Original Research

A comparative protein analysis of lung cancer, along with three controls using a multidimensional proteomic approach

Mahwish Saleem[1,2], Syed K Raza[3] and Syed G Musharraf[1]

[1]Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Science (ICCBS), University of Karachi, Karachi 75270, Pakistan; [2]Baqai Institute of Information Technology, Baqai Medical University, Karachi-74600, Pakistan; [3]College of Allied Health Professionals, Government College University Faisalabad, Faisalabad, Pakistan
Corresponding author: Syed G Musharraf. Email: musharraf@iccs.edu

Impact statement

A multistep proteomics fractionation strategy was developed and validated for the discovery of proteomic biomarkers which could be used as potential diagnostic biomarkers for monitoring the progression of disease in smokers and COPD patients towards lung cancer.

Abstract

Lung cancer is an important respiratory disease accounting for millions of deaths worldwide. Developments in proteomics techniques and mass spectrometry offer comprehensive answers to unravel the complexities of lethal diseases such as lung cancer at the molecular level. The current study focuses on the proteomic profiling of lung cancer and its comparison with other controls including chronic smoker (high-risk individuals), obstructive pulmonary disease (COPD), and healthy control. A multistep proteomic strategy was used on the pooled plasma of each group including depletion of seven most abundant proteins, 2D-SDS-PAGE separation followed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF-TOF-MS) analysis. Total 23 proteins were identified, and out of them only 7 proteins were found to be expressed in increased amounts in disease and smoker groups as compared to healthy group including haptoglobin, retinol binding protein 4 (RBP 4), alpha-1 antitrypsin, Ig lambda 2 chain C region, Ig alpha-1-chain C region, clusterin, transthyretin (TTR). Haptoglobin and alpha-1-antitrypsin were found to be sequentially increased in healthy control along with smoker, COPD, and lung cancer. The differentially expressed proteins might have a prognostic potential to be used in the progression of COPD to ultimately lung cancer.

Keywords: Lung cancer, MALDI-TOF mass spectrometry, two-dimensional gel electrophoresis

***Experimental Biology and Medicine* 2019; 244: 36–41. DOI: 10.1177/1535370219826525**

Introduction

Lung diseases come in the list of most common pathological diseases around the world, among which lung cancer and COPD are the most lethal diseases. Because of the last stage diagnosis and the inadequacy of treatment options, the five-year survival rate is not up to the mark.[1] The disease is the most prominent cause of deaths due to cancer, more common in males than in females, while it ranks fourth in all diagnosed cancers and second important cause which leads to death.[2] While on the other hand, COPD is a heterogeneous and multi-component disease of lung pathology and ranked fourth among the leading causes of morbidity and mortality worldwide. Etiological agent of both diseases is tobacco smoking and different environmental pollutants factor. The current diagnostic approaches in lung cancer include computed tomography (CT), positron emission tomography (PET) scan for the assessment of mediastinal adenopathies and the several needle aspiration methods for the investigation of metastatic site.[3,4] These technologies investigate the lung cancer mostly at the end stage of disease.

In clinical proteomics, there are valuable tools available for the detection of prognostic and diagnostic states of a disease. Diagnosis with proteins has already been known for the prediction of disease stage and for comprehensive understanding of diseases at their molecular level.[5] During the previous years, a few protein biomarkers had been detected in lung cancer including α-1 antitrypsin, carcinoembryonic antigen (CEA), squamous cell carcinoma antigen, and retinol binding protein (RBP). In other studies besides these proteins, neuron-specific enolase (NSE),

cytokeratin 19 (CYFRA-21–1), tumor M2-pyruvate kinase (PKM2), C-reactive protein (CRP), serum amyloid A (SAA), plasma kallikrein (KLKB1), haptoglobin β chain (Hpβ), complement component 9 (C9), carbohydrate antigen-125 (CA-125), alpha-fetoprotein, ferritin, carbohydrate antigen-19.9 (CA-19.9), haptoglobinα subunit, hepatocyte growth factor, α-1 acid glycoprotein 1 and 2, fibrinogen alpha chain, apolipoprotein, transthyretin, and Ras-p21 were also identified.[6–13]

A correlation study between the lung cancer, COPD, smoker, and healthy at the proteomic level has not been investigated yet. However, a correlation work between smoker, COPD, and lung cancer has been done on the basis of spirometry test and it suggests that about 50% of the lung cancer cases have concurrent moderate to severe COPD and 10% of chronic smokers get lung cancer.[14] In this study, we have applied a multidimensional fractionation approach to inquire the changes in protein composition between lung cancer, COPD, and smoker in comparison with healthy control in order to identify the differential protein pattern. Findings from this work are expected to be helpful for early diagnosis of lung cancer and COPD.

Materials and methods

Plasma collection and processing

This study was approved by the ethical committees of both participating institutes, Jinnah Postgraduate Medical Center (JPMC) and International Center for Chemical and Biological Sciences (ICCBS). All the participants and total 350 plasma samples of males were collected from the hospital, 50 healthy samples, 100 smokers, 100 COPD, and 100 lung cancers with filled written approval forms. All samples were collected from the control/patient under the age group of 20–80. The patient's samples of cancer were pathologically identified, proven lung cancer of common subtypes, mainly non-small cell lung cancer (NSCLC) mainly at their stage III and were not previously exposed to any treatment. Smokers included in our study have been smoking for more than 10 years. Neither healthy nor smoker individuals have lung diseases at the time of sample collection with normal vital signs. All participants were found to be negative for diabetes, human immunodeficiency virus (HIV) and hepatitis C. Lung cancer and chronic obstructive pulmonary disease (COPD) samples were acquired from the Oncology and Chest Unit at JPMC after approval from ethical committee, and on-paper informed-consent was taken from all participants in this study. Fasting plasma samples of male were collected from lung cancer, COPD patients, healthy smokers, and healthy volunteers. The standard protocol of human proteome organization (HUPO) was followed to process the individual samples;[15] 5 mL human blood was obtained by venipuncture into vacutainer blood collection tubes containing K_2-ethylene diamine tetra-acetic acid (K_2-EDTA). Plasma was separated for 10 min at 4°C by applying centrifugation at $2000 \times g$.

The individual samples of respective group were systematically pooled to make a pool of each separate group including healthy, smoker, COPD, and lung cancer. Pooled sample was further aliquoted for further processing and stored at −80°C. The estimation of protein concentration was performed with bicinchoninic acid (BCA) procedure according to the Kit protocol.[16]

Depletion of high-abundance proteins through affinity chromatography

Depletion of high-abundance proteins was performed through Affinity Chromatography Multiple Affinity Removal Column Human 7 (MARS Hu-7) column kit, purchased from Agilent (USA. leupeptin and pepstatin-A, Phenyl methanesulphonyl fluoride (PMSF), and EDTA was purchased from Sigma (Massachusetts, USA). Ultra free-MC 0.22 μm filters were purchased from Millipore (Massachusetts, USA).[17] Column has polyclonal antibodies against human transferrin, albumin, haptoglobin, IgA, IgG, α-1-antitrypsin, and fibrinogen. The unbound and bound fractions were collected after immunodepletion.

One- and two-dimensional gel electrophoresis

Next resultant unbound and bound fractions were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis (1D-SDS-PAGE) analysis to check the depletion efficiency. 1D SDS-PAGE analysis was conducted through electrophoresis cell (X cell Sure Lock system) (Invitrogen USA). Then proteins were precipitated using trichloroacetic acid (TCA)/acetone procedure in both bound and unbound fractions. The resultant precipitated proteins were then used further for 2D gel analysis.

ReadyPrep 2-D Starter Kit, Miniprotean IEF cell, pH 4–7 (7 cm), isoelectric focusing (IEF) focusing tray with lid, ReadyStrip IPG strips, mineral oil, electrode wicks, blotting filter papers, SDS-PAGE electrophoresis cell (miniprotean 3 ready gel), SDS gel staining trays, tris/glycine/SDS running buffer (TGS), nano-pur water and power supply appropriate for SDS-PSGE system were purchased from BioRad (USA). We used IPG strips (pH 4–7) for bound portion, and IPG strips (pH 3–10) for unbound portion. Precipitated proteins were dissolved in 125 μL of rehydration buffer (2% CHAPS, 10 mL of 8M urea, 0.2% (w/v) Bio-Lyte, 3–10 ampholytes, bromophenol blue and 50 mM dithiothreitol (DTT)) and loaded on IPG strips. After IEF, IPG strips were equilibrated with Equilibration Buffer I (2% SDS, 6M urea, 0.375M Tris-HCL (pH 8.8), 2% w/w DTT, 20% and glycerol) for 10 min and then in Equilibration Buffer II (2% SDS, 6 M urea, 0.375 M Tris-HCL (pH 8.8) and 20% glycerol) for 10 min before going to SDS-PAGE in second dimension. SDS-PAGE was performed using TGS running buffer at constant 200V for 40 min. Gels were stained with Colloidal blue staining kit and calibrated densitometer (GS-800) was used for the image acquisition of gels.

PDQUEST 2-D analysis software

The statistical analysis of two-dimensional SDS-polyacrylamide gel electrophoresis (2D-SDS-PAGE) images, such as spots detection and matching and applying filters was

performed with BioRad software PDQuest (version 8.0.1). The master gel was designed by the software in which all the spots from the gel (healthy, smoker, COPD and lung cancer). This master gel was used to compare all study groups. Two sets of statistical analysis were created: spots with student *t*-test score $\geq$95, and spot quantity with 2× increased or decreased expression.

MALDI analysis

Identified spots were excised and placed in the Eppendorf tube and the tryptic digestion of gel was carried out according to the defined protocol.[18,19] Digested proteins were analyzed using the matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF MS) instrument (Ultraflex III, Bruker Daltonics Germany). Samples (1 μL) were spotted on steel target plate using (1 μL) α-Cyano-4-hydroxycinnamic acid (HCCA) as a matrix in 50% acetonitrile and 0.1% TFA. The peaks profiles of plasma were acquired by flex Analysis version 3.0 (Bruker Daltonics). Instrument was calibrated with peptide calibrant standard I (Bruker Daltonics) in positive reflector mode between m/z 750 and 5000. Linear positive mode was applied to generate spectra with ion acceleration of 25 KV and the mass-to-charge ratio was set up between 800 and 10,000. Each spectrum was recorded over different position within the same spot and each spot consist of 200 shots per position and total 2000 laser shots was used. High gating strength was used to deflect the ion of a mass below 800 m/z by applying the electric potential of lens at 6 KV. The extraction was delayed at 100 ns with the sample rate of 0.5 GS/s and the detector gain was set to 7.5.

Raw data files of MALDI-TOF were searched against the Mascot search engine and the proteins were extracted from SWISS-PROT and NCBInr databases. Searching parameters for peptide mass fingerprinting (PMF) were set as follows: trypsin was defined as proteolytic enzyme with one missed cleavage. Carbamidomethylation of cysteine (C) was specified as fixed modification, whereas oxidation (M) was set as variable modification. Peptide mass tolerance was $\pm$ 0.5 Da with monoisotopic mass values.

Pathway analysis

The gene ontology (GO)-based analysis among the differentially expressed proteins was acquired using the European Molecular Biology Laboratory (STRING: EMBL) software version 10.5. This online software describes the connection between the proteins at the molecular, cellular, and biological levels.

Results and discussion

A pool plasma sample from each group including healthy ($n = 50$), smoker ($n = 100$), COPD ($n = 100$), and lung cancer ($n = 100$) were prepared and processed for further analysis.

Multistage fractionation and identification of proteins

A systematic fractionation strategy was used for the fractionation of blood samples. In the first step, we depleted the top seven abundance proteins (albumin, α1-antitrypsin, transferrin, fibrinogen, IgA, IgG, and haptoglobin) from the pooled plasma of healthy control, COPD, smokers, and lung cancer through immunoaffinity MARS (Hu-7) column (Figure S1). A chromatogram of bound and unbound portion of plasma shows a distinct separation of major- and lower-abundance of proteins. The separation efficiency of depleted portions was successfully examined through the one-dimensional gel electrophoresis (1D-GE) analysis (Figure S2). The enrichment of lower-abundance proteins was achieved on 1D gel. The prominent bands on 1D gel show high-molecular weight proteins. The apparently visible protein bands on 1D gel are the seven captured proteins of the bound fraction that showed no nonspecific binding using this low-resolution and low-sensitivity separation method.

After careful depletion of pooled plasma of healthy, COPD, smokers, and lung cancer, the bounded and unbounded fractions were subjected to protein enrichment via precipitation, followed by 2D electrophoresis analysis. The subsequent (2D-GE) maps of protein spots can be observed in Figures S3 and S4. For the comparison among the gel images of healthy, smoker, COPD, and lung cancer, a master gel was generated by PDquest software. The sets were compared separately through Boolean interception and their Boolean union was created. The bar graphs of these spots were generated, among these spots only those were chosen which showed a pattern of up- or down-regulation among the groups.

For protein profile analysis, we excised 108 gel spots of our interest from bound and unbound portion and exposed to MALDI-TOF MS, which led to the identification of differentially significant proteins in each group such as, healthy, smoker, COPD, and lung cancer. We identified 23 differential proteins, 8 proteins or their respective isoforms and subunits in bound and 15 proteins in unbound portion of all 4 groups (Figure S5). Table 1 shows the identified protein list. In this piece of study, we identified seven proteins that are significantly deregulated in each group, which includes transthyretin, clusterin, haptoglobulin, RBP 4, antitrypsin, Ig lambda-2 chain C region, and Ig alpha-1 chain C region.

Discussion

The results of 2D gel analysis exposed the differential expression and the changes in the concentrations of seven proteins (RBP 4, clusterin, haptoglobulin, transthyretin, Ig lambda-2 chain C region, alpha-1 antitrypsin, and Ig alpha-1-chain C region). Here, using the STRING:EMBL (Europeon Molecular Biology Laboratory) software, we found the inter- and intra-linkages of five identified protein only, because the STRING:EMBL version 10.5 did not recognize the rest of the two identified proteins: Ig lambda-2 chain C region and Ig alpha-1 chain C region. The evaluated direct involement among these five proteins indirect connections with other proteins is shown in Figure 1.

The linkages among the identified proteins are colored and labelled with their gene names. Potential interacting partners which were not identified by 2DE study are also

Table 1. List of identified proteins.

Serial no	Spot ID	Protein description	Mascot score	Observed MASS/pI	% cov.	Peptide match	Gene name	Protein identification
1	SSP 1101 b	Ig kappa chain C region	72	11773/5.58	71	5	IGKC	PMF
2	SSP 1308 b	Ig gamma-1 chain C region	139	36596/8.46	56	15	IGHG1	PMF
3	SSP 1703 b	Serotransferrin	211	79294/6.81	47	30	TF	PMF
4	SSP7106, 4102 b	Ig lambda 2 chain C region	62	11458/6.92	65	4	IGLC2	PMF
5	SSP 5603 b	Serum albumin	143	71317/5.92	36	20	ALB	PMF
6	SSP 3501,4502, 5401,5505 b	Ig alpha-1-chain C region	84	38486/6.08	34	8	IGHGA1	PMF
7	SSP 6211,7301, 5204,8301, 8303 b	Haptoglobulin	101	45861/6.13	37	12	HP	PMF
8	SSP 8501 b	Alpha-1-antitrypsin	71	46878/5.37	40	12	SERPINA 1	PMF
9	SSP 0502 a	Alpha-1-acid glycoprotein 2	72	23873/5.03	41	7	ORM2	PMF
10	SSP 1601 a	Alpha-2HS-glycoprotein	66	40098/5.43	17	6	AHSG	PMF
11	SSP 1502 a	Clusterin	81	53031/5.89	23	10	CLU	PMF
12	SSP 1702 a	Kininogen-1-variant	84	48906/6.29	32	15		NCBInr
13	SSP 3501 a	Haptoglobulin	117	45861/6.13	29	11	HP	PMF
14	SSP 4301 a	Retinol binding protein 4	64	23337/5.76	50	7	RBP4	PMF
15	SSP 3306 a	Apolipoprotein AI	119	30759/5.56	52	14	APOA1	PMF
16	SSP 3208 a	Transthyretin	60	15991/5.52	59	7	TTR	PMF
17	SSP 3903 a	Complement factor H	224	143680/6.21	16	17	CFH	PMF
18	SSP 5203 a	Haptoglobulin	81	45861/6.13	23	8	HP	PMF
19	SSP 5701 a	Hemopexin	129	52385/6.55	33	14	HPX	PMF
20	SSP 9701 a	Beta-2-glycoprotein	70	39581/8.34	30	9	APOH	PMF
21	SSP 7903 a	Complement factor B	64	86847/6.67	26	18	CFB	PMF
		Gelsolin	75	86043/5.90	26	17	GSN	PMF
22	SSP 8702 a	Serotransferrin	211	79294/6.81	36	26	TF	PMF
23	SSP 9505 a	Fibrinogen Beta chain	109	56577/8.54	43	18	FGB	PMF

Note: a: for unbound fraction.
b: for bound fraction.

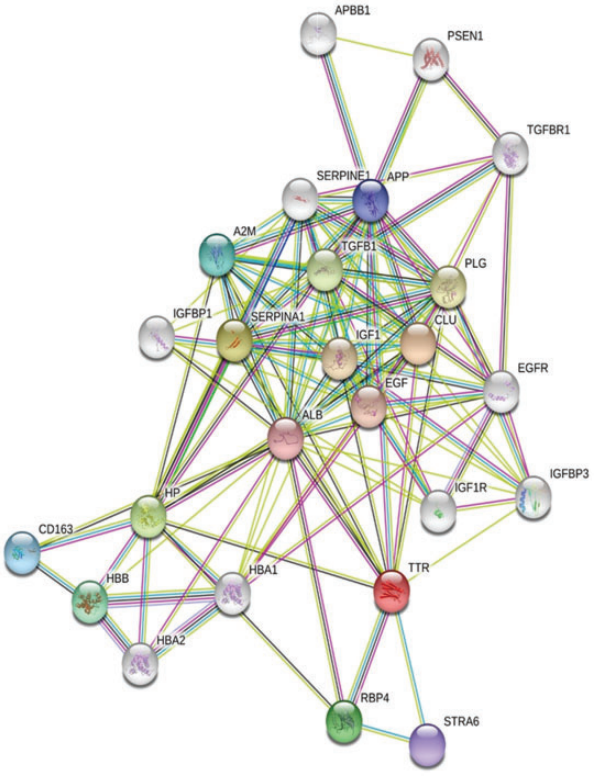

Figure 1. Interacting network among the differentially expressed proteins in healthy, smoker, COPD, and lung cancer. (A color version of this figure is available in the online journal.)

shown in colored with their gene names. Blue solid lines represent binding, black line represent the reaction, yellow line represent the expression, green line represent the activation, purple line represent the post tranlational modification.

We have seen by the graphs among the seven differentially expressed proteins the RBP 4, haptoglobulin, Ig alpha-1 chain C region, Ig lambda-2 chain C region, antitrypsin increased in healthy, COPD, and lung cancer, while the clusterin sequentially decreased in smoker, COPD, and lung cancer (Figures S6 and S7). In the previous paper, the protein expression of TTR, clusterin, haptoglobulin, immunoglobulin lambda chain, antitrypsin is overexpressed in lung cancer.[13,20,21] In our case, the increased expression of TTR, clusterin, haptoglobulin, immunoglobulin lambda chain and antitrypsin in smoker, COPD, and lung cancer in comparision with the normal is also observed. The elevated level of RBP 4 is reported previously in breast cancer.[22] In our case, the RBP 4 is also upregulated in smoker, COPD, and lung cancer. We have also noted that there is sequential increase of two proteins including haptoglobulin and alpha-1-antitrypsin from healthy, smoker, COPD to lung cancer.

The pathways which identified in the string analysis results were HIF-1, Fox O, P53, Rap1, Ras, p13K-AKT, MAPK, ERbB signaling pathways. The cummulative effects of these pathways are oxygen homeostasis, oxidative stress resistance, apoptosis, cell cycle control, glucose metabolism, cellular senesence, cell adhesion, cell polarity, cell–cell junction formation, differentiation,

cell proliferation, survival, cytoskeletal dynamism, cell motility, transcription, translation and growth migration. We deduce from the findings that disturbance of these signaling pathways might be the cause of deregulation and differential expression of proteins. In STRING, the proteins which are involved in the KEGG signaling pathways are the p53. There are number of stress signals involved in the activation of p53 which includes DNA damage, oxidative stress and activated oncogenes (Figure S8), and HIF (Figure S9); a hypoxia inducible factor 1 is a transcription factor that functions in oxygen homeostasis as a master regulator. The Forkhead box O (FOXO) signaling pathway belongs to the family of transcription factor that regulates the expression of genes in cellular physiological events (Figure S10). The Rap1 signaling pathway belongs to the small GTPase and is involved in signal transduction and in a number of communicating junctions such as cell–cell junction formation, cell adhesion, and cell polarity (Figure S11). On the other hand, the ras signaling pathways also belong to a class of protein GTPases which have functions in transmission of signals inside the cells and act as switches for other proteins which are involved in the growth migration, cell proliferation, survival, etc. The phosphotidyl 3′ Kinase (PI3K)-Akt signaling pathway is triggered by variety of cellular signals and toxic insults which regulates central cellular functions such as cell proliferation, transcription, translation, survival, and growth (Figures S12 and S13). Another one of the most conserved module of cascades is MAPK that is also involved in cellular proliferation, migration, and differentiation (Figure S14). The ErbB family belongs to receptor tyrosine kinases (RTKs) that couples the extracellular growth factor ligands with the internal cellular signaling pathways and are responsible for the regulation of a range of biological responses, including cellular differentiation, proliferation, cell survival, and motility (Figure S15).

Conclusion

The combination of gel- and non-gel-based fractionation strategy in proteomic was found to be very effective while analyzing the complex biological fluids, i.e. plasma. This study reveals a comparative proteomic profiling of lung cancer, COPD, smokers, and healthy groups. Total 23 proteins were identified among which up-fold of 7 proteins were found in lung cancer, COPD, and smokers in comparison to healthy group. Further mapping of these identified proteins disclosed that they are associated with three main biological processes including transportation, inflammation, and apoptosis. However, two proteins, i.e. haptoglobulin and alpha-1-antitrypsin correlate all groups in a sequence of healthy > smoker > COPD > lung cancer. After validation of these proteins in a large number of samples, these can serve as potential biomarkers to monitor the progression of smokers and COPD patients towards lung cancer.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; Mahwish Saleem, and Syed Kashif Raza conducted the experiments and wrote the manuscript, and Syed Ghulam Musharraf supervised the study and reviewed the manuscript.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Nadeem Rizvi (Head, Oncology Department, JPMC, Karachi) and healthy smokers and healthy non-smokers, for their kind cooperation in sample collection. Our special thanks are due to all CML patients who voluntarily gave their blood sample for this research project.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded by the Higher Education Commission (HEC project I.D. 2485).

REFERENCES

1. Youlden DR, Cramb SM, Baade PD. The international epidemiology of lung cancer: geographical distribution and secular trends. *J Thorac Oncol* 2008;**3**:819–31
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;**136**:E359–86
3. Rivera MP, Mehta AC. Initial diagnosis of lung cancer: ACCP evidence-based clinical practice guidelines. *CHEST J* 2007;**132**:131S–48S
4. Varela-Lema L, Fernandez-Villar A, Ruano-Ravina A. Effectiveness and safety of endobronchial ultrasound–transbronchial needle aspiration: a systematic review. *Eur Respir J* 2009;**33**:1156–64
5. Kisluk J, Ciborowski M, Niemira M, Kretowski A, Niklinski J. Proteomics biomarkers for non-small cell lung cancer. *J Pharm Biomed Anal* 2014;**101**:40–9
6. Okada M, Nishio W, Sakamoto T, Uchino K, Yuki T, Nakagawa A, Tsubota N. Prognostic significance of perioperative serum carcinoembryonic antigen in non-small cell lung cancer: analysis of 1,000 consecutive resections for clinical stage I disease. *Ann Thorac Surg* 2004;**78**:216–21
7. Schneider J. Tumor markers in detection of lung cancer. *Adv Clin Chem* 2006;**42**:1–41
8. Ferrigno D, Buccheri G, Giordano C. Neuron-specific enolase is an effective tumour marker in non-small cell lung cancer (NSCLC). *Lung Cancer* 2003;**41**:311–20
9. Xu M, Zhu M, Du Y, Yan B, Wang Q, Wang C, Zhao J. Serum C-reactive protein and risk of lung cancer: a case-control study. *Med Oncol* 2013;**30**:319
10. Bai J, He A, Zhang W, Huang C, Yang J, Yang Y, Wang J, Zhang Y. Potential biomarkers for adult acute myeloid leukemia minimal residual disease assessment searched by serum peptidome profiling. *Proteome Sci* 2013;**11**:39
11. Mo Ahn. J, Yoel Cho. J. Current serum lung cancer biomarkers. *J Mol Biomark Diagnos* 2013;**s4**:1–7
12. Anderson D, Hughes JA, Cebulska-Wasilewska A, Nizankowska E, Graca B. Ras p21 protein levels in human plasma from patients with chronic obstructive pulmonary disease (COPD) compared with lung cancer patients and healthy controls. *Mutat Res* 1998;**403**:229–35

13. Ding H, Liu J, Xue R, Zhao P, Qin Y, Zheng F, Sun X. Transthyretin as a potential biomarker for the differential diagnosis between lung cancer and lung infection. *Biomed Rep* 2014;**2**:765–9
14. Young RP, Hopkins RJ, Christmas T, Black PN, Metcalf P, Gamble GD. COPD prevalence is increased in lung cancer, independent of age, sex and smoking history. *Eur Respir J* 2009;**34**:380–6
15. Rai AJ, Gelfand CA, Haywood BC, Warunek DJ, Yi J, Schuchard MD, Mehigh RJ, Cockrill SL, Scott GB, Tammen H, Schulz-Knappe P, Speicher DW, Vitzthum F, Haab BB, Siest G, Chan DW. HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. *Proteomics* 2005;**5**:3262–77
16. Bainor A, Chang L, McQuade TJ, Webb B, Gestwicki JE. Bicinchoninic acid (BCA) assay in low volume. *Anal Biochem* 2011;**410**:310–2
17. Echan LA, Tang HY, Ali-Khan N, Lee K, Speicher DW. Depletion of multiple high-abundance proteins improves protein profiling capacities of human serum and plasma. *Proteomics* 2005;**5**:3292–303
18. de Roos B, Duthie SJ, Polley AC, Mulholland F, Bouwman FG, Heim C, Rucklidge GJ, Johnson IT, Mariman EC, Daniel H. Proteomic methodological recommendations for studies involving human plasma, platelets, and peripheral blood mononuclear cells. *J Proteome Res* 2008;**7**:2280–90
19. Bouwman F, Suylen D, Renes J, Mariman E. Evaluation and improving the success rate of protein identification by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2005;**19**:2465–8
20. Maciel CM, Junqueira M, Paschoal MEM, Kawamura MT, Duarte RLM, MdGdC C, Domont GB. Differential proteomic serum pattern of low molecular weight proteins expressed by adenocarcinoma lung cancer patients. *J Exp Ther Oncol* 2005;**5**:31–38
21. Langenfeld EM, Calvano SE, Abou NF, Lowry SF, Amenta P, Langenfeld J. The mature bone morphogenetic protein-2 is aberrantly expressed in non-small cell lung carcinomas and stimulates tumor growth of A549 cells. *Carcinogenesis* 2003;**24**:1445–54
22. Jiao C, Cui L, Ma A, Li N, Si H. Elevated serum levels of retinol-binding protein 4 are associated with breast cancer risk: a case-control study. *PLoS One* 2016;**11**:e0167498

(Received August 14, 2018, Accepted January 4, 2019)