Research Article

Proteomic identification of exosomal LRG1:
A potential urinary biomarker for detecting NSCLC

In the present research, we aimed to screen for non-small cell lung cancer (NSCLC)-related proteins in urinary exosomes by comparing urinary exosomes proteome of normal controls and NSCLC patients. Urinary exosomes were isolated by ultracentrifugation and identified by electron microscopy. Exosomal proteins were separated by 1-D SDS-PAGE and the differentially expressed bands between healthy controls and NSCLC patients ranging in size from 35 to 45 kD were cut from the gel. After tryptic digestion, 18 proteins were identified by nano-HPLC-chip-MS/MS. The differential expression of leucine-rich α-2-glycoprotein (LRG1) was further validated in urinary exosomes by Western blot and in lung tissue by immunohistochemistry. The LRG1 was found to be expressed at higher levels in urinary exosomes and lung tissue of NSCLC patients. These results suggested that LRG1 may be a candidate biomarker for non-invasive diagnosis of NSCLC in urine.

Keywords:
Immunohistochemistry / Non-small cell lung cancer / Proteome / Urinary exosomes / Western blot

1 Introduction

Lung cancer was a leading cancer-related cause of death in the late 20th century. Non-small cell lung cancer (NSCLC) accounts for 80–85% of lung cancers, and its 5-year survival rate is <15% [1]. The lack of satisfactory diagnostic indicators is an important reason for the poor clinical efficacy of NSCLC treatment. The current commonly used clinical markers for NSCLC include serum or pleural fluid carcinoembryonic antigen (CEA), fragment of cytokeratin 19 (CYFRA21-1), neuron-specific enolase (NSE), carbohydrate antigen 19-9 (CA19-9), and squamous cell carcinoma antigen (SCCA); however, the value of these markers in early diagnosis of lung cancer has not been determined is not clear. Because of difficulties in diagnosing NSCLC at an early stage, 70–80% of NSCLC patients are diagnosed during the middle or late phases of the disease when the cancer is inoperable [2]. For this reason, the search for new diagnostic biomarkers of NSCLC has become a pressing task.

Changes in urine protein composition not only reflect the status of the urinary system [3], but can also reflect the status of the circulatory system. As urine can be obtained noninvasively in large quantities, urinary proteomics has received increased attention [4, 5].

Exosomes are 30–100 nm vesicles coated with lipid bilayer membranes that are released into the extracellular environment by fusion of the multivesicular body with the membrane [6]. As exosomes can transmit many molecules intercellularly, they are considered a potential source of new biomarkers [7, 8]. Many types of cells can secrete exosomes, but exosomes from different cell types have different components [9–11]. Urinary exosomes, a low-density solid component secreted by renal epithelial cells, contain proteins, lipids, and micro-RNA. The protein content of urinary exosomes accounts for about 3% of the total protein in normal urine [12]. Proteins in urinary exosomes derived from the urinary and circulatory systems. Thus, these exosomes carry a wealth of physiological and pathological biomarker information, and changes in urinary exosome protein composition may reflect the pathological process of systemic and urinary system disease [13].

In this study, we aimed to provide new experimental evidence for the potential of using a comparative proteomics strategy to screen for NSCLC-related proteins in urinary exosomes for early clinical diagnosis of NSCLC.
2 Materials and methods

2.1 Urine samples collection

Normal urine samples were obtained from ten healthy volunteers (aged 50–70, five males and five females with no serious systemic disease, no history of medication for serious diseases in the past 2 years, no history of smoking, and females not in menstrual cycle at the time of collection). Fifty milliliters per subject was collected and mixed together. Urine samples from eight NSCLC patients who had not received chemotherapy (aged 50–70, four males and four females, grades I + II) were collected at the Department of Respiratory Medicine of the First affiliated hospital of Chongqing Medical University. Urine collection was approved by all patients and the ethics committee of the First Affiliated Hospital of Chongqing Medical University. Protease inhibitors were added (1.67 mL of 100 mM Na2EDTA, 2.5 mL of 11.5 mM PMSF, and 50 μL of 1 mM leupeptin for every 50 mL urine) immediately after collection to avoid proteolysis. The urine samples were stored on ice prior to centrifugation at 1000 g for 15 min at 4 °C. The precipitates were removed and the supernatants were stored at −20 °C until use.

2.2 Isolation of urinary exosomes

The thawed urine samples were centrifuged at 17 000 × g for 15 min at 4 °C and the supernatants were collected. Whole cells, large membrane fragments, and other debris were discarded. The supernatants were ultracentrifuged at 200 000 × g, 4 °C in a Hitachi Refrigerated Centrifuge (Hitachi, Tokyo, Japan) for 1 h to sediment the low-density fraction. The low-density sediments were resuspended in 0.01 mol/L of PBS solution [14, 15].

2.3 Negative staining electron microscopy

In total, 20–30 μL exosomes corpuscle was suspended on carriers copper grid and placed for 1 min at room temperature. Excess liquid was blotted with filter paper from the other side of the grid. The tryptic peptide mixture was eluted from the gel. The selected protein bands ranging in size from 35 to 45 kDa were excised manually from the gel and transferred into 0.5 mL siliconized Eppendorf tubes before destained by incubation in 75 mM ammonium bicarbonate/40% ethanol (1:1). After destaining, the gel pieces were incubated in a solution of 5 mM DTT/25 mM ammonium bicarbonate (volume sufficient to cover the gel) at 60% for 30 min. The gel pieces were cooled to room temperature and the liquid was discarded. For alkylation of proteins, the gel was incubated in a solution of 55 mM iodoacetamide at room temperature for 30 min, and then the gel pieces were washed, dehydrated in 100% ACN and dried. The gel pieces were then applied to 200-mesh nickel grids. After blocking with 5% BSA and washing, the grids were incubated with primary antibody solutions (1:50–1:500 dilutions) for rabbit polyclonal antibody to AQP-2 (Beijing Boisynthesis Biotechnology, Beijing, China) for 2 h at room temperature. As a control, samples were incubated with 0.01 mol/L PBS instead of primary antibody. Following PBS rinses, the grids were then exposed to species-specific anti-IgG antibodies conjugated to colloidal gold particles (5 nm) (Beijing Boisynthesis Biotechnology). After washing, membranes underwent negative staining with 0.5% uranyl acetate. Finally, the grids were examined with a PHILIPS-TECNAI 10 electron microscope operated at 80 kV after drying.

2.4 Immuno-electron microscopy of immunogold labeled exosomes

Urinary exosomes were fixed using 4% paraformaldehyde/0.25% glutaraldehyde in PBS for 1 h, dehydrated in graded ethanol and cut into ultra-thin sections (70–80 nm). Sections were then applied to 200-mesh nickel grids. After blocking with 5% BSA and washing, the grids were incubated with primary antibody solutions (1:50–1:500 dilutions) for rabbit polyclonal antibody to AQP-2 (Beijing Boisynthesis Biotechnology, Beijing, China) for 2 h at room temperature. As a control, samples were incubated with 0.01 mol/L PBS instead of primary antibody. Following PBS rinses, the grids were then exposed to species-specific anti-IgG antibodies conjugated to colloidal gold particles (5 nm) (Beijing Boisynthesis Biotechnology). After washing, membranes underwent negative staining with 0.5% uranyl acetate. Finally, the grids were examined with a PHILIPS-TECNAI 10 electron microscope operated at 80 kV after drying.

2.5 1-D SDS-PAGE separation of exosomal proteins

Twenty microgram exosomal proteins was separated under denaturing conditions in a 10% polyacrylamide gel. The SDS-PAGE gel was run in a Mini Protein 3 Cell (BIO-RAD, CA, USA) at 120 V for 2 h. After completion of the electrophoresis, the protein bands in the gel were visualized by Coomassie Blue staining and the images were acquired by an image scanner (BIO-RAD), which were operated by Quality One software (BIO-RAD).

2.6 In-gel digestion

The selected protein bands ranging in size from 35 to 45 kDa were excised manually from the gel and transferred into 0.5 mL siliconized Eppendorf tubes before destained by incubation in 75 mM ammonium bicarbonate/40% ethanol (1:1). After destaining, the gel pieces were incubated in a solution of 5 mM DTT/25 mM ammonium bicarbonate (volume sufficient to cover the gel) at 60% for 30 min. The gel pieces were cooled to room temperature and the liquid was discarded. For alkylation of proteins, the gel was incubated in a solution of 55 mM iodoacetamide at room temperature for 30 min, and then the gel pieces were dehydrated in 100% ACN and dried. The gel pieces were then swollen in 10 mL of 25 mM ammonium bicarbonate buffer containing 20 mg/mL modified sequencing grade trypsin (Promega, Madison, WI) and incubated overnight at 37 °C. The tryptic peptide mixture was eluted from the gel with 0.1% formic acid.

2.7 LC-MS/MS analysis

Peptides were resuspended in 25 μL 0.1% formic acid and 20 μL was used for each LC-MS/MS analysis. An Agilent 1200 series nanoflow HPLC system (Agilent Technologies, Palo Alto, CA, USA) was run in the trapping mode with an enrichment column (560.3 mm, 5 mm particles) and a Zorbax 300SB C18 analytical column (150 × 0.075 mm,
precursor ions and a tolerance of 7 Da for the fragment ions and a tolerance of ±0.7 Da for the fragment ions were used. Two missed cleavages were allowed. Carboxymethylated cysteines were set as fixed modification and oxidized methionine as variable modification. Spectrum Mill scores above 13 for complete proteins with a minimum score of 70% and a minimum scored peak intensity (SPI) for individual peptides were used as auto-validation criteria. These criteria are a good compromise between risking too many false positive if the values are set too low and the risk to miss real protein hits if the values are set too high. Auto-validation was necessary to ensure the same data analysis conditions for all the LC-MS/MS runs done in this study and to assure a fair comparison of the results.

2.9 Western blot

Protein concentrations were measured using Bradford method, and 50 μg proteins was separated on a 12% polyacrylamide gel. Electrophoresis was stopped when bromophenol reached the bottom of the gel. Proteins were transferred to PVDF membranes (Millipore, USA) using a semi-dry transfer device (BIO-RAD) for approximately 30 min. The membranes were washed once with distilled water, blocked with 5% skim milk for 3–5 h at room temperature, and incubated with monoclonal mouse anti-lucinuria-rich α-2-glycoprotein (LRG1) primary antibody (diluted 1:200, Abcam) at 4°C overnight. The membranes were then washed three times for 10 min with TBST (20 mM Tris-Cl, 140 mM NaCl, pH 7.5, 0.05% Tween-20) and incubated with horseradish peroxidase-labeled goat antiamouse IgG secondary antibodies (diluted 1:2000, Abcam) at room temperature for 5 h. After washing three times for 10 min in TBST, the membrane was visualized using the ECL method and a chemiluminescence imaging system (Millipore).

2.10 Immunohistochemistry staining

Paraffin-embedded tissues from 20 NSCLC patients and adjacent non-tumor lung tissues were obtained from the Department of Pathology of the First Affiliated Hospital of Chongqing Medical University, China. All samples were collected with informed consent. Table 1 lists the clinicopathological features of the 20 NSCLC patients. Deparaffinization and rehydration were carried out in a series of xylene and ethanol baths of decreasing concentration. The sections were then rinsed with water for 5 min, soaked for 5 min in 0.01 mol/L PBS, boiled in citrate buffer solution (pH = 6) for 20 min and cooled to room temperature.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gender</th>
<th>Age</th>
<th>Pathological type</th>
<th>Clinical stage</th>
<th>Lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>&lt;60 years</td>
<td>&gt;60 years</td>
<td>Squamous carcinoma</td>
</tr>
<tr>
<td>Number of patients</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

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Sections were washed twice for 3 min with PBS, incubated in 3% H₂O₂ in deionized water for 10 min, washed twice with PBS and incubated with normal goat serum for 20 min at room temperature. The serum was discarded, and sections were incubated with monochlonal mouse anti-LRG1 primary antibody (diluted 1:200, Abcam) or 0.01 mol/L PBS for 2 h in a 37°C water bath. Sections were then washed twice with PBS and incubated with goat antimouse secondary antibody for 30 min in a 37°C water bath. Sections were again washed twice with PBS, incubated with horseradish peroxidase-labeled streptavidin albumin working solution for 30 min in a 37°C water bath, washed twice with PBS, stained with DBA color reagent and washed with tap water. Sections were then counterstained with hematoxylin, dehydrated, hyaline and mounted.

### 2.11 Statistical analysis

SPSS 13.0 was used for statistical analysis. Statistical differences were evaluated using t-tests. \( p < 0.05 \) was considered statistically significant.

## 3 Results

### 3.1 Isolation and verification of urinary exosomes

Compared with freshly collected urine, we found that some exosomal components were lost when samples were frozen at \(-20°C\); however, exosomes frozen at \(-80°C\) were complete.

In this research, we employed ultracentrifugation to isolate urinary exosomes. To verify whether the sediments separated from urine were exosomes, the vesicles were visualized by qualitative negative staining electron microscopy. As shown in Fig. 1, analyzed by TEM, isolated urinary exosomes are relatively round vesicles ranging from approximately 30 to 100 nm in diameter. We next performed colloidal gold-labeled immune electron microscopy using a primary antibody against water channel protein-2 (AQP-2) and 5-nm colloidal gold-labeled goat anti-rabbit IgG as a secondary antibody (Fig. 1). Black colloidal gold particles were evenly distributed on the surface of the spherical bodies of the exosomes. Because of the high electron density of the exosomes, black spots were clearly visible (Fig. 1B); however, no colloidal gold particles were found on the surface of untreated exosomes (Fig. 1C).

### 3.2 1-D SDS-PAGE analysis

In this study, the molecular weight distribution of urinary exosomes proteins was observed by 1-D SDS-PAGE. As shown in Fig. 2A, the distribution of protein bands following gel electrophoresis was similar in uncentrifuged urine (Fig. 2A) and supernatant after centrifugation at 17 000 × g (Fig. 2B); however, the distribution of protein bands was different in exosome samples (Fig. 2C).

Urinary exosome samples contained highly abundant bands distributed between 90 and 110 kDa (Fig. 2A). In addition, exosome samples contained several clear bands between 30 and 60 kDa. Compared with lanes A and B, the exosome samples (lane C) contained significantly fewer bands with molecular weights < 30 kDa.

Electrophoresis results of urinary exosomes from NSCLC patients and healthy controls showed significant differences in protein composition between these two groups (Fig. 2B). In this study, we focused on analyzing the differential bands between 35 and 45 kDa. Quantity One histogram analysis (Fig. 2C) showed that the expression of proteins in the range of 35–45 kDa bands was increased in NSCLC patient samples, but the degree to which the intensity of these bands increased differed among the six NSCLC samples.

### 3.3 Protein identification by nano-HPLC-chip-MS/MS

MS/MS data were input into UniProtKB/SWISS-PROT and Homo Sapiens (Human) database. Eighteen proteins were identified in the 35–45 kDa bands of which eleven were identified in samples from the NSCLC patients, three were identified in samples from both normal subjects and NSCLC patients and four were identified in samples from normal patients. These 18 proteins are summarized in Table 2.

In this study, we focused on one LRG family member in urinary exosomes from NSCLC patients: LRG1. HPLC-chip-MS/MS identification results showed that sequences of eight peptide ions were consistent with sequences in the LRG1 primary structure. The mean peptide spectral intensity was 2.01e+007, and the coincidence rate with amino
acid residues was 20.2% (70/347). Figure 3 shows the MS/MS spectrum of peptide ion [GPLQLER] \textsuperscript{2} \textsuperscript{1} (200–206). A wealth of b/y ion information was found, suggesting high confidence in these identification results.

We next verified the presence of LRG1 in urinary exosomes from NSCLC patients by Western blot. Expression of LRG1 in lung cancer tissue and adjacent non-tumor lung tissue in NSCLC patients was detected by immunohistochemistry.

3.4 Validation of LRG1 in urinary exosomes by Western blot

The presence of LRG1 in urinary exosomes from NSCLC patients and healthy controls was validated by Western blot. Western blot analysis showed that LRG1 was expressed in the extracts of urinary exosomes from six NSCLC patients and six healthy controls; however, Quantity One histogram analysis showed that LRG1 expression was significantly increased (approximately sixfold) in the six NSCLC patients (Fig. 4).

3.5 Immunohistochemistry of LRG1 in lung tissue

Immunohistochemical staining was used to provide a semi-quantitative measure of LRG1 protein expression in tumor tissues. Both the intensity of clear brown granules in the cytoplasm and the percentage of stained tumor cells were evaluated. Five high-power microscopic fields (magnification, 200 ×) were selected randomly, and the cells in these

<table>
<thead>
<tr>
<th>Table 2. Proteins identified in selected bands of urinary exosomes in NSCLC patients and normal controls</th>
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</thead>
<tbody>
<tr>
<td>Protein name</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Zinc-(\alpha)-2-glycoprotein(AZGP1,ZAG,ZAGP1)</td>
</tr>
<tr>
<td>LRG1 precursor</td>
</tr>
<tr>
<td>Aspartate aminotransferase, cytoplasmic (GOT1)</td>
</tr>
<tr>
<td>Keratin 13 isoform b (KRT13)</td>
</tr>
<tr>
<td>Actin, cytoplasmic 2 (ACTG1, ACTB,ACTG)</td>
</tr>
<tr>
<td>Isoform 1 of Serpin B3 (SERPINB3)</td>
</tr>
<tr>
<td>IST1 homolog (KIAA0174)</td>
</tr>
<tr>
<td>Plasma serine protease inhibitor precursor (SERPIN5)</td>
</tr>
<tr>
<td>G-protein coupled receptor family C group 5 member B precursor (GPRC5b)</td>
</tr>
<tr>
<td>Sorbitol dehydrogenase (SORD)</td>
</tr>
<tr>
<td>Keratin type II cuticular Hb3 (KRT83)</td>
</tr>
<tr>
<td>Keratin, type I cuticular Hb3-II (KRT3B)</td>
</tr>
<tr>
<td>Haptoglobin precursor (HP)</td>
</tr>
<tr>
<td>AMBP protein precursor (AMBp)</td>
</tr>
<tr>
<td>cDNA FLJ77275 (KIAA0174)</td>
</tr>
<tr>
<td>N-acetyl-phosphatidylethanolamine-hydrorylating phospholipase D (NAPEPLD)</td>
</tr>
<tr>
<td>Protein sprouty homolog 1 (SPRY1)</td>
</tr>
<tr>
<td>Actin, (\alpha) skeletal muscle (ACTA1)</td>
</tr>
</tbody>
</table>

a) D, proteins expressed in NSCLC patients; N, proteins expressed in normal controls; N,D, proteins expressed in both NSCLC patients and normal controls.
fields were categorized as follows: negative staining (0% positive cells), “−”; weak positive staining (<10% positive cells), “+”; positive staining (10–60% positive cells), “++”; and strong positive staining (>60% positive cells), “+++.” As statistical analysis required binary variables (positive or negative), fields scored as “−” and “+” were defined as negative, and fields scored as “++” and “+++” were defined as positive [16]. The results of these analyses are summarized in Table 3 and shown in Fig. 5. Sixty-five percent of NSCLC specimens were positive for LRG1 staining (of 20 samples, 13 were positive, and among these, 6 were strongly positive and 7 were positive). On the contrary, 10% of adjacent non-tumor lung tissues were positive for LRG1 staining (of ten samples, one was positive). The rate of LRG1 staining in NSCLC specimens was significantly higher than in that of adjacent non-tumor tissue (p < 0.01).

4 Discussion

To extract exosomes, we first centrifuged urine at 17 000 × g for 15 min to remove whole cells, large membrane fragments and other debris and then froze the supernatant at −80°C. After enough sample volume was obtained, samples were centrifuged at 200 000 × g for 1 h to avoid the adsorption of exosomes by Tamm-Horsfall (THP) protein and to improve the absorption rate of exosomes. THP, which is produced by the Golgi apparatus in the ascending branch of Henry’s loop and in epithelial cells of the distal renal tubules, is the most abundant urinary glycoprotein. At low temperatures, it can easily wrap exosomes [17]. Performing the 17 000 × g centrifugation after freezing samples may result in precipitation and removal of THP-encased exosomes, and thus affect the extraction of exosomes.

As exosomes were isolated from urine with complex components, we identified urinary exosomes based on morphological and immunological characteristics. This identification of exosomes was mainly performed by Western blot and electron microscopy. Electron microscopy provided intuitive and reliable results. As the structure and size of exosomes can be observed under electron microscopy, it is the preferred method for the identification of exosomes. In this study, ultrastructural observation was performed under negative staining electron microscopy. Consistent with exosomes from other sources, qualitative electron microscopy negative staining images revealed that urinary exosomes were flat or spherical corpuscles composed of lipid bilayers. On the contrary, urinary
Exosomes, which had a diameter of about 60 nm, were smaller than exosomes from dendritic cells and tumor cells. We further characterized urinary exosomes by colloidal gold immune electron microscopy using a primary antibody that recognized water channel protein-2 (AQP-2), a cytoplasmic antigen located on the luminal side of the principal cell of the kidney collecting duct and within vesicles near the luminal side, which can be used as a marker of urinary exosomes [14]. Second antibody colloidal gold carries negative charge in alkaline environment and can absorb with antibodies, thus the antibody was labeled. Immune electron microscopy techniques are often divided into pre-embedding staining and post-embedding staining. Both methods have advantages and disadvantages for target antigen protection and preservation of ultrastructure. Post-embedding staining preserves ultrastructure well and does not result in staining problems. The positive results of post-embedding staining are reproducible, and double immunohistochemical staining can be performed using this method. For these reasons, we used post-embedding staining to visualize exosomes in this study.

1-D SDS-PAGE results showed that exosomes were selectively enriched in specific proteins. Glomeruli mainly filters proteins with molecular weight lower than 60 kD, suggesting that the 35–45 kD protein bands observed upon electrophoresis of urinary exosomes may be derived from the urinary system or the circulatory system. We thus focused our study on these bands. In this study, 1-D SDS-PAGE combined with HPLC-chip-MS/MS analysis were used to analyze differentially expressed proteins in the 35–45 kD bands. In these bands, 18 proteins were identified. The scores of peptides were much higher than the set value, indicating that the enzymatic peptide mixtures produced strong mass spectrometry signals after enrichment with a microliter-upgrade Zorbax 300SB-C18 column in HPLC-chip and separation with a microliter-upgrade Zorbax 300SB-C18 reversed column, thus enhancing the sequence analysis. The results in Table 2 also summarize that the urinary exosome proteome in NSCLC patients was different from that in healthy subjects. The protein content and the number of protein species of urinary exosomes from NSCLC patients were higher than those of urinary exosomes from healthy subjects.

Past research has shown that LRG1 is involved in important biological and pathological processes such as protein–protein interaction, signal transduction and cell adhesion. LRG1 is also expressed during granulocyte differentiation. In recent years, elevated levels of serum LRG were found in patients with liver cancer [18], lung cancer [19], and pancreatic adenocarcinoma [20]. Furthermore, Heo isolated and identified LRG1 in the serum of adenocarcinoma patients by lectin affinity chromatography and LC-MS/MS and concluded that it was a potential biomarker of lung cancer in serum [21]. Our results not only showed that LRG1 increased in the urinary exosomes of NSCLC patients (6/6), but also showed high expression in tumor tissue of NSCLC patients (positive rate of 65%, 13/20), suggesting that LRG1 in urinary exosomes in NSCLC patients may be derived from tumor tissues, and LRG1 may be a candidate marker for NSCLC-related tumors in urine. In this study, we used established analysis strategies to provide a new approach for identifying potential NSCLC-related biomarkers in urine at the sub-cellular level.

The authors have declared no conflict of interest.

5 References


