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Metabolomic study of serum, urine and bronchoalveolar lavage fluid based on gas chromatography mass spectrometry to delve into the pathology of lung cancer

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ABSTRACT

This study explores for the first time the combination of serum, urine and bronchoalveolar lavage fluid (BALF) to deep insight into the pathology of lung cancer (LC) using a metabolomic platform based on gas chromatography mass spectrometry (GC—MS). The study includes LC patients, healthy control group (HC) and a group of patients with noncancerous lung diseases (NCC) used as a control group respect to BALF because of the invasive nature this fluid collection.

The metabolomic platform was applied to serum, urine and BALF samples in order to compare the metabolomic profiles of these biological fluids and establish metabolic similarities and differences between them. The application of PLS-DA presented a clear classification of groups for all types of samples, indicating the existence of altered metabolites in LC. Twenty six and thirty one perturbed metabolites in the LC were annotated in the comparison of serum and urine samples. On the other hand, sixteen metabolites were altered in BALF of LC patients compared to NCC. The pathway analysis indicated that several amino acid metabolic routes were the most affected in LC. Finally, ROC curves were applied to the dataset and metabolites with an AUC value higher than 0.75 were considered as relevant in the progression of LC.

1. Introduction

It is well-known that the aggressive character of lung cancer (LC) causes more deaths than the combination of breast, prostate, colon and pancreatic cancers together [1]. LC is an unspecific disease difficult to discriminate from other lung disorders that leads to late diagnosis, being necessary to have safe and early diagnosis methods that allow to increase the averaged survival period of patients longer than five-years from 15% of cases to 85% [2]. Several biomarkers have been extensively used for the diagnosis of LC as carcinoembryonic antigen (CEA), cancer antigen 125, cytokeratin 19 fragment (CYFRA21-1), and neuron-specific enolase [3], but these biomarkers present low accuracy, which limit their utility for early detection of LC.

Screening techniques, such as low-dose computed tomography (LDCT) could reduce mortality due to LC by 20%, however, the incidence of high false-positive rates (96%) decrease the efficacy of the technique and complementary noninvasive and sensitive/specific biomarkers should be used in conjunction with LDCT for more accurate diagnosis [4]. Other radiologic diagnosis techniques, such as ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography FDG (PET/CT), have proved a high capability to detect cancerous lesions in the lung, despite their high cost makes these tests unable for general public annual screening under any healthcare system.

Consequently, a deeper knowledge of metabolites involved in the normal physiological function of human cells and organs and the suitable interpretation of theirs interactions is essential to understand pathological mechanisms of diseases and the searching of early diagnostic markers, and metabolomics is a very suitable

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tool for this purpose [5]. Metabolomics has emerged as a valuable complement to genomics and proteomics to provide information related to cellular metabolic processes that drive tumor formation and progression. Metabolomics allows evaluating the variability in the number and type of metabolites expressed in lung tumors, which are related to the type, stage, and, potentially, the response to drug treatment [6].

Therefore, the use of metabolic biomarkers to supplement imaging diagnosis is an interesting alternative to detect the onset of the disease, and the application of non-targeted metabolomic techniques for this purpose can provide new options in this field, since it can monitor the changes of these key-biomolecules in response to LC.

Several metabolomic platforms has been proposed in searching LC biomarkers based on NMR [7] and MS [2]. Generally, MS metabolomic approaches use couplings with HPLC [7] capillary electrophoresis [8] and gas chromatography [2,9], although direct infusion of the sample into the mass spectrometer has also been proposed [10].

Several human biofluids and tissue biopsies have been used in metabolomic studies [5] aimed to LC diagnosis, such as serum [2,11,12], plasma [13,14], urine [15], sputum [16], and lung tissues [2,17], but only a few authors had described the combined use of fluids or tissues for LC diagnosis purpose [2]. This complementary approach is useful because similar changes in both biofluids and resected tumors from cancer patients can reflect metabolic changes in the tumor itself. In addition, during oncogenesis the levels of some metabolites increase releasing into the blood, which results in higher levels of these metabolites in the serum. On the contrary, certain metabolites can be transferred from the blood to lung tumor promoting cancer proliferation [6]. So, the combined study of tumor tissues and biofluids allows not only the search for biomarkers for early diagnosis, but also as indicators of disease progression.

In connection to this, bronchoalveolar lavage is a fluid obtained during the exploratory study of lung patients (bronchoscopy) through instillation and later aspiration of liquid in one or more lung segments, which provide information about cellular and epithelial surface of the lower respiratory tract. It is estimated that BALF samples take a million cells (1% of the lung surface), providing about 1 ml of pulmonary secretions in the total recovered liquid [18]. Since BALF is in close interaction with lung tissue is a very representative sample of lung status, and presents some equivalence with lung biopsies.

Previous metabolomic studies based on BALF samples consider different lung diseases, such as asthma [19] or cystic fibrosis [20], but only one study reported by Callejón-Leblic et al. [21] focus on the use of this fluid in LC diagnosis.

The aim of the present study was to identify altered metabolites in three different fluids from LC patients, which could provide interesting information about the pathology of LC and the proposal of tentative biomarkers for early diagnosis of the disease. To our knowledge, this is the first study which presents the joint study of these three fluids (serum, urine and BALF) in the diagnosis of LC using a metabolomic platform based on gas chromatography mass spectrometry (GC–MS).

2. Material and methods

2.1. Sample collection

Blood and urine samples were collected from 32 lung cancer patients and 29 healthy people (HC) at the Pneumonology Area of Juan Ramón Jiménez Hospital (Huelva, Spain) from July 2011 to July 2013. In the same way, a total of 54 BALF samples (24 LC and 30 NCC) obtained by bronchoscopy, were collected. Because the invasive character of the technique used to obtain the BALF, it is not possible to get samples from healthy people. In addition, the 24 BALF samples from LC patients overlapped to 24 serum and urine samples from the same patients. The blood samples were obtained by venipuncture of the antecubital region, after 8 h of fasting, and collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system. The samples were immediately cooled and protected from light for 30 min to allow clot retraction. After centrifugation (2057 g for 10 min) serum, urine and BALF samples were aliquoted in Eppendorf tubes and frozen at −80 °C until analysis. Clinical data of LC, NCC and HC patients are shown in Supplementary Material (Table 1 from serum and urine samples and in Table 2 from BALF samples).

Table 1
Altered metabolites in serum from LC with VIP, sense of alteration, fold change (LC/HC), p-value from one-way ANOVA followed by Tukey test and AUC values.

Metabolite	Rt (min)	VIP	Disturbance sense	Fold change	*p* values	AUC
L-Valine*	4.15	1.5	↓	0.53	0.001	0.75
L-Glycine*	4.63	1.33	↓	0.91	0.022	0.76
Tartaric acid	5.02	1.2	↓	0.54	0.01	0.72
L-Serine	5.32	1.12	↓	0.67	0.012	0.67
L-Threonine	5.48	1.38	↑	1.54	0.009	0.69
Uridine*	5.82	1.31	↓	0.6	0.004	0.76
Malonic acid*	6.23	1.02	↑	1.02	0.002	0.75
L-Proline	6.45	1.39	↓	0.64	0.002	0.71
L-Cysteine	6.92	1.28	↑	1.9	0.039	0.64
L-Glutamine	7.40	1.57	↓	0.61	0.043	0.67
L-Phenilalanine	7.53	1.92	↑	1.03	0.022	0.73
Fructose*	7.57	2.24	↓	0.69	0.009	0.75
Phosphoric acid*	8.35	1.63	↓	0.82	0.005	0.77
Isocitric acid	8.57	2.27	↓	0.77	0.013	0.61
L-Asparagine*	8.77	2.57	↓	0.52	0	0.83
Inositol	8.87	1.66	↓	0.56	0.002	0.74
L-Ornithine	8.97	1.32	↓	0.7	0.018	0.65
Deoxy-glucose*	9.10	2.31	↑	4.24	0.046	0.8
Glucose*	9.38	2.74	↑	2.29	0.001	0.82
Palmitic acid	10.18	2.01	↓	0.94	0.037	0.53
Uric acid*	10.72	2.64	↓	0.38	0.003	0.79
Stearic acid*	11.38	1.82	↓	0.41	0.001	0.8
L-Cystine	11.88	1.48	↓	0.91	0.009	0.54
Myristic acid	12.45	2.42	↓	0.5	0.042	0.73
Margaric acid*	13.93	2.24	↑	0.42	0.012	0.78
Arachidonic acid	15.30	1.98	↓	0.65	0.02	0.67

* Metabolites with AUC higher than 0.75.

The work was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of Juan Ramón Jiménez Hospital and University of Huelva.

2.2. Reagents

All the solvents used were of HPLC-grade. Methanol, ethanol and pyridine were purchased from Aldrich (Steinheim, Germany). Formic acid was supplied by Merck (Darmstadt, Germany) and derivatizing agents, namely methoxylamine hydrochloride and *N*-methyl-**N**-(trimethylsilyl) trifluoroacetamide (MSTFA), as well as urease from *Canavalia ensiformis* (Jack bean) Type IX powder 50000–100000 units/g solid were obtained from Aldrich (Steinheim, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK).

2.3. Sample treatments

Serum samples were extracted following a procedure described by Bruce et al. [22]. In brief, 100 μl of serum were mixed with 400 μl of 1:1 MeOH/EtOH mixture in an Eppendorf tube and vortexed for 5 min at room temperature followed by centrifugation at 2057 g for 10 min at 4 °C to discard the pellet which contains the protein