Comparison of Urine Proteomes in Tumor-Bearing and Tumor-Resected Mice

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Abstract

Objective This study addressed the critical concern of clinical surgeons—the efficacy of solid tumor resection. The urinary proteome can sensitively reflect physiological changes in the organism. This study explored changes in the urinary proteome of mice with MC38 subcutaneous tumors following resection or non-resection. Methods An MC38 subcutaneous tumor-bearing mouse model was established, and mice were divided into a healthy control group, a complete resection experimental group, and a non-resection experimental group. Surgery was performed on the three groups of mice, and urine was collected on days 7 and 30 post-surgery. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify the urinary proteome, and differential proteins and related biological pathways were analyzed. Results (1) At 7 days post-surgery, the urinary proteome between the subcutaneous tumor complete resection group and the non-resection group could be used to screen 20 differential proteins capable of distinguishing the two groups, with biological processes including circadian rhythm, Notch signaling pathway, leukocyte intercellular adhesion, heterophilic cell adhesion via plasma membrane adhesion molecules, etc. (2) At 30 days post-surgery, the urinary proteome between the subcutaneous tumor complete resection group and the non-resection group could be used to screen 33 differential proteins capable of distinguishing the two groups, with biological processes including cell adhesion, complement activation and alternative pathway, immune system processes, angiogenesis, etc. (3) At 30 days post-surgery, no significant difference was observed between the subcutaneous tumor complete resection group and the healthy control group. Conclusion Changes in the urinary proteome can reflect tumor resection status.

Full Text

Comparison of Urine Proteomes from Tumor-Bearing and Tumor-Resected Mice

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Abstract

Objective: This study addresses the foremost concern of clinical surgeons—the efficacy of solid tumor resection. The urine proteome can sensitively reflect physiological changes in the body. We explored changes in the urine proteome of mice with MC38 subcutaneous tumors following tumor resection.

Methods: We established an MC38 subcutaneous tumor-bearing mouse model and divided mice into healthy control, complete tumor resection, and non-resection groups. Urine was collected on days 7 and 30 post-surgery. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to identify urine proteins, and differential proteins and related biological pathways were analyzed.

Results: (1) Seven days after surgery, 20 differential proteins distinguished the complete resection group from the non-resection group, enriched in biological processes including circadian rhythm, Notch signaling pathway, leukocyte cell-cell adhesion, and heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules. (2) Thirty days after surgery, 33 differential proteins distinguished the complete resection group from the non-resection group, enriched in biological processes including cell adhesion, complement activation and alternative pathway, immune system processes, and angiogenesis. (3) No significant differences were observed between the complete resection group and healthy control group at 30 days post-surgery.

Conclusion: Changes in the urine proteome can reflect tumor resection status.

Keywords: tumor; surgical resection; urine; proteome

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1 Introduction

Surgical resection combined with chemotherapy or radiotherapy represents the most common treatment for solid tumors, yet post-resection recurrence remains frequent. Whether a tumor has been completely resected is a primary concern for surgeons. All cells in the body depend on a stable internal environment for survival and function. Blood, as the critical provider of this internal environment for all tissues and organs, must maintain stability and homeostasis to protect organs from disruptive factors. In contrast, as an ultrafiltrate of blood,

urine lacks such stabilizing mechanisms. Consequently, urine can enrich and concentrate changes excreted from the body without homeostatic regulation, making it an excellent biological source for discovering biomarkers that reflect early disease-induced changes [1].

Our laboratory' s previous studies have demonstrated that in Walker 256 tumor-bearing rats, the urine proteome exhibits significant changes before palpable tumors develop, and these early urinary alterations can also be identified through differential abundance in advanced cancer stages [2]. Following tail vein injection of Walker-256 cells, rat urine proteome changes occurred on day 2, preceding the pathological changes of lung tumor nodules that appeared on day 4 [3]. Three days after Walker 256 cell implantation in rat tibia, 25 proteins showed significant changes in urine before clear lesions were detected by CT scan [4]. Three days after Walker-256 cell injection into rat liver, 12 differential proteins were identified, with 7 significantly associated with liver cancer, and identical tumor cells growing in different organs could be reflected in differential urinary proteins [5].

Clinical urine samples involve numerous confounding factors, and collecting early patient samples is time-consuming. Therefore, establishing a colorectal cancer mouse model can minimize potential interfering factors while enabling dynamic monitoring of disease onset and obtaining urine samples at sufficiently early time points before pathological or clinical manifestations appear, facilitating observation of tumor resection and recurrence [6].

In this study, we constructed a subcutaneous tumor model using mouse colon adenocarcinoma cell line MC38. After model establishment, subcutaneous tumor resection surgery was performed, and mouse urine was collected post-surgery for urine proteomic analysis using liquid chromatography-tandem mass spectrometry. The experimental workflow is shown in Figure 1. This study aims to explore changes in the urine proteome following MC38 subcutaneous tumor resection in mice.

Figure 1. Research workflow for studying urine proteomic profiles in mice with and without subcutaneous tumor resection.

2 Materials and Methods

2.1 Establishment of MC38 Subcutaneous Tumor Mouse Model

Fifteen male C57BL/6J mice (18–20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Animal License: SYXK(Beijing) 2017-0038). All animals were housed in a standard environment (22 ± 1°C, 50–70% humidity). After three days of acclimatization, mice were randomly divided into three groups: healthy control (n=5), complete tumor resection (n=5), and non-resection (n=5). MC38 cells (gifted by Professor Sheng Wang' s group at Beijing

University of Technology) were cultured in complete medium (DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin) until sufficient cells were available. When cells were in good condition, they were counted using trypan blue staining to ensure viable cell numbers greater than 5$×10^{6}$ cells/ml per tube before animal implantation.

The modeling procedure was as follows: mice were anesthetized with pentobarbital sodium at 10 mg/kg based on body weight. After anesthesia, mice were fixed on disposable sterile medical pads, hair at the injection site was removed, and the area was disinfected. A 1 ml syringe was used to aspirate aliquoted cells (PBS buffer for the control group) with an injection volume of 0.1 ml. MC38 cells were injected subcutaneously into the right hind limb of mice in the complete resection (n=5) and non-resection (n=5) groups. The healthy control group (n=5) received PBS buffer injection. Based on tumor growth status, surgery was performed on all three groups: complete tumor resection for the complete resection group; partial tissue and muscle resection without tumor removal for the non-resection group to ensure consistent wound size; and identical partial tissue and muscle resection for the healthy control group.

2.2 Urine Sample Collection

Urine was collected from experimental mice. During collection, all mice were individually placed in metabolic cages with fasting and water restriction. Urine was collected overnight for 12 hours, with a minimum volume of 1 ml per sample.

2.3 Urine Protein Extraction and Digestion

Proteins were extracted from mouse urine and digested with trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA). Digested peptides were desalted using HLB columns (Waters, Milford, MA) and dried using a vacuum concentrator. Samples were resuspended in 0.1% formic acid, diluted to 0.5 g/ L, and dried again before final resuspension in 0.1% formic acid. A pooled peptide sample was prepared from each sample and fractionated using a high pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific), dried, and resuspended in 0.1% formic acid for subsequent library construction. iRT standard (Biognosys) was added to all identified samples for retention time alignment.

2.4.1 Database Acquisition in Data-Dependent Acquisition Mode

Ten fractionated samples were analyzed using an EASY-nLC 1200 ultra-high performance liquid chromatography system coupled to an Orbitrap Fusion Lumos high-resolution mass spectrometer. Peptides dissolved in 0.1% formic acid were loaded onto a trap column (75 µm × 2 cm, 3 µm, C18, 100 Å) and separated on a reversed-phase analytical column (50 µm × 250 mm, 2 µm, C18, 100 Å) with a gradient of 4–35% mobile phase B (80% acetonitrile + 0.1% formic acid + 20% water) at 300 nL/min for 90 minutes. For automated and sensitive

signal processing, iRT calibration kit (Biognosys, Switzerland) was used at 1:20 v/v in all samples.

DDA-MS analysis of 10 fractions was performed with the following parameters: spray voltage 2.4 kV, Orbitrap primary resolution 60,000, scan range 350–1550 m/z, secondary scan range 200–2000 m/z, resolution 30,000, isolation window 2 Da, HCD collision energy 30%, AGC target 5e4, and maximum injection time 30 ms. Raw files were processed for library construction and analysis using PD (Proteome Discoverer 2.1, Thermo Fisher Scientific) software.

2.4.2 DIA Data Acquisition of Experimental Samples

Thirty-six experimental samples were analyzed in DIA-MS mode. Liquid chromatography parameters were identical to DDA library acquisition. Mass spectrometry parameters were as follows: primary full scan at 60,000 resolution with 350–1550 m/z range; secondary scans at 30,000 resolution with 36 isolation windows, HCD collision energy 30%, AGC target 1e6, and maximum injection time 50 ms. Window calculation: based on DDA search results, all identified peptides were sorted by m/z and divided into 36 groups, with each group' s m/z range defining the window width for DIA data acquisition.

2.5 Mass Spectrometry Data Analysis

Spectronaut X software was used to process and analyze mass spectrometry data. Raw files from DIA acquisition were imported for library searching. High-confidence protein criteria were peptide q-value < 0.01, with protein quantification based on the peak area of all fragment ions from secondary peptides.

2.6 Statistical Analysis

Group comparisons were performed using independent samples t-test. Differential protein screening criteria: fold change FC ≥ 1.5 or FC ≤ 0.67 between groups, P < 0.05.

2.7 Random Grouping Validation

To determine the likelihood of identified differential proteins arising by chance, random grouping validation was performed using the same screening criteria: FC ≥ 1.5 & ≤ 0.67, P < 0.01.

2.8 Functional Analysis of Differential Proteins

Total proteins were analyzed using orthogonal partial least squares discriminant analysis (OPLS-DA) on the Wukong platform (https://www.omicsolution.org/) [7]. Differential proteins were functionally enriched using the DAVID database (https://david.ncifcrf.gov/) with a significance threshold of P < 0.05.

3 Results

3.1 Animal Model Construction and Subcutaneous Tumor Growth

Tumors became palpable 7–8 days after subcutaneous injection in all three experimental groups, at which point surgical resection was performed. Urine was collected on postoperative days 7 and 30. The experimental timeline is shown in Figure 2.

Tumor growth indicated successful establishment in all 10 mice receiving MC38 cell injection. Tumor sizes (expressed as mean diameter) on days 7–8 are shown in Table 1. No recurrence was observed in the complete resection group up to 90 days post-surgery, confirming successful resection. The healthy control group showed no abnormalities.

Figure 2. Animal model construction process and urine collection time points.

Table 1. Tumor size (mean diameter)

Group	Size (mm)
Complete resection (n=5)	[data]
Non-resection (n=5)	[data]
Healthy control (n=5)	[data]

3.2.1 Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) of Urinary Proteins at Different Time Points

DIA quantitative analysis identified a total of 405 high-confidence proteins across all samples from the three experimental groups at two time points.

OPLS-DA analysis of total urinary proteins identified in the non-resection versus complete resection groups on postoperative day 7 (n=10) clearly separated the two groups (Figure 3a). R^2X, R^2Y, and Q^2Y values were 0.369, 0.993, and 0.788, respectively, indicating good model fit accuracy. Variable Importance for the Projection (VIP) calculation identified 20 differential proteins meeting the VIP $>$ 1.0 criterion, demonstrating strong discriminatory power for classifying the two groups.

Similarly, OPLS-DA analysis on postoperative day 30 (n=10) clearly separated the non-resection and complete resection groups (Figure 3b). R^2X, R^2Y, and Q^2Y values were 0.435, 0.968, and 0.766, respectively, with good model fit accuracy. VIP calculation identified 33 differential proteins meeting the VIP $>$ 1.0 criterion, indicating strong discriminatory power.

(a) (b) Figure 3. OPLS-DA analysis of total proteins in two groups at different time points.

3.2.2 Differential Proteins and Biological Pathways on Day 7 Between Complete and Non-Resection Groups

Twenty differential proteins were identified between complete and non-resection groups on day 7 post-surgery (Table 2), including 9 upregulated and 11 downregulated proteins, enriched in 6 biological pathways (Table 3). Biological processes included circadian rhythm, Notch signaling pathway, leukocyte cell-cell adhesion, and heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules. Studies have shown that circadian rhythm correlates with both wound healing and tumor growth in animals [8, 9]. Skin wound healing involves inflammation, leukocyte trafficking, and tissue remodeling, with some proteins participating in circadian-driven temporal coordination mechanisms. Notch signaling regulates multidimensional subcutaneous tumor behavior [10] and can function directly as an oncogene or tumor suppressor, influencing tumor cell proliferation, differentiation, apoptosis, and genomic instability [11]. The immune response against tumors is enhanced through leukocyte adhesion regulated by integrin ligands ICAM-1 and VCAM on lymphatic endothelial cells [12].

Table 2. Differential proteins on day 7 between complete and non-resection groups

Protein Accessions	Protein Descriptions	Fold change
Q62266	Cornifin-A	[value]
P00687	Alpha-amylase 1	[value]
Q91X17	Uromodulin	[value]
O35657	Sialidase-1	[value]
P0CG49	Polyubiquitin-B	[value]
Q80X71	Transmembrane protein 106B	[value]
Q60590	Alpha-1-acid glycoprotein 1	[value]
P81117	Nucleobindin-2	[value]
P18761	Carbonic anhydrase 6	[value]
Q07797	Galectin-3-binding protein	[value]
P06869	Urokinase-type plasminogen activator	[value]
Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	[value]
P29533	Vascular cell adhesion protein 1	[value]
P05533	Lymphocyte antigen 6A-2/6E-1	[value]
P0CW02	Lymphocyte antigen 6C1	[value]
Q9ESD1	Prostasin	[value]
P15947	Kallikrein-1	[value]

Protein Accessions	Protein Descriptions	Fold change
Q9Z0J0	NPC intracellular cholesterol transporter 2	[value]
O09051	Guanylate cyclase activator 2B	[value]
Q9EP95	Resistin-like alpha	[value]

Table 3. Biological pathways associated with day 7 differential proteins

Biological Process	P-value
circadian rhythm	1.33E-04
Notch signaling pathway	2.04E-04
ubiquitin homeostasis	[value]
excretion	[value]
leukocyte cell-cell adhesion	[value]
heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules	[value]

3.2.3 Differential Proteins and Biological Pathways on Day 30 Between Complete and Non-Resection Groups

Thirty-three differential proteins were identified between complete and non-resection groups on day 30 post-surgery (Table 4), with 13 upregulated and 20 downregulated proteins, enriched in 19 biological pathways (Table 5). Biological processes included cell adhesion, complement activation and alternative pathway, immune system processes, positive regulation of glucose metabolic process, mitochondrial morphogenesis, and angiogenesis. Literature indicates that cell adhesion and protein localization to the cell surface are closely related to tumor growth, particularly cadherins and integrins, which function as ligand-activated receptors and activate relevant signaling through physical environmental changes [13]. Immune-related pathways frequently change during tumor growth; for example, complement activation in the tumor microenvironment can enhance tumor growth and increase metastasis [14]. Evidence shows that changes in calcium-dependent cell adhesion molecules are associated with tumors, such as cadherin 1 (CDH1), whose downregulation can be used for diagnosis and prognosis of epithelial cancers [15]. Cadherin 13 (CDH13) regulates blood vessels during tumor growth and promotes neovascularization [15, 16]. Additionally, tumor cells secrete high levels of pro-angiogenic factors, contributing to abnormal vascular network formation, making tumor vasculature a key target for cancer therapy [17].

Table 4. Differential proteins on day 30 between complete and non-resection groups

Protein Accessions	Protein Descriptions	Fold change
Q8VED5	Keratin, type II cytoskeletal 79	[value]
P00687	Alpha-amylase 1	[value]
Q922U2	Keratin, type II cytoskeletal 5	[value]
Q61781	Keratin, type I cytoskeletal 14	[value]
P11591	Major urinary protein 5	[value]
Q6NXH9	Keratin, type II cytoskeletal 73	[value]
P03953	Complement factor D	[value]
O55186	CD59A glycoprotein	[value]
Q61581	Insulin-like growth factor-binding protein 7	[value]
P09803	Cadherin-1	[value]
Q9JJS0	Signal peptide, CUB and EGF-like domain-containing protein 2	[value]
P11589	Major urinary protein 2	[value]
P01132	Pro-epidermal growth factor	[value]
Q07456	Protein AMBP	[value]
P47878	Insulin-like growth factor-binding protein 3	[value]
P04186	Complement factor B	[value]
Q9WTR5	Cadherin-13	[value]
P11276	Fibronectin	[value]
Q61129	Complement factor I	[value]
Q921W8	Secreted and transmembrane protein 1A	[value]
O88968	Transcobalamin-2	[value]
Q91X72	Hemopexin	[value]
P0CW02	Lymphocyte antigen 6C1	[value]
Q8K4G1	Latent-transforming growth factor beta-binding protein 4	[value]
O09051	Guanylate cyclase activator 2B	[value]
P25119	Tumor necrosis factor receptor superfamily member 1B	[value]
Q9EP95	Resistin-like alpha	[value]
Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	[value]

Protein Accessions	Protein Descriptions	Fold change
P29533	Vascular cell adhesion protein 1	[value]
O08997	Copper transport protein ATOX1	[value]
Q91VW3	SH3 domain-binding glutamic acid-rich-like protein 3	[value]
Q4KML4	Costars family protein ABRACL	[value]
Q61646	Haptoglobin	[value]

Table 5. Biological pathways associated with day 30 differential proteins

Biological Process	P-value
cell adhesion	[value]
regulation of protein localization to cell surface	[value]
complement activation, alternative pathway	[value]
immune system process	[value]
cell activation	[value]
heat generation	[value]
positive regulation of lipid metabolic process	[value]
cellular response to lipid	[value]
negative regulation of lipid biosynthetic process	[value]
aging	[value]
calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules	[value]
negative regulation of lipid storage	[value]
positive regulation of glucose metabolic process	[value]
energy reserve metabolic process	[value]
negative regulation of insulin secretion involved in cellular response to glucose stimulus	[value]
negative regulation of gluconeogenesis	[value]
locomotor rhythm	[value]
mitochondrion morphogenesis	[value]
angiogenesis	[value]

3.2.4 Differential Proteins on Day 30 Between Complete Resection and Healthy Control Groups

Eight differential proteins were identified between complete resection and healthy control groups on day 30 post-surgery (Table 6), including 3 upregulated and 5 downregulated proteins, with no biological pathways enriched. To

determine whether identified differential proteins could arise randomly, urine proteome data from 10 mice (complete resection vs non-resection at day 30) were subjected to random grouping validation using the same criteria (FC $\geq$ 1.5 & $\leq$ 0.67, P $<$ 0.01). Results showed that among 125 random groupings, an average of approximately [value] differential proteins were identified. Thirty days after surgery, tumor-bearing mice with complete resection showed minimal differences in urinary protein levels compared to healthy mice without tumor inoculation.

Table 6. Differential proteins on day 30 between complete resection and healthy control groups

Protein Accessions	Protein Descriptions	Fold change
Q922U2	Keratin, type II cytoskeletal 5	[value]
P60710	Actin, cytoplasmic 1	[value]
O55186	CD59A glycoprotein	[value]
P11276	Fibronectin	[value]
P0CW02	Lymphocyte antigen 6C1	[value]
P06869	Urokinase-type plasminogen activator	[value]
Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	[value]
Q91WR8	Glutathione peroxidase 6	[value]

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Note: Figure translations are in progress. See original paper for figures.