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RESEARCH

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Lysosome-related proteins may have changes in the urinary exosomes of patients with acute gout attack

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Abstract

Background The autophagy–lysosome is intricately linked to the development of gout. At present, the diagnosis and monitoring of gout are mainly invasive tests, which cannot predict the occurrence of gout in the acute phase, and bring new pain to patients. This study focuses on the changes of lysosome-related proteins in urinary exosomes of patients with acute gout attack to explore the potential noninvasive biomarkers clinical application value.

Methods Urine samples were collected from the subject and exosomes were extracted. To explore the differentially expressed proteins in urinary exosomes among acute gout patients (AD group), intermittent gout patients (ID group) and normal controls (NC group) by DIA mass spectrometry. Urinary exosomal lysosome associated proteins were analyzed and receiver operating characteristic (ROC) curves of differentially expressed proteins were drawn to evaluate their clinical value in monitoring acute gout attack.

Results A total of 1896 proteins were detected between AD group and ID group, of which 121 proteins were differentially expressed ($FC > 1.5$ and $p < 0.05$). There were three lysosomal-related proteins differentially expressed in urinary exosomes between AD group and ID group. Compared with the ID group, the expression of Cathepsin Z (CTSZ) and AP-1 complex subunit beta-1 (AP1B1) was increased, while the expression of Lysosome-associated membrane glycoprotein 2 (LAMP2) was decreased in AD group. The ROC analysis showed that CTSZ, AP1B1 and LAMP2 had a strong ability to predict acute gout attack, with AUC of 0.826, 0.847 and 0.882, respectively.

Conclusions There are many specific protein changes in the urinary exosomes of patients with acute gout attack. The urinary exosomes of patients with acute gout attack may exhibit alterations in lysosome-related proteins, particularly CTSZ, AP1B1, and LAMP2, which may become potential biomarkers for monitoring acute gout attack.

Keywords Acute gout attack, Urine exosomes, Lysosome, Autophagy, Biomarker

Introduction

Gout is a metabolic disease caused by disorders of purine metabolism and (or) excretion of uric acid. Urate crystals are deposited in multiple tissues of the body, such as the synovial membrane, bursa, and cartilage, which can lead to acute inflammation [1]. According to research statistics, the global prevalence of gout is 0.1–10% and shows a continuous upward trend [2]. The prevalence of gout in China is also increasing, and the number of patients with hyperuricemia is far more than 17 million, gout has

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become the second largest metabolic disease after diabetes in China [3–5].

Gout mainly occurs in men. Patients may have no symptoms before the acute phase of gout. Patients are often awakened at night by joint pain and cannot tolerate the pain caused by gout. In addition, gout patients may experience joint dysfunction. All of these have brought huge trouble to gout patients. The inflammatory activity associated with gout can cause atherosclerosis and promote thrombosis [6]. An acute attack of gout can lead to a significant increase in the risk of heart and cerebral infarction, renal injury and heart failure. In addition, an acute attack of gout can also cause a variety of complications, such as diabetes, hyperlipidemia and metabolic syndrome in patients [7, 5, 8].

At present, the diagnosis and monitoring of gout mainly include invasive tests, such as serum uric acid, joint fluid and tophus composition analysis. These tests cannot predict the acute phase of gout, and repeated invasive tests create new suffering for gout patients. Urine, as an ultrafiltrate of blood, retains a large amount of biological information in plasma. Changes in urine composition, content and properties can reflect various metabolic states in the body. The whole process of urine collection is a non-invasive operation, which is easy to operate and has good patient compliance. The inhibitory effect of high abundance in urine is not obvious, and low abundance proteins that can be used as potential disease markers are more likely to be found in urine [9, 10]. Urinary exosomes are derived from diverse cells, contain rich biological information, and can reflect a variety of physiological and pathological states of the body with strong stability, which has great research and application value in the study of disease biomarkers.

Autophagy is the process by which cells utilize lysosomes to degrade damaged organelles and macromolecules. Autophagy plays an important role in the regulation of inflammatory response. It can regulate innate immune inflammatory response through a variety of ways [11]. Studies have found that innate immunity is involved in the occurrence and development of gout [12]. Studies have found that autophagy flux increases and autophagy is significantly activated in patients with acute gout attack [13, 14]. The relationship between autophagy lysosome and gout has gradually become a research hotspot.

In this study, we analyzed the changes of urinary exosomal lysosome associated proteins in gout patients and evaluated the ability of lysosome associated differentially expressed proteins to monitor acute gout attack, so as to provide a theoretical basis for non-invasive biomarker research for diagnosis and monitoring of acute gout attack.

Materials and methods

Study subjects

From January 2022 to July 2022, 24 patients diagnosed with gout from the outpatient department of Beijing Shijitan Hospital were enrolled in the study. Inclusion criteria: gout patients met the 2015 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) gout classification criteria, and according to the Chinese Guidelines for the Diagnosis and Treatment of Hyperuricemia and Gout, they were judged to have elevated blood uric acid (> 420 μmol/L for 2 times of fasting hematuria on different days). Exclusion criteria: excluding chronic diseases, such as blood diseases, thyroid diseases, tumors, liver and kidney dysfunction, patients taking drugs for a long time, and patients with positive urine protein test. Exclude urinary system diseases, including chronic kidney disease and urinary system infections, as well as other types of arthritis, such as infectious arthritis and rheumatoid arthritis. The 24 gout patients were divided into two groups. The acute gout attack group (AD group) is defined as 12 patients who are diagnosed with gout, have elevated blood uric acid and have swelling, pain and (or) tenderness of peripheral joints or bursa. Intermittent gout group (ID group) is defined as 12 patients with previous diagnosis of gout and current elevated blood uric acid but no obvious joint symptoms. In addition, 12 healthy people were recruited as the normal control (NC) group. All enrolled subjects were male.

All subjects provided informed consent prior to inclusion in the study and specimen collection. All procedures were carried out in accordance with the ethical standards of the Declaration of Helsinki and approved by the Ethics Committee of Beijing Shijitan Hospital.

Exosome extraction

Thirty milliliters of clean morning urine from the subjects were collected. Dead cells and debris in the urine samples were removed by centrifugation at 1500 *g* for 10 min and 10000 *g* for 30 min. The exosomes were then collected using the size exclusion SEC method (qEV10/35 nm, IZON, Shanghai, China). The morphology of exosomes was detected by transmission electron microscope (TEM). Exosome concentrations and sizes were determined by NanoSight nanoparticle tracking analysis (NTA); and the exosome markers and common exosome negative protein were analyzed by western blotting.

Urinary exosomes proteomics analysis

The samples were fractionated using a high pH reverse phase fractionator and measured in DDA mode. The mass spectrometer was operated on a quadrupole

Orbitrap mass spectrometer (Q Exactive HF-X, Thermo Fisher Scientific, Bremen, Germany) coupled to an EASY nLC 1200 ultra-high pressure system (Thermo Fisher Scientific) via a nano-electrospray ion source. The mass spectrometer was operated in data-dependent mode, collecting MS spectra in the Orbitrap mass analyzer (120,000 resolution, 350–1500 m/z range) with an automatic gain control (AGC) target of 3E6 and a maximum ion injection time of 80 ms. The most intense ions from the full scan were isolated with an isolation width of 1.6 m/z. Using normalized collision energy (NCE) values for fragmentation by higher-energy collisional dissociation (HCD) revealed results.

In contrast to DDA–MS, DIA–MS is based on the fragmentation of all precursor ions identified in a MS1 scan (350 to 1500 m/z), where fragment ions are accumulated in a fixed number of wide isolation windows (14 m/z to 312 m/z) that span the entire mass-to-charge ratio (m/z) range.

The identification and quantitation of protein

DDA-library and direct-DIA-library were generated, respectively. The mixed spectral library was used to search the MS data of single shot samples for final protein identification and quantification, for final protein identification and quantitation. All searches were performed against the human UniProt reference proteome of canonical and isoform sequences. Searches used carbamidomethylation as fixed modification and acetylation of the protein N-terminus, oxidation of methionines as variable modifications. Using 1% protein and precursor FDR, the protein mass in the sample was reported only when the protein passed the filter ("Q-value sparse" mode data filtering). All other settings were left default.

Statistics

All experimental data were presented as the mean ± standard deviation (SD), and statistical analysis was performed by GraphPad Prism (GraphPad, La Jolla, CA, USA) software. One-way ANOVA was used for the comparison between different groups of normal distribution data, and Kruskal–Wallis *H* test was used for the comparison between different groups of non-normal distribution data. $P<0.05$ was considered statistically significant.

Results

Clinical characteristics

The age of each group was matched, and the clinical data and relevant indicators are presented in Table 1. There were no significant differences in uric acid (UA), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) between the AD group and the ID group ($P>0.05$). Compared with the ID group, C-reactive protein (CRP) was significantly increased in AD group ($P<0.05$).

Table 1 Clinical characteristics of AD group and ID group

Characteristics	AD group ($n=12$)	ID group ($n=12$)	*P* value
Age (years)	43.08 ± 10.46	43.08 ± 10.46	> 0.05
CRP(mg/L)	9.52 ± 7.40	2.50 ± 1.77	< 0.05
UA(μmol/L)	512.33 ± 47.03	523.83 ± 62.74	> 0.05
TC(mmol/L)	4.98 ± 0.66	4.69 ± 0.76	> 0.05
TG(mmol/L)	1.58 ± 0.33	1.31 ± 0.41	> 0.05
HDL-C(mmol/L)	1.20 ± 0.153	1.28 ± 0.18	> 0.05
LDL-C(mmol/L)	3.33 ± 0.67	3.24 ± 0.71	> 0.05

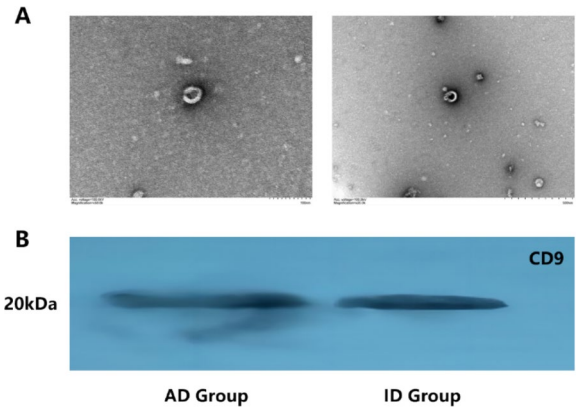

Fig. 1 Identification of urine exosomes

Characteristics of exosomes isolated from urine

Exosomes were isolated from the urine samples, and their morphology was detected using TEM. As shown in Fig. 1A, we observed a distinct double-membrane oval structure. NTA measurements revealed an average size of 148.5 nm for the exosomes. Western blotting was used to detect the presence of the exosome marker, the transmembrane protein CD9 (Fig. 1B).

Comparison of differentially expressed proteins in urinary exosomes among AD group and ID group

A total of 1896 proteins were detected between AD group and ID group, of which 121 proteins were differentially expressed (FC > 1.5 and $p<0.05$). Compared with the ID group, 98 proteins were up-regulated and 23 were down-regulated in the AD group (Table 2). The hierarchical clustering heatmap of differentially expressed proteins is shown in Fig. 2A and the volcano is shown in Fig. 2B

Table 2 Comparison of differentially expressed proteins in urinary exosomes among AD group and ID group

Comparisons	Upregulation	Downregulation	All
AD vs. ID	98	23	1896

Changes in the differentially expression of lysosomal-related proteins in urine exosomes

Fold change > 1.5 and *P* value < 0.05 were considered significant differences. We found that there were three lysosomal-related proteins differentially expressed between AD group and ID group (Table 3). Compared with the ID group, the expression of CTSZ and AP1B1 in the urinary exosomes of the AD group was increased, while the expression of LAMP2 was decreased (Fig. 3).

The functional analysis of differentially expression of lysosomal-related proteins in urinary exosomes among AD group and ID group

We used the STRING database to predict the interaction analysis of three differential proteins, as shown in Fig. 4A, these proteins have strong interactions. At the same time, we conducted a literature search and plotted the main mechanisms of action of three differential proteins in lysosomes. These three proteins play an important role in lysosomal activity (Fig. 4B).

Lysosomal-related proteins in urinary exosomes have clinical value for auxiliary monitoring of acute gout attack

The clinical value of lysosome-related proteins for auxiliary monitoring of acute gout attack is shown in Table 3 and Fig. 5. The area under the curve (AUC) of urinary exosome CTSZ was 0.826 (95% CI 0.653–0.999), and the AUC of urinary exosome AP1B1 was 0.847 (95% CI 0.677–1.000). The AUC of urinary exosomal LAMP2 was 0.882 (95% CI 0.749–1.000). In conclusion, urinary exosomal lysosomal-related proteins may be potential biomarkers for monitoring acute gout attack (see Table 4).

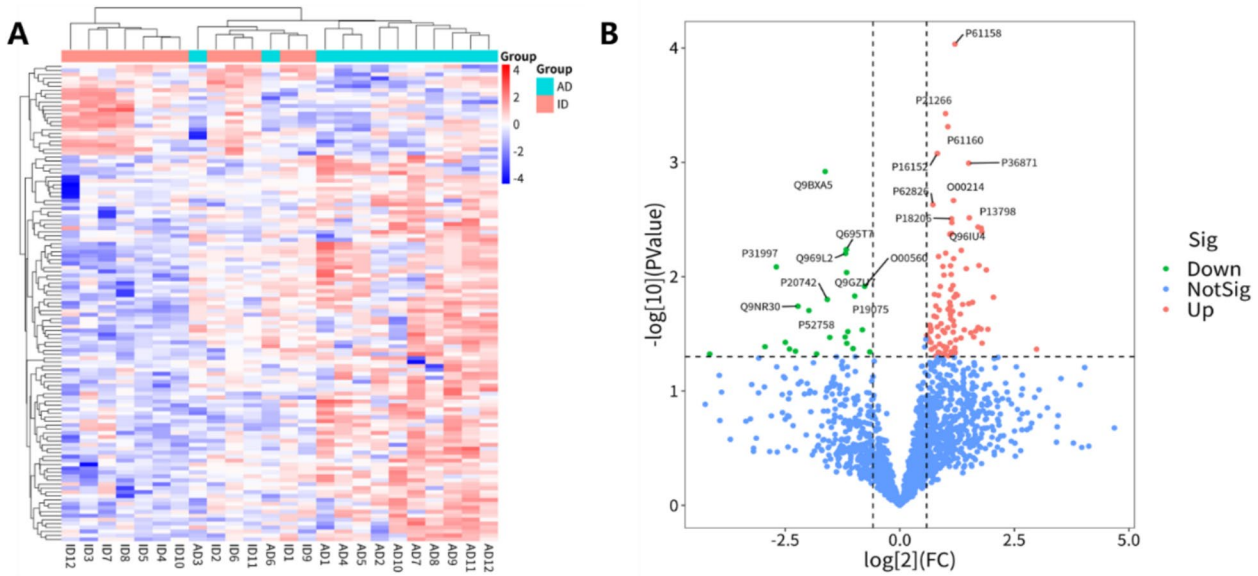

Fig. 2 Hierarchical clustering heatmap and the volcano of differentially expressed proteins in urinary exosomes among AD group and ID group

Table 3 Differentially expression of lysosomal-related proteins in urinary exosomes among AD group and ID group

UniProt-ID	Protein Name	Gene Name	FC	*P* value	Form of expression
Q9UBR2	Cathepsin Z	CTSZ	4.125	0.015	Up
Q10567	AP-1 complex subunit beta-1	AP1B1	1.950	0.010	Up
P13473	Lysosome-associated membrane glycoprotein 2	LAMP2	0.188	0.043	Down

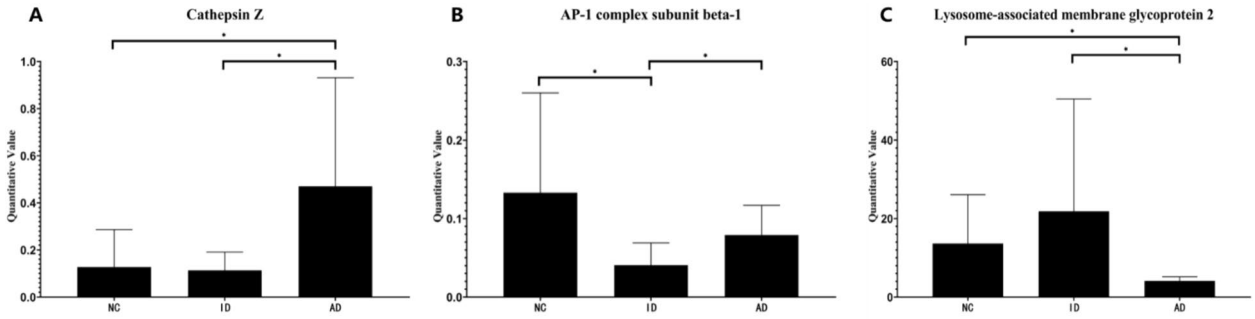

Fig. 3 Quantitative value of CTSZ (**A**), AP1B1 (**B**), LAMP2 (**C**). *, $P < 0.05$

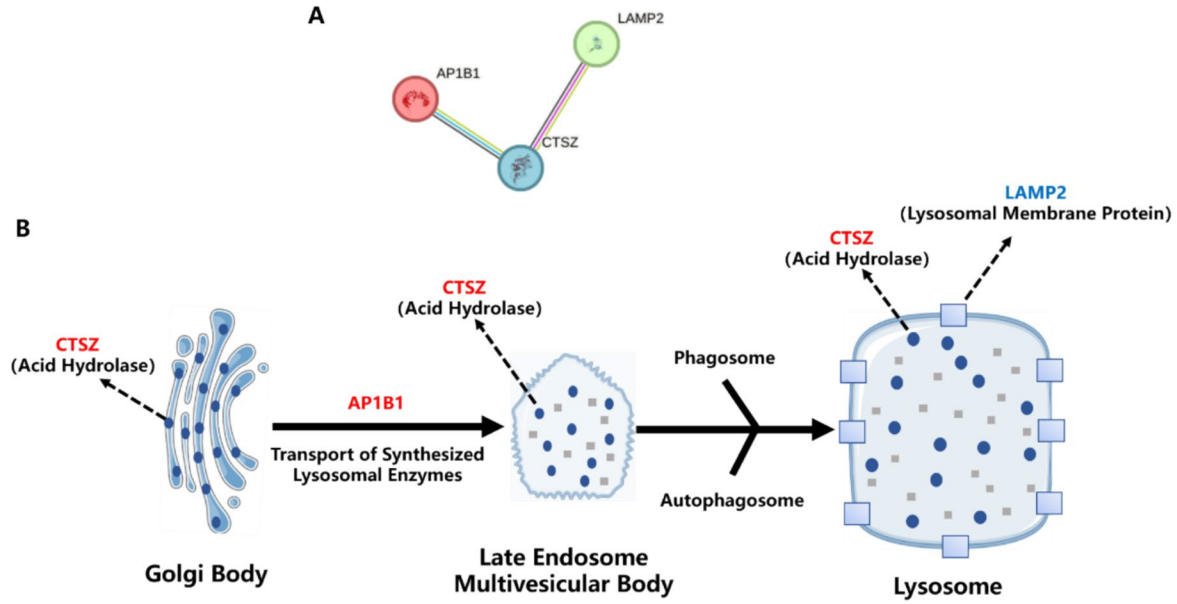

Fig. 4 Functional analysis of three differentially expression lysosomal-related proteins

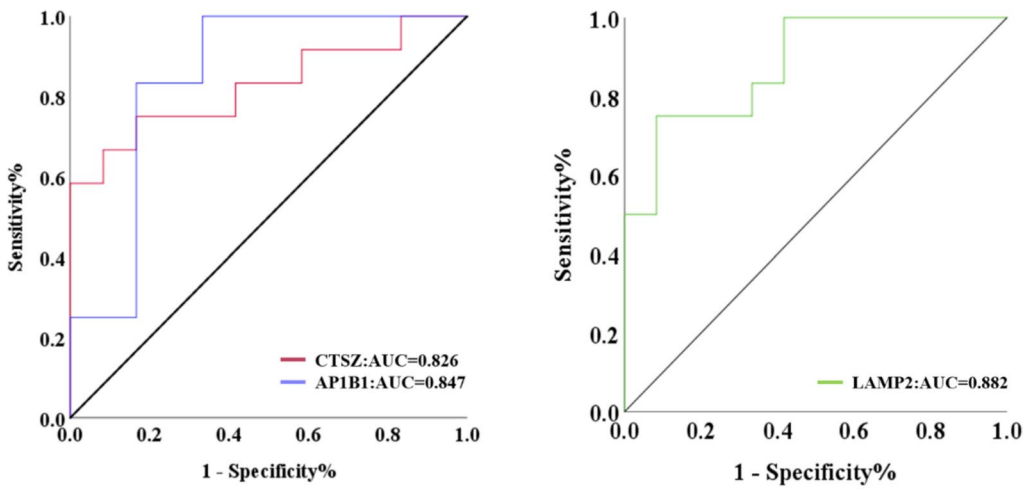

Fig. 5 ROC curve analysis of lysosomal-related proteins in urinary exosomes in monitoring of acute gout attack

Table 4 Lysosomal-related proteins clinical value for auxiliary monitoring of acute gout attack

Lysosomal-related proteins	AUC	95%CI	I value	Youden index
CTSZ	0.826	0.653–0.999	0.007	0.583
AP1B1	0.847	0.677–1.000	0.004	0.667
LAMP2	0.882	0.749–1.000	0.001	0.667

Discussion

Gout is the most common form of inflammatory arthritis, characterized by the deposition of monosodium urate in synovial fluid and other tissues[1]. The global incidence of gout patients is steadily rising, and gout has become a global public health problem [15]. Patients may not have any symptoms before the acute phase of gout. In the acute phase of gout, leukocytes phagocytose urate crystals and release inflammatory factors and hydrolytic enzymes that cause cell necrosis. The continuous release of inflammatory factors leads to articular chondrolysis and soft tissue injury. In addition, the patient is in extreme pain during this stage. Autophagy is a process of self-degradation of intracellular components that is highly conserved at the evolutionary level and plays an important role in various stress responses, such as inflammation [16]. In the process of autophagy, the body degrades and recycles the degradation products through the lysosomal pathway. Autophagy is the process of wrapping damaged or hypofunctional organelles and misfolded proteins that fuse with lysosomes and hydrolyse intracellular components [17].The findings of various studies have demonstrated an augmentation in autophagy flux and a significant activation of autophagy in individuals suffering from acute gout attack [13, 14].

In this study, we found significant changes in the protein composition of urinary exosomes in patients with acute gout attack. A total of 1896 proteins were detected between AD group and ID group, of which 121 proteins were differentially expressed (FC > 1.5 and $p < 0.05$). Compared with the ID group, 98 proteins were up-regulated and 23 were down-regulated in the AD group. We found that there were three lysosomal-related proteins differentially expressed between AD group and ID group. Compared with the ID group, the expression of CTSZ and AP1B1 in the urinary exosomes of the AD group was increased, while the expression of LAMP2 was decreased. These lysosome-related differential proteins are closely related to autophagy.

CTSZ is mainly found in lysosomes, and is an important acid hydrolase of lysosomes. It plays a proteolytic role and participates in the process of apoptosis [18, 19]. It has been confirmed that it plays a key role in the regulation of inflammation [20]. CTSZ enhances IL-1β production through the activation of integrin signaling pathways [21]. Studies have confirmed that CTSZ play a crucial role in the inflammatory response induced by monosodium urate (MSU) [22]. AP1B1 is a subunit of clathrin-associated adaptor protein complex1 (AP1), which recognizes sorting signals and assembles into transport vesicles in cells. AP1B1 is mainly responsible for the transport between the trans-Golgi network and endosomes, and plays an important role in the transport of lysosomal enzymes [23]. Studies have shown that adaptor proteins are closely related to pyroptosis and inflammation [24]. However, their role in MSU-induced inflammation requires further investigation. LAMP2, a lysosomal membrane-associated glycoprotein, is one of the key proteins in chaperone-mediated autophagy. It plays an important role in lysosomal biogenesis, lysosomal pH regulation and autophagy [25, 26]. Studies have shown that MSU can cause lysosome homeostasis disorder, resulting in changes in LAMP2 [27]. In addition, ROC analysis showed that CTSZ, AP1B1 and LAMP2 had a strong ability to predict acute gout attack, with AUC of 0.826, 0.847 and 0.882, respectively. At present, the diagnostic methods for gout are all invasive examinations and cannot predict the onset of the acute phase of gout. Collecting urine from patients is non-invasive and convenient. On the other hand, urinary exosomes contain rich biological information, which is of great research value. Urinary exosomal lysosome associated proteins may be potential non-invasive biomarkers for monitoring acute gout attack.

In this study, the expression changes of lysosome-related proteins, specifically CTSZ, AP1B1 and LAMP2, in urinary exosomes of patients with gout was analyzed. The expression changes of these three lysosome-related proteins may serve as potential non-invasive biomarkers for monitoring acute gout attack. However, further evaluation and validation with more samples are needed in the future.

Summary

We analyzed the urinary exosomal lysosome associated proteins in gout patients by DIA mass spectrometry and evaluated the ability of lysosome associated differentially expressed proteins to monitor acute gout attack, so as to provide a theoretical basis for non-invasive biomarker research for diagnosis and monitoring of acute gout attack.

Abbreviations

CTSZ	Cathepsin Z
AP1B1	AP-1 complex subunit beta-1
LAMP2	Lysosome-associated membrane glycoprotein 2
NC	Normal control
ROC	Receiver operating characteristic
ACR	American College of Rheumatology

EULAR	European League against Rheumatism
TEM	Transmission electron microscope
NTA	NanoSight nanoparticle tracking analysis
AGC	Automatic gain control
HCD	Higher-energy collisional dissociation
SD	Standard deviation
UA	Uric acid
TC	Total cholesterol
TG	Triglyceride
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
CRP	C-reactive protein
AUC	Area under the curve
AP1	Adaptor protein complex1

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Author contributions
Conceptualization, JTW. and MZ.; Methodology, NL. and MH.; Writing—original draft, JTW.; Writing—review & editing, JTW. NL. and MZ.All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Beijing Shijitan Hospital.

Informed consent
Informed consent was obtained from all subjects involved in the study.

Competing interests
The authors declare no competing interests.

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