Investigation of serum biomarkers in primary gout patients using iTRAQ-based screening

Y. Ying[1], Y. Chen[1], S. Zhang[2], H. Huang[3], R. Zou[3], X. Li[3], Z. Chu[3], X. Huang[1], Y. Peng[1], M. Gan[1], B. Geng[1], M. Zhu[1], Y. Ying[1], Z. Huang[2]

[1]Department of Rheumatology, and [2]Stem Cell and Regenerative Medicine Laboratory, Ningbo no. 2 Hospital, Zhejiang, China; [3]Medical School, Ningbo University, Zhejiang, China.

Abstract

Objective

Primary gout is a major disease that affects human health; however, its pathogenesis is not well known. The purpose of this study was to identify biomarkers to explore the underlying mechanisms of primary gout.

Methods

We used the isobaric tags for relative and absolute quantitation (iTRAQ) technique combined with liquid chromatography-tandem mass spectrometry to screen differentially expressed proteins between gout patients and controls. We also identified proteins potentially involved in gout pathogenesis by analysing biological processes, cellular components, molecular functions, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and protein-protein interactions. We further verified some samples using enzyme-linked immunosorbent assay (ELISA). Statistical analyses were carried out using SPSS v. 20.0 and ROC (receiver operating characterstic) curve analyses were carried out using Medcalc software. Two-sided p-values <0.05 were deemed to be statistically significant for all analyses.

Results

We identified 95 differentially expressed proteins (50 up-regulated and 45 down-regulated), and selected nine proteins (α-enolase (ENOA), glyceraldehyde-3-phosphate dehydrogenase (G3P), complement component C9 (CO9), profilin-1 (PROF1), lipopolysaccharide-binding protein (LBP), tubulin beta-4A chain (TBB4A), phosphoglycerate kinase (PGK1), glucose-6-phosphate isomerase (G6PI), and transketolase (TKT)) for verification. This showed that the level of TBB4A was significantly higher in primary gout than in controls (p=0.023).

Conclusion

iTRAQ technology was useful in the selection of differentially expressed proteins from proteomes, and provides a strong theoretical basis for the study of biomarkers and mechanisms in primary gout. In addition, TBB4A protein may be associated with primary gout.

Key words

iTRAQ, primary gout, biomarkers, proteome, TBB4A

Ying Ying, MSc
Yong Chen, MSc
Shun Zhang, MSc
Haiyan Huang, MSc
Rongxin Zou, MSc
Xiaoke Li, MSc
Zanbo Chu, MSc
Xianqian Huang, MSc
Yong Peng, MSc
Minzhi Gan, MSc
Baoqing Geng, MSc
Mengya Zhu, MSc
Yinyan Ying, MSc
Zuoan Huang, MSc

Please address correspondence to:
Dr Yong Chen,
41 Northwest Street,
Haishu District, Ningbo,
Zhejiang 315010, China.
E-mail: nbdeyycy@163.com

Received on October 22, 2017; accepted in revised form on January 9, 2018.

Funding: this study was funded by the Technology Project of Zhejiang Province (2015KYB346, 2017KY589).

Competing interests: none declared.

Introduction

Primary gout is an innate immunoreactive arthritis based on hyperuricaemia, which is stimulated by the deposition of monosodium urate crystals (MUS) in joints and connective tissue (1). Epidemiological studies have shown that the prevalence of primary gout and hyperuricaemia is rapidly increasing worldwide (2). The incidence of primary gout is 1.1% from 2000 to 2014 in inland China, while that of hyperuricaemia is 13.3% (3).

Life science research has entered the genomic era, so isobaric tags for relative and absolute quantitation (iTRAQ), with its characteristic high sensitivity, good reproducibility, and wide detection range, is a suitable technique to screen and identify proteins using a high-throughput approach (4-6). The objective of this study was to identify potential serum biomarkers of primary gout using iTRAQ combined with liquid chromatography-tandem mass spectrometry (LC-MS / MS) technology and to conduct additional experiments using enzyme-linked immunosorbent assay (ELISA) to verify the differentially expressed proteins identified in primary gout. Furthermore, we will focus on the pathogenesis pathways to explore the underlying mechanisms of primary gout.

Materials and methods

Screening of differentially expressed proteins

• *Populations and clinical data*

A total of 30 patients with primary gout and 30 controls were selected from Ningbo No. 2 Hospital, China between January 2015 and January 2016. The diagnosis of primary gout was in accordance with the ACR/EULAR primary gout classification criteria in 2015 (7). Controls had no history of autoimmune diseases, including primary gout, or cancer, haematopathy, hypertension, hepatopathy, nephropathy, diabetes, hyperlipidaemia, or other metabolic diseases.

All participants were unrelated Chinese Han male individuals who provided their written informed consent to participate. This experiment was approved by the Ethics Committee of Ningbo No. 2 Hospital, Ningbo University School of Medicine, and obeyed the ethical guidelines of the Declaration of Helsinki. Personal information and laboratory data, including age, sex, alanine aminotransferase (ALT), aspartate transaminase (AST), creatinine (CREA), uric acid (UA), glucose (GLU), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride levels (TG), and white blood cell (WBC) count were recorded by experienced rheumatologists.

• *Protein sample preparation and iTRAQ labelling*

Serum was extracted from peripheral blood stored in coagulant tubes within 6 h of collection, and kept at 80°C. Patients and controls were randomly divided into three groups: A1-A3 for the patients and B1-B3 for the controls. Serum albumin and immunoglobulin G were removed from blood samples using the ProteoExtract™ Albumin/IgG Removal Kit (Calbiochem, San Diego, CA).

Cysteine-blocking reagent was added to 100 μg of each protein sample to block disulfide bonds. The samples then underwent trypsin digestion, and the digested peptides were collected. iTRAQ reagent was added to each tube and incubated at room temperature for 2 h. Isotopes of 113, 114, 115, 116, 117, and 118 were separately labelled A1, A2, A3, B1, B2, and B3, respectively. Tubes were vortexed to mix, then centrifuged and the solution collected.

• *Two dimensional LC-MS/MS analysis*

Reversed-phase LC (RPLC) was carried out on the Agilent 1200 HPLC System (Agilent Technologies, China) using the following parameters: narrow-bore 2.1*150 mm 5 μm, with 215 nm and 280 nm UV detection. The first segment was collected at the 8th minute, and then segments were collected at 1 min intervals for the 9^{th}–51^{st} minutes. Three or four segments were mixed together to give a total of 10 segments. Online nano-RPLC used the EksigentnanoLC-Ultra™ 2D System (AB Sciex, Framingham, MA). Data acquisition was performed using the Triple TOF 5600 System (AB Sciex, Framingham, MA).

• *Bioinformatics analysis*
Trusted protein screening criteria were as follows: unused >1.3 and unique peptide ≥1. Invalid values and counter library data were removed. Differentially expressed protein screening criteria were as follows: fold-change, 1.2 times; *p*-values <0.05 were considered significantly different following *t*-test analysis of data conducted in triplicate. Results were shown on volcanic graphs. The functions of differentially expressed proteins were analysed through the Database for Annotation, Visualisation and Integrated Discovery (DAVID) and QuickGO database by Gene Ontology (GO), including biological processes (BP), cellular components (CC), and molecular function (MF). Results were shown on bar graphs. KEGG pathways were analysed using the KEGG database. The analysis of protein–protein interactions (PPI) was performed through the STRING database.

Verification of differentially expressed proteins

• *Populations and clinical data*
Primary gout patients (42 cases) and controls (42 cases) were selected from Ningbo No. 2 Hospital, China between January 2015 and January 2016. The rest is the same as part of 1.1.

• *Serum sample preparation and ELISA*
An ELISA assay kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., China) was used to measure differentially expressed protein levels. Peripheral venous blood (5 ml) was collected into coagulation tubes, and then the upper serum layer was extracted after 6 h and stored at -80 °C. Different concentrations of standard (50 μL) were added into standard wells, 10 μL of test sample and 40 μL sample dilution buffer were added into sample wells and nothing was added to blank wells, then 100 μL of detection antibody labelled with horseradish peroxidase (HRP) was added to all wells. The wells were sealed with sealing film and the plate was placed at 37°C in a water bath for 60 min. The liquid was discarded and each well was washed with washing solution five times. Then, 50 μL of each of substrates A and B was added and incubated at 37°C for 15 min in the dark, 50 μL of stop solution was added and the OD value of each well was measured at 450 nm by a microplate reader.

Table I. Demographic and clinical data of participants in screening tests.

Characteristic	Gout (n=30)	Control (n=30)	*p*-value
Age (years)	51.50 ± 13.62	33.60 ± 6.97	<0.0001
ALT (IU/L)	36.75 ± 31.94	28.80 ± 9.61	0.198
AST (IU/L)	23.00 ± 9.01	23.60 ± 4.12	0.743
CREA (μmol/L)	81.40 ± 16.52	75.08 ± 9.41	0.083
UA (μmol/L)	424.81 ± 157.63	351.35 ± 61.02	0.025
GLU (mmol/L)	6.17 ± 2.10	4.97 ± 0.28	0.003
TC (mmol/L)	5.45 ± 1.23	4.64 ± 0.52	0.009
HDL-C (mmol/L)	1.35 ± 0.31	1.50 ± 0.28	0.077
LDL-C (mmol/L)	2.78 ± 0.95	2.67 ± 0.43	0.618
TG (mmol/L)	2.71 ± 2.54	1.04 ± 0.37	0.007
WBC ($\times 10^9$/L)	10.67 ± 3.92	6.90 ± 1.64	<0.0001

ALT (alanine aminotransferase); AST (aspartate transaminase); CREA (creatinine); UA (uric acid); GLU (glucose); TC (total cholesterol); HDL-C (high-density lipoprotein-cholesterol); LDL-C (low-density lipoprotein-cholesterol); TG (triglyceride); and WBC (white blood cell count).

• *Detection of total protein*
A bicinchoninic acid (BCA) protein assay kit (Shanghai Bo-Yuan Biological Technology Co., LTD., China) was used to measure protein levels; 25 μL of A-G bovine serum albumin standard or protein sample to be tested was added into wells of a 96-well plate in triplicate. A 200 μL aliquot of BCA working solution was added to each well, which was then covered. After incubation at 37°C for 30 min, the absorbance was measured at 540-590 nm with a microplate.

Statistical analyses
Statistical analyses were carried out using SPSS v. 20.0 (SPSS Inc., Chicago, IL). Continuous variables were evaluated as means±standard deviation (SD), and the Student's *t*-test was used to assess differences in clinical data. Medcalc software was taken to carry on ROC curve analysis. Two-sided *p*-values <0.05 were deemed to be statistically significant for all analyses.

Results

Screening of differentially expressed proteins

• *Participant demographic and clinical characteristics*
All participants' clinical information data and *p*-values are listed in Table 1. Comparative analysis suggested that the variables of ALT, AST, CREA, HDL-C, and LDL-C had no statistical difference, while variables of age, UA, GLU, TC, TG and WBC count were significantly different among these two groups ($p<0.05$).

• *Mass spectrometry analysis*
A total of 369 proteins were quantified, of which 95 were differentially expressed in primary gout populations (50 up-regulated and 45 down-regulated) compared with controls (Supplementary Table).

• *Bioinformatics analysis*
The biological functions of these 95 differentially expressed proteins were analysed by DAVID and the QuickGO database, revealing three main gene ontologies: 1) 93 proteins (97.9%) were associated with biological processes, involving up to 2454 types of functions; of these, 1336 dominant functions were mainly involved in trauma, stress response, and coagulation; 2) 95 proteins (100%) were associated with the cellular component, involving 312 types of functions; of these, 138 dominant functions were mainly extracellular-related; 3) 95 proteins (100%) had 385 types of molecular functions; of these 186 were different, with protein binding being the main function. Table II listed the top 20 entries enrichment analysed by the three categories of enrichment analysis of biological processes, cell localisation and molecular function.
A total of 95 proteins (100%) were associated with 91 signalling pathways in the KEGG database, of which 24 dif-

Table II. The figure listed the top 20 categories of biological processes, cellular components and molecular function.

Biological process	Cell component	Molecular function
Response to wounding	extracellular organelle	protein binding
Response to stress	extracellular vesicle	actin binding
Wound healing	extracellular membrane-bounded organelle	enzyme binding
Haemostasis	extracellular exosome	peptidase regulator activity
Response to stimulus	extracellular region part	endopeptidase inhibitor activity
Phagocytosis	extracellular region	endopeptidase regulator activity
Blood coagulation	membrane-bounded vesicle	peptidase inhibitor activity
Coagulation	vesicle	identical protein binding
Regulation of biological quality	extracellular space	enzyme regulator activity
Regulation of body fluid levels	blood microparticle	cytoskeletal protein binding
Vesicle-mediated transport	focal adhesion	enzyme inhibitor activity
Defense response	cell-substrate adherens junction	antigen binding
Endocytosis	cell-substrate junction	serine-type endopeptidase inhibitor activity
Immune system process	membrane-bounded organelle	protein complex binding
Single-multicellular organism process	adherens junction	quaternary ammonium group binding
Platelet activation	anchoring junction	molecular function regulator
Multicellular organismal process	organelle	macromolecular complex binding
Response to external stimulus	cytosol	alcohol binding
Biological regulation	cell junction	ammonium ion binding
Immune response-regulating cell surface receptor	cell leading edge	antioxidant activity

Table III. Signalling pathway analysis of differentially expressed proteins.

Pathway ID	Pathway name	Associated proteins
hsa04610	Complement and coagulation cascades	ANT3↓, KLKB1↓, FA5↓, CO9↑, F13B↓, CO4B↑
hsa04810	Regulation of actin cytoskeleton	GELS↓, VINC↑, ACTB↑, ACTN1↑, COF1↑, PROF1↑, MOES↑, ACTG↑
hsa05132	Salmonella infection	ACTB↑, PROF1↑, LBP↓, ACTG↑, RAB7A↑
hsa05130	Pathogenic Escherichia coli infection	ACTB↑, 1433Z↑, TBB4A↑, ACTG↑
hsa05146	Amoebiasis	VINC↑, CO9↑, ACTN1↑, ILEU↑, RAB7A↑
hsa04145	Phagosome	ACTB↑, COR1A↑, TBB4A↑, ACTG↑, RAB7A↑, FCG3A↓
hsa05131	Shigellosis	VINC↑, ACTB↑, PROF1↑, ACTG↑
hsa01200	Carbon metabolism	ENOA↑, G3P↑, PGK1↑, G6PI↑, TKT↑
hsa00010	Glycolysis / Gluconeogenesis	ENOA↑, G3P↑, PGK1↑, G6PI↑
hsa04670	Leukocyte transendothelial migration	VINC↑, ACTB↑, ACTN1↑, MOES↑, ACTG↑

NOTE: ↑ represents up-regulated proteins; ↓ represents down-regulated proteins.

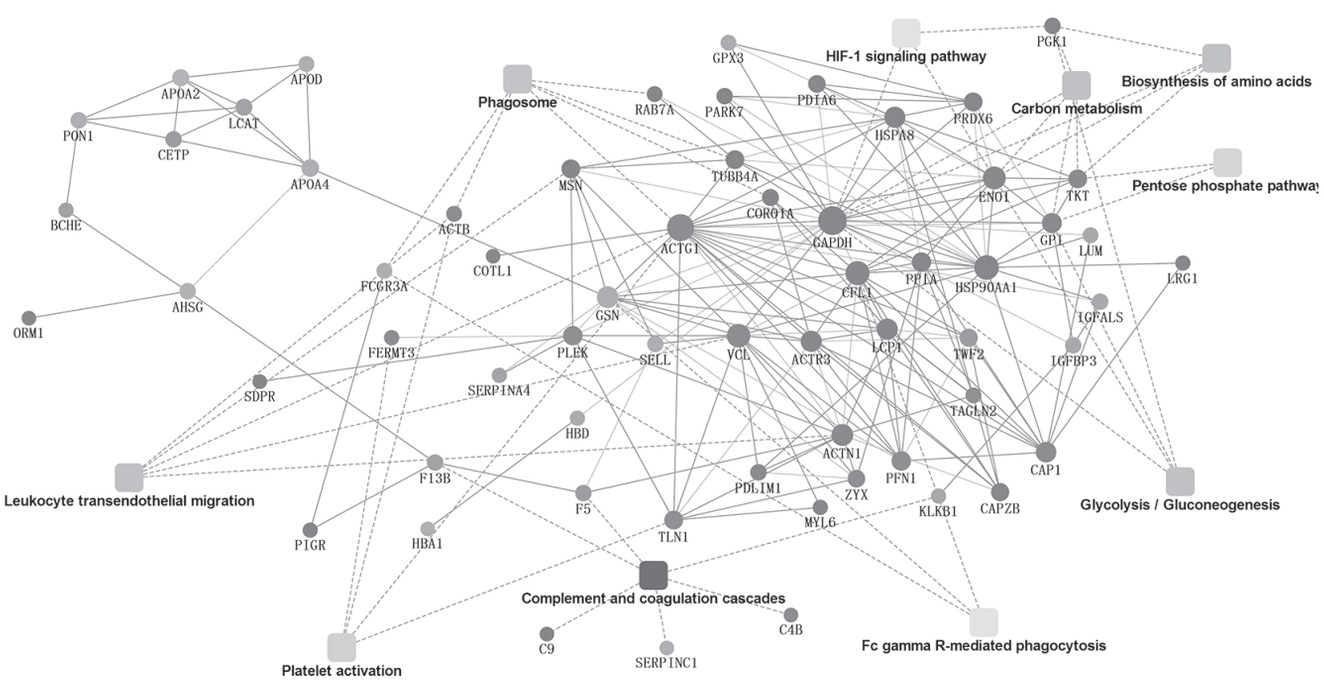

Fig. 1.

Table IV. Demographic and clinical data of participants in verification tests.

Characteristic	Gout (n=42)	Control (n=42)	*p*-value
Age (years)	52.62 ± 14.82	34.61 ± 7.07	<0.0001
ALT (IU/L)	37.76 ± 32.54	27.74 ± 9.73	0.234
AST (IU/L)	23.54 ± 9.32	23.54 ± 3.87	0.756
CREA (μmol/L)	81.58 ± 16.34	74.12 ± 8.42	0.082
UA (μmol/L)	428.82 ± 159.45	351.45 ± 62.02	0.028
GLU (mmol/L)	6.34 ± 2.12	5.09 ± 0.32	0.008
TC (mmol/L)	5.40 ± 1.34	4.73 ± 0.56	0.017
HDL-C (mmol/L)	1.34 ± 0.30	1.48 ± 0.23	0.070
LDL-C (mmol/L)	2.77 ± 1.73	2.63 ± 0.53	0.763
TG (mmol/L)	2.73 ± 2.58	1.08 ± 0.42	0.011
WBC ($\times 10^9$/L)	10.76 ± 3.97	6.92 ± 1.67	<0.0001

Table V. Comparison of differential proteins between primary gout group and healthy control group.

Proteins	Primary gout (n=42)	Healthy control (n=42)	*p*-value
ENOA	5.6 (2.9,10.1)*10^{-8}	5.0 (3.1,7.4)*10^{-8}	0.569
G3P	5.2 (4.0, 8.6)*10^{-9}	(6.3 ± 3.6)*10^{-9}	0.634
CO9	(5.6 ± 2.8)*10^{-4}	(5.8 ± 2.4)*10^{-4}	0.676
PROF1	(2.8 ± 0.8)*10^{-3}	(2.7 ± 0.6)*10^{-3}	0.592
LBP	(7.6 ± 1.6)*10^{-6}	(7.5 ± 1.1)*10^{-6}	0.749
TBB4A	(8.0 ± 2.3)*10^{-7}	(6.9 ± 2.0)*10^{-7}	0.023
PGK1	(2.2 ± 0.5)*10^{-8}	(2.2 ± 0.4)*10^{-8}	0.992
G6PI	(2.4 ± 0.5)*10^{-9}	(2.4 ± 0.5)*10^{-9}	0.707
TKT	(1.3 ± 0.2)*10^{-6}	(1.3 ± 0.2)*10^{-6}	0.791

Note: The data in the table is the difference in protein content/total protein (100%)

fered significantly from each other. The top 10 significant pathways and their related proteins were listed in Table III. Figure 1 showed the analysis of these protein-protein interactions through the STRING database.

After biological analysis (including BP, CC, MF, KEGG pathways and PPI), we found α-enolase (ENOA), glyceraldehyde-3-phosphate dehydrogenase (G3P), complement component C9 (CO9), profilin-1 (PROF1), lipopolysaccharide-binding protein (LBP), tubulin beta-4A chain (TBB4A), phosphoglycerate kinase (PGK1), glucose-6-phosphate isomerase (G6PI), and transketolase (TKT) were shown to play an important part in the pathogenesis of primary gout.

Verification of differentially expressed proteins

• *Participant demographic and clinical characteristics*

Table IV showed all patients and controls' clinical information. And analysis also suggested that the variables of ALT, AST, CREA, HDL-C, and LDL-C had no statistical difference, while variables of age, UA, GLU, TC, TG and WBC count were significantly different between patient group and control group ($p<0.05$).

• *Comparison of protein expression levels*

The level of each protein in the serum is expressed by the concentration of the protein divided by the concentration of the total protein. The level of TBB4A protein in primary gout samples was $(8.0\pm2.3) \times 10^{-7}$, while in the healthy controls it was $(6.9\pm2.0) \times 10^{-7}$ and the difference was statistically significant ($p=0.023$). However, the difference in levels of the other proteins between the two groups was not statistically significant ($p>0.05$; listed in Table V).

• *ROC curve analysis*

The ROC curve showed that the level of TBB4A protein predicted the status of primary gout with low accuracy (AUC (area under roc curve) = 0.649, $p=0.0142$). The sensitivity (SE) and specificity (SP) were 0.45 and 0.86, respectively, when the critical value was more than 9.21.

Discussion

The concept of the proteome was first proposed by two Australian academics in 1994, and refers to all proteins expressed in a biological entity or cell at a specific period of time and in a specific space (8, 9). iTRAQ technology is particularly useful for the study of proteomics. iTRAQ reagents comprise a binding group, a reporter group, and a balance group. Lysine side chains or N-terminal peptides can be tagged to the protein, which are then detected by mass spectrometry. This enables the identification of an unknown protein by qualitative and quantitative analysis, and the technique successfully overcomes the disadvantages of two-dimensional gel electrophoresis (2DE) technology including its time-consuming and laborious nature, and poor repeatability (10-12).

Previous studies have applied iTRAQ technology to screen out potential biomarkers of diseases such as osteoarthritis, rheumatoid arthritis, and cancer (13-15). Fan *et al*. used iTRAQ technology to identify differentially expressed proteins between cervical cancer and normal tissue, and suggested that G6PD, aldehyde dehydrogenase 3A1, signal transducer and activator of transcription 1, and heat shock protein beta-1 are potential proteins to target in cervical cancer treatment (16). De Seny *et al*. also identified differentially expressed proteins associated with osteoarthritis (V65 vitronectin, C3F peptide, CTAP-III, and M / Z3762 protein) using this approach (17).

The cause of primary gout is a disorder of purine metabolism and/or poor uric acid excretion combined with joint inflammation and loose connective tissue caused by MUS (18, 19). However, its pathogenesis is not clear. Proteomics analysis gives an overall view of protein expression levels and filters out those proteins that are differentially expressed between cases and controls. Our study detected 95 differentially expressed proteins between the primary gout and control groups, and found 9 proteins (ENOA, G3P, CO9, PROF1, LBP, TBB4A, PGK1, G6PI and TKT) were closely related to the pathogenesis of primary gout by bioinformatics

analysis. ENOA, G3P, PGK1, G6PI and TKT played a key role in glucose metabolism; CO9 played an important role in the inflammatory response; TBB4A, PROF1 and LBP were important composition of cells. This study confirmed that TBB4A protein in primary gout patients was more highly expressed than control group.

Microtubules, which consist of heterodimer of α-tubulin and β-tubulin and a small number of microtubule-binding proteins, are a major component of the cytoskeleton. Microtubules have the dual kinetic properties of polymerisation and depolymerisation, and play a major part in maintaining cell morphology, signal transduction, cell division, and substance transport. The β-tubulin subunit is divided into seven β-isotypes (β-I, β-II, β-III, β-IVa, β-IVb, β-V and β-VI) (20, 21).

A study reported in *Nature* showed that microtubules are specifically involved in the activation of the NALP3 inflammasomes (22). And NALP3 inflammasomes is a major driver of various autoimmune and autoimmune diseases, including gout, rheumatoid arthritis and lupus (23). Endogenous ASC is localised to mitochondria, the cytoplasm and the nucleus under resting conditions, while endogenous NALP3 is mainly located in the endoplasmic reticulum. During the activation of NALP3 mitochondria gradually approach the endoplasmic reticulum, leading to co-localisation of ASC on mitochondria with NALP3 inflammasomes on the endoplasmic reticulum. In addition to direct activation of NALP3 inflammation through outflow of reactive oxygen species and K^+, a necessary condition of NALP3 inflammatory activation is the microtubule-mediated proximity of ASC to NALP3 inflammasomes. Colchicine inhibits the transport of mitochondria, thus blocking the ASC approach to NALP3 inflammasomes. Therefore, the use of colchicine could effectively inhibit the activation of NALP3 inflammasomes and reduce the level of mature IL-1β during acute primary gout caused by MSU (24-25).

TBB4A protein (β-IVa) is a type of β-tubulin, and the level of TBB4A protein in the primary gout group was significantly higher than the healthy control group. The primary gout patients also had more NALP3 inflammatory activation and released more mature IL-1β. However, the mechanism regulating TBB4A protein expression in peripheral blood is still unknown. It may involve single nucleotide polymorphisms (SNPs) or epigenetics of the gene *TUBB4A* (26, 27) and further study is needed to elucidate this. Futhermore, it may help us to foster new treatments according to the high level of TBB4A protein in gout patients.

The proteins in the peripheral serum of primary gout patients were screened by iTRAQ technology combined with liquid chromatography-tandem mass spectrometry. The levels of the above nine proteins were different between the primary gout group and the healthy control group. However, the results were not the same when these proteins were examined in a larger number of samples and only the level of TBB4A was different between the two groups. This may be related to the following reasons:

1. Selection bias – the selected samples were different between screening tests and verification tests;
2. iTRAQ combined with LC-MS / MS technology had higher sensitivity than ELISA technology;
3. the larger the sample number, the higher the accuracy. Therefore, it will be necessary to increase sample sizes when undertaking further analyses of these proteins in the future.

ROC curves are usually used to identify the effects of diagnostic indicators of disease and to find the best diagnostic critical value (28, 29). An AUC range from 0.5 to 0.7 means a lower accuracy, while a range of 0.7 to 0.9 means higher accuracy and more than 0.9 means the highest accuracy. In other words, the closer the AUC is to 1, the better the diagnostic value. In our research, ROC curve analysis result showed that TBB4A protein had lower accuracy for diagnosis of primary gout. When the level of TBB4A protein was more than 9.21, the sensitivity and specificity were maximal at the same time.

In summary, 369 proteins were differentially screened by iTRAQ combined with LC-MS/MS technology. TBB4A was confirmed as a differentially expressed protein between primary gout and control groups in a larger sample using ELISA technology. This protein may play an important role in the pathogenesis of primary gout but further studies to elucidate the regulatory mechanism of TBB4A protein expression in peripheral blood are required. And, it may also help us to find out novel treatments of gout.

References

1. STEWART S, DALBETH N, VANDAL AC, ROME K: The first metatarsophalangeal joint in gout: a systematic review and meta-analysis. *BMC Musculoskelet Disord* 2016; 17: 69.
2. RODDY E, ZHANG W, DOHERTY M: The changing epidemiology of gout. *Nat Clin Pract Rheumatol* 2007; 3: 443-9.
3. LIU R, HAN C, WU D *et al*.: Prevalence of hyperuricemia and gout in mainland China from 2000 to 2014: a systematic review and meta-analysis. *Biomed Res Int* 2015; 2015: 762820.
4. ZHANG S, MERCADO-URIBE I, HANASH S, LIU J: iTRAQ-based proteomic analysis of polyploid giant cancer cells and budding progeny cells reveals several distinct pathways for ovarian cancer development. *PLoS One* 2013; 8: e80120.
5. ORVISKY E, DRAKE SK, MARTIN BM *et al*.: Enrichment of low molecular weight fraction of serum for MS analysis of peptides associated with hepatocellular carcinoma. *Proteomics* 2016; 6: 2895-902.
6. KOLTAI T: Clusterin: a key player in cancer chemoresistance and its inhibition. *Onco Targets Ther* 2014; 7: 447-56.
7. ZHANG W, DOHERTY M, PASCUAL E *et al*.: EULAR evidence based recommendations for gout. Part I: Diagnosis. Report of a task force of the Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT). *Ann Rheum Dis* 2006; 65: 1301-11.
8. WILKINS MR, PASGUALI C, APPEL RD *et al*.: From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnology* 1996; 14: 61-5.
9. WASINGER VC, CORDWELL SJ, CERPA-POLJAK A *et al*.: Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis* 1995; 16: 1090-4.
10. ZHANG YX, HAN QQ, GUO QX, ZHANG S: Physiological and proteomic analysis reveals the different responses of Cunninghamialanceolata seedlings to nitrogen and phosphorus additions. *J Proteomics* 2016; 146: 109-21.
11. MA S, SUN Y, ZHAO X, XU P: Recent advance in high accuracy iTRAQ for quantitative proteomics.*Sheng Wu Gong Cheng Xue Bao* 2014; 30: 1073-82.
12. CHAHROUR O, COBICE D, MALONE J: Stable isotope labelling methods in mass spectrometry-based quantitative proteomics. *J Pharm Biomed Anal* 2015; 113: 2-20.

13. CUI Y, GAO D, LINGHU E *et al*.: Epigenetic changes and functional study of HOXA11 in human gastric cancer. *Epigenomics* 2015; 7: 201-13.
14. TANG L, YE H, HONG Q *et al*.: Elevated CpG island methylation of GCK gene predicts the risk of type 2 diabetes in Chinese males. *Gene* 2014; 547: 329-33.
15. CHEN H, YU Y, RONG S, WANG H: Evaluation of diagnostic accuracy of DNA methylation biomarkers for bladder cancer: a systematic review and meta-analysis. *Biomarkers* 2014; 19: 189-97.
16. FAN G, WRZESZCZYNSKI KO, FU C *et al*.: A quantitative proteomics-based signature of platinum sensitivity in ovarian cancer cell lines. *Biochem J* 2015; 465: 433-42.
17. DE SENY D, SHARIF M, FILLET M *et al*.: Discovery and biochemical characterisation of four novel biomarkers for osteoarthritis. *Ann Rheum Dis* 2011; 70: 1144-52.
18. TAO JH, ZHANG Y, LI XP: P2X7R: a potential key regulator of acute gouty arthritis. *Semin Arthritis Rheum* 2013; 43: 376-80.
19. HAHN J, KNOPF J, MAUERÖDER C, KIENHÖFER D, LEPPKES M, HERRMANN M: Neutrophils and neutrophil extracellular traps orchestrate initiation and resolution of inflammation. *Clin Exp Rheumatol* 2016; 34 (Suppl. 98): S6-8.
20. SIMONS C, WOLF NI, MCNEIL N *et al*.: A de novo mutation in the β-tubulin gene TUBB4A results in the leukoencephalopathy hypomyelination with atrophy of the basal ganglia and cerebellum. *Am J Hum Genet* 2013; 92: 767-73.
21. ROMANIELLO R, ARRIGONI F, BASSI MT, BORGATTI R: Mutations in α- and β-tubulin encoding genes: implications in brain malformations. *Brain Dev* 2015; 37: 273-80.
22. HOWARD J, HYMAN AA: Dynamics and mechanics of the microtubule plus end. *Nature* 2003; 422: 753-8.
23. ZHONG Z, SANCHEZ-LOPEZ E, KARIN M: Autophagy, NLRP3 inflammasome and auto-inflammatory/immune diseases. *Clin Exp Rheumatol* 2016; 34 (Suppl. 98): S12-16.
24. MISAWA T, TAKAHAMA M, KOZAKI T *et al*.: Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. *Nat Immunol* 2013; 14: 454-60.
25. KARDON JR, VALE RD: Regulators of the cytoplasmic dynein motor. *Nat Rev Mol Cell Biol* 2009; 10: 854-65.
26. SHRIDHAR K, WALIA GK, AGGARWAL A *et al*.: DNA methylation markers for oral precancer progression: A critical review. *Oral Oncol* 2016; 53: 1-9.
27. TAKAHASHI T: Interleukin 28B genetic polymorphism and hepatitis B virus infection. *World J Gastroenterol* 2014; 20: 12026-30.
28. KRASNIQI X, BERISHA B, GASHI M, KOÇINAJ D, JASHARI F, VINCELJ J: Influence of apelin-12 on troponin levels and the rate of MACE in STEMI patients. *BMC Cardiovasc Disord* 2017; 17: 195.
29. SEO JY, SUH CH, JUNG JY, KIM AR, YANG JW, KIM HA: The neutrophil-to-lymphocyte ratio could be a good diagnostic marker and predictor of relapse in patients with adult-onset Still's disease: A STROBE-compliant retrospective observational analysis. *Medicine* (Baltimore) 2017; 96: e7546.