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ORIGINAL ARTICLE

Comparative proteomical and metalloproteomical analyses of human plasma from patients with laryngeal cancer

Clarissa P. C. Gomes · Mirna S. Freire · Bruno R. B. Pires · Érico A. R. Vasconcelos · Thales L. Rocha · Maria de Fátima Grossi-de-Sá · Taia M. B. Rezende · Ângela Mehta · Rinaldo W. Pereira · Bernardo A. Petriz · Aparecido D. da Cruz · Igor C. Pescara · Octávio Luiz Franco

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Abstract Laryngeal cancer is a significant disease worldwide, which presents an increasing incidence. Two contrasting ideas of the immune system role during cancer development are accepted: (1) it fights tumor cells, and (2) it aids tumor progression. Thus, there is no clear understanding about the immune response in laryngeal cancer. Furthermore, since tobacco is the main cause of laryngeal cancer and it contains various carcinogenic components, including metallic elements, these may play a role on cancer development. Plasmas of patients with laryngeal cancer and of healthy smokers were evaluated by 2D gel electrophoresis and mass spectrometry. Proteins were detected on every gel around pH 4.0–10.0 from molecular mass of 10–60 kDa. Few differences were found among cancer and control patients. However, three spots gathered between pI 7.3 and 7.6 with different molecular masses appeared exclusively in cancer profiles. From ten spots identified, six correspond to immune system components, including the three differential ones. The latter were observed only in cancer patients. The presence of several trace elements in the identified proteins was determined by inductively coupled plasma mass spectrometry, where chromium was increased in all proteins analyzed from patients with cancer. This study reinforces the importance of the immune response as target in the understanding and treatment of laryngeal cancer and the possibility that chromium is important in the carcinogenic progress.

Keywords Laryngeal cancer · Immunology · Proteomics · Human plasma · Trace elements

C. P. C. Gomes · M. S. Freire · B. R. B. Pires · T. M. B. Rezende · R. W. Pereira · B. A. Petriz · O. L. Franco (✉)
Centro de Análises Proteômicas e Bioquímicas, Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, SGAN Quadra 916, Av. W5 Norte, Módulo C, 70, Brasília, DF 790-160, Brazil
e-mail: ocfranco@gmail.com

É. A. R. Vasconcelos · T. L. Rocha · M. de Fátima Grossi-de-Sá · Â. Mehta
Embrapa (Brazilian Agricultural Research Corporation) Recursos Genéticos e Biotecnologia, Brasília, Brazil

É. A. R. Vasconcelos
Universidade de Brasília, Brasília, Brazil

A. D. da Cruz
Universidade Católica de Goiás, Pró-Reitoria de Pós-Graduação e Pesquisa, Goiânia, Brazil

I. C. Pescara
Laboratório de Espectroscopia Atômica, Hospital da Universidade Católica de Brasília, Brasília, Brazil

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a significant disease worldwide, and accounts for 90% of all malignancies of the larynx. Much of its increasing incidence is related to tobacco and alcohol consumption, which have a clear synergistic effect [1]. Given the few signs and symptoms of early-stage disease, these tumors are usually discovered in advanced-stages, when more severe treatments are needed, often leaving patients with debilitating functions The survival rates for advanced-stage disease are less than 50% [2].

Tobacco and tobacco smoke contain several metallic elements associated to cancer development [3]. The

number of studies showing that trace elements have important roles in various biological processes, such as activation or inhibition of enzymes and competition with other elements for the metalloprotein's active site, for example, is constantly increasing [4]. Therefore, it is possible to infer that these elements have direct or indirect effect on tumor development process [5].

On the other hand, it is known that the immune system responds to tumors but in a lower magnitude compared to its response to infectious agents [6]. Contradicting the widely accepted view of the immune system response [7], there are more recent studies showing it actually contributes to tumor growth [8]. The idea that the immune system mounts a defense against cancer development is opposed by the idea that persistent inflammatory reactions facilitate tumor progression. Usually, the sufferer's immune system almost always fails to eliminate the tumor [9], and tumor suppression mechanisms vary. Hahne et al. [10] reported that melanoma cells bear on their surface a molecule (Apo-1 or Fas) that induces immune cells to initiate apoptosis. Recently, Markieweski et al. [9] demonstrated that the complement system indeed contributes to mechanisms that promote the growth of malignant tumors. In their study, deficiency of complement proteins C3, C4 or C5a receptor associates with slowing of tumor growth.

Much remains to be uncovered about this disease, while the literature regarding LSCC proteomics is relatively scarce. Recent advances in proteomic technologies have the potential to lead to better understanding of the pathology of laryngeal cancer, its immunology, diagnosis and aid drug discovery for treatment [11]. There has been an increase in proteomic studies related to cancer in order to enlighten our understanding of this pathology. Much of this is due to the strong relation between individual differences of protein expression and cellular activity, and the possibility to identify changes in protein patterns between different states of the organism, such as in health and disease [12]. Typical medical studies are based on body fluids, which represent complex mixtures of proteins. The most complex human proteome is the blood plasma, since it contains different proteins synthesized by all body tissues [13]. The identification by proteomic techniques of antigens involved in tumor immunity can provide insights into the reaction of the host to the tumor [12]. Furthermore, determining the concentrations of trace elements in samples of healthy and cancer patients may help the understanding of the etiology, diagnosis and prognostic of different cancers [14].

Given this paradoxical function of the immune system in different models, to our knowledge, there is no clear understanding about the immune response in laryngeal cancer. In the present work, our aim is to focus on plasma proteomics, where most components of the immune system are present as well as metallic elements [13]. The inherent dynamic nature of the proteome reflects physiological and pathological changes related to cell activity, which allows the monitoring of the course of the disease. For this reason and for the readiness and availability of blood, plasma was used to investigate laryngeal cancer proteomics. Thus in this study, plasmas of different patients with LSCC and of healthy smoking people were evaluated by a combo two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) following mass spectrometry (MS) identification in order to search for differences between them, making a plasma protein profile for this type of cancer. Selected proteins from 2D-PAGE profiles were also analyzed by inductively coupled plasma mass spectrometry (ICP-MS) in order to detect metals bound to them.

Materials and methods

Patient characteristics and plasma collection

Plasma samples were obtained from four patients with LSCC, on the same tumor staging (T2: localized tumor with metastasis in one lymph node but no distant metastasis) according to the classification of malignant tumors, TNM [15]. These patients correspond to male and female smokers of ages between 59 and 73 years. The six control samples came from healthy people from the same age interval, from both sexes and with the same smoking habits of the LSCC patients. All individuals reported habits of consuming alcohol regularly. Plasma was obtained by centrifuging (1,000 g) blood collected with sodium heparin (Vacuette 4 mL, Greiner Bio-One, Rio de Janeiro, Brazil) at low temperature (4°C). Immediately after collection, 1 mM of proteinase inhibitor cocktail (Sigma) was added. The protocol of this experiment was approved by the Associação de Combate ao Câncer em Goiás (*Goiás Association of Cancer Action*) Ethics Committee (June 30, 1998), and all patients signed informed consent forms.

Sample processing and 2D-PAGE profiling

Plasma samples (500 μg) quantified with BCA (BioAgency, São Paulo, Brazil) and Qubit fluorescence standard kit (Invitrogen) assays, according to manufacturer's instructions, were subjected to the *2-D Clean-Up Kit* (Amersham Biosciences, Piscataway, USA) according to the manufacturer's protocol. Samples were than hydrated for 11 h with a solution containing 2 M thiourea, 7 M urea, 1% dithiothreitol (DTT), 2% CHAPS, 1% IPG-buffer and traces of bromophenol blue. Samples were then applied to 18 cm pH 3–10 linear IPG strips (GE Healthcare Life Sciences, Sweden). Isoelectric focusing at the *Ettan*

IPGphor 3 (GE Healthcare Life Sciences) was carried out for 1,000 V during the first 2.5 h, then increased linearly from 1,000 up to 10,000 V for 3 h. Finally, it remained at 10,000 V for 1 h. 12.5% polyacrylamide gels containing 1.5 M Tris–HCl pH 8.8, 10% sodium dodecyl sulfate (SDS), 10% ammonium persulfate (APS), 10% tetramethyl-ethylenediamine (TEMED) and Milli-Q water were used. Electrophoresis was performed at 5 W per gel overnight at 15°C using *Ettan DALTsix Vertical System* (GE Healthcare Life Sciences). The broad range isoelectric point marker (GE Healthcare Life Sciences) was also used for posterior pI identification on gels as well as molecular weight markers (GE Healthcare Life Sciences). Each protein sample was analyzed in triplicate.

Image analysis

Gel image analysis was performed with the BioNumerics software v. 4.5 (Applied Maths). Comparisons were made between gel images of cancer and control samples. First, calibration with a gray scale was necessary to transform gray levels into values for each pixel of the gel picture. A calibration curve from the software was used. All gel pictures were analyzed as Tiff files. The 12 gel images were placed in one folder and the wizard detection method proposed by the software was used for spot detection. Automatically detected spots were manually checked, and some of them were manually added or removed according to the size (>0.2), shape (circular) and density (>2 pixel cm^{-1}). Following the detection procedure, the normalization step was carried out to attribute a common protein identity for identical spots from different gel images. For this procedure, a reference gel was constructed and automatically matching options of BioNumerics software were used. Proteins detected in all gel images were automatically added to the reference gel. Spots that appeared on every gel (used as controls) and spots exclusive of cancer samples were selected for analysis by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF-TOF MS).

Protein purification and mass spectrometry

Protein spots were excised from Coomassie blue-stained gels using a scalpel, and further destained and digested with sequencing-grade Trypsin Gold (Promega, Madison, USA), according to Shevchenko et al. [16]. Briefly, spots were treated with acetonitrile until dehydrated before adding ammonium bicarbonate solution with DTT and incubated at 56°C. After 1 h, this solution was substituted for one containing iodoacetamide for 45 min at room temperature. Spots were washed with 100 mM ammonium bicarbonate solution and dehydrated with acetonitrile before being rehydrated with a solution containing 600 ng of trypsin. After overnight incubation, the supernatant was collected with water containing acetonitrile and trifluoroacetic acid.

Peptides derived from tryptic digestion were analyzed by two methodologies: MALDI TOF-TOF MS/MS and nano-liquid chromatography electrospray ionization (LC–ESI) MS/MS. Most samples were identified by the first methodology. Peptides were analyzed in MALDI TOF-TOF Ultra Flex II (Bruker Daltonics) MS and de novo sequenced using CID and LIFT acquired MS/MS spectral data. The identification of protein spots (2, C1–C3 and C5–C7) was performed by sequence searches in public databases using the Blastp program.

Peptides corresponding to spot numbers 1, 3 and C4 were analyzed by nano-LC–ESI–MS/MS UltiMate 3000 system (Dionex Corporation, Sunnyvale, CA, USA) interfaced to the QSTAR Pulsar mass spectrometer (Applied Biosystems, Foster City, CA, USA). The high performance liquid chromatography (HPLC) used was an Ultimate 3000 system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a PepMap100 C-18 trap column (300 μm × 5 mm) and PepMap100 C-18 analytic column (75 μm × 150 mm). The gradient was ($A = 0.1\%$ formic acid in water, $B = 0.08\%$ formic acid in acetonitrile) 8–30% B from 0 to 25 min, 80% B from 25 to 30 min, 8% B from 30 to 50 min. The flow rate was 300 nL/min. A HCTultra ETDII (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of *m/z* 350–1,500, and MS/MS spectra in information-dependent data acquisition over the mass range of *m/z* 100–2,800. The voltage between ion spray tip and spray shield was set to 1,600 V. Drying nitrogen gas was heated to 150°C and the flow rate was 7 L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). Searches were done by using the MASCOT 2.2.04 (Matrix Science, London, UK) against latest Swissprot database for protein identification. The MASCOT program used the probability-based Mowse Score and a threshold of $P < 0.05$. Only matches to human proteins showing a significant score were considered reliable and therefore determined as positive identifications according proteomics previous studies [17, 18]. Protein identification and modification information returned from MASCOT were manually inspected and filtered to obtain confirmed protein identification list.

Trace element analysis

Spots selected from 2D-PAGE experiments that were digested with trypsin to be identified by MS were analyzed by inductively coupled plasma mass spectrometry (ICP-MS 810-MS, Varian Mulgrave, Australia) to determine the concentration of the trace elements calcium (Ca), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), selenium (Se), lead (Pb), cobalt (Co) and zinc (Zn). Trypsinized samples were diluted with 65% nitric acid and digested at microwave SPEX (MX 350) for 30 min. Each sample was subjected directly to ICP-MS, and reference carbon solution was used to calibrate the equipment. Reference solutions for each metal were also used.

Results and discussion

2-D protein maps

LSCC is a multifactorial disease influenced by environmental and lifestyle-related factors. Our control group was represented by healthy smokers. The upper aero digestive tract is the first compartment of the human body that has contact with the harmful components of the tobacco smoke. The passage of smoke, whether in an active or passive smoker determines some alterations in the aero digestive area, is directly exposed to the harmful action of the substances present in smoke. The tobacco contains more than 50 components, including aromatic polycyclic hydrocarbon, tobacco-specific nitrosamines, aromatic amines, aldehydes and free radicals that have important role in carcinogenesis, being the main responsible substances for the neoplasic buccal epithelium transformation. One of the first steps in the development of head and neck neoplasia may be the link between these compounds and mutations of the mucosal cell's DNA [19].

Human plasma samples from patients with LSCC and of smoking health controls were obtained through approved protocols. The low incidence of this kind of tumor was reflected in our small number of samples [20]. The 2D-PAGE profiling of these samples was done in three technical and four biological replicates. The gel images obtained were compared against each other using the spots' volume with the software BioNumerics. After the normalization procedure, where identical spots on different gel images were selected, it was possible to identify spots exclusive to cancer patients.

In order not to eliminate or degrade any protein, plasma samples were used instead of serum. Besides, plasma is not only the primary clinical specimen but also represents the largest and deepest version of the human proteome present

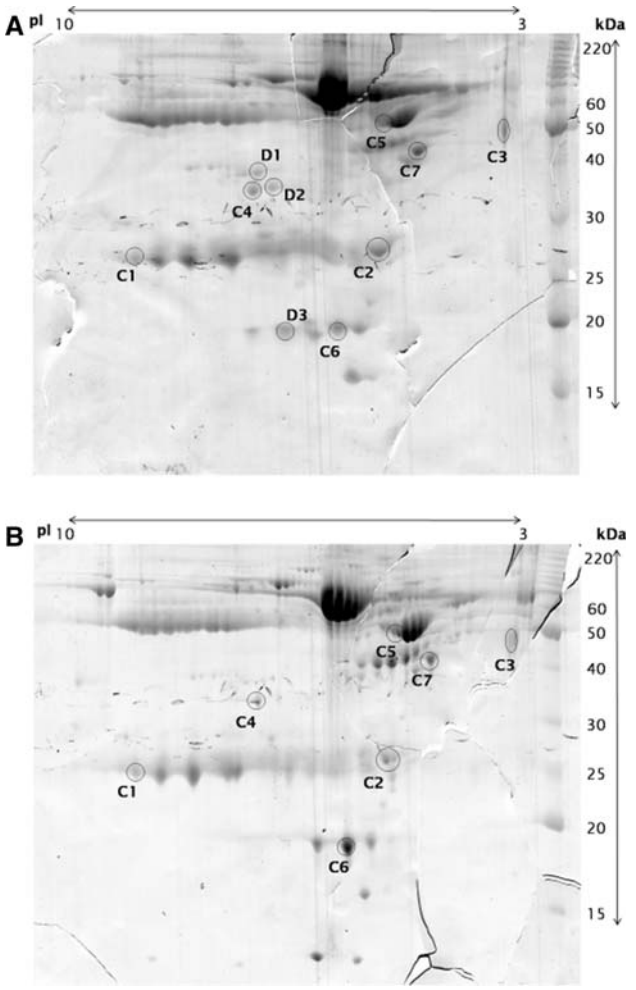

Fig. 1 2D map of plasma with cancer (**a**) and without cancer (**b**). Identified proteins are *circled* and identified with their corresponding spot number. Each gel was carried in technical and biological triplicates. 2D gels were Coomassie blue stained

in any sample; in addition to the classical "plasma proteins", it contains all tissue proteins (as leakage markers) plus very numerous distinct immunoglobulin sequences, and it has an extraordinary dynamic range in that more than ten orders of magnitude in concentration separate albumin from the rarest proteins now measured clinically [13].

In every 2D gel, protein spots with molecular mass of 10–60 kDa and pH ranging from 4 to 10 were observed (Fig. 1). Profiles from normal samples showed over 50 spots, out of which 34 were considered well defined (Fig. 1b). Profiles from samples with cancer showed the same amount of spots, but 45 were considered as distinguished spots (Fig. 1a). Most proteins were detected on every gel from pI 4.4 to 7.0, and around molecular weight of 53 and 60 kDa, forming a conglomerate especially around pI 6.4. According to this mass' characteristics and comparison with breast cancer serum profile available in ExPASy's Swiss-2DPAGE [21], these proteins correspond

Table 1 Identification of differential (D) and control (C) trypsinized protein spots from 2D gels

Spot number	Mowse score	Peptide sequence	Sequence mode	Protein identification	Access number (UniProt)	Identified pI	Identified MW (kDa)
D1	220	KNQVSLTCLVKG	De novo/CID	Ig gamma-3 chain C region	P01860	7.61	44.86
D2	294	KLGQYASPTAKR	De novo/CID	Complement component C4a	P0C0L4	7.39	42.56
D3	422	KDSTYSLSSTLTLSKA	De novo/CID	Ig kappa chain C region	P01834	7.28	20.34
C1	70	KSGTASVVCLLNNFYPRE	De novo	Ig kappa chain C region	P01834	9.7	32.23
C2	56	RTHLAPYSDELRQ	De novo	Proapolipoprotein A-I	P02647	5.67	32.12
C3	82	KEQLGEFYEALDCLRI	De novo	Alpha-1-acid glycoprotein precursor	P02763	3.5	49.95
C4	398	RVEYGFQVKV	De novo/CID	Complement component C4a	P0C0L4	7.73	41.88
C5	88	KITPNLAEFAFSLYRQ	De novo	Alpha-1-antitrypsin precursor	P01009	5.22	52.32
C6	56	KLPECEADDGCPKPPEIAHGYVEHSVRY	De novo	Haptoglobin Hp2	P00738	6.39	20.65
C7	71	KVYAYYNLEESCTRF	De novo	Complement component C3	P01024	4.97	47.34

MOWSE score is based on the number of peptide mass sequenced with the corresponding at the database (MASCOT, http://www.matrixscience.com/help/scoring-help.html). Sequencing mode de novo was done by MALDI-TOF and CID by LC–ESI–MS

to albumins. The same pattern was also found when comparing the profiles here obtained with the one of human plasma obtained by Anderson and Anderson [22]. From the similarity of the gels in this work and the maps available, let us infer the identity of some previously identified proteins.

To restrict the major proteins in our study, we focused in the differentiated proteins expressed between the control and experimental groups. Few differences were found among the cancer and control patients (Fig. 1). There were spots found differentially expressed in many samples, but were not exclusive of cancer or control gels. However, the appearance of three spots named D (for differential) 1, 2 and 3 gathered between pI 7.3 and 7.6 and molecular masses of 20, 42 and 44 kDa in all gels from patients with cancer suggests the expression of proteins either caused by the cancer or that may aid in the disease development (Fig. 1a). These three spots—D1, D2 and D3—along with seven others that appeared on every gel—called C (for control) 1–7—were treated with trypsin and identified by MALDI TOF-TOF de novo mass spectrometry and nano-LC–ESI–MS/MS.

MS protein identification

A total of ten spots were identified by MS (Table 1), including the three differential ones. All identified proteins are typically found in plasma, being six of them immune system components. Among control spots, named C and the cognate number, is spot C2, which was identified as the

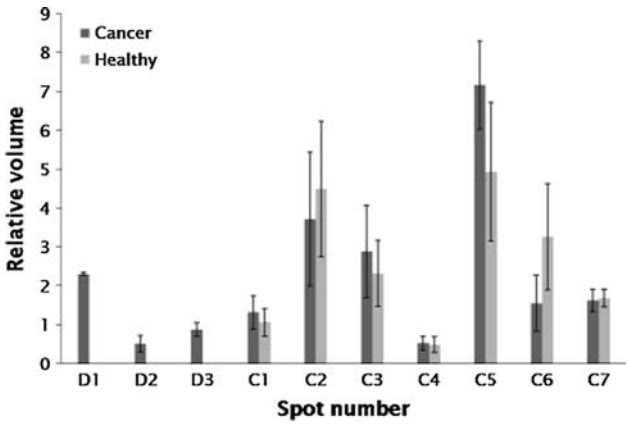

Fig. 2 Histogram of sequenced spots from samples with and without cancer according to their volume. *Vertical bars* correspond to standard deviation

proapolipoprotein A-I, a precursor of major apoprotein A-I (apoA-I). This protein binds to lipids to form high density lipoproteins (HDL), which is relatively abundant in plasma. ApoA-I is a cofactor for lecithin:cholesterol acyltransferase (LCAT), responsible for the formation of most cholesterol esters on HDL. It stabilizes the structure of HDL and also promotes efflux of cholesterol from cells [23]. Apolipoproteins have been linked to several diseases such as Alzheimer [24] and atherosclerosis [25]. In the present study, the protein spot number C2 analyzed by volume counting the pixel numbers suggests a slightly higher expression of proapolipoprotein A-I in healthy patients (Fig. 2). Differences on the expression of apolipoproteins

have been reported in pancreatic [26] and liver cancer [27]. In pancreatic cancer serum, proapolipoprotein C-II and apolipoprotein C-III_1 were up- and down-regulated, respectively, suggesting their use as possible diagnostic markers for the disease [26]. ApoA-I and apoA-II show lower values in serum from patients with liver cancer [27].

Moreover, spot C3 corresponds to alpha-1-acid glycoprotein (AGP), also called orosomucoid, and is a serum protein derived from the liver. Orosomucoid concentrations increase under certain conditions, such as inflammation, pregnancy, after surgery and cancer, since it belongs to the acute phase proteins. It has been reported that there is a significant homology between AGP protein amino acid sequence with immunoglobulin (Ig) G and that it is needed to maintain the capillary permeability required for homeostasis [28]. The suggestion that AGP levels reflect disease activity and may reflect tumor burden was also observed by Ganz et al. [29]. AGP plasma levels of active lung cancer patients were elevated compared with cancer patients with disease remission (considered inactive). In this study, the normalization of AGP during chemotherapy correlated with a prolonged relapse-free survival [29].

The spot numbered C5 was identified as pre-alpha-1-antitrypsin, a major serine protease inhibitor in human plasma. This glycoprotein belongs to the serpin family of protease inhibitors, and is produced mainly in the hepatocytes. Its main function is to protect tissues from enzymes of inflammatory cells, especially neutrophil elastase [30]. The disorder of this enzyme, called alpha-1-antitrypsin deficiency, is caused by a single base mutation and prevents its export from the hepatocyte, causing a decrease of the protein concentration in the plasma. Thus, it is associated with chronic liver disease and premature emphysema [30], and according to some research, it is also related to cancer [31]. Alpha-1-antitrypsin deficiency causes chronic liver disease and hepatocellular carcinoma in adults, although little is known about the latter [32]. Study by Yang et al. [33] evaluated this risk factor in the development of lung cancer, demonstrating that people with alpha-1-antitrypsin deficiency has twice the risk of developing lung cancer than those without it.

Control spot C6 was attributed as haptoglobin (HPT), which belongs to the family of acute-phase proteins, whose synthesis is induced by several cytokines during inflammatory processes [34]. A major function of haptoglobin is to bind free plasma hemoglobin to form a stable complex, and thereby prevent hemoglobin-induced oxidative tissue damage [34]. HPT has also been shown to be an angiogenic agent [35], activating endothelial cell growth and differentiation. The experiments presented here based on the spot volume show a higher expression in patients without laryngeal cancer (Fig. 2). On the other hand, this protein has been found over expressed in serum of patients with solid tumors such as ovarian and small-cell lung cancer [36].

Once our control group was represented by healthy smokers, the immune response was not completely activated by this stimulus, although we observed the expression of some inflammatory proteins. Laryngeal tissue samples were used in many proteomic analysis studies with different scopes of protein expression. Sewell et al. [37], for example, observed that the differentially expressed proteins were represented by stratifin, S100 calcium-binding, protein A9, p21-ARC, stathmin, and enolase. In our study, the differentiated proteins were represented by immune response components. The immune response occurs in loco and also in a systemic level. In this way, in our study, the remaining identified spots correspond to proteins of the immune system: Ig gamma-3 chain C region, complement component C4a, Ig kappa chain C region and complement component C3 (spots D1, D2 and C4, D3 and C1, C7, respectively). The differential spots were all in this category.

Another component of the immune system with an important role in the inflammatory response, including cancer, is the complement system, which is made up of many plasma proteins. Complement is a key link between the innate and adaptive immunity, since it can be activated by both [38]. Complement proteins are activated by cleavage, where some fragments get attached to pathogen or host cell surfaces and others are released to act as inflammatory factors. For example, the complement proteins C3a, C4a and C5a enhance inflammatory reactions by stimulating dilation of arteries, releasing histamine from mast cells and basophils and attracting neutrophils by chemotaxis [8]. Activation of the complement system causes tumor-cell destruction by inducing lysis and promoting cell-mediated killing [39].

On the other hand, Markiewski et al.'s [9] study contradicts this common thought, showing evidence that certain immune responses by complement can give the tumor a growth advantage. They demonstrated that the complement system indeed contributes to mechanisms that promote the growth of malignant tumors. In their study, deficiency of complement proteins C3, C4 or C5a receptor associates with retardation of tumor growth. Such impairment was observed in C4-deficient mice together with deposition of C1q in tumor tissue, indicating activation of complement through the classical pathway during tumor development. They concluded that complement activation and C5a signaling generated an immunosuppressive environment by efficiently recruiting myeloid-derived suppressor cells (MDSCs) into tumors to suppress the CD8+ T cell-mediated antitumor response.

Cancer cells can be viewed from an immunologic perspective as altered self-cells that escaped normal

growth-regulating mechanisms. The possibility that specific immune responses can eliminate tumors is widely accepted, and has been subject of several researches [7, 40]. Kús et al. [41] observed that scanning electronic microscopy of primary larynx tumors revealed that these tumors are infiltrated by cells involved in the immune responses. These cells are located within and also in surrounding cancer infiltrations and are represented by: lymphocytes, monocytes and macrophages. Gabriel et al. [42] verified the presence of immune response components in the laryngeal tissue samples by immunohistochemical staining. They observed that the CD 43 lymphocytic infiltration indicate a prognostic value for determining a shorter survival time and the possibility of lymph node metastases in patients with recurrences of cancer. These findings confirm that the human organism, until certain degree, is able to fight against malignant tissue. Tumor-reactive antibodies found in the serum of people with cancer support the role of the T and B lymphocytes as members of the immune surveillance team against tumors [40].

Regarding the immunoglobulins' expression present in the laryngeal blood cancer patients, Vlock et al. [43] observed that patients with moderately or poorly differentiated tumors had significantly higher antibody titers when compared with patients with well-differentiated tumors. In this study, they also observed that patients with higher levels of circulating immune complexes were less likely to respond to chemotherapy. No correlations were noted between immune complex levels and stage of disease, nodal status, site of disease, recurrence, or survival. These results support the fact that the formation of tumor-associated immune complexes in patients with squamous cell carcinoma of the head and neck is associated with a decreased response to chemotherapy.

In our study, we observed that C3 and C4 complement components are present in both cancer and non-cancer plasma samples. The presence of these components, according to Markiewski et al. [9], in smoking healthy patients may contribute to tumor growth. Based on our results, it seems that the active immune response against the tumor was represented by the T and B lymphocytes activities based on the immunoglobulin's expression in the cancer patients' samples.

Since there is a tremendous molecular heterogeneity in all human cancers and proteins are cell's function mediators, the study of proteins changes that result from a pathological lesion, would appear to be a rich source of potential cancer markers. According to our study and to current literature, a specific serum biomarker for laryngeal cancer diagnosis was not found, although the literature reports biomarkers for different kinds of cancer, such as nasopharyngeal. Cho et al. [44] identified two isoforms of serum amyloid A proteins as biomarkers to monitor relapse of nasopharyngeal carcinoma. They observed a dramatic serum amyloid A protein increase, which correlated with relapse and a drastic fall correlated with response to salvage chemotherapy. Surprisingly, despite the close localization of the nasopharynx and the larynx, our study did not find the same biomarkers for laryngeal and nasopharyngeal cancers. Our study confirms the tobacco role in the development of laryngeal cancer and reinforces the immune response importance as target in cancer understanding and treatment.

Trace element analysis

Aliquots of the trypsinized proteins submitted to MS were also analyzed by ICP-MS for selected metals. Most elements, Ca, Cd, Co, Cu, Fe, Mg, Mn, Mo, Se and Zn, were below detection level by this technique, where Cr and Pb were the only ones observed in all samples. The average concentration of Cr and Pb between patients for each spot was determined separately.

The relative amount of Pb detected was low in most spots; however, spot C6 (haptoglobin) and C7 (complement component C3) showed higher levels among healthy patients (data not shown). The values found here were not in accordance with study by Pasha et al. [45] where the concentration of Pb was higher in plasmas of cancer patients. These results might be due to different amounts of Pb in the diet of the patients, once this is the main source of Pb intake.

Cr was found in all three differential spots (Fig. 3a). For the common spots, higher amounts of Cr were found in all samples from patients with cancer, except in spot C6 (haptoglobin), where the same concentration was found in samples from both cancer and healthy patients (Fig. 3b). The maximum increase was of 3,050% for spot C4 (complement component C4a). Cr has a long history of industrial use, such as welding and plating and is accepted as a mutagenic agent [46], being the respiratory tract the primary target organ once inhalation is the main route of entry into the body [47]. Its exposure causes gene mutations, cell cycle arrest, apoptosis and neoplastic transformation [14]. Epidemiological and experimental evidences show that occupational exposure to Cr is associated with lung cancer [47, 48]. Studies show a relation between high concentrations of Cr with accelerated tumor growth [45]. Others relate this metal with greater susceptibility to develop nasal and respiratory cancers [49]. Significant higher levels of Cr in scalp hair of patients with malignant tumors add to the evidences that it increases cancer risk [50].

Both elements detected in this work are found in tobacco and its smoke, which are a complex mixture of thousands of components, adding to near 5,000 already isolated and

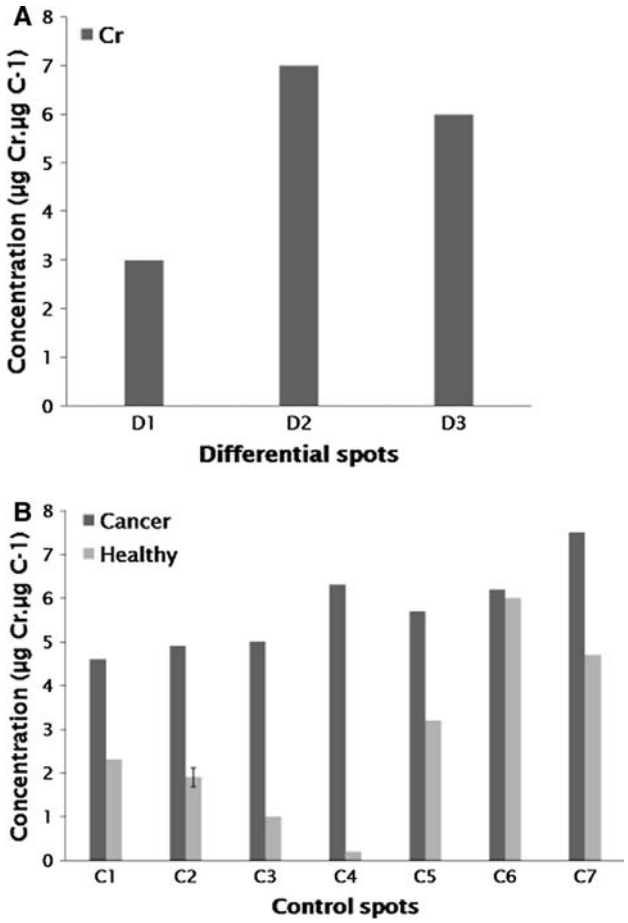

Fig. 3 Average of Cr concentration at each differential spot (1, 2 and 3) of patients with cancer (**a**) and average of Cr concentration for common spots in samples of patients with and without cancer (**b**). All samples were obtained from trypsinized proteins of the 2D gel spots. *Vertical bars* correspond to standard deviation

identified. Among these, 69 are known to cause cancers [51]; therefore, neoplasms induced by tobacco smoking have a more complex etiology due to the presence of various carcinogenic agents [48]. Once there are evidences of the role trace elements play in biological processes, it is possible to infer that the chromium found in the samples analyzed in this work has direct or indirect effect on the carcinogenic process, including the development of LSCC.

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