

## Early candidate biomarkers in urine of Walker-256 lung metastasis rat model

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### Abstract

Cancer metastasis accounts for the majority of deaths by cancer. Detection of cancer metastasis at its early stage is important for the management and prediction of cancer progression. Urine, which is not regulated by homeostatic mechanisms, reflects systemic changes in the whole body and can potentially be used for the early detection of cancer metastasis. In this study, a lung metastasis of a Walker-256 rat model was established by tail-vein injection of Walker-256 cells. Urine samples were collected at days 2, 4, 6 and 9 after injection, and the urinary proteomes were profiled using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The urinary protein patterns changed significantly with the development of Walker-256 lung metastasis. On the fourth day, lung metastasis nodules appeared. On the sixth day, clinical symptoms started. On days 2, 4, 6 and 9, 11, 25, 34 and 44 differential proteins were identified in 7 lung metastatic rats by LC-MS/MS. Seventeen of these 62 differential proteins were identified on the second day, and 18 of them were identified on the fourth day. The differential urinary proteins changed significantly two days before lung metastasis nodules appeared. Differential urinary proteins differed in Walker-256 lung metastasis rat models and Walker-256 subcutaneous rat models. A total of 9 differential proteins (NHRF1, CLIC1, EZRI, AMPN, ACY1A, HSP7C, BTD, NID2, and CFAD) were identified in 7 lung metastatic rats at one or more common time points, and these 9 differential proteins were not identified in the subcutaneous rat model. Seven of these 9 differential proteins were associated with both breast cancer and lung cancer, eight of

the nine were identified on the second day, and 8 of the nine can be identified on the fourth day; these early changes in urine were also identified with differential abundances at late stages of lung metastasis. Our results indicate that (1) the urine proteome changed significantly, even on the second day after tail-vein injection of Walker-256 cells and that (2) the urinary differential proteins were different in Walker-256 lung metastatic tumors and Walker-256 subcutaneous tumors. Our results provide the potential to detect early breast cancer lung metastasis, monitor its progression and differentiate it from the same cancer cells grown at other locations.

**Keywords:** cancer biomarkers, urine proteome, breast cancer metastasis, early detection

## Introduction

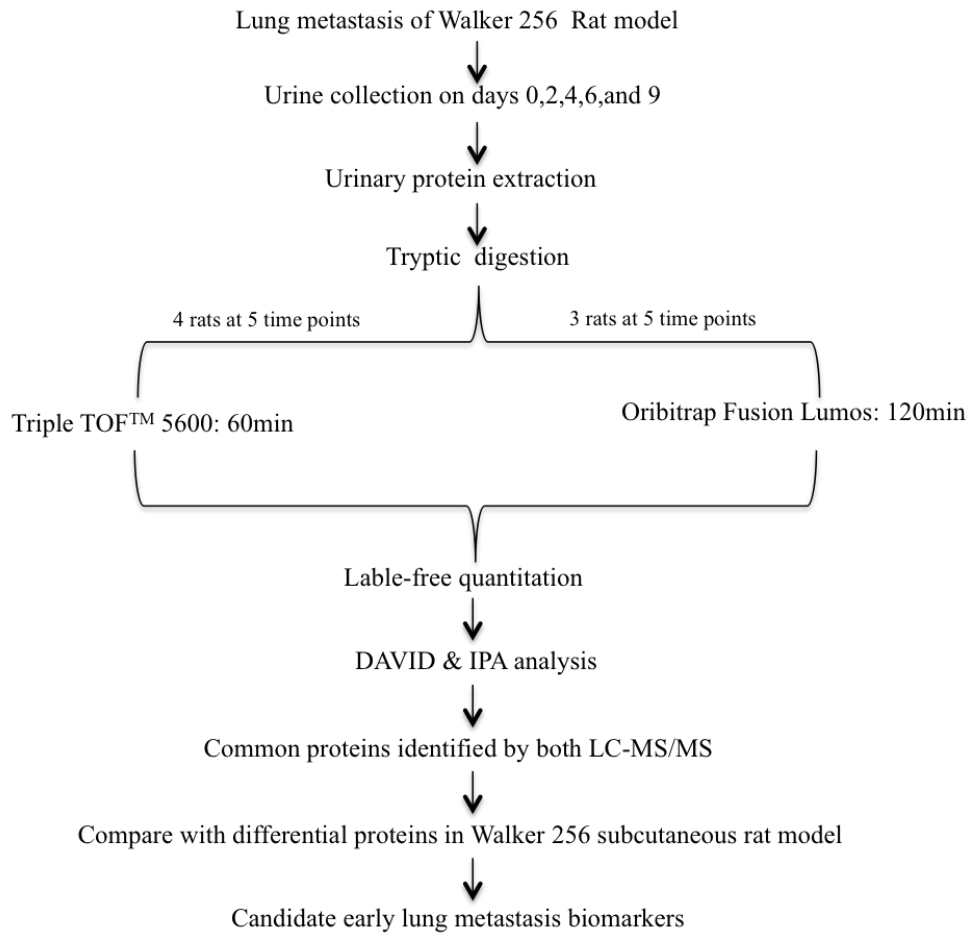
Cancer metastasis is a process in which cancer cells are disseminated from primary tumor tissue to different sites through blood vessels and lymphatic vessels. Lung, brain, bone and liver are the common metastatic organs in cancer patients[1]. Distant organ metastasis accounts for most cancer morbidity and mortality and nearly 90% of cancer death[2] and is usually accompanied by a poor 5-year survival rate as well as limited treatment strategies[3]. Due to special lung-specific immunoregulatory mechanisms, tumor colonization occurs more readily in an immunologically permissive environment[4]. Therefore, many cancer metastases such those of as breast cancer and malignant melanoma occur more easily in lung. The early detection of cancer metastasis is still elusive, as finding and predicting specific distant metastatic organs is difficult, especially in early-stage cancer without clinical symptoms. Therefore, the early detection of cancer metastasis can significantly improve the survival rate and effective therapies of cancer patients and also helps in monitoring cancer metastasis progression in time.

Biomarkers are measurable changes associated with the physiological or pathophysiological processes of disease and usually derive from tissue, blood and tumor cells [5]. Because of the homeostatic mechanisms in the internal environment, the levels of important factors in blood tend to be stable to protect the stability of the internal environment[6]. In addition, without the control of homeostatic mechanisms, urine can accumulate systemic changes from the whole body and thus has the potential to reflect the small pathophysiological changes of disease[7]. Therefore, urine has the potential to reflect

early changes in disease. However, whether time-course analyses of urine proteins can reveal reliable cancer metastasis biomarkers at different stages of cancer metastasis is unclear, as urinary proteins are easily affected by complicated factors such as medicine and diet, especially in human samples. Therefore, using a small number of animal models can help to determine the direct relationship between urine protein changes and related diseases such as cancer metastasis because the genetic and environmental factors are minimized [8]. In addition, determining an exact description of cancer metastasis is very helpful for the identification of cancer metastasis biomarkers, especially in early stages.

Various studies have applied urinary proteomics to discover cancer biomarkers for the early diagnosis and monitoring of cancer[9-11]. However, most of these studies used clinical urine samples from breast cancer patients who had already had distant metastases to viscera or bone[10]. It is difficult to clinically collect the exact early stages of breast cancer lung metastasis samples. Using animal models renders the exact starting point of cancer lung metastasis available, which is very helpful in the identification of biomarkers in the early stage of cancer lung metastasis.

Walker-256 cells are mammary gland carcinoma cells[12], and the Walker-256 lung carcinoma metastasis rat model is a well-known cancer lung metastasis rat model for studies of lung metastasis progression, such as evaluating the effects of some drugs on the development of Walker-256 lung metastases [13]. In this study, the Walker-256 lung carcinoma metastasis rat model was established by tail-vein injection of Walker-256 tumor cells. Urine samples were collected from lung metastasis rat models on days 2, 4, 6, and 9 for further urine proteome analysis. By comparing the differential proteins of Walker-256 lung carcinoma metastasis rats and subcutaneous rats[9], early lung metastasis associated urine biomarkers was identified. The workflow of this research is presented in Figure 1.



**Figure 1. Workflow of urinary proteomic discovery in this study. Urine samples were collected on days 2, 4, 6, and 9.**

## Materials and methods

### Experimental animals

Male Wistar rats ( $150 \pm 20$  g) and Sprague-Dawley (SD) rats ( $70 \pm 20$  g) were purchased from the Beijing Vital River Laboratory Animal Technology Co, Ltd. All animals were housed with free access to a standard laboratory diet and water with controlled indoor temperature ( $22 \pm 1^\circ\text{C}$ ) and humidity (65 ~ 70%) and a 12 h/12 h light-dark cycle. All animal protocols governing the experiments in this study were approved by the Institute of Basic Medical Sciences Animal Ethics Committee, Peking Union Medical College (Approved ID: ACUC-A02-2014-008). The study was performed according to the guidelines developed by the Institutional Animal Care and Use Committee of Peking Union Medical College. All efforts were made to minimize suffering.

### **Rat model establishment**

The Walker-256 lung carcinoma metastasis rat model was established as reported previously[13]. The Walker-256 carcinosarcoma cells were purchased from the Cell Culture Center of the Chinese Academy of Medical Sciences (Beijing, China). Briefly, male SD rats were used for ascitic tumor cell cultivation. After two cell passages, the Walker-256 tumor cells were collected, centrifuged, and resuspended in phosphate-buffered saline (PBS) for the following establishment of rat models. The cell viability was assessed by the trypan blue exclusion test. Walker-256 cells were stained with 0.4% trypan blue solution and then counted using a hemocytometer. Their viability was approximately 95% before the tail-vein injection.

Male Wistar rats were randomly divided into two groups: the Walker-256 lung carcinoma metastasis group (n = 10) and the control group (n = 4). The Walker-256 lung carcinoma metastasis group was injected with  $2 \times 10^6$  viable Walker-256 cells in 100  $\mu$ L of PBS by tail-vein injection. The control group was tail-vein injected with the same volume of PBS. The animals were anesthetized with sodium pentobarbital solution (4 mg/kg) before the tail-vein injection.

### **Lung histopathology**

For histopathology, rats were sacrificed on days 2, 4, 6, and 9 by using an overdose of sodium pentobarbital anesthetic. The whole lung tissue was fixed in 4% formalin fixative and embedded in paraffin.

Then, the paraffin sections (4- $\mu$ m thick) were stained with hematoxylin and eosin (HE) to reveal the metastatic nodules.

### **Urine collection and protein extraction**

First, the rats were accommodated in metabolic cages for 2-3 days for urine sample collecting. Then, the urine samples were collected from each rat (from either the lung metastasis group or the control group) on days 2, 4, 6, and 9 after Walker-256 cell or PBS tail-vein injection. Each rat was placed in metabolic cages with free access to water and without food to avoid contamination overnight for the collection of the urine samples in the following 12 h.

Urine samples were centrifuged at 12,000 g for 30 min at 4°C immediately to remove cell debris. Then, the supernatants were precipitated with three volumes of ethanol at -20°C for 2 h. After centrifugation at 12,000 g for 30 min, the pellets were resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, and 25 mmol/L DTT) at 4°C for 2 h. Finally, after centrifugation at 4°C and 12,000 g for 30 min, the supernatants of each sample were measured by using the Bradford assay. The protein samples were stored at -80°C for later use.

### **SDS-PAGE analysis**

After Walker-256 cell tail-vein injection, 25 µg of protein from each sample on days 2, 4, 6, and 9 and the control group on day 2 was added to loading buffer (50 mmol/L Tris-HCl, pH 6.8, 50 mol/L DTT, 0.5% SDS, and 10% glycerol). Then, all these protein samples were incubated at 98°C for 10 min. The urine protein samples from randomly selected Walker-256 lung carcinoma metastasis rats were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### **Protein digestion and peptide preparation**

Urine protein samples of Walker-256 lung carcinoma metastasis rats on days 2, 4, 6, and 9 and the control group on day 2 were randomly selected for proteomic analysis. Proteins were digested with trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, Wisconsin, USA) by using filter-aided sample preparation methods, as reported previously[14]. Briefly, 100 µg of proteins were loaded onto 10-kD cutoff filter devices (Pall, Port Washington, NY) and washed with UA (8 M urea in 0.1 M Tris-HCl, pH 8.5) at 14,000 g for 40 min at 18°C twice. Then, 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub> was added to wash the protein. Each urinary protein was subsequently denatured with 20 mM DTT at 37°C for 1 h and then alkylated with 50 mM iodoacetamide (IAA) for 40 min in the dark. After being washed twice with UA and 3 times with 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub>, the denatured proteins were resuspended with 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub> and digested with trypsin (enzyme to protein ratio of 1:50) at 37°C for 12-16 h. Finally, the collected peptide mixtures were desalted using Oasis HLB cartridges (Waters, Milford, MA) and then dried by vacuum evaporation (Thermo Fisher Scientific, Bremen, Germany).

### **LC-MS/MS analysis**

Digested peptides were re-dissolved in 0.1% formic acid to a concentration of 0.5  $\mu\text{g}/\mu\text{L}$ . Then, 2  $\mu\text{g}$  of each sample was transferred to a reversed-phase microcapillary column using a Waters ultra-performance liquid chromatography (UPLC) system, and peptides were separated on a 10-cm fused silica column. The elution from the fused silica column was performed in 60 min with a gradient of 5%–28% buffer B (0.1% formic acid and 99.9% acetonitrile (ACN)); flow rate, 0.3  $\mu\text{L}/\text{min}$ ). The peptides were analyzed using an AB SCIEX (Framingham, MA, US) Triple TOF 5600 mass spectrometry (MS) system. Samples from four Walker-256 lung carcinoma metastasis rats at 4 time points and four control rats were randomly chosen for this study. Each sample was analyzed twice.

Digested peptides were re-dissolved in 0.1% formic acid to a concentration of 0.5  $\mu\text{g}/\mu\text{L}$ . For analysis, 1  $\mu\text{g}$  of each peptide from an individual sample was loaded onto a trap column and separated on a reverse-phase C18 column (50  $\mu\text{m} \times 150 \text{ mm}$ , 2  $\mu\text{m}$ ) using the EASY-nLC 1200 HPLC system (Thermo Fisher Scientific, Waltham, MA). The elution for the analytical column lasted 120 min at a flow rate of 300 nL/min. Then, the peptides were analyzed with an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA). MS data were acquired in high-sensitivity mode using the following parameters: data-dependent MS/MS scans per full scan with top-speed mode (3 s), MS scans at a resolution of 120,000 and MS/MS scans at a resolution of 30,000 in the Orbitrap, 30% HCD collision energy, charge-state screening (+2 to +7), dynamic exclusion (exclusion duration 30 s), and a maximum injection time of 45 ms.

### **Database searching and label-free quantitation**

The MS/MS data of Walker-256 lung metastasis rat samples were searched using Mascot software (version 2.4.1, Matrix Science, London, UK) against the SwissProt rat database (released in February 2017, containing 7992 sequences). For Triple TOF 5600<sup>TM</sup>, the parameters were set as follows: the fragment ion mass tolerance was set to 0.05 Da, and the parent ion tolerance was set to 0.05 Da. The search allowed two missed cleavage sites in the trypsin digestion. The carbamidomethylation of cysteines was considered a fixed modification, and the oxidation of methionine and deamidation of asparagine were considered variable modifications. For the Orbitrap Fusion Lumos, the parent ion tolerance was set to 10 ppm, and the fragment ion mass tolerance was set to 0.02 Da. Carbamidomethylation of cysteine was set as a fixed modification, and the oxidation of methionine

was considered a variable modification. The specificity of trypsin digestion was set for cleavage after K or R, and two missed trypsin cleavage sites were allowed.

Peptide and protein identification was further validated by Progenesis LC-MS/MS software (version 4.1, Nonlinear, Newcastle upon Tyne, UK) and Scaffold (version 4.7.5, Proteome Software Inc., Portland, OR). For Progenesis, the acquired data from the MS scans were transformed and stored in peak lists using a proprietary algorithm. Features with only one charge or more than five charges were excluded from the analyses. Protein abundance was calculated from the sum of all unique peptide ion abundances for a specific protein in each run. The normalization of abundances was required to allow comparisons across different sample runs by this software. For further quantitation, all peptides of an identified protein were included. Proteins identified by more than one peptide were retained. For Scaffold, peptide identifications were accepted at a false discovery rate (FDR) of less than 1.0% by the Scaffold Local FDR algorithm, and protein identifications were accepted at an FDR less than 1.0% with at least two unique peptides. Comparisons across different samples were performed after normalization of total spectra using Scaffold software. Spectral counting was used to compare protein abundances at different time points according to a previously described procedure.

### **Gene ontology and ingenuity pathway analysis**

All proteins identified to be differentially expressed between the control and lung carcinoma metastasis rats were assigned a gene symbol using DAVID [15] and analyzed by Gene Ontology (GO) based on biological process, cellular component and molecular function categories. The biological pathway analysis of differential proteins analyzed at four time points were performed by IPA software (Ingenuity Systems, Mountain View, CA, USA)

### **Statistical analysis**

Statistical analysis was performed with GraphPad Prism version 7.0 (GraphPad, San Diego, CA). Comparisons between data from samples of four time points were conducted using repeated-measures one-way ANOVA followed by multiple comparison analysis with the least significant difference (LSD) test. Group differences resulting in  $P < 0.05$  were considered statistically significant.

### **Results and discussion**



### Body weight changes of Walker-256 lung carcinoma metastasis rat model

From 6 days after the tail-vein injection of Walker-256 cells, the average body weight of lung carcinoma metastasis rats was lower than that of the control rats (Figure 2), and reduced food intake was also observed in lung carcinoma metastasis rats. On day 9 after the tail-vein injection of Walker-256 cells, the body weight of lung carcinoma metastasis rats was significantly lower than that of the control group. Therefore, we believed that days 2 and 4 were early time points during Walker-256 lung metastasis.

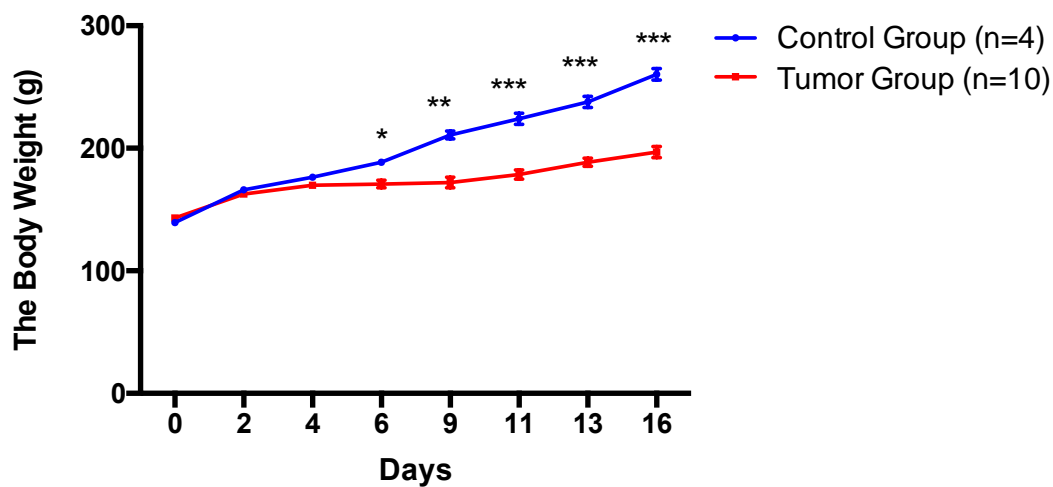
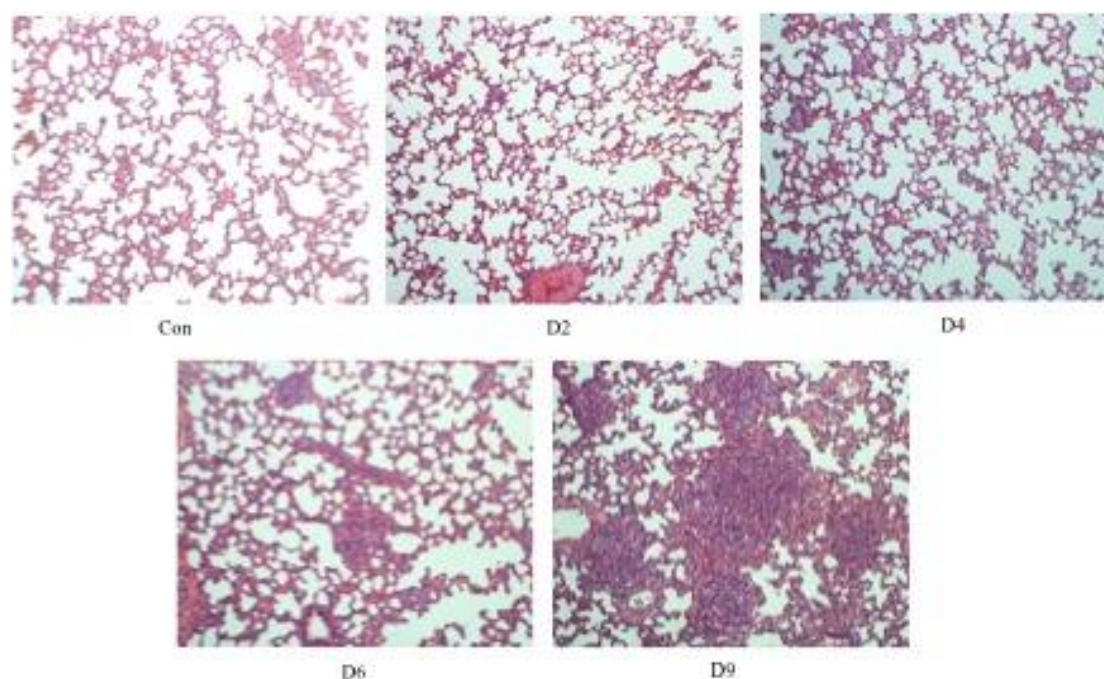


Figure 2. The body weight changes of Walker-256 lung carcinoma metastasis rats.

### Pathological changes in Walker-256 lung metastasis rat model

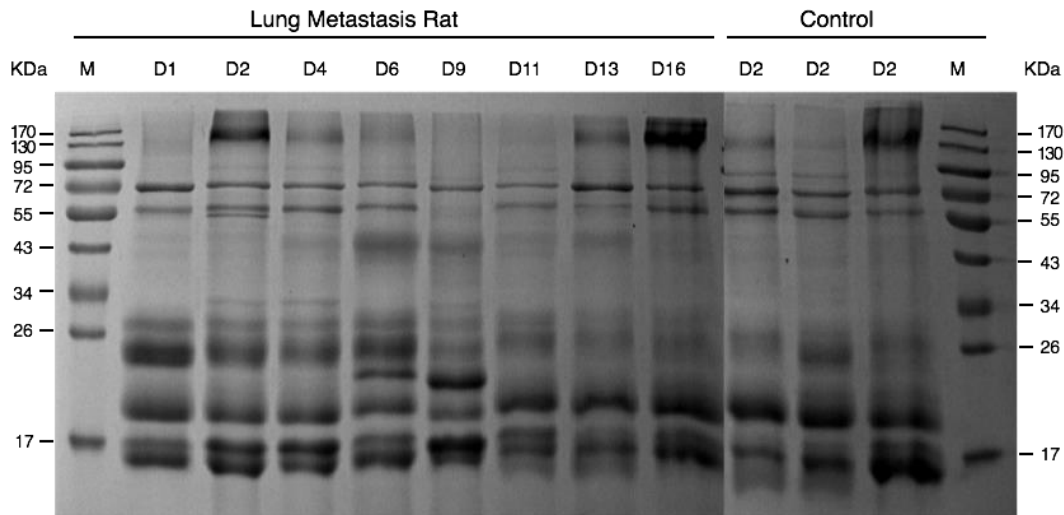
The pathological changes in the Walker-256 lung carcinoma metastasis rats at different time points are shown in Figure 3. The lung metastasis nodules appeared on day 4, and their number and volumes increased in Walker-256 lung carcinoma metastasis rats during lung metastasis progression. In addition, the metastatic Walker-256 cells arranged closely in lung metastatic rats, and the majority of cells showed round or elliptic morphologies accompanied by poor differentiation, while their nuclei were large, irregular, and hyperchromatic. More importantly, the lung metastasis nodules were scattered throughout the lung parenchyma, while the invasion of Walker-256 cells destroyed the lung tissue structure and the alveolar structure.



**Figure 3. Pathological changes in Walker-256 lung metastatic rats. The magnification was 100× for the images of H&E staining.**

#### **SDS-PAGE of Walker-256 lung metastasis rat model**

Urine samples collected at different time points from Walker-256 lung carcinoma metastasis rats were separated by 12% SDS-PAGE. As shown in Figure 4, the patterns in urine sample proteins from a representative Walker-256 lung metastasis rat changed significantly during lung metastatic progression (days 1, 2, 4, 6, 9, 11, 13 and 16). Similar patterns were observed in another 6 rats, suggesting consistent lung metastatic progression in the chosen rats. It can be seen in Figure 4 that on days 6 and 9, the protein band intensities increased significantly, which is consistent with the times at which the body weight changed.

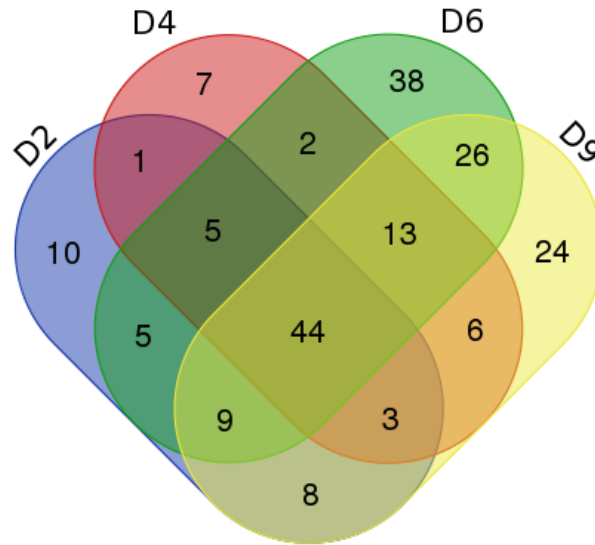


**Figure 4. Dynamic protein patterns in the urine of Walker-256 lung carcinoma metastasis rats.**

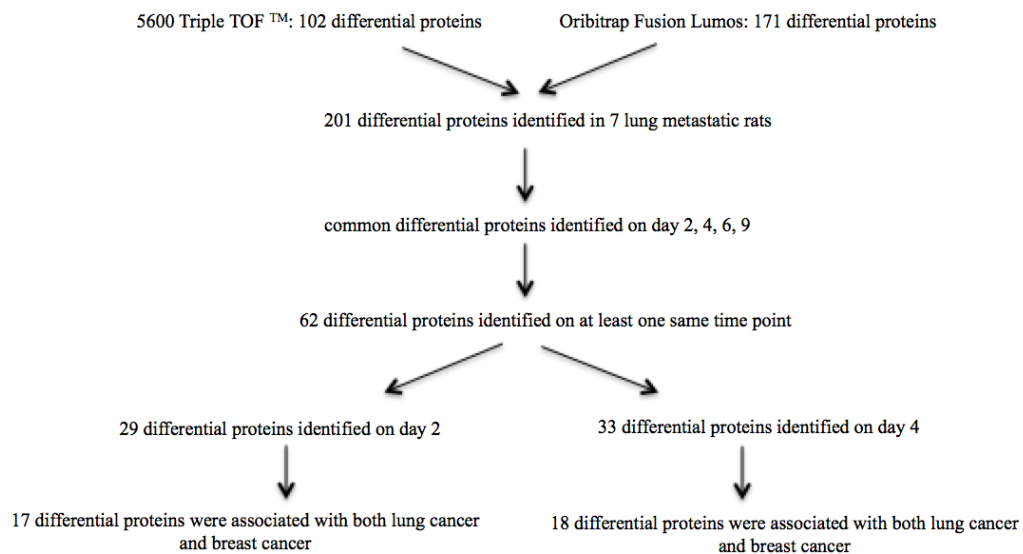
### **The urine proteome was significantly different in the Walker-256 lung metastasis rat model**

A total of 263 urinary proteins were identified with at least two peptides by using a Triple TOF 5600<sup>TM</sup> mass spectrometer, and 839 urinary proteins were identified with <1% FDR at the protein level with at least two peptides by using Orbitrap Fusion Lumos. The differential proteins were screened by the following criteria: fold change  $\geq 1.5$  or  $\leq 0.67$ , confidence score  $\geq 200$ ,  $P < 0.05$  for differences in the protein level between the metastasis rat model and the control group, protein spectral counts or the normalized abundance from every rat in the high-abundance group greater than those in the low-abundance group, and the average spectral count in the high-abundance group  $\geq 4$ . By using these screening criteria, 102 differential proteins were identified by using the Triple TOF 5600<sup>TM</sup> mass spectrometer, and 171 differential proteins were identified by using the Orbitrap Fusion Lumos.

The overlap of differential proteins identified at different stages in 7 lung metastatic rats is shown by a Venn diagram (Figure 5). There were 85, 81, 142, and 133 differential proteins on days 2, 4, 6, and 9 after tail-vein injection of Walker-256 cells, respectively. In addition, 11, 25, 34, and 44 proteins that were differentially expressed in all 7 lung metastatic rats were identified by two mass spectrometers on days 2, 4, 6, and 9, respectively. There were 62 differential proteins that differed at one or more of the same time points and were identified by using two mass spectrometers. Among these 62 differential proteins, the levels of 29 differential urinary proteins changed significantly on the second day and before lung metastasis nodules appeared, indicating their potential roles in the early detection of lung cancer metastasis (Table 1). A data processing flowchart is presented in Figure 6.



**Figure 5. Evaluation of the overlap of differential proteins identified at different metastatic phases in 7 lung metastatic rats.**



**Figure 6. Steps for processing the data from 7 lung metastatic rats.**

Some of these 29 differential proteins have been reported to be clinical lung cancer biomarkers and also associated with breast cancer metastasis. For example, (1) galectin-3-binding protein (LG3BP) is a candidate biomarker for the diagnosis of large-cell neuroendocrine lung carcinoma[16], and it can also induce galectin-mediated tumor cell aggregation to increase the survival of cancer cells in the

bloodstream during the metastasis [17]. As another example, (2) neutrophil gelatinase-associated lipocalin (NGAL) is a potential biomarker for early stages of lung tumorigenesis[18]. Additionally, the stroma-secreted NGAL promotes breast cancer metastasis in vitro and in vivo, thereby contributing to tumor progression[19]. (3) The baseline soluble intercellular adhesion molecule 1 (ICAM-1) levels in serum were evaluated as an additional prognostic factor in patients with small cell lung cancer (SCLC). In addition, the soluble protein ICAM-1 may also be a predictive marker for an objective response during chemotherapy for patients with extensive disease ( $p = 0.001$ )[20]. In breast cancer, ICAM-1 can also activate intracellular signaling pathways in cancer cells, leading to enhanced cell motility, invasion and metastasis[21]. (4) In advanced non-small cell lung cancer (NSCLC) patients, the pretreatment serum C-reactive protein (CRP) was associated with a poor outcome of treatment with pemetrexed[22]. In addition, a positive association between pre-diagnostic high-sensitivity CRP (hs-CRP) was reported with breast cancer risk[23]. (5) Apolipoprotein E (ApoE) levels significantly increased in the pleural effusion of patients with NSCLC, which serves as a potential marker for the diagnosis of malignant pleural effusions (MPEs) as well as the differential diagnosis of MPE in NSCLC[24]. Additionally, apolipoprotein E expression promoted lung adenocarcinoma proliferation and migration, which can be a potential survival marker in lung cancer[25]. In breast cancer, the serum level of apolipoprotein E was also reported to correlate with disease progression and poor prognosis[26]. Other differential proteins are annotated in Table 1.

According to our results, we suggest that it is more appropriate to use a protein panel for a biomarker, as the specificities of single protein biomarkers are not significant enough.

**Table 1. The differential proteins identified at days 2 and 4 in 7 lung metastatic rats by using two mass spectrometers**

Accession	Protein names	ANOVA P		Trend	5600 fold change				Lumos fold change				Lung cancer	Breast cancer
		5600	Lumos		D2	D4	D6	D9	D2	D4	D6	D9		
LG3BP_RAT	Galectin-3-binding protein	0	0.0079	↑	14.58	9.55	3.24	2.69	4.41	3.75	2.02	1.90	[16, 27, 28]	[17, 29, 30]
EZRI_RAT	Ezrin	6.43187E-09	0.12	↑	2.40	3.00	2.83	2.77	3.04	4.28	3.19	3.74	[31-33]	[34, 35]
GGT1_RAT	Gamma-glutamyltranspeptidase 1	4.01137E-06	0.00081	↑	2.62	2.30	-	1.96	2	2.00	-	2	-	[36]
CLIC1_RAT	Chloride intracellular channel protein 1	1.55997E-10	0.12	↑	7.71	11.55	6.61	10.41	-	6.72	4.22	3.78	[37]	[38]
MOES_RAT	Moesin Neutrophil	5.54445E-13	0.3	↑	7.26	10.54	6.27	20.80	-	4.80	3.70	4.16	[39]	[40-42]
NGAL_RAT	gelatinase-associated lipocalin	1.16041E-12	< 0.00010	↑	-	2.12	91.74	60.14	-	3.43	12.43	12.45	[18, 43]	[19]
CO4_RAT	Complement C4	6.63924E-08	0.0086	↑	2.05	1.97	3.31	2.0	-	3.29	3.81	2.391	[44-46]	[47]
A1AG_RAT	Alpha-1-acid glycoprotein	2.01394E-09	0.0019	↑	-	2.65	6.10	10.09	-	2.01	2.04	3.84	[48-50]	[51]
NEP_RAT	Neprilysin	0.000298879	0.074	↑	2.08	2.15	-	-	2.32	2.53	1.90	1.96	-	[52]
ABHEB_RAT	Alpha/beta hydrolase domain-containing protein 14B	2.08722E-14	< 0.00010	↑	1.90	1.51	12.49	2.21	1.97	-	4.13	-	-	-
NHRF1_RAT	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	5.07818E-06	0.006	↑	-	5.16	-	3.66	3.97	4.17	3.23	5.18	[53]	[54-58]

PIGR_RAT	Polymeric immunoglobulin receptor	1.08377E-08	0.035	↑	2.143	1.67	1.74	1.58	-	1.55	-	1.76	[59, 60]	-
NHRF3_RAT	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	2.93346E-06	0.12	↑	-	4.02	-	3.565564 55	-	2.29	2.35	2.80	-	-
ROB1_RAT	Protein RoBo-1	1.44534E-07	0.073	↑	2.16	2.44	2.98	2.81	-	1.97	-	2.29	-	[61]
HSP7C_RAT	Cluster of heat shock cognate 71 kDa protein	9.28777E-09	0.63	↑	2.41	2.34	1.81	2.18	-	-	1.72	1.67	[62]	[63]
ICAM1_RAT	Intercellular adhesion molecule 1	6.86884E-12	0.55	↑	-	1.69	2.53	1.63	-	-	1.94	1.88	[20, 64]	[21]
ACY1A_RAT	Aminoacylase-1A	1.31825E-07	0.3	↑	2.66	3.55	2.38	2.45	-	-	2.99	2.72	[58]	[65]
AMPN_RAT	Aminopeptidase N	1.12905E-07	0.2	↑	2.12	2.53	-	1.53	-	1.61	-	-	[66-69]	[70-72]
MTND_RAT	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase	5.66214E-15	0.00012	↑	2.26	1.58	4.65	1.78	-	-	4.08	-	-	-
THIO_RAT	Thioredoxin	7.78253E-09	0.00024	↑	-	-	17.86	-	2.22	2.15	6.37	2.12	[73]	[74]
GSTO1_RAT	Glutathione S-transferase omega-1	1.99611E-07	0.011	↑	3.06	2.96	11.98	3.88	-	-	6.4	-	[75]	[76]
CRP_RAT	C-reactive protein	4.59968E-08	0.012	↓	-	0.41	-	0.32	0.63	-	0.28	0.291	[22, 77]	[23]
CATC_RAT	Dipeptidyl peptidase 1	0.002482636	0.0018	↓	0.62	0.62	0.57	-	0.33	0.30	0.33	0.30	-	-
APOE_RAT	Apolipoprotein E	2.72005E-14	0.0014	↓	0.44	-	0.26	0.38	0.49	-	0.37	0.55	[24, 25, 78]	[26]
FETUB_RAT	Fetuin-B	1.9689E-10	0.00019	↓	-	0.17	0.14	0.07	-	0.59	0.36	0.39	-	-
EGF_RAT	Pro-epidermal growth factor	1.9762E-14	0.0073	↓	0.39	0.52	0.423	0.30	0.66	-	-	0.58	-	-

VTDB_RAT	Vitamin D-binding protein	1.43844E-06	0.0012	↓	-	0.38	0.43	-	0.36	0.34	0.39	0.46	[79, 80]	[81]
ALBU_RAT	Serum albumin	8.57146E-08	0.0015	↓	-	0.35	-	0.33	0.58	0.53	0.63	0.67	[82, 83]	[84]
BTD_RAT	Biotinidase	2.45435E-06	0.0049	↓	-	0.60	0.53	0.49	0.60	0.53	-	0.37	-	[85]
CALB1_RAT	Calbindin	1.34388E-10	0.064	↓	-	0.47	0.43	0.21	-	-	0.64	0.38	-	-
MXRA8_RAT	Matrix-remodeling-associated protein 8	5.70928E-05	0.11	↓	0.65	0.66	0.52	0.56	-	-	0.58	0.62	-	-
NID2_RAT	Nidogen-2	4.09459E-08	0.038	↓	0.47	-	-	-	0.44	-	-	-	[86, 87]	[88]
KLK1_RAT	Kallikrein-1	1.68021E-12	0.22	↓	0.40	0.36	0.33	0.32	-	0.62	-	-	-	-
CFAD_RAT	Complement factor D	0.002928967	0.33	↓	-	0.60	0.59	-	-	-	0.64	-	[89]	
APOA4_RAT	Apolipoprotein A-IV	4.0156E-08	< 0.00010	↓	-	-	-	0.48	0.10	0.09	0	0.13	[90]	[91]



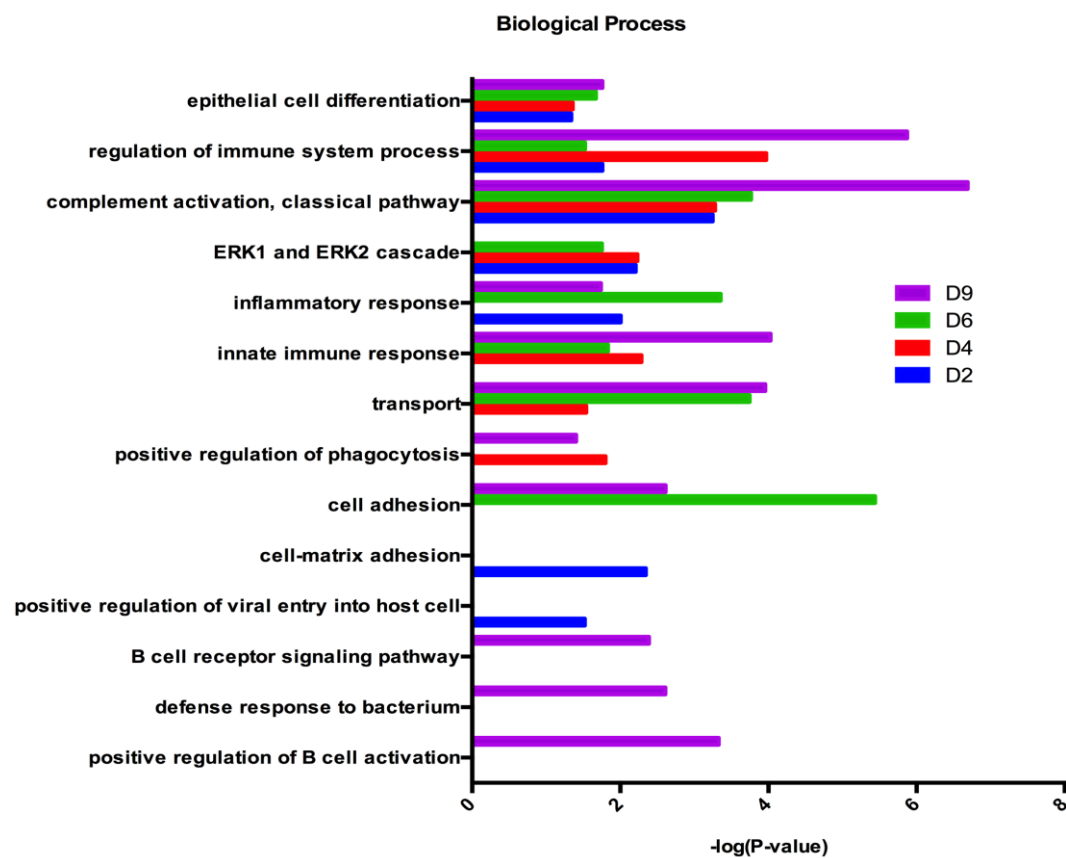
### **Functional analysis of differential urine proteins in Walker-256 lung carcinoma metastasis rats**

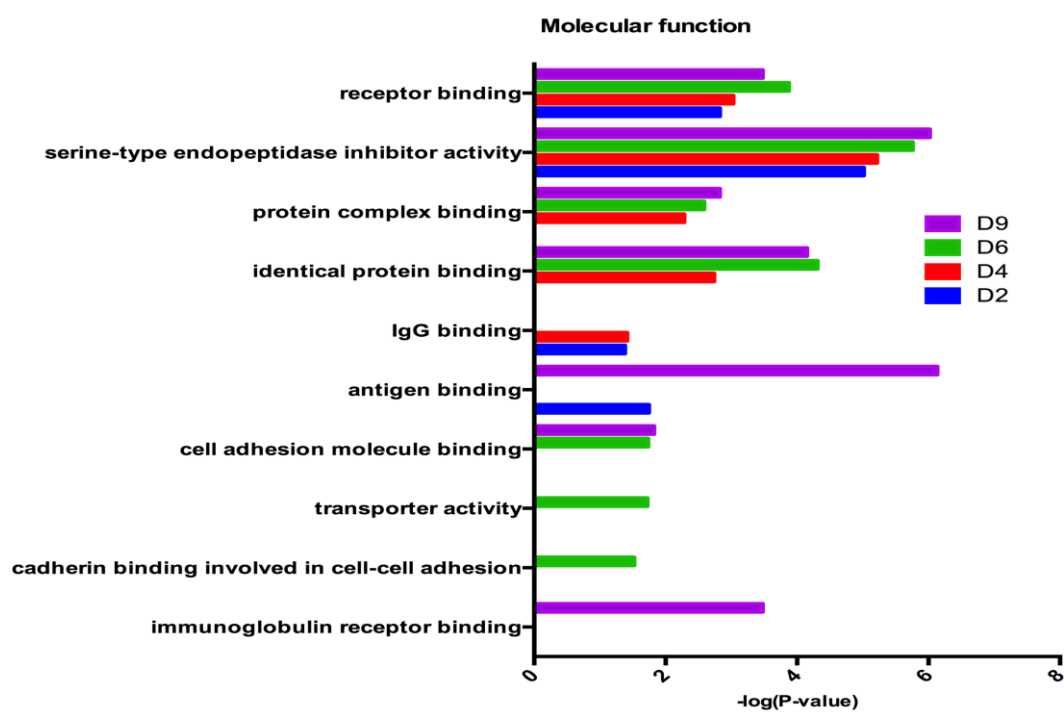
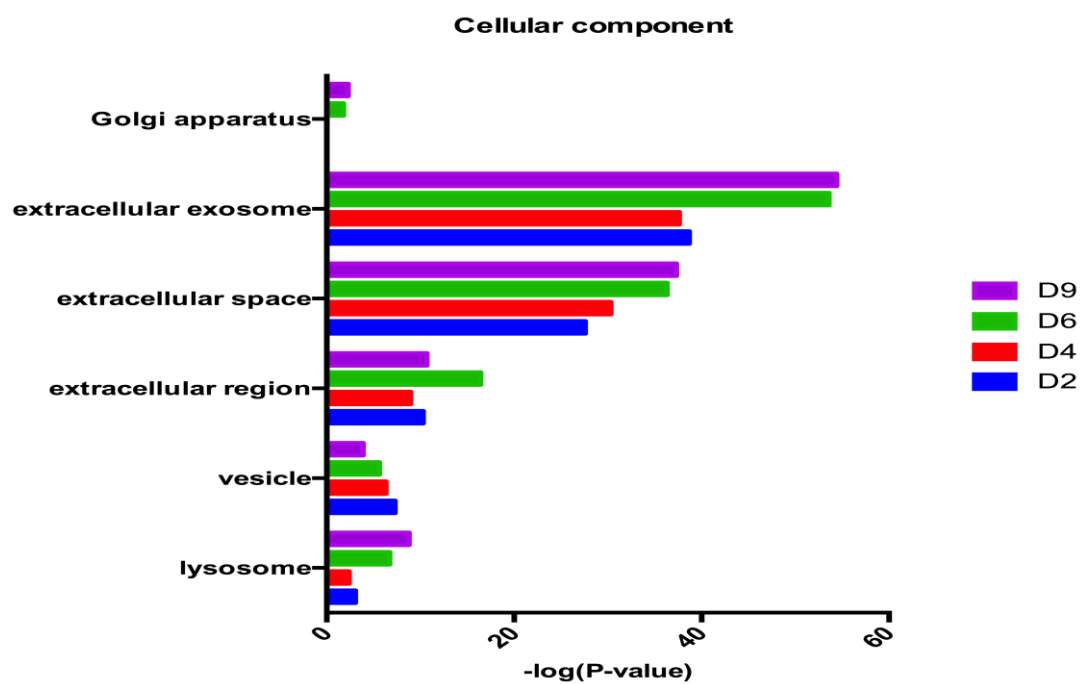
The functional annotation of differential proteins was performed by using DAVID[15]. Differential proteins at different metastatic time points were classified into biological process, molecular function, and molecular components (Figure 7). In the biological processes, epithelial cell differentiation, the regulation of immune system processes, and classical complement activation pathway were overrepresented on days 2, 4, 6 and 9. The ERK1 and ERK2 cascade was overrepresented on days 2, 4 and 6. The innate immune response and transport were overrepresented on days 4, 6 and 9. The cell adhesion was overrepresented on days 6 and 9. Interestingly, proteins representing the B cell receptor signaling pathway, the defense response to bacteria and the positive regulation of B cell activation appeared on day 9 (Figure 7A). The majority of these biological processes were reported to be associated with breast cancer metastasis or lung cancer. For example, the increasing levels of ERK1 and ERK2 were associated with breast cancer initiation, growth, and metastasis[92]. The persistent complement activation was reported for tumor cells in breast cancer, which consistent with the timing of its overrepresentation in this study[93]. The transport and cell adhesion processes were both overrepresented on days 6 and 9, which indicated the severe metastasis during lung tumor progression. Interestingly, on day 9, proteins representing the B cell activation process became differentially expressed, indicating that a candidate antibody may be produced in this period. However, it may be too late for these antibodies to overcome Walker-256 cells and to stop the metastasis.

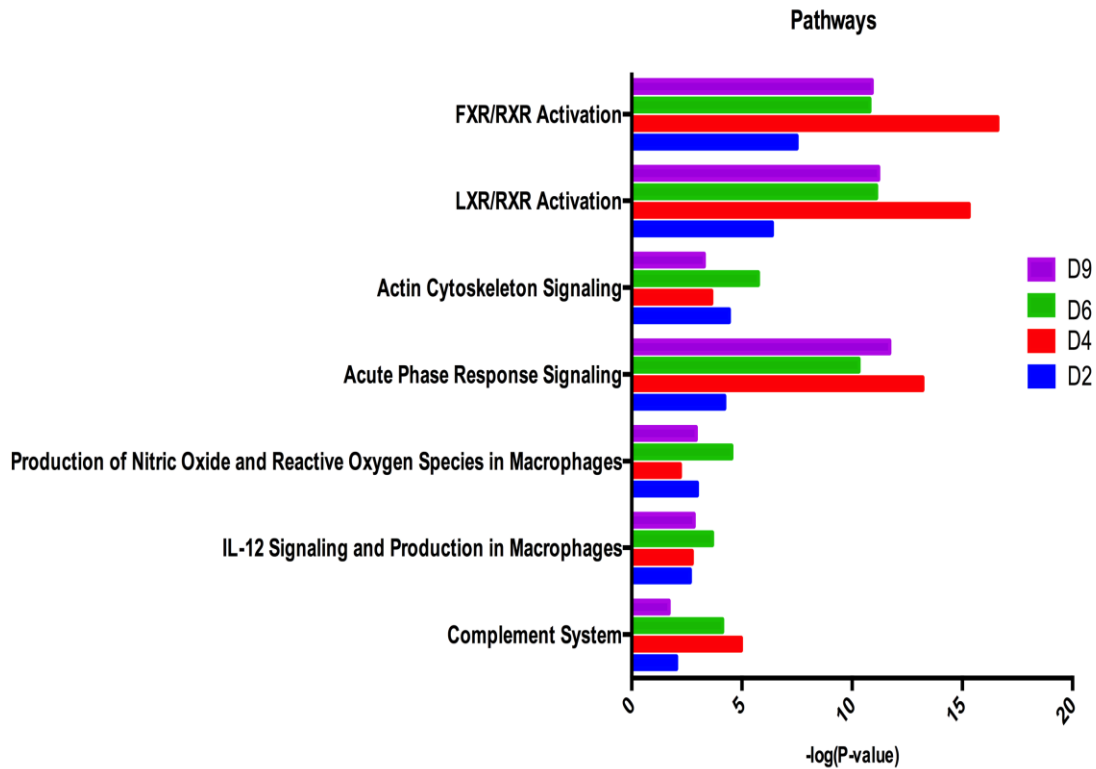
The majority of differential proteins in the cellular component category came from extracellular exosomes, the extracellular space, the cellular region, and vesicles. Only a small number of differential proteins were derived from organelles, such as the Golgi apparatus (Figure 7B). This result is consistent with the source of normal urine. In the molecular function category, receptor binding and serine-type endopeptidase inhibitor activity were overrepresented at all time points, while identical protein binding, protein complex binding were overrepresented on days 4, 6, and 9. The transporter activity and cell adhesion molecule binding were both overrepresented on day 6, which is consistent with the cell adhesion and transport process protein differential expression on day 6. On day 9, immunoglobulin receptor binding was overrepresented, but its representation was still consistent with

that of the B cell receptor signaling pathway and the positive regulation of B cell activation on day 9 (Figure 7C). It is noteworthy that this molecular function did not appear before day 9.

To identify the major biological pathways involved with the differential urine proteins, we used IPA for canonical pathway enrichment analysis. FXR/RXR activation, LXR/RXR activation, actin cytoskeleton signaling, acute-phase response signaling, IL-12 signaling and production in macrophages, the production of nitric oxide and reactive oxygen species in macrophages, and the complement system were significantly enriched during the whole metastatic progression (Figure 7D). This result indicated that the differential proteins were indeed associated with lung metastatic development.



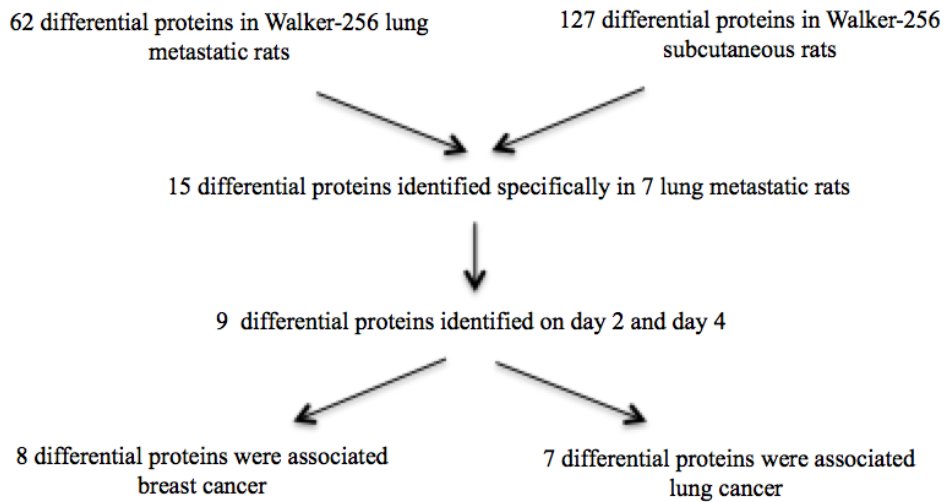




**Figure 7. Functional analysis of differential proteins during Walker-256 lung metastatic development. (A) Biological process. (B) Cellular component. (C) Molecular function. (D) Pathways**

### **Comparison of differential urine proteins in Walker-256 lung carcinoma metastasis rats and Walker-256 subcutaneous rats**

There were 15 differential proteins identified specifically when these 62 differential proteins common to 7 lung metastatic rats at one or more of the same time points were compared with Walker-256 subcutaneous model data that our laboratory published before[9]. The comparison procedure is presented in Figure 8. Nine of these 15 differential proteins (NHRF1, CLIC1, EZRI, AMPN, ACY1A, HSP7C, BTD, NID2, and CFAD) were identified at the early stages (day 2 or 4) of lung metastatic development to have homologous human proteins, and their levels continued to change during later lung metastatic stages, suggesting that these proteins have indeed participated in cancer lung metastasis development (Table 2). In addition, eight of these nine differential proteins have been reported to be associated with breast cancer, especially metastasis, while seven of the nine (NHRF1, CLIC1, EZRI, AMPN, ACY1A, HSP7C, and NID2) have been referenced in lung cancer, indicating their roles in the early detection of breast cancer lung metastasis.



**Figure 8. The procedure comparing urine proteins differentially expressed in Walker-256 lung carcinoma metastasis rats and Walker-256 subcutaneous rats**

Seven of these nine differential proteins were associated with both lung cancer and breast cancer, while two of them (BTD and CFAD) were reported in lung or breast cancer. (1) The high expression of Na(+)/H(+) exchange regulatory cofactor NHE-RF1 (NHRF1) is a potential marker of aggressiveness in NSCLC[53]. In addition, the loss of nuclear NHERF1 expression is associated with reduced survival and may serve as a prognostic marker for the routine clinical management of breast cancer patients[54]. Additionally, the expression of NHRF1 can define an immunophenotype of grade 2 invasive breast cancer associated with poor prognosis[57]. (2) Chloride intracellular channel protein 1 (CLIC1) is closely associated with the occurrence and development of lung adenocarcinoma and may thus be used as an effective marker for predicting the prognosis of lung adenocarcinoma[37]. In addition, CLIC1 was also reported to be a potential serological marker for the early detection of breast cancer[38]. (3) Ezrin is an early biomarker for the early diagnosis of lung cancer[33] and a potential prognostic marker of progression in NSCLC[94-96]. In addition, tumor-associated macrophages (TAMs) promote the ezrin phosphorylation-mediated epithelial-mesenchymal transition (EMT) in lung adenocarcinoma through FUT4/LeY-mediated fucosylation[97]. In breast cancer, ezrin regulates focal adhesion and invadopodia dynamics by altering calpain activity to promote breast cancer cell invasion and metastasis[98]. Additionally, ezrin is correlated with cortactin, which facilitates the EMT in breast cancer metastases[35]. (4) The expression of aminopeptidase N (AMPN)/CD13 was reported as a

potential unfavorable factor in predicting the efficacy and prognosis of post-operative chemotherapy in NSCLC patients, especially in lung adenocarcinoma patients[66, 67]. Additionally, a high level of circulating AMPN/CD13 was reported to be an independent prognostic factor in patients with NSCLC[69]. In breast cancer patients, the expression of APN/CD13 can serve as a poor prognostic factor in the evaluation of breast cancer prognosis[70]. (5) Aminoacylase-1A is not expressed in SCLC[58]. In addition, the expression level of aminoacylase-1A in human MCF-7 breast cancer cells was altered by 17 $\beta$ -estradiol (E2) treatment and so a might be potent target for treating breast cancer patients[65]. (6) The expression level of cluster of heat shock cognate 71 kDa protein was changed significantly when lung cancer cells were treated with periplocin, which revealed molecular mechanisms underlying the anti-cancer effects of periplocin on lung cancer cells[62]. In MCF-7 and MDA-MB-231 breast cancer cell lines, heat shock cognate 71 kDa protein was reported to be identified as specific phthalic acid-binding proteins [63]. (7) Loss of nidogen-2 significantly promotes lung metastasis of melanoma cells[86]. In human breast cancer specimens, expression of the extracellular protease ADAMTS1 (A disintegrin and metalloprotease with thrombospondin repeats 1) was downregulated, and nidogen proteolysis was partially inhibited, which has implications for vessel integrity[88]. (8) Complement factor D (CFD)/adipsin was overexpressed in BHGc7 cells cultured in conditioned medium, and BHGc7 cells were the first establishment of permanent circulating tumor cell (CTC) lines from blood samples of advanced stage SCLC patients[89]. (9) Biotinidase is a potential serological biomarker for the detection of breast cancer[85].

The presence of some other differential proteins, identified only by Triple TOF 5600<sup>TM</sup> or Orbitrap Fusion Lumos, cannot be ignored. When the metastasis model data were compared with the Walker-256 subcutaneous data, these differential proteins were screened by the following criteria: (1) only identified at early metastatic stages (days 2 and 4); (2) did not contain the differential proteins annotated in Table 2; (3) all these differential proteins had corresponding homologous proteins; and (4) all these differential proteins exhibited the same trend during the whole lung metastatic periods. The differential lung metastatic proteins only identified in these two mass spectrometers were annotated in Supplement Tables 1 and 2, respectively. There were 17 differential proteins identified by using Triple TOF 5600<sup>TM</sup>, and 13 of these 17 differential proteins have been reported to be associated with lung

cancer and breast cancer. Specifically, 10 of them were reported in both lung cancer and breast cancer, while 3 were associated with either lung cancer or breast cancer. There were 24 differential proteins identified by using the Orbitrap Fusion Lumos. Fifteen of these 24 differential proteins are associated with lung cancer and breast cancer. Specifically, 13 of them were reported in both lung cancer and breast cancer, while 2 were associated with either lung cancer or breast cancer. Interestingly, some identified differential proteins were associated with either lung cancer or breast cancer, indicating their novel potential roles as early candidate biomarkers in monitoring Walker-256 lung metastasis progression.

However, in our study, we found that identifying differential proteins by two different mass spectrometers yielded differences. Therefore, we suggest that the use of different mass spectrometers should be considered when conducting clinical applications. Overall, this study was preliminary, and our results indicate that (1) the urine proteome changed significantly, even on the second day after the tail-vein injection of Walker-256 cells and that (2) the urinary differential proteins were different between Walker-256 lung metastatic tumors and Walker-256 subcutaneous tumors. Our results provide a potential possibility to detect early breast cancer lung metastasis, monitor its progression and differentiate it from the same cancer cells grown at other locations.

**Table 2. Walker-256 lung metastasis differential proteins specifically identified on days 2 and 4**

Accession	Protein name	ANOVA P		Trends	5600 fold change				Lumos fold change				Lung cancer	Breast cancer
		5600	Lumos		D2	D4	D6	D9	D2	D4	D6	D9		
NHRF1_RAT	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	5.08E-06	0.006	↑	-	5.16	-	3.66	3.97	3.91	3.23	4.91	[53]	[54, 57]
CLIC1_RAT	Chloride intracellular channel protein 1	1.56E-10	0.12	↑	7.71	11.55	6.61	10.41	-	6.29	4.00	3.71	[37]	[38]
EZRL_RAT	Ezrin	6.43E-09	0.12	↑	2.40	3.00	2.83	2.77	3.10	4.32	3.39	3.95	[33, 94-97]	[35, 98]
AMPN_RAT	Aminopeptidase N	1.13E-07	0.2	↑	2.12	2.53	-	1.53	-	1.57	-	-	[66, 67, 69]	[70]
ACY1A_RAT	Aminoacylase-1A	1.32E-07	0.3	↑	2.66	3.55	2.38	2.45	-	-	2.99	2.72	[58]	[65]
HSP7C_RAT	Cluster of heat shock cognate 71 kDa protein	9.29E-09	0.63	↑	2.41	2.34	1.81	2.18	-	-	1.72	1.67	[62]	[63]
BTD_RAT	Biotinidase	2.45E-06	0.0049	↓		0.60	0.53	0.49	0.56	0.53	-	0.37	-	[85]
NID2_RAT	Nidogen-2	4.09E-08	0.038	↓	0.47	-	-	-	0.44	-	-	-	[86]	[88]
CFAD_RAT	Complement factor D	0.0029	0.33	↓	-	0.60	0.59	-	-	-	0.64	-	[89]	-



**Supplement Table 1. The lung metastasis differential proteins specifically identified with a Triple TOF**

5600 <sup>TM</sup>									
Accession	Protein description	Trend	ANOVA	Average fold change				Lung cancer	Breast cancer
			P	D2	D4	D6	D9		
ACTG_RAT	Actin, cytoplasmic 2	↑	6.10E-08	2.54	3.18	1.79	3.35	[99-101]	[102, 103]
PRDX6_RAT	Peroxiredoxin-6	↑	1.31E-08	3.30	-	17.61	7.25	[104, 105]	[106]
CK054_RAT	Ester hydrolase C11orf54 homolog	↑	3.23E-09	3.29	-	6.95	10.07	-	-
IF6_RAT	Eukaryotic translation initiation factor 6	↑	1.02E-08	2.23	-	6.56	2.57	[107]	-
CD14_RAT	Monocyte differentiation antigen CD14	↑	3.30E-05	-	1.65	1.67	1.96	[108, 109]	[110, 111]
VDAC1_RAT	Voltage-dependent anion-selective channel protein 1	↑	2.07E-08	-	30.97	24.51	18.64	[112]	[113]
F16P1_RAT	Fructose-1,6-bisphosp hatase 1	↑	3.51E-05	-	4.41	4.79	5.99	[114]	[115, 116]
ILEUA_RAT	Leukocyte elastase inhibitor A	↑	4.59E-05	2.34	-	11.10	-	[33]	-
MEP1B_RAT	Meprin A subunit beta	↑	1.04E-05	1.79	-	-	-	-	-
ALDOB_RAT	Fructose-bisphosphate aldolase B	↑	0.000111 701	-	2.14	-	-	[117]	[118]
EF1A1_RAT	Elongation factor 1-alpha 1	↑	0.002499 31	-	1.76	-	-	[119]	[120, 121]
AMPE_RAT	Glutamyl aminopeptidase	↑	6.40E-06	-	1.93	-	-	-	-
PIP_RAT	Prolactin-inducible protein homolog	↓	2.10E-06	0.47	0.53	0.29	0.26	-	[122-124]
GGH_RAT	Gamma-glutamyl hydrolase	↓	2.07E-14	-	0.39	0.52	0.39	[125]	[126]
PRVA_RAT	Parvalbumin alpha	↓	0.000182 114	-	0.30	-	0.36	-	-
FETUA_RAT	Alpha-2-HS-glycoprot ein	↓	1.73E-08	-	0.48	-	0.35	[127, 128]	[129-131]
TRFE_RAT	Serotransferrin	↓	1.60E-05	-	0.36	-	-	[132, 133]	[134, 135]

**Supplement Table 2. The lung metastasis differential proteins specifically identified with an Orbitrap**

**Fusion Lumos**

Accession	Protein description	Trend	ANOVA	Average fold change				Lung cancer	Breast cancer
			P	D2	D4	D6	D9		
CLIC4_RAT	Chloride intracellular channel protein 4	↑	0.12	23.5	20	22	-	[136]	[38, 137]
PPIA_RAT	Peptidyl-prolyl cis-trans isomerase A	↑	0.079	3.39	-	2.46	2.87	[138, 139]	[140, 141]
GP2_RAT	Pancreatic secretory granule membrane major glycoprotein GP2	↑	0.046	4.76	-	3.18	4.66	-	-
NKG2D_RAT	NKG2-D type II integral membrane protein	↑	0.016	-	7.89	8.33	8.11	-	-
ENOA_RAT	Alpha-enolase	↑	0.022	1.77	-	1.56	-	[142, 143]	[144]
EF1A1_RAT	Elongation factor 1-alpha 1	↑	0.12	3.03	-	4.31	-	[119]	[120, 121]
ACTB_RAT	Actin, cytoplasmic 1	↑	0.27	2.30	-	2.29	-	[99, 101, 145]	[102, 103]
RHOA_RAT	Transforming protein RhoA	↑	0.26	7.25	-	5.42	-	[146, 147]	[148, 149]
BASP1_RAT	Brain acid soluble protein 1	↑	0.22	3.33	-	-	4.46	-	-
ACY3_RAT	N-acyl-aromatic-L-amino acid amidohydrolase	↑	0.24	-	4.30	-	3.43	-	-
HS90B_RAT	Heat shock protein HSP 90-beta	↑	0.38	8.27	-	-	-	[150]	[151]
CYC_RAT	Cytochrome c, somatic Voltage-dependent	↑	0.052	3.27	-	-	-	-	-
VDAC1_RAT	anion-selective channel protein 1	↑	0.25	10.67	-	-	-	[112]	[113]
GDIR1_RAT	Rho GDP-dissociation inhibitor 1	↑	0.028	-	2.92	-	-	[152]	[153]
PRDX1_RAT	Peroxiredoxin-1	↑	0.62	-	3.43	-	-	[154, 155]	[156]
GPC5C_RAT	G-protein coupled receptor family C group 5 member C	↑	0.24	-	2.79	-	-	[157]	-
RET4_RAT	Retinol-binding protein 4	↓	0.0015	0.28	0.31	0.28	0.31	[158]	[159]
PPT2_RAT	Lysosomal thioesterase PPT2	↓	0.088	0.59	0.62	0.36	-	-	-
APOH_RAT	Beta-2-glycoprotein 1	↓	0.035	-	0.39	0.45	0.44	[160]	[161, 162]
NEUR1_RAT	Sialidase-1	↓	< 0.00010	0.60	-	-	0.14	-	-
MINP1_RAT	Multiple inositol polyphosphate phosphatase 1	↓	0.053	0.47	-	-	0.23	-	-
PCOC1_RAT	Procollagen C-endopeptidase enhancer 1	↓	0.025	0.40	-	-	0.43	-	-
HRG_RAT	Histidine-rich glycoprotein	↓	0.42	-	0.33	0.42	-	-	[163]

PDIA1_RAT	Protein disulfide-isomerase	↓	0.1	0.45	-	-	-	[164]	[38, 165]
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## Conflict of Interest/Disclosure Statement

The authors have no conflict of interest to report.

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