4%) for p16 and in three (3.1%) for RASSF1A. Methylated genes only found in sputum accounted for 38.3% and only in BW in 41.7%, and in both 20.0%. In the 11.2% of the patients studied, HGD and a hypermethylated gene were present, while for the 55.1% of the sample only one of both was detected and for the rest of the subjects (33.6%), none of the risk factors were observed.
Conclusions: Our data determines DNA aberrant methylation panel in bronchial secretions is present in a 61.2% and HGD is found in 15.6%. Although both parameters have previously been identified as risk factors for lung cancer, the current study does not find a significative association between them. The study also highlights the importance of BW as a complementary sample to induced sputum when analyzing gene aberrant methylation.

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1. Introduction

In Europe lung cancer is the second most common type of malignant tumor in men and the third in women, and remains the leading cause of cancer mortality in both sexes [1]. The overall five year survival rate for lung cancer remains one of the lowest for all solid cancers and has been estimated to be 13.0% in Europe [2]. It has been

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* Corresponding author at: Feixa Llarga, s/n. 08907 L'Hospitalet de Llobregat, Barcelona, Spain.
E-mail address: arosell@bellvitgehospital.cat (A. Rosell).

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demonstrated that screening with three annual low-dose chest computed tomography (CT) can reduce mortality for lung cancer in 20.0%, in a population of 55–74 year olds with a history of cigarette smoking of at least 30 pack-years. Nevertheless, a total of 96.4% of the non calcified solitary nodules found in low-dose CT were false positives. This resulted not only in an high consumption of financial resources, but also in patient anxiety, radiation exposure, invasive biopsies or surgery, along with associated pain, disability and, rarely, death [3].

In this setting, accurate and definitive diagnostic tools for determining true positive lesions are desirable at the first round. Flexible bronchoscopy is limited by its sensitivity, ranging from 34% to 88% [4], depending on the size of the lesion and its location. Even with newer bronchoscopic navigation systems, sensitivity for lung cancer is not higher than 70% for peripheral lesions [5]. Bronchoscopy diagnostic yield can be empowered by simultaneously obtaining surrogate biomarkers such as the presence of bronchial dysplasia, by means of autofluorescence or narrow band imaging [6], as well as genetic and epigenetic abnormalities from normal bronchial epithelium [7–9].

The transcriptional silencing of genes by CpG island methylation is recognized as a crucial component in lung cancer initiation and progression [10–12]. This epigenetic event is, together with mutation and loss of heterozygosity, a tumor inactivation mechanism of tumor suppressor genes. It has been reported that the number of genes involved in the onset of neoplasia that are methylated is equal to or greater than those in relation to genes inactivated by mutations. Some examples are the genes involved in cell cycle regulation (p16), apoptosis (death associated protein kinase 1 [DAPK1]) and signal transduction (ras effector homolog 1 [RASSF1A]) [13,14]. The predictive ability of gene methylation for lung cancer was analyzed by Belinsky et al. In a cohort of high risk lung cancer patients, it was observed that methylation of three or more genes from a panel of 14 genes in sputum collected within 18 months of diagnosis was associated with a 6.5-fold increase in the risk for lung cancer [15]. Recently, new genes are being discovered through DNA methylation microarray platforms. Sandoval et al. have found the prognostic value of a DNA methylation signature of stage I Non Small Cell Lung Carcinoma (NSCLC). Aberrant methylation of HIST1H4F, PCDHGB6, NPBWR1,ALX1, and HOXA9 confer a bad prognosis, with statistically significant shorter relapse-free survival [16].

The main objective of this study was to establish the relationship between bronchial dysplasia and aberrant methylation genes in the respiratory secretions of a sample of patients at risk of lung cancer. The second endpoint was to determine the complementariness of sputum and bronchial washing methylation results.

2. Methods

A cross-sectional multicenter study that included patients with a high risk for cancer was conducted at two universitary hospitals from a health area with 500,000 inhabitants each. The study is part of an ongoing project to evaluate the predictive value of a panel of methylated genes to detect dysplasia progression in COPD patients (CIDIS cohort). The eligibility criteria were: more than 30 cigarette packs per year (current and former smokers who quit smoking less than 15 years ago), chronic obstructive pulmonary disease (COPD), between 45 and 75 years old and able to sign the informed consent form. Patients were excluded if they were diagnosed with lung cancer during recruitment, if less than one year had passed since curative surgery of larynx or lung cancer, in cases of respiratory failure (Pa02 < 60 mmHg with 0.21 FiO2), severe COPD (FEV1 < 35%), chemotherapy, thoracic radiotherapy, systemic corticosteroids, or any contraindication for flexible bronchoscopy or sputum induction.

Patients attending the participant hospitals' outpatient pulmonology clinics who met the eligibility criteria, were consecutively recruited between July 2008 and June 2012. At the initial visit, informed consent was signed by the patient and information on demographic variables was gathered. Induced sputum was collected prior to autofluorescensce bronchoscopy following standard procedure. Briefly, 15 min after salbutamol inhalation (200 μg), normal saline (0.9%) and then hypertonic saline (3%, 4%, and 5%), nebulized by an ultrasonic nebulizer (Ultra-Neb 2000, DeVilbiss Heathcare Inc, Somerset, USA), were inhaled for each concentration over a period of seven minutes. Subjects were encouraged to cough deeply after the seven minute intervals and the all produced sputum was processed for cytology examination. Data were prospectively recorded through a web-based case report form.

2.1. *White light and autofluorescence bronchoscopy*

Autofluorescence bronchoscopy (AFB) was performed with a videobronchoscope SAFE 3000 from Pentax (Tokyo, Japan) in twin mode, combined simultaneously with white light bronchoscoy (WLB). All abnormal lesions detected by white light bronchoscopy, AFB or by both were recorded, and biopsy specimens were taken from those lesions. All bronchial biopsies were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin and classified according to the World Health Organization (WHO) lung cancer classification [17] as metaplasia, dysplasia (mild, moderate or severe) or carcinoma in situ (CIS) by two independent pathologists. In case of disagreement, a second simultaneous round was performed until consensus was achieved.

2.2. *Molecular analysis*

Peripheral blood, sputum and bronchial washing samples were collected from each clinical control and frozen at −20 °C until the end of the study. Peripheral blood samples (obtained in EDTA tubes for plasma) were spun for ten minutes at 1800 rpm at room temperature and the plasma carefully transferred into new tubes. The cell-free supernatants were re-centrifuged for 10 min at 1800 rpm to avoid lymphocyte contamination and were transferred to a new tube and stored at −20 °C. The sputum and bronchial washings (BW) of each patient were treated first with Sputazol® (0.1% dithiothreitol in phosphate buffer; Oxoid Unipath, Basingstoke, Hampshire, United Kindom, Europe), and after centrifugation they were transferred to a new tube and stored at −20 °C. For DNA isolation from fluids, we used the QIAmp DNA Blood Mini Kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA), but using for plasma a total of 800 microliters of serum and eluting the sample in 60 microliters of DNAse-RNase free distilled water. Molecular analysis was performed on DAPK1, RASSF1A and p16 genes. Gene promoter methylation status was assessed using Methylation-Specific PCR (MSP) and DNA bisulfite modification was performed according to previously described methods [18]. MSP reaction was performed with primers specific for each gene for either methylated or unmethylated DNA as described in Table 1. In all PCR reactions, negative and positive controls were included for each pair of specific primers: the DNA obtained from lymphocytes was used as unmethylated control, and methylated modified DNA (CpGenome™ Universal Methylated DNA, Chemicon, Millipore, Massachusetts, USA) was used as methylated control.

2.3. *Statistical analysis*

A descriptive analysis of demographic and clinical variables was performed. The mean and standard deviation (SD) of continuous variables and the percentage distribution of categorical variables were calculated. Molecular results were analzed by gene (DAPK1,

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