# Urine Proteome of AOM/DSS-Induced Inflammatory Murine Colorectal Cancer Model

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## Abstract

Abstract: [Objective] The urinary proteome can sensitively reflect physiological changes in the organism and provide clues for the development of colorectal cancer induced by inflammation. [Method] This study utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS) to screen differential urinary proteins and perform biological pathway analysis in an AOM/DSS-induced colorectal cancer mouse model, aiming to identify patterns at the biological pathway level and investigate the progression of the inflammation-cancer model in colorectal cancer development. [Results] (1) Changes in the urinary proteome could reflect pathophysiological changes in experimental mice. Differential proteins from all experimental mice were enriched in previously reported common biological pathways related to inflammation and tumor, including: acute-phase response, cell adhesion, innate immune response, positive regulation of protein kinase B signaling, positive regulation of B cell activation, and glucose metabolism-related pathways, with immune-related responses persisting throughout. (2) Experimental mice exhibited different disease progression, with different urinary proteome profiles at different stages. The single-mouse independent analysis approach employed in this study can provide clues for disease progression in the inflammation-cancer mouse model and also serve as a reference for future personalized and precise disease analysis methodologies.

## Full Text

## Urine Proteome Changes in a Mouse Model of AOM/DSS-Induced Colorectal Cancer

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[**Objective**] The urine proteome sensitively reflects physiological changes in the body and can provide clues for the development of colorectal cancer caused

by inflammation. [**Method**] This study used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to screen differential urine proteins in a mouse model of AOM/DSS-induced colorectal cancer and analyze the associated biological pathways, aiming to explore the disease progression process and identify patterns at the pathway level. [**Results**] (1) Urine proteome changes reflected the pathophysiological alterations in experimental mice. Common biological pathways related to inflammation and tumors were enriched across all mice, including acute phase response, cell adhesion, innate immune response, positive regulation of protein kinase B signaling, positive regulation of B cell activation, and glucose metabolism-related pathways, with immune-related responses persisting throughout the process. (2) Disease progression varied among experimental mice, and urine proteome profiles differed at different disease stages. The single-mouse longitudinal analysis approach used in this study provides clues for understanding disease progression in inflammation-cancer models and offers a reference for future personalized and precision disease analysis methods.

**Keywords:** Proteomics; Urine; Inflammation-driven tumor; Colorectal cancer

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Colorectal cancer (CRC) is a common malignant tumor of the gastrointestinal tract. According to GLOBOCAN 2020 statistics[1], there were over 1.9 million new CRC cases and approximately 935,000 deaths in 2020, accounting for about one-tenth of all cancer cases and deaths. The incidence and mortality of CRC in China are also increasing annually[2]. Colorectal malignancies develop from aberrant crypt foci that gradually evolve into precursor lesions known as colon polyps. The progression from precancerous lesions to cancer generally takes 5-10 years, providing a critical time window for early diagnosis and clinical intervention[3]. Currently, colonoscopy is the recognized gold standard for CRC screening[4], but it requires complex preparation and carries anesthesia risks. The immunochemical fecal occult blood test (FIT) is also used for CRC screening with high sensitivity for CRC but limited sensitivity for precancerous lesions. Additional techniques including sigmoidoscopy, CT colonography, and multi-target stool FIT-DNA testing can be used for CRC screening under specific conditions, yet all share the limitation of limited sensitivity for precancerous lesions[5]. Current societal guidelines recommend CRC screening for individuals

over 50 years old[5], though the age recommendation is trending downward due to the increasing incidence in younger populations[6].

CRC can occur spontaneously or as a long-term complication of chronic intestinal inflammation such as Crohn' s disease and ulcerative colitis. Research shows that many environmental causes and risk factors for cancer are associated with some form of chronic inflammation. The body can promote malignant progression of transformed cells by recruiting and activating inflammatory cells, and inflammation can lead to immunosuppression, creating a favorable environment for tumor development[7]. This study utilizes the chemical carcinogen azoxymethane (AOM) and the pro-inflammatory agent dextran sulfate sodium (DSS) to establish a tumor induction model that mimics inflammation-driven non-hereditary CRC development[8] [Figure 1: see original paper].

**[Figure 1: see original paper] Research workflow for urine proteome analysis in the AOM/DSS-induced colorectal cancer mouse model**

All cells in the human 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 balance to protect organs from disruptive factors. In contrast, as an ultrafiltrate of blood, urine does not require or possess homeostatic mechanisms. Therefore, urine can enrich changes excreted from the body without homeostatic regulation, enabling it to reflect early disease-induced changes and serving as an excellent biological source for biomarker discovery[9].

Our laboratory' s research has shown that in a Walker-256 intracranial tumor transplantation model[10], urine proteome exhibited significant differences that could distinguish normal from experimental rats before radiological tumor detection. Urine proteome changes appeared on day 2 after tail vein injection of Walker-256 cells, earlier than the pathological changes of lung tumor nodules that appeared on day 4[11]. In rats with Walker-256 cells implanted in the tibia, 25 proteins showed significant changes in urine on day 3, before obvious lesions were detected by CT[12]. In nude mice subcutaneously implanted with patient-derived colorectal tumor tissue, differential protein screening led us to conclude that immunodeficient animals may not be suitable models for identifying early immune-related tumor biomarkers in urine[13]. All these studies used transplantation models, and the appearance of early immune-related tumor biomarkers in urine may be due to the exogenous nature of transplanted tumors. In this study, we selected an induced inflammation-cancer model to better simulate the process of human primary tumor development. Additionally, we wanted to specifically investigate whether immune-related pathways would appear in urine during primary tumor development.

## 2 Materials and Methods

### 2.1 Establishment of the AOM/DSS-Induced Colorectal Cancer Mouse Model

Male C57BL/6N mice (18-20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed under standard conditions (room temperature 22 ± 1°C, humidity 65-70%). Azoxymethane was purchased from Sigma, and dextran sulfate sodium was obtained from [supplier name missing in original]. After three days of acclimation, mice were randomly divided into two groups: control group (6 mice) and experimental group (10 mice). The AOM/DSS-induced colorectal cancer model was established as follows: mice were weighed, and the experimental group received intraperitoneal injection of AOM at 10 mg/kg in the right lower abdomen, while the control group received 0.9% saline. During weeks 1, 4, and 7 post-injection, experimental mice received 2.5% DSS solution ad libitum for 7 days, while controls received normal drinking water [Figure 2: see original paper].

**[Figure 2: see original paper] AOM/DSS induction process for the colorectal cancer mouse model**

### 2.2 Pathological Examination of AOM/DSS-Induced Mice

At weeks 1, 4, 7, and 11 of the modeling process, experimental and control mice were euthanized. Distal colon tissues were rapidly collected and fixed in 4% paraformaldehyde. The tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) to observe pathological changes.

### 2.3 Urine Protein Extraction and Digestion

Urine samples from four experimental mice were selected for mass spectrometry analysis based on the criterion that urine was successfully collected at weeks 0, 1, 3, 4, 6, 7, 9, 10, and 11 using metabolic cages. Proteins were extracted from urine samples of mice #1-4 and digested with trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA). Peptides were desalted using HLB columns (Waters, Milford, MA) and dried in 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 all samples and fractionated using a high pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific). After drying, fractions were resuspended in 0.1% formic acid for database construction. iRT standards (Biognosys) were added to all samples for retention time alignment.

### 2.4 LC-MS/MS Tandem Mass Spectrometry Analysis

**(1) Data-Dependent Acquisition (DDA) for Database Generation**
Ten fractionated samples were analyzed using an EASY-nLC 1200 UHPLC system coupled to an Orbitrap Fusion Lumos mass spectrometer. Peptides dis-

solved 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. The iRT calibration kit (Biognosys, Switzerland) was used at 1:20 v/v in all samples for automated, sensitive signal processing. DDA-MS analysis of the 10 fractions was performed with the following parameters: spray voltage 2.4 kV, Orbitrap MS1 resolution 60,000, scan range 350-1550 m/z, MS2 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 using Proteome Discoverer 2.1 (Thermo Fisher Scientific) for database searching.

**(2) DIA Data Acquisition for Experimental Samples**
Thirty-six experimental samples were analyzed in DIA-MS mode with identical LC parameters. MS parameters were as follows: MS1 scan at 60,000 resolution, 350-1550 m/z; MS2 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 DIA acquisition window width.

### 2.5 Mass Spectrometry Data Analysis

Spectronaut X software was used to process and analyze mass spectrometry data. DIA raw files were imported for library searching. High-confidence proteins were defined by peptide q-value $< 0.01$, with protein quantification based on the summed peak areas of all fragment ions from secondary peptides.

### 2.6 Statistical Analysis

For each mouse, data from weeks 1, 3, 4, 6, 7, 9, 10, and 11 were compared to week 0 using independent samples t-test. Differential proteins were filtered using fold change criteria ($FC \geq 1.5$ or $FC \leq 0.67$) and $P < 0.05$.

### 2.7 Functional Analysis of Differential Proteins

Differential proteins were subjected to functional enrichment analysis using the DAVID database (https://david.ncifcrf.gov/) with a significance threshold of $P < 0.05$.

# 3 Results

## 3.1 Pathological Analysis

**(1) Week 1**
Experimental mice #5 and #6 and control mouse #1 were euthanized. No obvious pathological changes were observed in the distal colon segments of either experimental or control mice. Histological examination revealed clear fold structures, intact mucosal epithelium with normal columnar epithelial cells, abundant intestinal glands in the lamina propria with numerous goblet cells, uniformly stained muscular layers with normal, regularly arranged muscle fibers, and no significant inflammation [FIGURE:5-7].

**(2) Week 4**
Experimental mice #7 and #8 and control mouse #2 were euthanized. While experimental mouse #7 and control mice showed no obvious pathological changes, mouse #8 exhibited initial inflammatory lesions. Histology showed locally reduced intestinal glands in the mucosal layer replaced by hyperplastic connective tissue (black arrows) with mild lymphocytic infiltration (yellow arrows) and partial inflammatory cell infiltration into the submucosa (red arrows) [FIGURE:8-10].

**(3) Week 7**
Experimental mice #9 and #10 and control mouse #3 were euthanized. Control mice showed no obvious pathological changes, while experimental mouse #9 showed initial inflammatory lesions and mouse #10 showed severe inflammation. Histological findings included locally reduced intestinal glands replaced by hyperplastic connective tissue with mild lymphocytic infiltration in mouse #9, and multiple areas of reduced glands with hyperplastic connective tissue replacement, mild lymphocytic infiltration, local edema, loose connective tissue arrangement, and widened gland spacing in mouse #10 [FIGURE:11-13].

**(4) Week 11**
At the end of the 11-week induction period, the four experimental mice with complete time-point data showed varying degrees of pathological changes in the distal colon. Histology revealed multiple mucosal ulcers with destroyed intestinal epithelium, necrotic and dissolved glands in the lamina propria replaced by hyperplastic granulation tissue (black arrows), abundant lymphocyte and neutrophil infiltration in the lamina propria (red arrows) with mild submucosal infiltration, and substantial squamous cell carcinoma with high nuclear-cytoplasmic ratios and rare mitotic figures (yellow arrows) in mouse #1. Mouse #2 showed large amounts of squamous cell carcinoma with high nuclear-cytoplasmic ratios and frequent mitoses (black arrows), mild lymphocytic infiltration, local gland necrosis replaced by granulation tissue (red arrows), surrounding edema with loose connective tissue, and abundant lymphocyte and plasma cell infiltration. Mouse #3 exhibited similar carcinoma features, while mouse #4 showed multiple dilated intestinal glands in the mucosal layer (black arrows) and mild lymphocytic infiltration at the base of the lamina propria and submucosa (yellow

arrows) [FIGURE:14-17].

### 3.2 Urine Proteome Change Analysis

Pathological results indicated that the inflammatory and carcinogenic processes varied among the 10 mice during the 11-week AOM/DSS induction. Since urine responds sensitively to disease-induced changes, comparing the four mice as a group against controls might mask genuine changes due to large inter-individual variation. Therefore, we employed a single-mouse longitudinal approach, analyzing urine from the four experimental mice collected at weeks 0, 1, 3, 4, 6, 7, 9, 10, and 11, comparing proteomic profiles at each time point to week 0 (pre-AOM injection) to identify consistent urine proteome signatures predictive of the inflammation-to-cancer progression.

The number of differential proteins identified at each time point for each mouse is shown in , with all differential proteins listed in Supplementary Table 1. Functional enrichment analysis of differential proteins from mouse #1 at each time point using DAVID revealed significantly altered biological pathways (Supplementary Table 2). Acute phase response was enriched at weeks 1, 3, 4, 6, 7, 9, 10, and 11. Drug-induced inflammatory activation initiates a complex systemic early defense system, triggering soluble mediators such as cytokines, acute phase proteins, and chemokines that promote neutrophil and macrophage migration to inflammatory sites[14]. Other significantly altered pathways included: (1) Cell adhesion, which plays a crucial role in controlling colonic transepithelial permeability and is involved in thrombosis, wound healing, and cell-matrix interactions during inflammation, particularly in the inflammatory tumor microenvironment during tumorigenesis[15,16]; (2) Immune-related pathways, which emerged prominently at week 9, including positive regulation of phagocytosis[17], innate immunity[18], positive regulation of B cell activation[19], and classical complement activation[20], confirming that inflammation-induced CRC can activate the immune system; (3) Key signaling pathways such as Notch signaling, which mediates direct cell-cell interactions and whose abnormal activation in CRC cells promotes tumorigenesis and correlates with disease severity[21,22], and positive regulation of protein kinase B (PKB) signaling, which plays a vital role in PI3K-mediated tumorigenesis and is central to epithelial-mesenchymal transition required for cancer cell invasion and migration[23-25]; (4) Oxidative stress response, which is critical in tumor metastasis, epithelial-mesenchymal transition, and tumor microenvironment formation, with redox changes in signaling pathways and transcription factors potentially initiating CRC and causing drug resistance[26]; (5) Glucose metabolism pathways, which are simultaneously perturbed during tumor growth[27]; (6) Proteolysis, associated with drug-induced intestinal inflammation[28]; and (7) Circadian rhythm, which plays an important role in gastrointestinal physiology, with circadian-related proteins frequently altered in colonic malignancies affecting tumor cell phenotype and cancer progression[29].

Functional enrichment analysis for mouse #2 (Supplementary Table 3) similarly

showed acute phase response enrichment at weeks 3, 4, 7, 9, 10, and 11[14], confirming inflammation-induced damage. Mouse #2 also exhibited significant changes in cell adhesion[15,16], immune system activation and regulation[17-20], positive regulation of PKB signaling[23], oxidative stress response[26], glucose metabolism regulation[27], and proteolysis[28]. Additionally, mouse #2 showed enrichment of cytokine response pathways at weeks 1, 3, 4, 7, 9, 10, and 11, with cytokine networks in intestinal cells being key mediators of tissue homeostasis, inflammation, and tumorigenesis. Cytokines such as TNF and IL-6 are classical core participants in CRC[30]. At week 10, response to interleukin-1 emerged; IL-1$\alpha$ and IL-1$\beta$, primarily derived from macrophages and monocytes, are more strongly associated with acute and chronic inflammation than any other cytokine family[31]. N-linked protein glycosylation appeared at weeks 3, 4, 6, 9, 10, and 11, with abnormal protein glycosylation potentially serving as a prognostic tool for CRC[32].

Mouse #3 showed enrichment of acute phase response at weeks 3, 4, and 9 only (Supplementary Table 4), with fewer time points compared to other mice. However, mouse #3 still exhibited significant changes in cell adhesion[15,16], immune system processes and regulation[17-20], positive regulation of PKB signaling[23], glucose metabolism regulation[27], and proteolysis[28], though overall differential proteins and enriched pathways were fewer.

Mouse #4 showed acute phase response enrichment at all time points (weeks 1, 3, 4, 6, 7, 9, 10, and 11), indicating persistent inflammatory responses throughout induction. Notably, immune-related pathways were more abundant and appeared earlier in mouse #4 than in the other three mice, emerging as early as week 3 with classical complement activation[20], immune system processes[17], innate immune response[18], positive regulation of B cell activation[19], and cytokine response[30], while Notch signaling appeared only at week 11[21,22]. Oxidative stress response[26] and circadian rhythm pathways[29] were also significantly altered.

Overall, mice #1-4 all showed enrichment of acute phase response[14], cell adhesion[15,16], immune system activation and regulation[18-20], positive regulation of PKB signaling[23], glucose metabolism regulation[27], and proteolysis[28] during induction. Common biological processes enriched in three of the four mice included oxidative stress response[26], phagocytosis[17], classical complement activation pathway[20], inflammatory response, cytokine response[30], cellular response to interleukin-1[31], circadian rhythm pathway[29], and N-linked protein glycosylation[32].

These results demonstrate that: (1) urine proteome changes reflect pathophysiological alterations in experimental mice, with common pathways across the four mice including acute phase response, cell adhesion, phagocytosis, positive regulation of PKB signaling, positive regulation of B cell activation, glucose metabolism-related pathways, and proteolysis; (2) disease progression differed among experimental mice, with urine proteome profiles varying at different stages. Compared to transplantation models, we found that immune-related

pathways also appear in induced models. Notably, even in genetically similar, co-housed model animals, individual differences exist in carcinogen-induced CRC, suggesting that inter-individual variation in human CRC is even more substantial. This implies that group analysis of samples may mask individual differences in biomarker research, while single-subject analysis better reflects precision and personalized detection approaches. If applied clinically, combined with clinical biobanking, single-subject analysis represents a future direction for disease analysis.

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**Author Contributions**

Heng Ziqi and Gao Youhe: conceived the study and designed the research; Heng Ziqi: performed experiments; Heng Ziqi: analyzed data; Heng Ziqi: drafted the manuscript; Gao Youhe: revised the final manuscript.

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