Analysis of Post-translational Modifications in the Urinary Proteome of Patients with Metabolic Associated Fatty Liver Disease

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Abstract

Metabolic Associated Fatty Liver Disease (MAFLD) is a chronic liver disease with high global prevalence, and its progression poses significant hazards. Based on publicly available raw urine proteomics data, this study conducted a comparative analysis of differential characteristics of urine protein post-translational modifications (PTMs) among healthy control group, mild hepatic steatosis group (MRI-PDFF 5%–10%), and severe hepatic steatosis group (MRI-PDFF $>$ 10%). The results revealed that a total of 281 differential modifications were identified between the mild and healthy groups, 445 differential modifications between the severe and healthy groups, and 181 differential modifications between the mild and severe groups. Many proteins exhibiting differential modifications have been reported to play functional roles or undergo alterations in MAFLD, with six of these proteins simultaneously showing changes in both expression levels and modification status across mild and severe groups. The findings demonstrate that urine proteome PTMs in patients with mild and severe hepatic steatosis differ from those of healthy individuals, offering a novel perspective for MAFLD diagnosis and mechanistic investigation.

Full Text

Analysis of Post-Translational Modifications in the Urinary Proteome of Patients with Metabolic-Associated Fatty Liver Disease (MAFLD)

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Abstract

Metabolic-associated fatty liver disease (MAFLD) is a highly prevalent chronic liver disease worldwide, and its progression poses substantial risks. Based on publicly available raw urinary proteome data, this study comparatively analyzed the differential characteristics of post-translational modifications (PTMs) in urinary proteins among healthy controls, mild hepatic steatosis group (MRI-PDFF 5%–10%), and severe hepatic steatosis group (MRI-PDFF > 10%). The results showed that a total of 281 differential modifications were identified between the mild steatosis group and the healthy control group, 445 differential modifications between the severe steatosis group and the healthy control group, and 181 differential modifications between the mild and severe steatosis groups. Among these, multiple proteins with differential modifications have been reported to function or undergo changes in MAFLD, and six of these proteins exhibited simultaneous alterations in both expression levels and modification status in both mild and severe steatosis groups. The findings indicate that the urinary proteome PTMs of patients with mild or severe hepatic steatosis differ from those of healthy individuals, providing a novel perspective for the diagnosis and mechanism exploration of MAFLD.

Key words: Metabolic-associated fatty liver disease (MAFLD); Urinary proteome; Post-translational modifications (PTMs)

Introduction

Metabolic-associated fatty liver disease (MAFLD) is the most prevalent chronic liver disease globally, defined as hepatic lipid accumulation exceeding 5% of liver weight in the absence of alcohol abuse, viral hepatitis, or other etiologies, and directly associated with metabolic abnormalities such as obesity, insulin resistance, or type 2 diabetes [1]. Since its first identification in 1987, the global prevalence of MAFLD has risen dramatically, currently affecting approximately 25% of the adult population worldwide [2]. From 1990 to 2021, the disease burden among adolescents and young adults increased significantly, with the estimated global prevalence in the 15-39 age group rising by 75.31% compared to 1990, representing a major public health challenge [3].

MAFLD progression carries substantial risks, advancing from simple hepatic steatosis to non-alcoholic steatohepatitis, and further to liver fibrosis, cirrhosis, and even increased risk of hepatocellular carcinoma, while also being closely associated with extrahepatic complications [4]. Beyond the liver, MAFLD affects multiple organ systems and regulatory mechanisms, contributing to increased cardiovascular disease, cardiac complications, and chronic kidney disease [5]. The primary lipid abnormality in MAFLD is elevated triglycerides, with pathological mechanisms involving multi-stage dysregulation including hepatic lipid metabolism imbalance, oxidative stress activation, and increased reactive oxygen species production leading to hepatocyte injury [6].

Urine is not regulated by homeostatic mechanisms, enabling it to more sensi-

tively capture dynamic changes in physiological and pathological states. Urinary proteomics offers distinct advantages through non-invasive sampling, continuous monitoring capabilities, and enrichment of low-molecular-weight proteins and peptides, providing crucial technical support for systematic analysis of physiological and pathological processes [7]. In recent years, advances in liquid chromatography-mass spectrometry-based proteomics have enabled high-precision identification of low-abundance modified proteins in urine [8]. Protein post-translational modifications (PTMs) refer to the enzymatic addition of chemical groups following protein synthesis, with common types including phosphorylation, acetylation, methylation, and ubiquitination. These modifications regulate critical biological processes such as cell signaling transduction and metabolic pathway activation by altering protein structure, activity, or localization [9]. As key molecular mechanisms regulating cellular function, PTMs play important regulatory roles in the pathological processes of many diseases. In MAFLD, PTMs abnormalities have been confirmed to be closely associated with disease progression. For example, phosphorylation of AMP-activated protein kinase (AMPK) at Thr172 activates lipid oxidation and inhibits lipogenesis [10]. Conversely, phosphorylated NF-kB drives inflammatory cytokine production, exacerbating steatosis and fibrosis [11]. Acetylation enhances the DNA-binding affinity of sterol regulatory element-binding protein-1c (SREBP1c) for lipid biosynthesis genes, leading to triglyceride accumulation [12]. However, systematic studies on urinary proteome PTMs in MAFLD patients remain limited.

Currently, MAFLD screening and severity assessment primarily rely on serological markers and imaging methods. Magnetic resonance imaging proton density fat fraction (MRI-PDFF), as an emerging quantitative imaging technique, can accurately and precisely assess whole-liver triglyceride content and steatosis degree, and has been adopted as an endpoint indicator in multiple early clinical trials and observational studies [13]. Clinically, MRI-PDFF values between 5% and 10% are typically defined as mild hepatic steatosis, while values >10% define severe steatosis, which also serves as an inclusion criterion for interventional clinical trials [14].

Therefore, this study investigated differences in urinary proteome PTMs between MAFLD patients classified by MRI-PDFF as mild or severe hepatic steatosis and healthy individuals, to explore protein modification changes at different disease stages and provide novel perspectives for MAFLD diagnosis and related mechanisms.

2.1 Data Sources and Sample Information

The raw urinary proteome data for MAFLD and healthy groups in this study were derived from a cross-sectional study conducted at West China Hospital of Sichuan University and stored in the ProteomeXchange Consortium database (accession: PXD026333). The original study recruited 57 participants; this study utilized 27 urine samples from the discovery set for in-depth analysis. All participants were divided into three groups based on MRI-PDFF results: healthy

control group (n=7), mild hepatic steatosis group (MRI-PDFF 5%-10%, n=8), and severe hepatic steatosis group (MRI-PDFF > 10%, n=12). MAFLD diagnosis strictly followed international guidelines, and all participants were excluded for other liver diseases, renal insufficiency, cancer, or other comorbidities that could confound results.

2.2 Sample Preparation and Mass Spectrometry Acquisition

The sample processing and mass spectrometry acquisition workflow for the data used in this study is summarized as follows: Urine samples were preprocessed by centrifuging midstream morning urine to remove debris, followed by concentration, reduction, and alkylation using 10 kDa ultrafiltration tubes, and dual enzymatic digestion with trypsin and Lys-C. For LC-MS analysis, digested peptides were separated using an EASY-nLC 1200 liquid chromatography system and analyzed by data-independent acquisition (DIA) on an Orbitrap Exploris 480 mass spectrometer equipped with a FAIMS interface. The raw mass spectrometry data had undergone quality control by the original research team.

2.3 Open PTM Search with pFind

pFind Studio software (version 3.2.1, Institute of Computing Technology, Chinese Academy of Sciences) was used for unrestricted modification searching of each sample' s raw mass spectrometry data with default parameters. The database was the *Rattus norvegicus* protein database downloaded from UniProt (https://www.uniprot.org) with version date September 2024. Instrument type was set to HCD-FTMS, enzyme to trypsin, enzyme specificity to full, maximum missed cleavages to 2. Mass error tolerance for both precursor and fragment ions was set to $±$20 ppm, with open search mode. The false discovery rate (FDR) filtering threshold at the peptide level was set to 1%.

2.4 Bioinformatic Analysis of Protein PTMs

Following unrestricted modification searching, modification identification results (PROTEIN files) were obtained for each sample. Python script pFind_{{{protein}}*{{contrast}}}*{script} was acquired from GitHub (https://github.com/daheitu/scripts_{{{for}}*{{pFind3}}}*{protocol}.io) to integrate modification identification information across samples. Differentially modified proteins were then screened by comparing experimental and control groups using the criteria: fold change (FC) $\geq$ 1.5 or $\leq$ 0.67, and P-value $<$ 0.05 by heteroscedastic independent t-test. Proteins containing differential modifications were annotated and functionally queried using the UniProt database, with relevant literature retrieved through PubMed (https://pubmed.ncbi.nlm.nih.gov) for further analysis of potential functions.

3.1 Comparative Analysis of Urinary Proteome PTMs Between Mild Steatosis and Healthy Groups

Comparison of urinary proteome PTMs between the mild hepatic steatosis group and healthy group identified 281 differential modifications across 161 proteins, with details provided in Supplementary Table 1. Among the 82 proteins with modifications showing FC ≥ 10 or ≤ 0.01, literature search in PubMed revealed that 32% of these proteins or their family members have been reported to be associated with MAFLD, hepatic steatosis, or lipid metabolism. Due to space limitations, only selected examples are listed below, with modified proteins and relevant literature detailed in Table 1 .

P19440, Glutathione hydrolase 1 proenzyme (GGT1) (FC=0, P=3.82E-02), has been identified as a protein significantly associated with metabolic dysfunction-associated steatotic liver disease (MASLD) risk. Specifically, elevated GGT1 levels correlate with increased MASLD risk and may serve as an early predictive marker for MASLD development, suggesting its important role in fatty liver pathogenesis [16].

P19652, Alpha-1-acid glycoprotein 2 (FC=0, P=4.65E-02), functions as an acute-phase response protein playing important roles in inflammation and metabolic disorders. Studies have found its levels significantly correlate with hepatic steatosis severity [18].

Q08380, Galectin-3-binding protein (FC=0, P=4.88E-02), shows increased expression in serum and visceral adipose tissue of high-fat diet mice, with high expression in VAT macrophages. Its levels significantly correlate with metabolic syndrome incidence, and increased expression in adipose tissue may be associated with lipid metabolism disorders [21].

Q03154, Aminoacylase-1 (FC=23.63, P=3.54E-02), demonstrates significantly increased expression in NAFLD patients. Research indicates this protein' s increased expression in hepatic lipid droplets may be related to metabolic adjustments and fatty liver development [27].

P07288, Prostate-specific antigen (PSA) (FC=∞, indicating detection in mild steatosis group but not healthy group, P=4.79E-02), shows decreased expression in NAFLD patient livers. PSA deficiency exacerbates diet-induced triglyceride accumulation by enhancing lipogenesis and suppressing fatty acid β-oxidation. Additionally, PSA can reduce oxidative stress and lipid overload by stabilizing NRF2 protein expression and activating the NRF2 signaling pathway [31].

P25311, Zinc-alpha-2-glycoprotein (FC=∞, P=4.92E-02), plays an important role in lipid metabolism and alleviates NAFLD by negatively regulating tumor necrosis factor-α (TNF-α), potentially reducing fat accumulation by inhibiting lipogenic enzymes and thereby regulating body weight [36].

3.2 Comparative Analysis of Urinary Proteome PTMs Between Severe Steatosis and Healthy Groups

Comparison between severe hepatic steatosis and healthy groups identified 445 differential modifications across 183 proteins, with details in Supplementary Table 2. Among 112 proteins with modifications showing FC ≥ 10 or ≤ 0.01, 36% were found to be associated with MAFLD, hepatic steatosis, or lipid metabolism through PubMed literature search.

P07195, L-lactate dehydrogenase B chain (LDHB) (FC=0.08, P=2.45E-02), has acetylation closely associated with NAFLD progression. P300/CBP-associated factor-mediated LDHB K82 acetylation significantly reduces LDHB activity, impairing hepatic lactate clearance and causing lactate accumulation, which exacerbates lipid deposition and inflammatory responses through activation of histone hyperacetylation [44].

P04004, Vitronectin (FC=19.25, P=4.02E-03), deficiency significantly attenuates hepatic fibrosis in a non-alcoholic steatohepatitis (NASH)-induced mouse model [30].

P17900, Ganglioside GM2 activator (FC=∞, P=1.15E-02), can bind and transfer various lipid molecules including gangliosides GM2, GM1, GM3, and at least one phosphatidylglycerol. This protein functions not only as a cofactor for β-hexosaminidase A in GM2 hydrolysis in lysosomes but also as a lipid transport protein inside and outside cells [35].

Q99519, Sialidase-1 (FC=∞, P=2.51E-02), shows significantly increased expression and activity in obese patients or obese mice, with its activity inhibition markedly reducing lipid accumulation in livers of high-fat diet or obese mice [51].

P12821, Angiotensin-converting enzyme (ACE) (FC=∞, P=2.65E-02), research demonstrates that the ACE2/Angiotensin-(1-7)/Mas axis can ameliorate hepatic steatosis by activating the Akt signaling pathway [54].

P02751, Fibronectin (FC=∞, P=3.88E-02), circulating plasma levels correlate with tissue insulin sensitivity and promote obesity-related metabolic disorders. Studies indicate increased fibronectin expression in adipose tissue and liver is associated with insulin resistance and fatty liver development [59].

P02788, Lactotransferrin (FC=∞, P=4.37E-02), improves hepatic lipid metabolism by reducing fatty acid synthesis and increasing lipolysis, significantly ameliorating high-fat, high-cholesterol diet-induced NAFLD in mice [64].

Q8IWU5, Extracellular sulfatase Sulf-2 (FC=∞, P=4.56E-02), in a high-fat, high-cholesterol, high-fructose diet-induced NAFLD mouse model, SULF2 knockout significantly attenuated diet-induced weight gain, dyslipidemia, steatohepatitis, and hepatic fibrosis. SULF2 expression increased significantly in wild-type mice, while knockout mice showed reduced weight gain and dyslipi-

demia, indicating SULF2 plays an important role in NAFLD development and its knockout can significantly mitigate steatohepatitis and fibrosis progression [66].

Q07654, Trefoil factor 3 (FC=∞, P=4.69E-02), can alleviate high-fat diet-induced hepatic steatosis in mice by increasing peroxisome proliferator-activated receptor-α-mediated fatty acid oxidation [68].

Due to space limitations, only selected examples are listed, with modified proteins and relevant literature detailed in Table 2 .

3.3 Comparative Analysis of Urinary Proteome PTMs Between Mild and Severe Steatosis Groups

Comparison between mild and severe hepatic steatosis groups identified 181 differential modifications across 112 proteins, with details in Supplementary Table 3 . Among 54 proteins with modifications showing FC $\geq$ 10 or $\leq$ 0.01, 33% were associated with MAFLD, hepatic steatosis, or lipid metabolism.

P04066, Tissue alpha-L-fucosidase (FC=0, P=3.83E-02), levels positively correlate with metabolic syndrome and its five components—central obesity, hypertriglyceridemia, low HDL cholesterol, hypertension, and elevated fasting glucose. Elevated alpha-L-fucosidase levels represent an independent risk factor for NAFLD and may serve as a potential biomarker [70].

P19835, Bile salt-activated lipase (FC=0, P=4.92E-02), plays a dominant role in neonatal fat digestion and is present in breast milk, facilitating infant fat absorption [72].

Q9HD89, Resistin (FC=0, P=4.99E-02), a cytokine secreted by adipocytes and macrophages, shows increased hepatic expression with liver injury progression and significantly higher serum levels in NAFLD patients compared to controls [73].

P10451, Osteopontin (OPN) (FC=14, P=3.61E-02), macrophage-derived OPN is protective in NASH by regulating inflammatory responses and lipid metabolism to attenuate NASH progression [74].

3.4 PTM Analysis Across Healthy, Mild Steatosis, and Severe Steatosis Groups

Analysis of urinary proteome PTMs across the three comparison groups (healthy vs. mild, healthy vs. severe, mild vs. severe) revealed that key proteins closely associated with MAFLD pathogenesis—including insulin-like growth factor-binding protein complex acid-labile subunit (ALS), galectin-3-binding protein (Gal-3BP), patatin-like phospholipase domain-containing protein 3 (PNPLA3), AMP-activated protein kinase (AMPK), sterol regulatory element-binding protein 1c (SREBP1c), carbohydrate response element-binding protein (ChREBP), and acetyl-CoA carboxylase (ACC)—all exhibited PTMs across

experimental groups, but the modification types differed significantly from previously reported functional PTMs [77]. Specifically, previous studies have confirmed that AMPK Thr172 phosphorylation, SREBP1c acetylation and phosphorylation, ChREBP Lys672 acetylation, and ACC Ser79 phosphorylation directly regulate hepatic lipid metabolism, inflammatory activation, and fibrosis progression [78][79][80][81]. In contrast, our unrestricted modification search revealed that modifications of these proteins primarily involved basic types such as oxidation, dehydration, deamidation, and pyroglutamation, without detecting the functionally relevant PTMs previously reported to be directly involved in MAFLD pathological regulation.

3.5 Comparison of Differentially Expressed Proteins and PTMs

We performed cross-study correlation analysis between differentially expressed proteins from the original literature (screening criteria: $p<0.05$) and differentially modified proteins identified in this study through unrestricted modification searching (screening criteria: FC ≥ 1.5 or ≤ 0.67, $P<0.01$). Venn diagram visualization of the intersection and union between these datasets (Figure 1 [Figure 1: see original paper]) revealed six proteins showing significant changes in both differential expression and modification: Thyroxine-binding globulin, Immunoglobulin heavy constant gamma 2, Peptidase inhibitor 16, Ceruloplasmin, Alpha-1B-glycoprotein, and Alpha-1-acid glycoprotein 1. This suggests these proteins may regulate protein function through PTMs during MAFLD pathogenesis, representing key molecular nodes linking abnormal gene expression to phenotypic alterations. Among them, Thyroxine-binding globulin, Ceruloplasmin, and Alpha-1-acid glycoprotein 1 have been previously implicated in MAFLD and hepatic steatosis [25][82][83].

Figure 1 [Figure 1: see original paper] Venn diagram of differentially expressed proteins from the original literature and differentially modified proteins from this study (Note: DEPs = differentially expressed proteins from original literature; HC-M = differentially modified proteins between mild steatosis and healthy groups; HC-S = differentially modified proteins between severe steatosis and healthy groups; M-S = differentially modified proteins between severe and mild steatosis groups).

This study systematically investigated PTMs differences in urinary proteomes among healthy controls, mild hepatic steatosis, and severe hepatic steatosis MAFLD patients using publicly available urinary proteomics data through unrestricted modification searching and bioinformatic analysis, providing a novel molecular perspective for MAFLD pathological mechanism dissection and diagnosis. Protein concentration changes and PTMs represent complementary information that can characterize subtle, short-term interventions from two distinct dimensions. While the original literature focused on protein expression level differences, this study examined modification status changes, with integrated analysis of both providing additional clues for understanding MAFLD molecular networks.

From the modification profile perspective, our study revealed significant heterogeneity in urinary protein modifications across different MAFLD pathological stages: 281 differential modifications (161 proteins) between mild steatosis and healthy groups, 445 differential modifications (183 proteins) between severe steatosis and healthy groups, and 181 differential modifications (112 proteins) between mild and severe groups. These results suggest that urinary protein PTM changes correlate with MAFLD severity, and numerous differentially modified proteins have been confirmed in previous studies to be directly related to MAFLD, hepatic steatosis, or lipid metabolism, further validating our findings.

Notably, while we identified multiple PTM types in key MAFLD regulatory proteins (e.g., AMPK, SREBP1c), we did not detect the functionally relevant modification sites reported previously. This discrepancy may stem from two factors: first, the unique nature of urine samples, where proteins primarily represent metabolic byproducts or tissue-secreted products whose modification profiles may reflect degradation status or transport processes; second, incomplete functional annotation in the current literature for most PTMs identified in this study in the context of MAFLD, which objectively constrains the depth of specific modification event analysis. These results demonstrate that MAFLD-related core proteins exhibit specific modification signatures in urine samples, and the enrichment of basic modification types may reflect systemic responses to hepatic metabolic dysregulation, offering new leads for exploring non-invasive biomarkers and modification regulatory mechanisms in MAFLD. Future studies should expand sample sizes and incorporate multi-center, longitudinal cohort data to enhance result reliability and clinical translational value.

In summary, this study systematically analyzed differential modification characteristics of urinary proteomes from healthy individuals, mild hepatic steatosis, and severe hepatic steatosis MAFLD patients from a PTMs perspective, identifying numerous proteins with altered PTMs associated with MAFLD, providing novel clues for disease diagnosis and mechanism exploration.

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