One-to-Many Urine Proteome Comparison Provides Clues for Fever of Unknown Origin

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Abstract

Fever of unknown origin (FUO) refers to fever without identifiable etiology. Despite continuous advancements in medical diagnostic technologies, FUO remains a pressing clinical challenge. Urine serves as a route for excreting metabolic waste from the organism. Unaffected by homeostatic regulatory mechanisms, urine can reflect subtle physiological changes earlier and with greater sensitivity than blood. In this study, we employed a one-to-many urine proteome comparison approach on collected urine samples—specifically, a comparative analysis method contrasting one patient sample against a cohort of healthy controls—to identify disease-associated biological pathways through differential proteins, thereby providing clues and evidence for the clinical diagnosis of FUO. The results demonstrated that biological pathways enriched in differential proteins were associated with fever, and that the urine proteome could effectively differentiate patients from healthy individuals. Moreover, the one-to-many research methodology can offer personalized insights for FUO patients and represents a promising approach for investigating unknown diseases in patients.

Full Text

Using One-to-Many Urine Proteome Comparison to Provide Clues for Fever of Unknown Origin

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Abstract:

[**Objective**] To provide diagnostic evidence and clues for patients with fever of unknown origin (FUO) through urine proteomics analysis.

[**Methods**] Urine samples from FUO patients were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a one-to-many analytical approach, comparing each individual patient against a group of healthy controls to identify differential proteins and their associated biological pathways. One-to-many analysis refers to comparative analysis of one sample against multiple controls.

[**Results**] Biological pathways related to fever were observed in urine samples, including LXR/RXR activation, FXR/RXR activation, and acute phase response signaling, demonstrating that urine can clearly distinguish disease from health status. Meanwhile, each patient' s analysis yielded different results, highlighting the necessity of one-to-many analysis.

[**Conclusions**] The combination of urine proteomics and one-to-many analysis can provide clues for FUO and may also be applied to the exploration of any unknown disease.

Keywords: proteomics; urine; fever of unknown origin

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0 Introduction

Fever is a common clinical manifestation in medicine. Since the concept of fever of unknown origin (FUO) was first proposed in 1961 [1], it has remained a challenging problem in medical diagnosis for the past 60 years. FUO is defined as an illness lasting more than three weeks with multiple temperature elevations above 38.3°C, where repeated investigations over at least one week fail to establish a diagnosis.

According to literature reports, more than 200 different etiologies can cause fever, including bacterial or viral infections and neoplastic diseases [2]. Despite continuous advancements in diagnostic technologies, uncertainties persist in establishing definitive diagnoses for febrile patients. Without identifying the causative agent, targeted treatment is impossible, while indiscriminate medication may delay proper management and waste medical resources. Given these

challenges, developing novel diagnostic approaches that can provide clues and facilitate diagnosis and verification for febrile patients represents an important task.

Urine serves as a pathway for excreting metabolic waste from the body. Without homeostatic regulatory mechanisms, urine can reflect subtle physiological changes earlier and more sensitively than blood [3]. Numerous studies have demonstrated that urine is an excellent source of disease biomarkers. For instance, in a Walker-256 tumor-bearing rat model established by subcutaneous injection of cancer cells, urine proteins were found to change with tumor growth [4]. Additionally, studies using tail-vein injection of Walker-256 cells revealed that urine proteomes differ when tumors grow in different organs [5], demonstrating the diagnostic discrimination capability of urine. Furthermore, research in a drug-induced chronic pancreatitis rat model showed that pancreatitis-related urine proteins changed significantly before pathological changes became visually apparent [6], reflecting the early and highly sensitive nature of urine. These findings provide a basis for early diagnosis using urine.

In this study, we employed a one-to-many analysis approach, comparing each individual patient against a group of healthy controls. We argue that for unknown diseases, it is difficult to group patients for analysis because disease uncertainty prevents us from establishing definitive criteria for patient grouping. In clinical practice, physicians typically assess disease status based on how an individual differs from the general population—in other words, comparing one person against all normal individuals, where deviations from the normal range suggest possible disease. Although normal individuals exhibit variations, these fluctuations remain within a normal range. Through internal normalization, parameters closer to the truly normal state can be obtained. When multiple proteins in an unknown disease patient show differences from this parameter, we can identify abnormal biological processes or even damaged organs through bioinformatics analysis of these differential proteins, thereby inferring the nature of the unknown disease. Therefore, we performed label-free quantitative proteomics analysis on urine from each FUO patient, aiming to identify disease-related biological pathways through differential proteins and provide clues and evidence for clinical diagnosis of FUO.

1.1 Patient Sample Collection

A total of 13 urine samples were collected from 13 febrile patients admitted to Beijing China-Japan Friendship Hospital, all of which were morning urine specimens. This study was based on the reuse of discarded samples from the clinical laboratory, a process that did not involve any patient identity information. We did not influence any patient treatment nor recommend any clinical or auxiliary examinations. All knowledge generated from this study remains at the research stage for obtaining clues about FUO only.

Two urine samples (Nos. 1 and 2) were excluded due to abnormal test results

and clinically verified proteinuria, which did not meet our sampling criteria. All patients had fever at admission (body temperature $ $37.3°C). Patient temperature records on the day of urine collection are shown in Table 1 . All participants signed informed consent forms, and the study was approved by the Ethics Committee of China-Japan Friendship Hospital (Approval No.: 2019-42-K30).

1.2 Urine Sample Processing

Urine Protein Extraction: Collected urine samples were stored at -80°C until use. For protein extraction, 4 mL of urine sample was thawed and centrifuged at 12,000×g for 20 minutes at 4°C to remove cellular debris. The supernatant was mixed with 20 mM dithiothreitol (DTT, Sigma) and heated in a metal bath at 99.2°C for 10 minutes. After cooling to room temperature, 50 mM iodoacetamide (IAA, Sigma) was added, mixed well, and incubated at room temperature in the dark for 40 minutes. Following the reaction, samples were transferred to new centrifuge tubes and mixed thoroughly with four volumes of pre-cooled absolute ethanol, then placed at -20°C for 24 hours to precipitate proteins. After precipitation, samples were centrifuged at 10,000×g for 30 minutes at 4°C, the supernatant was discarded, and the protein pellet was air-dried. The dried protein pellet was resuspended in appropriate lysis buffer (8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, and 25 mmol/L DTT). After centrifugation, the supernatant was retained and protein concentration was determined using the Bradford method.

Urine Protein Digestion: The FASP method was used for on-membrane digestion of urine proteins [7]. A total of 100 g of urine protein was loaded onto a 10 kD ultrafiltration tube (Pall, Port Washington, NY, USA). The membrane was washed twice with UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) and 25 mmol/L NH_4HCO_3 solution. Trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio for digestion and incubated overnight at 37°C. After overnight incubation, the digested filtrate was collected by centrifugation as the peptide mixture. The collected peptide mixture was desalted using Oasis HLB solid-phase extraction columns (Waters, Milford, MA) and vacuum-dried before storage at -80°C.

1.3 LC-MS/MS Tandem Mass Spectrometry Analysis

Peptides were resuspended in 0.1% formic acid, and peptide concentration was determined using a BCA assay kit. Peptide concentration was diluted to 0.5 g/ L. One microgram of peptide sample was separated using a Thermo EASY-NLC1200 liquid chromatography system with the following parameters: 90-minute elution time with a gradient of mobile phase A (0.1% formic acid) and mobile phase B (80% acetonitrile). Eluted peptides were detected using an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) in data-independent acquisition mode.

1.4 Data Analysis

Raw data files from the mass spectrometer were converted to mgf format using Proteome Discoverer 2.1 software (Thermo Fisher Scientific, USA) and imported into Mascot software for database searching. Database: Swissprot; Species: Homo sapiens (Human). Mass spectrometry data were searched against proteins with the following parameters: trypsin digestion allowing 2 missed cleavages; cysteine carbamidomethylation as fixed modification; methionine oxidation and protein N-terminal acetylation as variable modifications; mass tolerance of 0.05 Da for both precursor and fragment ions; precursor ion mass tolerance of 10 ppm. After searching, dat format files were exported and protein quality control and scoring were performed using Scaffold software with parameters set as: protein FDR 1%, peptide FDR 1%, at least 2 unique peptides per protein, normalization by total spectral counts, and subsequent analysis based on normalized secondary spectral counts.

1.5 Statistical Analysis

We applied spectral counting for quantitative screening of differential proteins. Screening criteria: fold change $ $2 or $ $0.5, paired t-test P-value after correction <0.05. Subsequent functional analysis of differential proteins was performed through Ingenuity Pathway Analysis software and literature searches in the Pubmed database.

2.1 Individual Patient Urine Proteome Analysis

(1) Patient F1 Urine Proteome Analysis

Comparison of Patient F1' s sample with the 8 healthy control samples identified 108 differential proteins, including 85 upregulated and 23 downregulated proteins. Differential proteins were screened using criteria of fold change (FC) $ $2 or $ $0.5 and P<0.05. Detailed information on these proteins is provided in Supplementary Table 1.

Ingenuity Pathway Analysis of the differential proteins revealed canonical pathways shown in Table 2 , with P<0.05 considered significant. Enriched pathways included LXR/RXR activation, acute phase response signaling, antigen presentation, FXR/RXR activation, phagosome maturation, folate polyglutamylation, serine and glycine biosynthesis, sucrose degradation, and protein ubiquitination. According to literature reports, acute phase response signaling is associated with fever [8]; LXR/RXR (liver X receptor/retinoid X receptor) and FXR/RXR (farnesoid X receptor/retinoid X receptor) are typically involved in biological and pathological pathways regulating glucose and lipid homeostasis and inflammatory responses [9]; both antigen presentation and phagosome maturation are closely related to immune processes. Additionally, Patient F3 was in an acute febrile state (temperature 38.8°C) at the time of sample collection. These results demonstrate that changes reflected in urine proteins are consistent with

clinical manifestations, and we can identify clues about fever from urine.

(2) Patient F2 Urine Proteome Analysis

Comparison of Patient F2' s sample with the 8 healthy control samples identified 174 differential proteins, including 131 upregulated and 43 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 3 , with P<0.05 considered significant. Enriched pathways included phagosome maturation, antigen presentation, LXR/RXR activation, PD-1/PD-L1 cancer immunotherapy pathway, Th1 and Th2 activation pathways, FXR/RXR activation, acute phase response signaling, virus entry via endocytic pathways, NK cell signaling, and B cell development. In addition to inflammation-related pathways mentioned previously, Patient F2 showed more virus- and immune-related signals, such as NK cell and B cell signaling pathways, and virus entry via endocytosis, suggesting that Patient F2' s fever was likely caused by viral invasion. Subsequent searches for viral sequences did not identify a clear pathogenic virus, requiring further investigation.

(3) Patient F3 Urine Proteome Analysis

Comparison of Patient F3's sample with the 8 healthy control samples identified 65 differential proteins, including 50 upregulated and 15 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 4 , with P<0.05 considered significant. Enriched pathways included acute phase response signaling, LXR/RXR activation, FXR/RXR activation, complement system, phagosome maturation, airway pathology in chronic obstructive pulmonary disease, bladder cancer signaling, inhibition of matrix metalloproteases, atherosclerosis signaling, uracil degradation, thymine degradation, thyroid hormone biosynthesis, ovarian cancer signaling, and tumor microenvironment. Again, the most significant pathways were inflammation-related.

(4) Patient F4 Urine Proteome Analysis

Comparison of Patient F4' s sample with the 8 healthy control samples identified 440 differential proteins, including 279 upregulated and 161 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 5 , with P<0.05 considered significant. Enriched pathways included clathrin-mediated endocytosis signaling, epithelial adherens junction signaling, epithelial adherens

junction remodeling, BAG2 signaling pathway, synaptogenesis signaling pathway, reelin signaling in neurons, glycolysis, and the same acute inflammation-related pathways observed in F1, F2, and F3 samples.

(5) Patient F5 Urine Proteome Analysis

Comparison of Patient F5' s sample with the 8 healthy control samples identified 184 differential proteins, including 143 upregulated and 41 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 6 , with P<0.05 considered significant. Enriched pathways included phagosome maturation, chondroitin sulfate degradation, ephrin receptor signaling, iron homeostasis signaling pathway, role of MAPK signaling in promoting influenza pathogenesis, sphingosine-1-phosphate signaling, axonal guidance signaling, and Rho signaling.

(6) Patient F6 Urine Proteome Analysis

Comparison of Patient F6' s sample with the 8 healthy control samples identified 456 differential proteins, including 298 upregulated and 158 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 7 , with P<0.05 considered significant. Enriched pathways included LXR/RXR activation, FXR/RXR activation, phagosome maturation, acute phase response signaling, coagulation system, antigen presentation pathway, intrinsic prothrombin activation pathway, hepatic fibrosis/hepatic stellate cell activation, crosstalk between dendritic cells and natural killer cells, neuroprotective role of THOP1 in Alzheimer' s disease, extrinsic prothrombin activation pathway, Th2 pathway, Th1 and Th2 activation pathways, mechanisms of viral exit from host cells, atherosclerosis signaling, dendritic cell maturation, and B cell development.

(7) Patient F7 Urine Proteome Analysis

Comparison of Patient F7' s sample with the 8 healthy control samples identified 170 differential proteins, including 153 upregulated and 17 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 8 , with P<0.05 considered significant. Enriched pathways included phagosome maturation, LXR/RXR activation, FXR/RXR activation, lactose degradation, phagosome formation, atherosclerosis signaling, clathrin-mediated endocytosis signaling, IL-12 signaling and production in macrophages, PD-1/PD-L1 cancer immunotherapy pathway, Th1 and Th2 activation pathway, sphingosine-

1-phosphate signaling, vitamin C transport pathway, remodeling of epithelial adherens junctions, production of nitric oxide and reactive oxygen species in macrophages, caveolar-mediated endocytosis signaling, and L-carnitine biosynthesis.

(8) Patient F8 Urine Proteome Analysis

Comparison of Patient F8' s sample with the 8 healthy control samples identified 563 differential proteins, including 460 upregulated and 103 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 9 , with P<0.05 considered significant. Enriched pathways included hepatic fibrosis/hepatic stellate cell activation, LXR/RXR activation, phagosome maturation, intrinsic prothrombin activation pathway, atherosclerosis signaling, acute phase response signaling, iron homeostasis signaling pathway, NK cell signaling, apelin liver signaling pathway, antigen presentation pathway, FXR/RXR activation, Th1 and Th2 activation pathways, caveolar-mediated endocytosis signaling, Th2 pathway, complement system, and others.

(9) Patient F9 Urine Proteome Analysis

Comparison of Patient F9's sample with the 8 healthy control samples identified 36 differential proteins, including 32 upregulated and 4 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 10 , with P<0.05 considered significant. Enriched pathways included primary immunodeficiency signaling, ovarian cancer signaling, B cell development, autoimmune thyroid disease signaling, communication between innate and adaptive immune cells, neuroprotective role of THOP1 in Alzheimer' s disease, superoxide radicals degradation, dendritic cell maturation, role of NFAT in regulation of immune response, systemic lupus erythematosus signaling, relaxin signaling, glutaryl-CoA degradation, acute phase response signaling, GNRH signaling, B cell receptor signaling, role of JAK1, JAK2 and TYK2 in interferon signaling, and NRF2-mediated oxidative stress response.

(10) Patient F10 Urine Proteome Analysis

Comparison of Patient F10' s sample with the 8 healthy control samples identified 409 differential proteins, including 272 upregulated and 137 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 11 , with P<0.05 considered significant. Enriched pathways included LXR/RXR ac-

tivation, FXR/RXR activation, acute phase response signaling, phagosome maturation, antigen presentation pathway, extrinsic prothrombin activation pathway, mechanisms of viral exit from host cells, ascorbate recycling, Wnt signaling pathway, atherosclerosis signaling, caveolar-mediated endocytosis signaling, and NK cell signaling.

(11) Patient F11 Urine Proteome Analysis

Comparison of Patient F11' s sample with the 8 healthy control samples identified 294 differential proteins, including 217 upregulated and 77 downregulated proteins. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05. Detailed information is provided in Supplementary Table 1.

Ingenuity Pathway Analysis revealed canonical pathways shown in Table 12 , with P<0.05 considered significant. Enriched pathways included acute phase response signaling, antigen presentation pathway, neuroprotective role of THOP1 in Alzheimer' s disease, FAT10 signaling pathway, PD-1/PD-L1 cancer immunotherapy pathway, polyamine regulation in colon cancer, BAG2 signaling pathway, protein ubiquitination pathway, phagosome maturation, LXR/RXR activation, B cell development, inhibition of ARE-mediated mRNA degradation pathway, Th2 pathway, neuroinflammation signaling pathway, role of IL-17A in psoriasis, and complement system.

2.2 Group Analysis of Patient Urine Proteomes

A total of 11 patient and 8 healthy control urine samples were collected for mass spectrometry analysis, identifying 2,812 proteins (FDR <1%) with at least two unique peptides required for protein identification. After screening, 129 differential proteins were identified, including 20 upregulated and 109 downregulated proteins. Detailed information is provided in Supplementary Table 1. Differential proteins were screened using criteria of FC$ $2 or $ $0.5 and P<0.05.

Ingenuity Pathway Analysis was performed on the differential proteins, with P<0.05 considered significant. As shown in Table 12, enriched pathways included STAT3 signaling pathway, SPINK1 pancreatic cancer pathway, interleukin-15 production, and phagosome maturation. Literature reports indicate that the STAT3 signaling pathway is closely related to autophagy [10], while interleukin-15 and phagosome maturation are integral to the immune system.

Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed on all proteins. The OPLS-DA score plot is shown in Figure 1 [Figure 1: see original paper]. The healthy and patient groups showed clear separation, indicating that urine proteins can reflect disease status differences. However, substantial intragroup variation was observed among patients, demonstrating significant individual differences in clinical samples. Comparing the IPA results from individual and group analyses reveals that group analysis masks individ-

ual uniqueness, providing theoretical support for the importance of individual analysis.

2.3 Comparison Between Individual and Group Analysis

By comparing one-to-many and group analysis approaches, we observed that group analysis results cannot represent the characteristics of any individual FUO patient. Group analysis only reveals commonalities among FUO patients, but each FUO patient is an independent case that cannot be grouped for analysis based solely on fever symptoms. Moreover, the analysis results for all 11 FUO patients were different. Notably, both one-to-many and group analyses revealed significant differences between FUO patients and healthy controls in urine, providing a theoretical basis for clinical diagnosis using urine. This further suggests that for specific patients, having a control group of normal individuals matched for age, sex, and medication history would be more appropriate than using the same control group for everyone. Comparison with a properly matched control group should yield more accurate results.

3 Conclusion

In this study, we used LC-MS/MS label-free quantitative methods to screen for differential urine proteins in 11 individual FUO samples and analyze their biological pathways. We found that urine proteomics can distinguish patients from healthy individuals.

Furthermore, this study proposes that the one-to-many approach can provide personalized clues for FUO patients. Individual differences in clinical samples are easily masked by group analysis, whereas one-to-many analysis better highlights patient individuality and represents a promising method for exploring clues to unknown diseases in the future.

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Author Contribution Statement

indicates co-first authors

Lilong Wei, Chenyang Zhao, Youhe Gao: Sample provision, research concept, study design
Chenyang Zhao, Jing Wei: Experiment execution
Chenyang Zhao, Jing Wei: Data analysis
Chenyang Zhao: Manuscript drafting
Youhe Gao: Final manuscript revision

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