Journal of Pharmaceutical and Biomedical Analysis 100 (2014) 369–380

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Metabolomics provide new insights on lung cancer staging and discrimination from chronic obstructive pulmonary disease

Stanislaw Deja[a], Irena Porebska[b], Aneta Kowal[b], Adam Zabek[c], Wojciech Barg[d], Konrad Pawelczyk[e], Ivana Stanimirova[f], Michal Daszykowski[f], Anna Korzeniewska[b], Renata Jankowska[b], Piotr Mlynarz[c,*]

[a] Faculty of Chemistry, Opole University, Pl. Kopernika 11a, 45-040 Opole, Poland
[b] Department and Clinic of Pulmonology and Lung Cancers, Wroclaw Medical University, Grabiszynska 105, 53-439 Wroclaw, Poland
[c] Department of Bioorganic Chemistry Wrocław University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland
[d] Department of Physiology, Wroclaw Medical University, T. Chalubinskiego 10, 50-368 Wroclaw, Poland
[e] Department and Clinic of Thoracic Surgery, Wroclaw Medical University, Grabiszynska 105, 53-430 Wroclaw, Poland
[f] Institute of Chemistry, The University of Silesia, Szkolna 9, 40-006 Katowice, Poland

ARTICLE INFO

Article history:
Received 22 April 2014
Accepted 13 August 2014
Available online 21 August 2014

Keywords:
COPD—chronic obstructive pulmonary disease
Lung cancer
Metabolomics
^{1}H NMR spectroscopy
Metabolic fingerprinting

ABSTRACT

Chronic obstructive pulmonary disease (COPD) and lung cancer are widespread lung diseases. Cigarette smoking is a high risk factor for both the diseases. COPD may increase the risk of developing lung cancer. Thus, it is crucial to be able to distinguish between these two pathological states, especially considering the early stages of lung cancer. Novel diagnostic and monitoring tools are required to properly determine lung cancer progression because this information directly impacts the type of the treatment prescribed. In this study, serum samples collected from 22 COPD and 77 lung cancer (TNM stages I, II, III, and IV) patients were analyzed. Then, a collection of NMR metabolic fingerprints was modeled using discriminant orthogonal partial least squares regression (OPLS-DA) and further interpreted by univariate statistics. The constructed discriminant models helped to successfully distinguish between the metabolic fingerprints of COPD and lung cancer patients (AUC training = 0.972, AUC test = 0.993), COPD and early lung cancer patients (AUC training = 1.000, AUC test = 1.000), and COPD and advanced lung cancer patients (AUC training = 0.983, AUC test = 1.000). Decreased acetate, citrate, and methanol levels together with the increased *N*-acetylated glycoproteins, leucine, lysine, mannose, choline, and lipid (CH_3—($C\underline{H}_2$)*n*—) levels were observed in all lung cancer patients compared with the COPD group. The evaluation of lung cancer progression was also successful using OPLS-DA (AUC training = 0.811, AUC test = 0.904). Based on the results, the following metabolite biomarkers may prove useful in distinguishing lung cancer states: isoleucine, acetoacetate, and creatine as well as the two NMR signals of *N*-acetylated glycoproteins and glycerol.

1. Introduction

Lung cancer diagnostics, treatment, and aftercare monitoring remain a great challenge in oncology. This highly preventable disease is the most frequent cancer worldwide (e.g., 1.38 million people diagnosed with lung cancer died in 2008) [1]. Despite the progress made in molecular oncology, lung cancer prognosis remains poor (15% five-year survival rate) [2], underscoring the need for new treatment regimens based on highly specific biological therapies and further development of diagnostic tools. The unfavorable prognosis of lung cancer is attributed to the following observations: early stage lung cancer is asymptomatic and clinical symptoms typically appear in the late stages of this disease. Usually, patients with lung cancer are in advanced age and concomitant diseases limit available method of treatment. Positive screening results based on low-dose computed tomography (LDCT) offers hope for improving this situation, but this procedure is still not a routine clinical practice [3]. The important problem related to LDCT results is overdiagnosis [4]. The discrimination between more or less aggressive as well as benign or malignant tumour is still not well established in LDCT [5].

* Correspondence to: Bioorganic Group, Department of Chemistry, Wroclaw University of Technology, Wybrzeże Wyspianskiego 27, 55-093 Wroclaw, Poland. Fax: +48 713204597.
E-mail address: piotr.mlynarz@pwr.wroc.pl (P. Mlynarz).

http://dx.doi.org/10.1016/j.jpba.2014.08.020
0731-7085/

The need for a simple, cheap, and readily available screening test that could serve as a stand-alone screening function or improve the specificity of low-dose computed tomography remains.

The most common type of lung cancer is non-small cell lung cancer (NSCLC), which accounts for approximately 85% of all lung cancer cases, followed by small cell lung cancer (SCLC), which accounts for approximately 15% of cases [6–8]. Other types of lung cancer are relatively rare.

The tumor node metastasis (TNM) staging system is widely accepted for NSCLC classification. Patients are classified according to four stages (I to IV). However, marked heterogeneity among patients, especially in the third stage, is observed in clinical practice. Patients within group III can be further divided into two sub-groups IIIA and IIIB; however, considerable controversy remains regarding this subdivision, which is primarily related to the treatment of N2 (IIIA) and T4 (IIIB) patients [9]. Combined treatment, including surgery and chemotherapy or chemoradiotherapy, produces unsatisfactory long-term survival for stage III. In stage IV patients, the results of chemotherapy and target treatment are still disappointing. Adverse events resulting from combined treatment are common, thereby explaining why some patients are unable to complete the planned therapy. This issue is of particular concern in stage IV patients. Some of the patients rapidly decline, whereas others on the same treatment regimen exhibit longer survival rates. Therefore, proper classification of lung cancer patients is important to prescribe the appropriate type of treatment and new predictive markers are expected.

In addition to lung cancer, chronic obstructive pulmonary disease (COPD) is a common lung disease. Cigarette smoking is a common risk factor for NSCLC and COPD. COPD is thought to increase the risk of lung cancer [10] and is even considered an independent risk factor [11]. Patients diagnosed with lung cancer often experience COPD symptoms [12]. Based on increasing data in the literature, inflammation is thought to be involved in the pathogenesis of both diseases [13–15].

Chronic inflammation is a potential factor associated with tumor development. The following examples typify the evolution of chronic inflammation to cancer: infection (e.g., *Helicobacter pylori* in gastric carcinoma), chronic immune-mediated inflammatory disorders (e.g., inflammatory bowel disease in colorectal cancer) as well as the development of lung cancer from COPD [16]. Therefore, COPD may increase the risk of developing lung cancer from chronic inflammation regardless of cigarette smoking. However, recent studies have implicated smoking as the primary risk factor for lung cancer despite the fact that COPD is strongly associated with lung cancer [17]. Cigarette smoke induces not only inflammation, but also contains genotoxic factors. All these elements together result in accumulation of genetic errors, which are essential for lung cancer development. Recently recommended prevention strategies highlight the importance of smoking cessation in the prevention of COPD and lung cancer. Secondarily, these measures seek to prevent the development of lung cancer in COPD patients [16]. Therefore, a chemically measurable indicator that differentiates between these two states is needed.

Both pathological states should be reflected in qualitative and quantitative molecular changes in the substrate and products of disrupted biochemical pathways at the cellular level. Thus, these disease hallmarks should be reflected in the chemical composition of body fluids. Data-rich analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), have the potential to generate a highly informative metabolic fingerprint during single measurements. Instead of relying on one biomarker for classification, it is possible to combine two or more biomarkers to enhance classification power. The combination allows for more accurate diagnoses, reflecting the biochemical pathways perturbed by pathogenesis. A multi-biomarker approach enables to distinguish between similar diseases [18]. Therefore, metabolomic-based studies have been extensively used for disease recognition, prognosis, predicting response to treatment and recovery monitoring. Recently, numerous COPD and lung cancer studies have revealed the great potential of metabolomic-based approaches in disease diagnostics and stratification [19–25]. Moreover this molecular technique, based on laboratory analysis of small blood sample, is minimally invasive for patient.

Considering the above aspects of NSCLC, the primary goal of our study was to perform a metabolomic analysis and generate metabolic fingerprints of NSCLC patients from various well-defined disease stages and to compare them with COPD patients. The metabolic data were used to construct discriminant models to aid in the differentiation of COPD patients from NSCLC patients at various stages. In addition, we sought to identify potential metabolic biomarkers related to these differences. The development of diagnostic models may be potentially useful in a clinical setting, providing valuable information concerning patient diagnosis, stratification and monitoring.

2. Materials and methods

2.1. Clinical population

In total, 77 non-small cell lung cancer (NSCLC) and 22 COPD patients were included in this study. For this study, the term "lung cancer" refers to non-small cell histological types. These patients were hospitalized in the Department of Pulmonology and Lung Cancer or Thoracic Surgery at Wroclaw University of Medicine from August 2011 to March 2013. A COPD diagnosis was made according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [26], which considers clinical symptoms and the spirometry test. Lung cancer diagnoses were made on the basis of pathological exams of tissue specimens obtained either from diagnostic bronchoscopy or curative surgery. NSCLC patients were classified according to the VII edition of the International Association for the Study of Lung Cancer (IASLC) TNM system [27]. The stage of patients undergoing therapeutic surgery was pathological, and the remaining cases were clinical. NSCLC patient characteristics are presented in Table 1.

All of the COPD patients were in a stabilization period without symptom exacerbation. Lung cancer patient sera were collected before disease treatment (surgery, radio- or chemotherapy), whereas the pharmacological treatment of the coexisting diseases was continued. During sample collection, COPD patients were treated according to disease stage and concomitant diseases. The study protocol was approved by the ethics committee of Wroclaw University of Medicine, and all of the patients provided written informed consent (KB-12/2010 and KB-263/2013).

2.2. NMR spectroscopy sample preparation

Blood samples (9 mL each) were collected in the morning and 12 h after the last meal and centrifuged at 4000 rpm for 10 min at 4 °C. The serum samples were then immediately frozen and stored at −80 °C until further analysis. Prior to the metabolomic experiments, the serum samples were thawed at room temperature and vortexed. Next, the mixtures containing 200 μL of serum and 400 μL of saline solution (0.9% NaCl in 15% D_2O) were mixed again [28]. After centrifugation (15000 rpm for 10 min), a 550-μL aliquot of each sample supernatant was subsequently transferred to a 5-mm NMR tube. Samples were maintained at 4 °C before measurement.