Quantitative Proteomics of Bronchoalveolar Lavage Fluid in Lung Adenocarcinoma

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Abstract. *Background: The most commonly reported primary lung cancer subtype is adenocarcinoma, which is associated with a poor prognosis and short survival. Proteomic studies on human body fluids such as bronchoalveolar lavage fluid (BALF) have become essential methods for biomarker discovery, examination of tumor pathways and investigation of potential treatments. Aim: This study used quantitative proteomics to investigate the up-regulation of novel proteins in BALF from patients with primary lung adenocarcinoma in order to identify potential biomarkers. Materials and Methods: BALF samples from individuals with and without primary lung adenocarcinoma were analyzed using liquid chromatography–mass spectrometry. Results: One thousand and one hundred proteins were identified, 33 of which were found to be consistently overexpressed in all lung adenocarcinoma samples compared to non-cancer controls. A number of overexpressed proteins have been previously shown to be related to lung cancer progression including S100-A8, annexin A1, annexin A2, thymidine phosphorylase and transglutaminase 2. Conclusion: The overexpression of a number of specific proteins in BALF from patients with primary lung adenocarcinoma may be used as a potential biomarker for lung adenocarcinoma.*

Lung cancer is one of the most commonly diagnosed types of cancer in the world. It is the leading cause of cancer mortality, being responsible for approximately 1.4 million deaths annually (1, 2) Overall prognosis in lung cancer remains poor despite incremental advances in treatment and still only 10-15% of patients survive five years or longer from diagnosis (3). Adenocarcinoma is the most frequently reported subtype of lung cancer in many countries (4, 5). Advances in the knowledge of the molecular pathways that relate to malignancy have opened up new methods for lung cancer treatment, particularly for adenocarcinoma, where molecular characterisation has led to the use of agents with high levels of antitumor activity. For example, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are being used to treat patients with metastatic lung adenocarcinoma and active mutation in *EGFR* (6, 7). As lung adenocarcinoma has been histologically described to be the most variable and heterogeneous subtype of lung cancer (4), urgent further research is needed in this area to understand its onset and progression.

Recently, quantitative proteomics has been utilised as a new technique to investigate the complex proteins present in the tissue of patients with cancer compared to non-cancer controls (8). Extensive efforts have been directed towards examining protein dynamics, which eventually determine cell behaviour (9). Cellular proteins mostly function as components of protein complexes rather than as single polypeptides. Therefore, characterising the structure and dynamics of multiprotein complexes is crucial to understanding and possibly manipulating the tumour microenvironment *via* various therapies (10). These investigations are aimed at understanding the proteins and protein interactions that may lead to treatments that can favour good patient outcomes as well as developing valuable biomarkers (9).

Proteomic studies of human body fluids such as bronchoalveolar lavage fluid (BALF) have already delivered vital information concerning alterations in protein expression and secretion in a range of pulmonary disorders including lung cancer (8, 11, 12). Alterations in protein expression have been shown in sarcoidosis, cystic fibrosis, hypersensitivity pneumonitis, and chronic obstructive pulmonary disease, as well as lung cancer (11-15). BALF is sampled during bronchoscopy of patients with a range of

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pulmonary disorders and provides a sample of physiological fluid from the pulmonary compartment (16, 17). BALF has been commonly used to obtain inflammatory cells (alveolar macrophages, neutrophils and monocytes) and other soluble components that are present in alveoli (17). Proteins that are found in BALF are secreted from epithelial and inflammatory cells as well as being derived from the bloodstream (17). An advantage of using BALF samples in proteomic analysis is the low protein concentration in the fluid. This increases the chances of detecting more lung cancer biomarkers due to the absence of highly abundant and non-specific proteins that potentially mask lung-derived molecules in other physiological fluids such as serum (11, 17). Thus, the proteomic analysis of BALF can be utilised to investigate important pathophysiological functions that relate to a particular pulmonary disease, such as lung cancer (11). However, not all proteins that are present in BALF can be identified using mass spectrometry, given that the dynamic range of BALF protein abundance is estimated to be around 10^{10}, while the resolving power of mass spectrometry proteomics is limited to 10^2-10^4 (18).

In the present study, our aim was to identify biological processes, cellular components and molecular functions in BALF samples from patients with and without adenocarcinoma of the lung using quantitative proteomics. We also categorised the up-regulated proteins and their biological processes, cellular components and molecular functions in patients with adenocarcinoma of the lung. Identification of de-regulated proteins in patients with primary lung adenocarcinoma may provide increased knowledge regarding the tumour microenvironment, cancer pathways and potential biomarkers.

Materials and Methods

Study participants. BALF samples were collected prospectively from patients with presumptive lung cancer undergoing diagnostic bronchoscopy through the Department of Respiratory and Sleep Medicine, Austin Health, Heidelberg, VIC, Australia. Human Ethics approval was received from HREC Austin Health H2007/02814 and RMIT University Human Research Ethics Committee ASEHAPP 15-13 and informed consent of all participants was obtained. The patient demographic is presented in Table I. Staging was applied in this study using the new TNM (tumour, node, metastases) staging system (seventh edition) for lung cancer (19).

BALF processing. BALF was sampled at the time of diagnostic fibre-optic bronchoscopy using instilled aliquots of normal saline pre-warmed to 37˚C. In the case of patients with lung cancer, the samples were taken from an adjacent uninvolved lobe. In the case of non-cancer controls, the sample was from the right middle lobe or lingula lobe. The BALF samples were collected in Falcon tubes, immediately placed on ice and stored at 4˚C until processing. Cells were collected by centrifuging the BALF at 500 × *g* for 6 min at 4˚C. The cells were then washed twice in 0.1% Bovine serum albumin/Phosphate buffered saline (PBS) (w/v) at 4˚C and counted in a haemocytometer using trypan blue 0.2% (w/v) (Sigma-Aldrich, St. Louis, MO, USA) staining.

Protein sample isolation. The protein samples were isolated using the PARIS kit (Protein and RNA Isolation System; Ambion, Life Technologies Australia, Mulgrave, VIC, Australia). The pellet was washed twice with PBS and cell disruption buffer was added (at least 300 μl) for ≥10^6 cells. The lysate was incubated on ice for 5-10 minutes to ensure complete cell disruption before further processing of the sample. Protein samples were then stored at −80˚C and kept on ice when in use.

Protein sample preparation. The protein samples (150 μl) were mixed and washed twice with 750 μl of ice-cold acetone (Merck Millipore, Darmstadt, Germany) and then incubated overnight at −20˚C. They were then centrifuged at 13,148 × *g* for 10 min at 4˚C and the supernatant discarded. The pellets were carefully layered with ice-cold acetone (750 μl) and then centrifuged at 17,968 × *g* for 10 min at 4˚C and the supernatant discarded. The resultant pellets were dissolved in 100 μl of 8 M urea (Merck Millipore, Darmstadt, Germany) and 50 mM triethyl ammonium bicarbonate (TEAB) (Sigma-Aldrich, St. Louis, MO, USA) with alternate sonication and vortexing. Protein estimation was carried out using the micro BCA assay kit (Thermo Scientific, Rockford, IL, USA) and concentration was adjusted to 100 μg/100 μl using 8 M urea and 50 mM TEAB buffer. Reduction was carried out using tris-2-carboxyethyl phosphine (Thermo Scientific, Rockford, IL, USA) to a final concentration of 10 mM and incubated at 37˚C for 30 min. Alkylation was carried out with iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 55 mM and incubated for 45 min in the dark. The urea was diluted to a 1 M final concentration using 25 mM TEAB prior to digestion. Digestion was then carried out using sequencing grade-modified trypsin (Promega, Madison, WI, USA) at a ratio of 1 μg trypsin: 40 μg protein overnight at 37˚C.

The peptide mixture samples were collected after overnight incubation and acidified using formic acid to a final concentration of 1% (v/v). Solid-phase extraction clean-up of samples to remove any traces of primary amine-containing molecules that could react with formaldehyde was carried out using Oasis HLB cartridges based on the manufacturer's instructions (Waters, Milford, MA, USA). The eluted samples were partially dried using CentriVap Centrifugal Vacuum Concentrators (Labconco, Kansas City, MO, USA) for 20 minutes followed by overnight freeze drying (Virtis Benchtop SLC Freeze Dryer) (SP Scientific, Warminster, PA, USA).

2-plex dimethylation labelling. Dimethyl labelling was carried out as described elsewhere (20). Peptide samples were first resuspended in 400 μl of 100 mM TEAB. To 100 μl of the buffer, 4 μl of 4% (v/v) CH_2O and CD_2O were added to the samples to be labelled with light and heavy dimethyl, respectively. This was followed by addition of 4 μl of 0.6 M $NaBH_3CN$ to both the light and intermediate label. The samples were incubated in a fume hood for one hour at room temperature (15-22˚C) while mixing using a bench top test tube mixer. The labelling reaction was quenched by adding 16 μl of 1% (v/v) ammonia solution then the samples were briefly mixed and centrifuged in a fume hood. For further reaction quenching, 8 μl of formic acid was added to the samples on ice to acidify the samples. The differentially labelled samples were then mixed at a 1:1 ratio and analysed using liquid chromatography–mass spectrometry (LC-MS/MS).

LC-MS/MS and data analysis. The dimethyl-labelled samples were analysed on a LTQ Orbitrap Elite (Thermo Scientific, Rockford, IL, USA) instrument coupled to an Ultimate 3000 RSLC nanosystem (Dionex) (Thermo Scientific, Rockford, IL, USA). A nanoLC system was equipped with an Acclaim Pepmap nano-trap column (Dionex – C_{18}, 100 Å, 75 μm × 2 cm) and an Acclaim Pepmap analytical column (Dionex C_{18}, 2 μm, 100 Å, 75 μm × 15 cm) running in 3-80% CH_3CN containing 0.1% formic acid gradient over 25 min. The LTQ Orbitrap Elite mass spectrometer was operated in the data-dependent mode, whereby spectra were acquired first in positive mode at 240,000 resolution followed by high-energy collisional dissociation at 15,000 resolution. Ten of the most intense peptide ions with charge states ≥2 were isolated and fragmented using normalized collision energy of 35 and activation time of 0.1 ms (high-energy collisional dissociation).

The Orbitrap MS data were analysed using Proteome Discoverer (Thermo Scientific version 1.4, Rockford, IL, USA) with the Mascot search engine (Matrix Science version 2.4, Boston, MA, USA) against the Uniprot database maintained at the Bio21 institute, University of Melbourne, Australia (currently containing 26 617 536 sequences). Search parameters were precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.2 Da. Carbamidomethyl of cysteine was set as fixed modification and dimethyl labelling (light and medium at +28.0313 and +32.0564, respectively) at the peptide N-terminus and lysine set as variable modifications. Trypsin with a maximum of 0 missed cleavage was used as the cleavage enzyme. A false discovery rate threshold of 1% was applied and identification of two or more unique peptides and two or more peptides were required for positive identification and quantification, respectively. A two-fold differential expression was chosen as being significant.

Results

Lung adenocarcinoma alters BALF protein expression. The number of proteins that were consistently identified in all samples was 1,100. The main biological processes that were associated with BALF proteins were metabolic processes (~19%), regulation of biological processes (~13%), response to stimulus (~12%) and transport (~9%). Additionally, the main cellular components or compartments found to be linked to BALF proteins were the cytoplasm (~23%), cell membrane (~14%), cytosol (~9%), nucleus (~8%), extracellular (~8%) and organelle lumen (~8%), respectively. For molecular functions, protein binding (~28%), catalytic activity (~22%), nucleotide binding (~11%), metal ion binding (~9%) and RNA binding (~5%) were found to be the leading molecular functions that BALF proteins displayed (Figure 1).

In the present study, 1,100 proteins were identified, out of which 33 were found to be up-regulated consistently in all studied lung adenocarcinoma samples compared to non-cancer controls (Table II). Eighteen percent of the up-regulated proteins were expressed at levels 3- to 5-fold higher in BALF from patients with lung adenocarcinoma compared to that from non-cancer controls. The remaining up-regulated proteins (81%) were found to have a higher fold change, with 5- to 11-fold increase in BALF from patients with adenocarcinoma compared to non-cancer controls. The top five up-regulated proteins were galectin-1, ADP/ATP translocase 2, 78 kDa glucose-regulated protein, cystatin-B and carbonic anhydrase II, respectively (Table II). The biological processes, cellular components and molecular functions of all 33 up-regulated proteins were also shown in this study (Table III).

Table I. *Demographic details of patients with lung adenocarcinoma and controls*.

	n	Age (years) Mean±SD	Gender	Smoking status	Tumour stage
			M/F	N/Ex/S	I/II/III/IV
Controls	8	60±8.71	3/5	3/2/3	
Patients	8	68.1±7.56	5/3	1/2/5	2/2/1/3

n: Number; SD: standard deviation; M: male; F: Female; N: non-smoker; Ex: Ex-smoker; S: smoker.

Discussion

Non-small cell lung carcinoma (NSCLC) was previously classified as one group with no further subtypes; however, a new pathological classification of lung cancer was described in 2011 by the International Association for the Study of Lung Cancer, the American Thoracic Society and the European Respiratory Society (21). The alteration of lung cancer classification was significant because it has been recognised that different lung cancer subtypes respond differently to treatment. For example, metastatic lung adenocarcinoma which expresses EGFR or Kirsten rat sarcoma (KRAS) can be treated with biological agents that are not as yet helpful for other subtypes, *e.g*. squamous cell carcinoma (6, 7). Lung adenocarcinoma is the most common subtype of lung cancer, with high variability and heterogeneity in its histological features (4, 5, 22).

Therefore, it is essential to use new techniques to improve our knowledge over the complexity of the molecular environment in lung adenocarcinoma. Quantitative proteomics is a new technique that has been recently utilised to investigate the protein dynamics in lung cancer and other pulmonary disorders (8). To date, most of the published literature has focused on plasma samples, cell lines, or on squamous lung carcinoma and not on lung adenocarcinoma (8). In this study, BALF was used as the source of proteins to be studied and we focused on revealing biological processes, cellular components and molecular functions of all identified proteins, as well as examining the impact of lung adenocarcinoma on protein expression comparing cancer samples to non-cancer controls using quantitative proteomics.

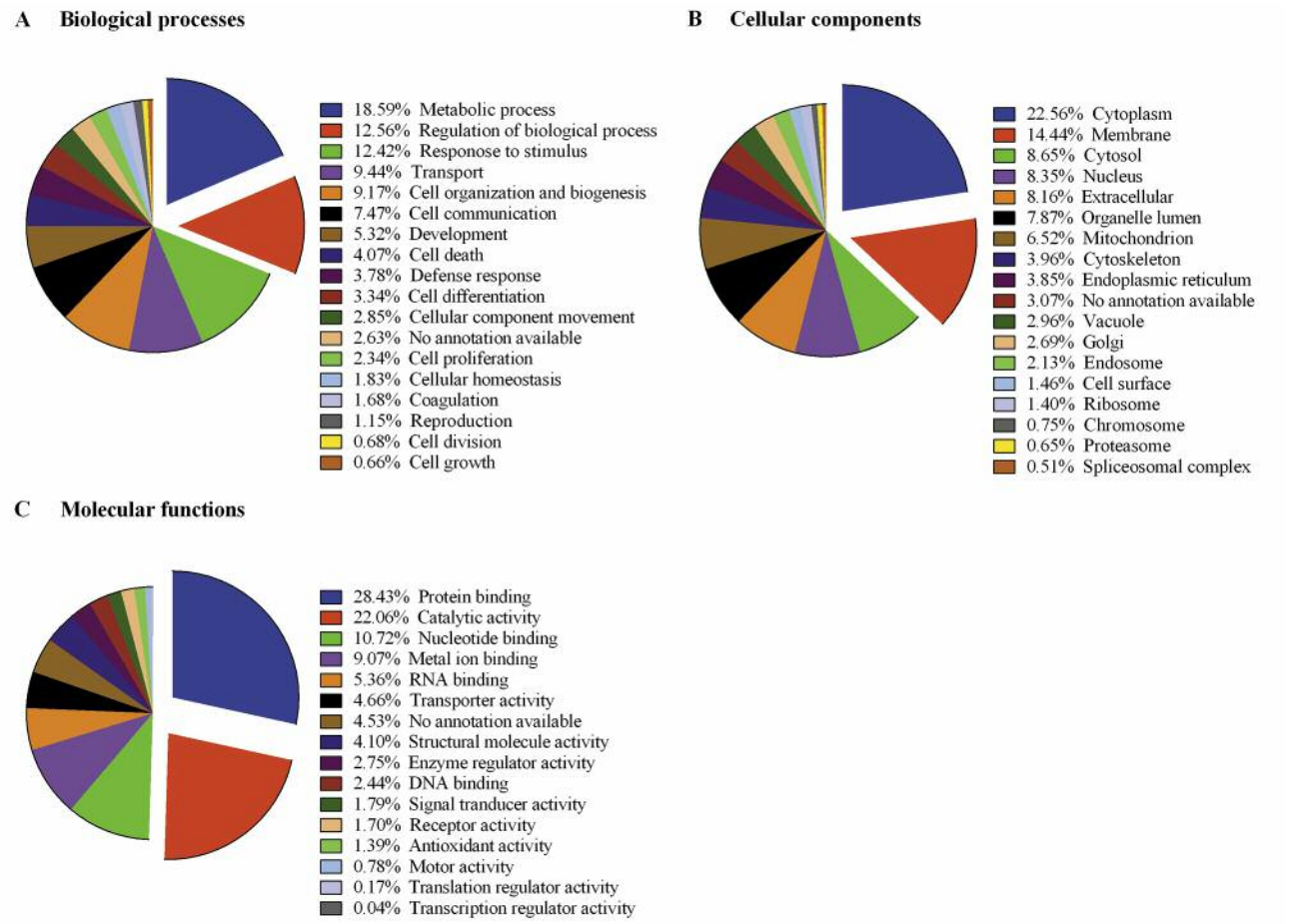

Figure 1. *Distribution and classification of all proteins that were identified in bronchoalveolar lavage fluid (BALF) samples. The pie charts display the profiles of (A) biological processes, (B) cellular components and (C) molecular functions of all proteins that were identified in all BALF samples from patients with lung adenocarcinoma and non-cancer controls.*

Traditional biochemical methods investigate only one or a few proteins, while proteomics has the ability to identify thousands of proteins and provide comprehensive proteomic information (23, 24). Being able to access all of this information can be significant in understanding the molecular mechanisms underlying lung adenocarcinoma.

In this study, 1100 distinct proteins were identified and their biological processes, cellular components and molecular functions were stated. Previous studies identified a similar number of up-regulated proteins in BALF samples (18, 23). Biological processes, cellular components and molecular functions have been examined in normal, allergic asthma, lung cancer cell lines and pulmonary squamous cell carcinoma (18, 23, 25, 26). In our study, a large number of biological processes were identified as being associated with BALF proteins such as metabolism, regulation of biological processes, response to stimuli, transport, cell proliferation, cell growth and cell differentiation. Numerous cellular components were found to be associated to BALF proteins, such as cytoplasm, membrane, cytosol, nucleus, extracellular, organelle lumen and cell surface. The molecular functions that were recognised as being associated with BALF proteins included protein binding, catalytic activity, nucleotide binding, metal ion binding, DNA binding and RNA binding.

Up-regulated proteins that were associated with lung adenocarcinoma samples are shown in Table II. Thirty-three proteins were found to be consistently up-regulated in all lung adenocarcinoma patient samples compared to non-cancer controls. These proteins were considered significant as they had a minimum two-fold increased expression and two or more unique peptides and peptide counts. A number of overexpressed proteins have been shown to be related to lung cancer progression, including S100-A8, annexin A1, annexin A2, thymidine phosphorylase (TYMP) and transglutaminase

Table II. *List of up-regulated proteins in bronchoalveolar lavage fluid from patients with lung adenocarcinoma versus non-cancer controls.*

No.	Protein name[a]	Protein description[a]	MW (kDa)[a]	#Peptide count	#Unique peptides	Fold change[b]
1	ACTN4_HUMAN	Alpha-actinin-4	104.8	19	8	3.095
2	ADT2_HUMAN	ADP/ATP translocase 2	32.8	9	2	10.611
3	ANXA1_HUMAN	Annexin A1	38.7	12	12	6.283
4	ANXA2_HUMAN	Annexin A2	38.6	17	17	5.799
5	B4DIT7_HUMAN	Transglutaminase 2	68.6	11	11	7.073
6	B4DJV2_HUMAN	Citrate synthase	50.4	5	4	5.681
7	B4DQJ8_HUMAN	6-Phosphogluconate dehydrogenase, decarboxylating	51.8	6	6	4.774
8	B4DW52_HUMAN	cDNA FLJ55253, highly similar to actin, cytoplasmic 1	38.6	13	7	4.949
9	B4E1F5_HUMAN	cDNA FLJ57475, highly similar to pulmonary surfactant-associated protein B	38.5	5	5	8.734
10	B7Z7A9_HUMAN	Phosphoglycerate kinase B	41.4	9	9	7.414
11	C9JGI3_HUMAN	Thymidine phosphorylase	46.1	9	9	4.928
12	CAH2_HUMAN	Carbonic anhydrase-II	29.2	9	9	9.888
13	CATA_HUMAN	Catalase	59.7	16	16	8.358
14	CLIC1_HUMAN	Chloride intracellular channel protein 1	26.9	5	5	6.092
15	CX7A2_HUMAN	Cytochrome c oxidase subunit 7A2, mitochondrial	9.4	2	2	7.147
16	CYTB_HUMAN	Cystatin-B	11.1	3	3	10.051
17	EFHD2_HUMAN	Swiprosin-1	26.7	5	5	6.037
18	ENOA_HUMAN	Alpha-enolase	47.1	14	11	6.060
19	F8W0P2_HUMAN	HLA class II histocompatibility antigen, DR alpha chain	26.9	6	5	6.313
20	GRP78_HUMAN	78 kDa glucose-regulated protein	72.3	15	13	10.385
21	H4_HUMAN	Histone H4	11.4	3	3	8.292
22	LEG1_HUMAN	Galectin-1	14.7	4	4	11.386
23	LKHA4_HUMAN	Leukotriene A-4 hydrolase	69.2	22	22	6.851
24	MYH9_HUMAN	Myosin-9	226.4	20	20	4.034
25	O60744_HUMAN	Thioredoxin delta 3	9.3	2	2	8.664
26	PLSL_HUMAN	Plastin-2	70.2	21	21	5.640
27	PROF1_HUMAN	Profilin-1	15.0	6	6	8.870
28	Q53FJ5_HUMAN	Prosaposin	58.1	9	9	9.982
29	Q5TCU6_HUMAN	Talin 1	257.9	23	23	7.410
30	Q6DC98_HUMAN	Lamin-B1 protein	38.1	6	5	7.298
31	S10A8_HUMAN	Protein S100-A8	10.8	4	4	8.512
32	SAMH1_HUMAN	SAM domain and HD domain-containing protein 1	72.2	10	10	4.769
33	TAGL2_HUMAN	Transgelin-2	22.4	9	9	9.572

MW: Molecular weight; #peptide count: sum of peptide count; #unique peptides: sum of unique peptides; A: lung adenocarcinoma; N:non-cancer controls; HLA: human leukocyte antigen; DR: D related; SAM: sterile alpha motif; HD: homodimer. [a]Accession number of proteins, protein name and molecular weight from the Uniprot database. [b]Fold difference in expression in patients with cancer versus controls.

2 (TG2) (27-30). In our case, a number of overexpressed proteins (Table II) were previously identified as being up-regulated in peripheral cholangiocarcinoma, including alpha-actinin-4 and 78 kDa glucose-regulated protein (31).

S100-A8 is an important protein and recent studies have focused on its critical role in tumour growth, progression and invasion. It was also proposed to be both a potential therapeutic target and an indicator for tumour progression (27, 32). In this study, the expression of S100-A8 was increased in lung adenocarcinoma samples compared to non-cancer controls. S100-A8 is a low molecular weight member of the S100 protein family which is characterised by the presence of two calcium ion Ca^{2+} binding EF-hand motifs (33). It is naturally present at high concentration in the cytoplasm of monocytes and neutrophils and has been shown to play an essential role in lung tumour growth, progression and invasion (27, 33). In previous studies, the up-regulation of S100-A8 was shown in lung adenocarcinoma human tissue and in BALF of patients with acute respiratory distress syndrome (27, 34). S100-A8 has been established as a pro-inflammatory mediator in chronic and acute inflammation (35). It has also been correlated to tumour progression in other cancer types, including kidney and breast cancer (36, 37). Another study also confirmed the role of S100-A8 protein in promoting cell migration and invasion by the activation of P38 mitogen-activated protein kinases and nuclear factor kappa-B in gastric cancer cells (32).

Table III. *The molecular functions, cellular components and biological processes of the proteins that were identified as being overexpressed in lung adenocarcinoma.*

	Protein name	Protein description	Molecular functions	Cellular components	Biological processes
1	ACTN4_HUMAN	Alpha-actinin-4			
2	ADT2_HUMAN	ADP/ATP translocase 2			
3	ANXA1_HUMAN	Annexin A1			
4	ANXA2_HUMAN	Annexin A2			
5	B4DIT7_HUMAN	Transglutaminase 2			
6	B4DJV2_HUMAN	Citrate synthase			
7	B4DQJ8_HUMAN	6-Phosphogluconate dehydrogenase, decarboxylating			
8	B4DW52_HUMAN	cDNA FLJ55253, highly similar to actin, cytoplasmic 1			
9	B4E1F5_HUMAN	cDNA FLJ57475, highly similar to pulmonary surfactant-associated protein B			
10	B7Z7A9_HUMAN	Phosphoglycerate kinase B			
11	C9JGI3_HUMAN	Thymidine phosphorylase			
12	CAH2_HUMAN	Carbonic anhydrase II			
13	CATA_HUMAN	Catalase			
14	CLIC1_HUMAN	Chloride intracellular channel protein 1			
15	CX7A2_HUMAN	Cytochrome *c* oxidase subunit 7A2, mitochondrial			
16	CYTB_HUMAN	Cystatin-B			
17	EFHD2_HUMAN	Swiprosin-1			
18	ENOA_HUMAN	Alpha-enolase			
19	F8W0P2_HUMAN	HLA class II histocompatibility antigen, DR alpha chain			
20	GRP78_HUMAN	78 kDa glucose-regulated protein			
21	H4_HUMAN	Histone H4			
22	LEG1_HUMAN	Galectin-1			
23	LKHA4_HUMAN	Leukotriene A-4 hydrolase			
24	MYH9_HUMAN	Myosin-9			
25	O60744_HUMAN	Thioredoxin delta 3			
26	PLSL_HUMAN	Plastin-2			
27	PROF1_HUMAN	Profilin-1			
28	Q53FJ5_HUMAN	Prosaposin			
29	Q5TCU6_HUMAN	Talin 1			

Table III. *continued*

Table III. *continued*

	Protein name	Protein description	Molecular functions	Cellular components	Biological processes
30	Q6DC98_HUMAN	Lamin-B1 protein			
31	S10A8_HUMAN	Protein S100-A8			
32	SAMH1_HUMAN	SAM domain and HD domain-containing protein 1			
33	TAGL2_HUMAN	Transgelin-2			

HLA: human leukocyte antigen; DR: D related; SAM: sterile alpha motif; HD: homodimer. **Colour definitions**: Molecular functions: protein binding, metal ion binding, catalytic activity, nucleotide binding, structural molecule activity, enzyme regulator activity, RNA binding, DNA binding, transporter activity, motor activity, signal transducer activity, antioxidant activity; Cellular components: cytoskeleton, extracellular, nucleus, cytoplasm, mitochondrion, organelle lumen, membrane, endosome, cell surface, cytosol, vacuole, endoplasmic reticulum, golgi, chromosome; Biological processes: response to stimulus, transport, coagulation, regulation of biological process, cell death, cell organization and biogenesis, cellular component movement, metabolic process, cell communication, defense response, cell differentiation, development, cell proliferation, reproduction development, cellular homeostasis, reproduction, cell division, cell growth.

Other proteins up-regulated here have been shown to be associated with tumour growth and treatment resistance, such as annexin A1 and A2 (28). These proteins belong to the annexin superfamily of calphobindin and play vital physiological roles in cytoskeletal movement, regulating cell growth and forming ion channels (38). Annexin A1 and A2 have been shown to participate in tumor drug resistance in lung adenocarcinoma both *in vivo* and *in vitro* (28). A number of studies have indicated that high annexin expression is important for progression of lung cancer (*e.g.* NSCLC and lung adenocarcinoma) and suggested annexin to be a potential prognostic and diagnostic factor, and therapeutic target for new lung cancer drug development (28, 39-41). In fact, circulating antibodies to annexin A1, and another protein named DEAD box protein 53, have been used as biomarkers for early lung cancer diagnosis (40). Our results revealed annexin A1 and A2 proteins to be overexpressed in patients with lung adenocarcinoma compared to non-cancer controls.

Another protein up-regulated in lung adenocarcinoma is TYMP, also known as platelet-derived endothelial cell growth factor. Overexpression of TYMP has been shown in lung cancer, including lung adenocarcinoma (29, 42). It has been reported that high TYMP expression is associated with tumour growth, invasiveness, increased microvessel density, metastasis and poor prognosis in lung cancer (29, 42, 43). It has been also suggested by many studies as a potential biomarker for poor prognosis and a novel target for treatment of lung adenocarcinoma and other types of cancer (42, 44, 45). Interestingly, TYMP is one of the major catabolic enzymes of 5-fluorouracil used in cancer chemotherapy and its level in NSCLC tissue correlates to the efficacy of such treatment (46).

TG2 has been linked to invasion and metastasis in different cancer types, including breast, ovarian cancer and lung cancer (30, 47, 48). It is a multifunctional protein that plays an essential role in drug resistance in NSCLC (49, 50). A previous study verified that increased TG2 expression is associated with increased invasion and migration in NSCLC cells *in vitro* and suggested TG2 to be a promising prognostic marker (30). Our study confirmed the association of elevated expression of TG2 protein in the BALF fluid of patients with lung adenocarcinoma compared to non-cancer controls.

Other overexpressed proteins such as Chloride intracellular channel protein 1, transgelin-2, catalase, carbonic anhydrase II, galectin-1, and Lamin-B1 have been shown to be promising new lung cancer biomarkers (12, 50-53). Carbonic anhydrase II protein has been suggested to be a potential biomarker for early diagnosis for colorectal cancer and as a significant prognostic factor in gastrointestinal stromal tumor (54, 55). Another carbonic anhydrase family member (CA IX) has been associated with lung adenocarcinoma progression and poor prognosis (56).

This study not only identified proteins that are associated with lung cancer progression, but also proteins that have been suggested to play anticancer functions and can thus be used as an indication of good tumour prognosis or as potential biomarkers for tumour aggressiveness, such as profilin-1, and prosaposin (57-59). Identifying proteins with pro-tumour functions as well as others with antitumour functions may

validate the previous proposal that some of the inflammation cells *e.g.* macrophages, found in BALF samples play a dual role in the tumour microenvironment (60). Using proteomics to study BALF samples in lung cancer is therefore a promising technique that can be utilised to discover new biomarkers, treatment targets and prognostic and diagnostic indicators.

Our study does have a number of limitations, which include a small sample size, which needs to be expanded to confirm these results. Individual proteins shown to be up-regulated in proteomics studies are recommended to be validated using other techniques such as enzyme-linked immunosorbent assay and western blot. Despite the broad coverage of proteomics technique, proteomics still has a tendency to detect proteins with higher abundance and larger molecular weight. Unfortunately, even the depletion of high-abundance proteins in BALF has been associated with protein sample loss (61). Thus, further improvements in BALF sample preparation protocols and proteomic technology are highly desirable.

In conclusion, the impact of lung adenocarcinoma on protein expression was examined in BALF samples and compared to non-cancer controls using quantitative proteomics. Eleven hundred proteins were identified, of which 33 were found to be up-regulated consistently in all studied lung adenocarcinoma samples compared to non-cancer controls. The up-regulation of specific proteins in BALF of lung adenocarcinoma patients may be used as a potential biomarker for lung adenocarcinoma. Using proteomic techniques to study BALF samples in patients with lung cancer and other pulmonary disorders is a promising technique that may be utilised to identify new biomarkers, treatment targets and prognostic and diagnostic indicators.

Conflicts of Interest

The Authors wish to confirm that there are no known conflicts of interest associated with this work.

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