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A novel combined score of biomarkers in sputum may be an indicator for lung cancer: A pilot study

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ABSTRACT

Background: Lung cancer is a leading cause of morbidity and mortality worldwide and there is an urgent need for sensitive, specific, and reliable biomarkers.
Methods: The study population included 60 patients (31 with lung cancer and 29 with chronic obstructive pulmonary disease [COPD]) and thirty healthy individuals comprised the control group. Measurements of neutrophil, beclin-1, VEGF, ICAM, VCAM, and TNF-alpha levels in induced sputum were analyzed as possible biomarkers for lung cancer.
Results: Neutrophil, beclin-1, VEGF, ICAM and TNF-alpha levels of lung cancer patients differed significantly compared to those of COPD patients and healthy controls. A novel combined-score was created which was found to increase the likelihood to belong to the cancer group by 70% (odds-ratio 1.70 CI = 1.310–2.224,$p < 0.001$).
Conclusion: Biomarkers of autophagy, angiogenesis and inflammation in lung-cancer patients are significantly different from controls, and combination of these markers may be an indicator for lung cancer.

1. Introduction

Lung cancer is one of the leading causes of cancer-related death worldwide [1]. Despite significant advances in surgical techniques and medical targeted treatment and immunotherapy, the 5- year survival rate in patients with lung cancer remains poor with an overall 5-year survival rate of 4–17% [2] The extremely poor prognosis associated with lung cancer is related to the difficulty of early diagnosis and high incidence of regional or distant metastasis and the occurrence of treatment resistance.

While age, family history of lung cancer and occupational exposures are factors that may lead to lung cancer, cigarette smoking is the main risk factor. Recent results from the National Lung Screening Trial have shown that annual screening of high-risk smokers with low-dose computed tomography of the chest can reduce lung cancer mortality by 20.0% [3], however one major caveat of this important study was a very high incidence of false positive results, which may lead to high screening costs and unnecessary invasive procedures, if such a screening program is applied. Therefore there is an urgent and growing need to develop and validate biomarkers that can both help identify those smokers at highest risk who are most likely to benefit from screening.

Since autophagy, inflammation and angiogenesis are an integral part of lung cancer pathogenesis and propagation [4–9], we aim to examine a combination of markers of these processes as an indicator for lung cancer, in the novel milieu of induced sputum. Induced sputum was chosen as a non-invasive method to sample the immune cells and cytokines in the lung environment. These markers cannot be sample or not accurate in blood or breathe condensate.

Abbreviations: ANOVA, Analysis of Variance; ATS, American Thoracic Society; BMI, Body Mass Index; CI, Confidence Interval; COPD, Chronic Obstructive Pulmonary Disease; ECOG, Eastern Cooperative Oncology Group; ELISA, Enzyme-Linked Immunosorbent Assays; FEV1, Forced Expiratory Volume 1 s; ICAM, Intracellular Adhesion Molecule; NSCLC, Non-Small Cell Lung Cancer; ROC, Receiver Operating Characteristic; SCLC, Small Cell Lung Cancer; SD, Standard Deviation; TNF alpha, Tumor Necrosis Factor Alpha; VCAM, Vascular Cell Adhesion Molecule; VEGF, Vascular Endothelial Growth Factor

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2. Methods

2.1. Study population

The study population included 60 patients (31 with lung cancer and 29 with chronic obstructive pulmonary disease [COPD]) recruited from the outpatient clinic of the institute of pulmonary diseases in the Tel-Aviv Sourasky Medical Center, between Feb. 2013 and Jan. 2016. Thirty healthy individuals comprised the control group. Each participant had undergone a comprehensive medical evaluation, spirometry and sputum induction. Patients with comorbidities such as known cardiovascular disease, other active malignant disease in the last 5 years, asthma, known allergy to salbutamol and an Eastern Cooperative Oncology Group (ECOG) performance status > 1 were excluded. Written informed consent for the use of the samples and data was obtained from all participants prior to enrollment, and the study was approved by the institutional Ethics Committee and complied with the principles of the Declaration of Helsinki (TLV-07-316).

2.2. Spirometry

Pulmonary function tests were performed by a Masterlab spirometer (Masterlab E. Jaeger, Wurzburg, Germany). The measurements were made with standard protocols according to the American Thoracic Society (ATS) guidelines [10]. The results are expressed as % of predictive values. The best of three consecutive measurements was chosen.

2.3. Sputum induction and processing

Sputum induction was conducted in all three groups according to a standard method as previously described elsewhere [11]. Lung cancer patients underwent pulmonary function testing and sputum induction 1–3 weeks after their diagnosis, prior any treatment for their disease.

Briefly, nebulized 3% saline was administered through an ultrasonic nebulizer which the subjects inhaled for 15 min. The selected plugs were treated with 0.1% dithiothreitol (Sputolysin; Calbiochem Corp., San Diego, CA, USA). The supernatant was divided into aliquots and stored at −80 °C. Cell pellets were resuspended in RPMI medium. Cytospins were prepared and 300 non squamous cells (immune cells) were deferentially counted using cytospin Giemsa stain slides. The percentage of each cell type (macrophages, neutrophils, lymphocytes, eosinophils and mast cells) was calculated in three different fields. For detection of human VEGF, VCAM, ICAM, TNF-alpha we used Human VersaMAP Mulitplex(R&D system), and for beclin 1 levels, sandwich enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's instructions (USCN Life Sciences Inc.) with a minimum detection limit of 0.156 ng/ml, values represent mean concentrations of two independent experiments.

2.4. Statistics

Continuous variables were evaluated for normal distribution using histograms and Q-Q plots. Normally distributed continuous variables were described as mean and standard deviation (SD). All data were summarized and displayed as mean ± standard deviation (SD) for the continuous variables and as number of patients plus the percentage in each group for categorical variables. Normally distributed measures were analyzed using One-way ANOVA with the Dunnett's T3 post Hoc adjustments for multiple comparisons. Measures with irregular distributions were analyzed using the non-parametric Kruskal-Wallis 1-way ANOVA (k samples) with multiple comparisons. For all categorical variables the Chi-Square statistic was used to assess the statistical significance between the three groups (cancer, COPD, control).

A binary logistic regression model was used to address odds ratio for cancer, and a Receiver Operating Characteristic (ROC) curve was used to describe sensitivity and specificity levels of our combined score to detect cancer.

All the above analyses were considered significant at $p < 0.05$ (two tailed). The statistical package for the Social Sciences (SPSS) version 22 was used to perform all statistical evaluation (SSPS Inc., Chicago, IL, USA).

Table 1
Characteristics of participants.

	Controls	COPD patients	Lung cancer patients	P value*
n	30	29	31	
Age, years	65.4 (10.7)	66.1 (11.0)	69.9 (9.2)	0.187
Gender,% male	56.7	31.0	61.3	0.043
BMI, kg/m2	24.7 (3.6)	25.5 (2.9)	25.0 (5.7)	0.740
Present smoker, %	13.3	27.6	77.4	< 0.001
Pack years	1.7 (4.9)	17.8 (44.1)	50.2 (35.7)	< 0.001
FVC, %	100.1 (12.4)	85.9 (20.6)	79.4 (21.3)	< 0.001
FEV1, %	101.8 (11.3)	77.1 (16.3)	72.8 (23.2)	< 0.001
FEV1/FVC	0.83 (0.1)	0.6 (0.14)	0.74 (0.13)	0.018
FEV25–75, %	105.3 (29.9)	61.4 (27.0)	52.6 (32.9)	< 0.001

COPD = chronic obstructive pulmonary disease; BMI = body mass index; FVC = forced vital capacity; FEV1 = flow expiratory volume in one second; FEV25–75 = flow expiratory volume 25–75.
* P value significant at < 0.05.

2.5. Results

The clinical and pathological features of the studied subjects are summarized in Table 1. Of note, our COPD group persists of higher percentage of females than the other two groups, in order to exclude gender bias all statistical analysis were adjusted to gender. As expected lung functions were lower in COPD and lung cancer patients compared to control group. The lung cancer patients had significantly lower flow expiratory volume in one second (FEV1) values than the COPD patients, probably because higher current smoking status and pack/years in this group (Table 1).

Fourteen of the lung cancer patients (45%) had adenocarcinoma, 13 (42%) had squamous cell carcinoma, and 4 (13%) had small cell lung cancer (Table 2). Cancer patients were staged according to TNM staging system. The type and stage of cancer did not influence the results. Differential sputum cell counts are shown in Fig. 1.The Neutrophil counts differed significantly, and they were found to be increased in the COPD and lung cancer patients compared to the controls ($p < 0.001$). The Macrophage counts also differed significantly, and were found to be decreased in lung cancer patients compared to the controls ($p = 0.035$). Lymphocyte and eosinophil counts were similar for the 3

Table 2
Characterization of lung cancer patients.

Parameter	Type	n	%
Histology	Adenocarcinoma	14	45
	Squamous cell carcinoma	13	42
	Small cell lung cancer	4	13
Stage	NSCLC 1	5	16
	NSCLC 2	0	0
	NSCLC 3	10	32
	NSCLC 4	12	39
	SCLC limited disease	0	0
	SCLC extensive disease	4	13
Localization	RUL	10	32
	RML	1	3
	RLL	6	19
	RUL	7	23
	LLL	7	23

NSCLC = non small cell lung cancer; SCLC = small cell lung cancer; RUL = right upper lobe; RML = right middle lobe; RLL = right lower lobe;LUL = left upper lobe; LLL = left lower lobe.