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Platelet linoleic acid is a potential biomarker of advanced non-small cell lung cancer

Javier de Castro [a], Marina C. Rodríguez [b], Vicenta S. Martínez-Zorzano [c], Marcial Llanillo [d], Jesús Sánchez-Yagüe [d,*]

[a] Radiology Service, Santísima Trinidad Foundation Hospital, Paseo de Carmelitas 74, 37007 Salamanca, Spain
[b] Neumosalud, Santísima Trinidad Foundation Hospital, Paseo de Carmelitas 74, 37007 Salamanca, Spain
[c] Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain
[d] Department of Biochemistry and Molecular Biology, University of Salamanca, Edificio Departamental. Lab. 106, Plaza Doctores de la Reina s/n; 37007 Salamanca, Spain

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ABSTRACT

New parameters that could be used as tumor markers for lung cancer would be valuable. Our aim was to analyze the fatty acid profiles of total lipids from erythrocytes and platelets from patients with advanced non-small cell lung cancer (NSCLC), chronic obstructive pulmonary disease (COPD) and asthma to reveal the fatty acids that could be used as NSCLC biomarkers. In our study, 50, 15 and 15 patients with advanced NSCLC, COPD and asthma and 50 healthy subjects were enrolled. Fatty acid profiles were investigated using gas chromatography/mass spectrometry followed by ROC (receiver operating characteristics) curves analysis to gain information about biomarkers. Sialic acid (SA) and cytokeratins were measured by the thiobarbituric acid and immunoradiometric methods respectively. Useful fatty acid markers were as follows: erythrocytes, 22:0 and linoleic acid (LA, 18:2n6); platelets, 16:0, 18:0, and LA. At the cutoff value to obtain maximum accuracy, the best biomarker was platelet LA, with higher diagnostic yields than the commonly used markers SA or cytokeratins (100%, 76%, 75% and 86% sensitivity, specificity, positive predictive value and accuracy, respectively). These findings suggest that platelet LA might be used as a biomarker of NSCLC in relation to different aspects of the disease process that now needs to be explored.

Introduction

Lung cancer is the leading cause of cancer-related death among men in Spain (Monge-Corella et al., 2008), and the leading cause of cancer-related mortality around the world (Jemal et al., 2007). Although serum biomarkers that could aid clinicians would be of great value, currently no blood test for lung cancer is available. There is therefore a growing interest in finding new substances or parameters that could be used as tumor markers for lung cancer, or neoplastic diseases in general, in order to gain more knowledge about these diseases and improve therapeutic approaches.

Although to our knowledge there is no reliable tumor marker for lung cancer, two different kinds of serum molecules that have been proposed to be useful for the diagnosis and/or management of lung cancer patients are sialic acids and cytokeratins, mainly TPS (tissue polypeptide-specific antigen) and Cyfra 21-1 (the cytokeratin fragment recognized by the KS 19-1 and BM 19-21 antibodies) (Buccheri and Ferrigno, 2001a; Patel et al., 1989, 1995; Stringou et al., 1992).

Furthermore, metabolic profiling has increasingly been used as a probe in disease diagnosis and pharmacological analysis (Chen and Hofestadt, 2006). Essential fatty acids play an important role in complex metabolic reactions, and essential polyunsaturated fatty acids (PUFA) appear to be one of the critical targets in the complex metabolic stages that lead to, or are associated with, cancer. Although evidence of the alterations in lipid and fatty acid metabolism in cancer patients is well documented (Mikirova et al., 2004; Mosconi et al., 1989; Newcomer et al., 2001; Pala et al., 2001), the potential use of fatty acid profiles, especially from erythrocytes and platelets, as markers in clinical oncology has not been addressed, and only recently has plasma fatty acid metabolic profiling assessed by gas chromatography/mass spectrometry (GC/MS) been used to detect biomarkers of type 2 diabetes mellitus (Yi et al., 2006).

It is known that blood cells are significantly affected by malignant disease, and in fact, two common complications in cancer, which are especially prevalent in patients with non-small cell lung cancer (NSCLC) (Groopman and Itri, 1999), are anemia and a prothrombotic state. In lung cancer platelet activation occurs (Prisco et al., 1995), altering erythrocyte morphofunctionality (Brittain et al., 2001; Santos et al., 1991). Evidence supporting the role of erythrocytes in inflammatory disease, and by extension in malignancy, has also been reported (Crawford et al., 2004). Moreover, we have previously described that advanced NSCLC is associated with changes in the fatty acids from peripheral erythrocytes and platelets (de Castro et al., 2006, 2008), with changes in some erythrocyte membrane proteins, including band 3 and glycophorins (Hernández-Hernandez et al., 2006), and with increased protein oxidation in platelets (de Castro

* Corresponding author. Fax: +34 923294579.
E-mail address: sanyaj@usal.es (J. Sánchez-Yagüe).

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et al., 2006). Also, we have recently proposed that the fatty acid profiles of phospholipid species in erythrocytes and platelets from patients with advanced NSCLC could provide an additional test for the diagnosis and/or management of NSCLC patients (de Castro et al., 2008), although that study only represented a primary screening for potential individual phospholipid fatty acid biomarkers but did not include benign inflammatory lung pathologies. Accordingly, in the present work, we analyzed the fatty acid profiles of total lipids in erythrocytes and platelets from patients with advanced NSCLC, chronic obstructive pulmonary disease (COPD), and asthma to check which of those fatty acids might eventually be used as a biomarker. For comparison, we also examined previously described markers for lung carcinoma such as sialic acid (FSA, free sialic acid; TSA, total sialic acid; BSA, glycoconjugate-bound sialic acid; TSA/TP, total sialic acid/total protein; BSA/TP, glycoconjugate-bound sialic acid/total protein) and the cytokeratins TPS and Cyfra 21-1.

Materials and methods

Subjects

We studied 50 patients with a clear histological diagnosis of primary NSCLC (35 with squamous cell carcinoma and 15 with adenocarcinoma) according to the World Health Organization's (WHO) classification. The eligibility criteria included the following: no previous chemotherapy or radiotherapy; ECOG performance status ≤ 2; adequate bone marrow, liver, renal and cardiac function; no known brain metastasis; no previous malignancy; and no serious concurrent medical illness. The staging of the tumors, performed according to the TNM staging system of the International Union Against Cancer (UICC), was IIIA, IIIB and IV for 9, 28 and 13 patients, respectively. For TNM staging, all patients underwent a computed tomographic (CT) scanning of the chest and upper part of the abdomen, a bone scintigraphy, and a brain CT or magnetic resonance imaging. All subjects were current smokers. We also studied 15 patients with COPD recruited during a moderate-to-severe exacerbation. The entry criteria for the patients with COPD included the following: a) a FEV_1/FVC (forced expiratory volume in 1 second/forced vital capacity) ratio of <0.7 and a FEV_1 of $<80\%$ of the predicted value, b) smoking history of >20 pack-years, and c) no history suggestive of asthma. All subjects were current smokers. The subjects maintained their medication (β_2 agonists, anticholinergics and inhaled corticosteroids). Pulmonary function parameters (FVC and FEV_1) were measured by means of a Masterscreen Body spirometer (Jaeger). β_2 Agonists and anticholinergics were suspended 12 hours before the procedure. Fifteen patients with asthma were also studied. These patients were recruited during a moderate exacerbation (Global Initiative for Asthma GINA guidelines). The patients underwent a complete clinical examination and chest x-ray to exclude the presence of concomitant acute illness such as pneumonia. The minimum criteria for the diagnosis of an asthma exacerbation included intense subjective breathlessness, audible wheezing on auscultation and a morning peak expiratory flow (PEF) $<70\%$ of the personal best value. The patients had no history of smoking. All these subjects were well informed of the purpose of the present study and fully agreed to participate in it. The survey was performed after approval by our local institutional review boards at the University of Salamanca and the Santísima Trinidad Foundation Hospital in Salamanca. We also studied 50 healthy male and female volunteers as controls, whose age, body weight, blood lipids, blood pressure and body mass index (BMI) were equivalent to those of the patient groups (Table 1). These control subjects had smoking habits similar to those of NSCLC or COPD patients (Table 1). No further control group of non-smokers was included in the study because no differences in erythrocyte or platelet lipids depending on smoking were detected in healthy subjects (data not shown). Regarding this, it has also been described that although smoking cause an increase in oxidative stress, the systemic oxidant/antioxidant imbalance in COPD is probably independent of smoking (Calikoglu et al., 2002). Blood samples were taken the day after the patients were informed of their illness (NSCLC) or at the time of consultation. All individuals were free of ischemic heart disease or metabolic disease that might influence blood cell lipid contents (Prisco et al., 1986; Prisco et al., 1989). No altered energy metabolism attributable to symptoms such as anorexia/cachexia, nausea and vomiting able to elicit oxidative stress was present in the patients. None of the individuals had received drugs that might interfere with platelet or erythrocyte functions, clotting or fibrinolytic activity within the 3 months preceding blood sampling. None of the individuals had extreme dietary habits; they all lived in the same geographical area (The Autonomous Regional Community of Castilla & Leon), and all of them were following a typical Mediterranean diet with similar dietary.

Table 1
Characteristics of control subjects and patients.

Variable	Controls ($n=50$)	COPD ($n=15$)	Asthma ($n=15$)	NSCLC ($n=50$)
Age (years)	60 ± 5	68 ± 5	59 ± 10	69 ± 5
Weight (kg)	68 ± 10	67 ± 12	68 ± 11	69 ± 12
Mean arterial pressure (mm Hg)	91 ± 10	95 ± 5	96 ± 3	95 ± 5
Platelets ($\times10^3$ ml)	190 ± 54	250 ± 63	230 ± 14	260 ± 69
Erythrocytes ($\times10^6$ ml)	5.2 ± 0.9	4.6 ± 0.8	4.4 ± 0.1	4.7 ± 0.9
Total cholesterol (mg/dl)	185 ± 47	190 ± 25	200 ± 10	175 ± 25
HDL-cholesterol (mg/dl)	50 ± 11	53 ± 10	59 ± 14	53 ± 10
Triacylglycerols (mg/dl)	80 ± 33	90 ± 23	74 ± 9	90 ± 27
Smoking history (pack-years)	>19	>20	0	≥20
BMI (kg/m^2)	25.4 ± 4.2	25.0 ± 2.8	25.6 ± 5.1	24.6 ± 5.1
FEV1, % predicted	88 ± 5	45 ± 19	70 ± 15	NA
FEV1/FVC ratio (%)	85 ± 7	57 ± 5	74 ± 9	NA
Illness duration (years)	NA	19 ± 6	15 ± 7	NA

Values given as mean ± S.D.
BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FVC, forced. vital capacity; NA, not applicable; pack-years, number of cigarettes smoked per day × number of years smoked/20.

Blood sampling and isolation of serum, plasma, erythrocyte and platelets

Two blood samples were drawn from all the individuals. One sample was allowed to coagulate at room temperature, and after centrifugation, serum was obtained. The other one corresponded to heparinized blood samples. The latter were centrifuged at $1000\times g$ for 5 min to obtain a lower phase (erythrocytes) and an upper phase (platelet-rich plasma). Two-thirds of the platelet-rich plasma was then centrifuged at $2600\times g$ for 10 min. The platelet pellet was washed three times with 3 volumes of a solution of 150 mM NaCl, 5 mM sodium phosphate buffer, pH 8.0 (PBS). After washing, platelets were resuspended in 10 mM HEPES, pH 6.5, 0.2 mM EGTA, 5 mM KCl and 5.5 mM glucose in order to obtain platelet homogenates (Hernández-Hernández et al., 1999; Hernández-Hernández et al., 2005). Isolated erythrocytes were washed as indicated above for platelets. The serum or plasma was stored at −80 °C until tested.

Biochemical measurements in serum and plasma

β-Thromboglobulin (β-TG) and platelet factor 4 (PF4) were measured in plasma by the ELISA technique (Boehringer Mannheim Italy, Milan, Italy) following the manufacturer's instructions.

Sialic acids and cytokeratins were measured in serum. FSA was determined by means of the thiobarbituric acid method (Warren, 1959) according to the procedure described by Aminoff (1961). TSA was quantified by the same procedure after hydrolysis of the samples in five volumes of 0.1N H_2SO_4 at 80 °C for 1 hour. BSA was calculated as the difference between TSA and FSA. The levels of Cyfra 21-1 were