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FBP2 and *Talin-1* are potential protein markers for Mongolian medicine symptom evaluation in viral infectious diseases

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Abstract

Background: Influenza, measles, and mumps are common viral infectious diseases in Mongolia. The traditional Mongolian medicine (TMM) classified them as warm disease, and still plays a major role in the diagnoses and treatments.

Methods: To interpret the connotation of the complex theoretical system in TMM with scientific technique, in this study, a high throughput mass spectrometry was used to identify potential protein markers of TMM symptom types. Fifty venous blood samples were drawn from influenza, measles and mumps patients. Differential proteins between samples of patients diagnosed as immature and mature heat in TMM were detected by mass spectrometry.

Results: After proteomics analysis, 1500 proteins and 7619 polypeptides were identified and 1323 in total showed differential expression between those 2 symptom types; then enrichment analysis of the differentially expressed proteins revealed the significant biological functions related to the differentially expressed proteins, including cardiomyopathy, several bacterial and parasitic infections, bacterial invasion of epithelial cells, insulin signaling pathway, and regulation of actin cytoskeleton. The network analysis showed that *FBP2* and *Talin-1* were critical points and might determine the evolution directions of TMM warm disease symptom.

Conclusions: This study suggests that the identified core differential proteins may be regarded as potential biomarkers, and benefit to evaluate the evolutionary tendency of TMM warm disease symptoms.

Abbreviations: AVP = TNF-α and arginine vasopressin, CRP = C-reactionprotein, GO = gene ontology, HCD = high-energy collision-induced dissociation, IL-1 = interleukin-1, IL-6 = interleukin -6, LCMS = liquid chromatography-mass spectrometry, MS = mass spectrometry, PGE2 = prostaglandin E 2, PPI = protein-protein interactions, TCEP = tetracalcium phosphate, TEAB = tetraethl-ammonium bromide, TMM = The traditional Mongolian medicine, TNF-α = tumor necrosis factor α.

Keywords: *FBP2*, *Talin-1*, the traditional Mongolian medicine, viral infectious diseases

1. Introduction

Influenza, measles, and mumps are most important viral infectious diseases in humans. Young children are at high risk of viral infection, and the clinical diseases can be a viral reservoir during epidemic phase.[1] However, the above diseases are still not completely preventable by vaccine[2] and remain public threats to human health in Mongolia. Since the 12th century, through a lengthy process of development, Mongolian medicine has formed a systematic theory of febrile diseases and has accumulated extensive experience in prevention and treatment. In traditional Mongolian medicine (TMM), influenza, measles, and mumps are classified as warm diseases, and divided the course of diseases into immature and mature heat period to improve the emergency and treatment. Immature heat is a common stage in infection immunity and other fever-related diseases, meaning an initiating stage of the pathological process after pathogenic microorganisms' infection, and is the critical period of diagnosis and treatment. The symptom of patients at immature heat stage may have a headache, white coated tongue, and turbid urine or temperature fluctuations. The symptom of mature heat is the second stage of disease development, showing signs of body temperature continues rising, intolerable thirst, general weakness, dyspnea, cough, or yellow coated tongue and urine.

Recent years, the diagnosis and treatment of traditional Chinese medicine theory have been scientifically proven in a variety of diseases, such as lung cancer,[3] coronary heart disease,[4] and showed a unique advantage especially in the long-term treatment

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of infectious diseases.[5–7] And our preliminary study results in an animal model with rabbit show that, during the evolution from immature heat to mature heat, tumor necrosis factor α (TNF-α) and C-reactionprotein (CRP) showed continues increases. Furthermore, after the treatment with TMM decoction, the expressions of interleukin-1 (IL-1), interleukin-6 (IL-6), TNF-α and arginine vasopressin (AVP) were up-regulated, and the prostaglandin E 2 (PGE2) was restrained, suggesting the effect of TMM decoction as delaying infection, reducing body temperature and shortening the course of disease.

However, although the detection of protein markers in plasma is a commonly used medical diagnostic method of infectious disease,[8] and the use of high-throughput screening of protein markers has played an important role in the scientific diagnosis of traditional Chinese medicine,[9] current screenings are still not able to distinguish TMM warm disease symptoms. Thus, it is necessary to adopt reliable methods or determine additional disease status indicators to make accurately diagnose of immature and mature heat in TMM. The purpose of the work is to establish a new method for distinguishing TMM warm disease stage with scientific technique. To create this new approach, venous blood samples were drawn from influenza, measles and mumps patients who were diagnosed as immature and mature heat, then the statistics are processed by mass spectrometry and proteomics analysis. As the result, *FBP2* and *Talin-1* could be critical points that have the potential to be evolution directions of TMM warm disease symptom. The proteins processed by function and network analysis was interpreted the connotation of the complex theoretical system of TMM with scientific technique. In future work, it will important to provide more direct evidence of the potential protein markers between those 2 symptom types.

2. Methods

2.1. Diagnostic

Diagnostic criteria for influenza refer to the "diagnostic Efficacy Standards for Mongolian Medical Diseases" and the "Ministry of Health of the People's Republic of China" develop diagnostic criteria. Diagnostic criteria for carbuncle refer to the "Health Standards of the People's Republic of China". Diagnostic criteria for measles refer to the "Measures for the Implementation of the Law of the People's Republic of China on the Prevention and Control of Infectious Diseases". The 22 control samples were from the people without other systemic infectious diseases and secondary diseases. In addition, the people had not been vaccinated within 1 month and a secondary blood collection was necessary. Once diagnosed, the case was ruled out.

2.2. Blood samples

Five mL untreated plasmas samples, from 50 patients (age range 18–65, no gender limitation) diagnosed at the immature and mature heat stage of influenza, measles, and mumps, were obtained from vein respectively. The blood was drawn into 5 mL purple top (plasma; K_2-EDTA anticoagulant) vacutainers. Tubes were gently mixed by inversion 8 to 10 times immediately after blood collection to evenly distribute the anticoagulant additive. After centrifuged at 2000 g for 10 min, the separated plasma was carefully aspirated, aliquoted, and stored at −80°C until analysis. The protocol for the present study was informed consents from all patients were acquired. Blank blood was collected under the same study procedures from healthy volunteers.

2.3. MS analysis of isolated plasma

Firstly, the high abundance proteins in plasma were removed in consideration of their cover to the low content disease-specific markers in plasma. The plasma samples were inactivated by 30 min incubation at 56°C and centrifuged at 14000 g for 5 min. The supernatants were aspirated and added to filter pipes in ProteoPrep 20 Plasma Immunodepletion LC Column (Sigma). After centrifuged at 14000 g and 4°C for 5 min, 100 μL of the filtered samples were diluted into 1 mL by PBS and purified by ProteoPrep 20 Plasma Immunodepletion LC Column. The effluent was collected as low and high abundance protein samples according to the manufacturer's guidelines. Then the samples were added into respectively 3k tubular ultrafiltration device, centrifuged at 7500 g and 4 °C until the final volume to 100 μL, 8 time volume replaced with 8 M urea solution (with 0.1% SDS, 1 × PI, 1 mM PMSF), and finally centrifuged at 7500 g and 4 °C until the volume less than 100 μL. Protein content was evaluated using a BCA assay (Pierce). The sample quality was authenticated by 10% to 20% SDS-page gradient gel electrophoresis.

The isolated plasma samples were labeled with TMT-10 tags (Pierce). 100 μg low abundance proteins from each sample were added into tubes with 8 M urea, 0.1% SDS and 45 mM TEAB respectively, and replenished with LCMS grade ultra-pure water to 100 μl. After added 5 μL 200 mM TCEP, the mixture oscillated at 700 rpm and 55 °C for 1 h. Then the temperature was adjusted to 25 °C immediately after the reaction. 5 μL 375 mM iodoacetamide was added, and the mixture was continuing to oscillate in dark at 700 rpm and 25°C for 30 min. After added 660 μL precooled acetone, the mixture was precipitated at −20 °C overnight. The acetone was removed by centrifuged at 10000 g and 4 °C for 15 min. The precipitates were washed by 0.8 mL precooled 90% acetone and air dried at room temperature. The precipitated proteins were dissolved by 100 μL 100 mM TEAB and digested by 2.5 μL 1 mg/mL trypsin at 37°C overnight. Then samples were centrifuged at 10000 g for 3 min to separate supernatant. The 0.8 mg TMT10 labeling reagent (with 41 acetonitrile) was mixed with aspirated supernatant and reacted at 700 rpm for 2 h at 25 °C. To end the labeling, 8 μL 5% hydroxylamine was added and oscillated at 700 rpm and 25 °C for 15 min.

The TMT-10 labeled samples were vacuum concentrated at 45 °C and dissolved by C18 chromatographic buffer (2% acetonitrile, 98% water, pH = 10). The solution was adjusted to pH = 10 by 5% ammonium hydroxide and centrifuged at 10,000 g for 5 min to separate supernatant. The C18 chromatographic column was pre-processed by the chromatographic buffer for 25 min. The effluent and graded components were collected every minute. Then the graded components were vacuum concentrated and dissolved by loading buffer (96% water, 4% acetonitrile and 0.1% formic acid). After centrifuged at 10,000 rpm for 5 min, the supernatants were tested by LC-MS (Dionex NCS3500 HPLC system and Q Exactive mass spectrometer). A full mass spectrometry (MS) scan (350–1600 m/z range) was processed. The 20 most abundant ions were selected and fragmented by high-energy collision-induced dissociation (HCD) for the determination of secondary mass spectrometry.

2.4. Statistical analysis of MS data

Data files were exported from the Proteome Discoverer software and performed against a proteomics database (human-refseq-20140303-71465s) downloaded from NCBI. After chromatogram classification, the samples labeled by TMT-10 were divided

into 20 components. The filter parameter of analysis software in database search was 5% FDR. Only unique peptides were used in protein spectrum quantification, and the up-regulated or down-regulated for 1.5 times would be identified as a significant change.

To detect the biological function term of differential proteins, significant enrichment analysis was performed between differential proteins and all quantitative proteins (protein background). Firstly, the protein background was mapped to the database to calculate the number of proteins for each term; then hypergeometric test was performed to find the functional items that significantly enriched in the differential proteins comparing with the protein background. The calculation formula is:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

Of which, N was the number of proteins with annotation information in the protein background, and n was the number of different proteins in N. M was the number of proteins annotated to functional items in the protein background, and m was the number of differential proteins annotated to functional items. In addition, *P-value ≤.05 was set as the cut-off criterion for functional items with significant enrichment in the differential proteins.*

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used to identify potential functions and signal transduction pathway of differential proteins. The protein-protein interactions (PPI) network of overlapping genes in influenza, measles and mumps groups was constructed by stringDB.[10]

3. Results

3.1. Quality of protein separation of blood samples for mass spectrometric detection

As shown in Figure 1A, most of the peptides were distributed in 7 to 20 amino acids, consistent with the general rule of trypsin digestion and HCD fragmentation. Among them, the peptides less than 5 amino acids were too small to produce effective sequence identification. And peptides larger than 20 amino acids were not suitable for HCD fragmentation due to their high mass and charge number. Thus, the distribution of the peptides length identified by mass spectrometry was in accordance with the quality control requirements. In quantitative analysis, a protein corresponding to multiple peptide segments (or corresponding

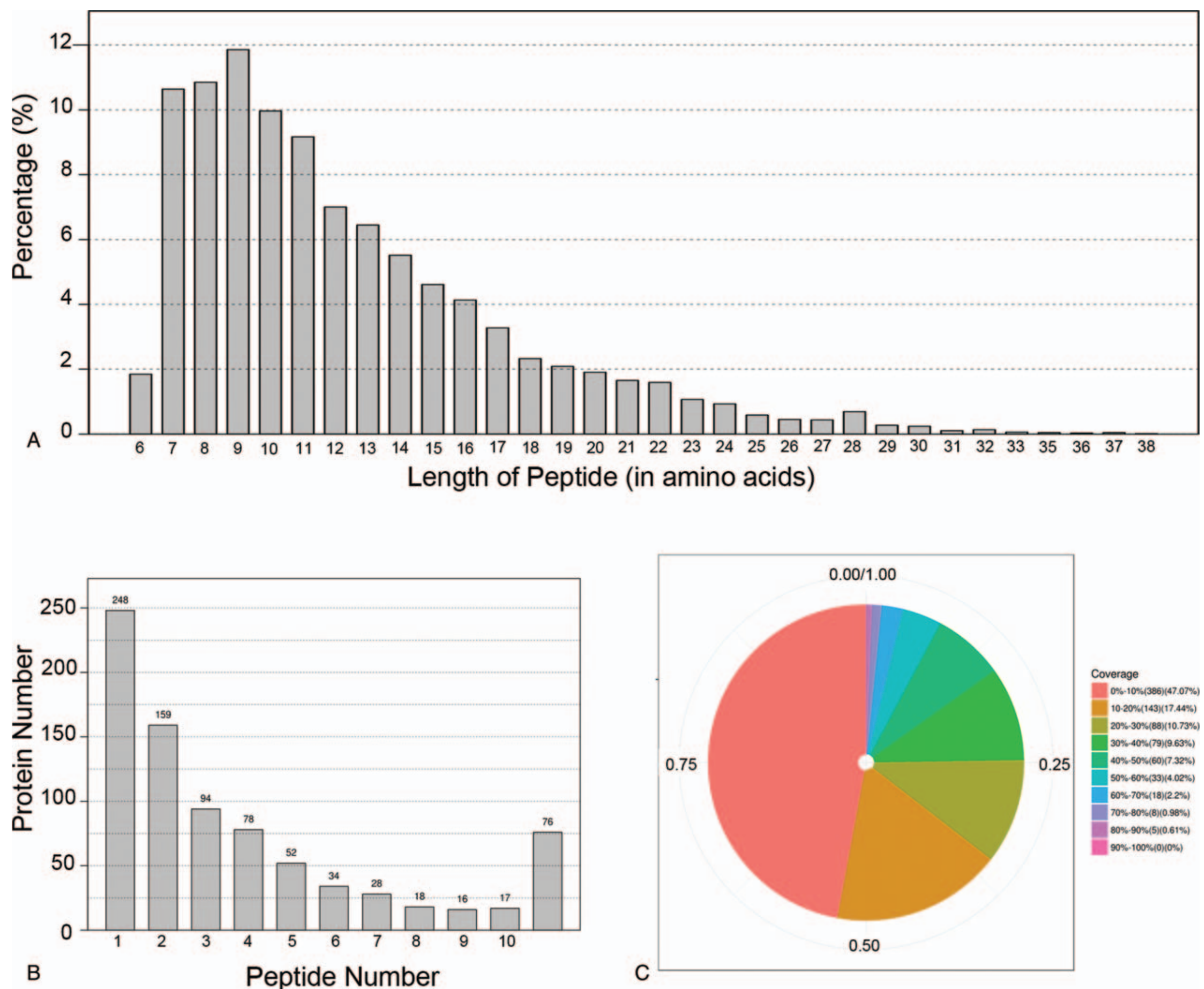

Figure 1. Quality of protein separation of blood samples for mass spectrometric detection. A. Percentage of peptides length in amino acids; B. Protein numbers of different peptide numbers; C. Protein coverage.

multiple spectrum diagrams) is beneficial to increase the accuracy and credibility of quantitative results. The peptide segment with higher abundance would be first scanned by mass spectra in the mass spectrometry analysis method based on shotgun (also known as bottom-up). Therefore, protein coverage is positively correlated with abundance in the sample. In this experiment, most proteins correspond to more than two peptide segments (Fig. 1B). The coverage of most proteins was below 20%, and major of the whole protein group of cells were medium and low abundance proteins with low coverage in the identification results of mass spectrometry. Furthermore, Supplementary Figure A and B, http://links.lww.com/MD/C708 showed that the first-order error was in ±10 ppm indicating the high accuracy of protein qualitative and quantitative analysis. Less proteins distributed below 10 kDA, and the proteins over 10 kDA had a uniform distribution. This result indicated that no significant bias appeared between proteins of different molecular weight during the sample preparation, and the proteins above 100 kDA were remained in the preparation process.

3.2. Differential protein screening and significant enrichment analysis

Under the condition of 5% FDR, 1500 proteins, 41,632 times of peptides and 7619 kinds of peptides were identified and quantified. Comparing with normal group, 154 differentially expressed proteins of influenza patients in immature heat (97 up-regulated and 57 down-regulated); 147 of influenza patients in mature heat (93 up-regulated and 54 down-regulated); 231 of measles patients in immature heat (159 up-regulated and 72 down-regulated); 208 of measles patients in mature heat (144 up-regulated and 64 down-regulated); 166 of mumps patients in immature heat (107 up-regulated and 59 down-regulated); 163 of mumps patients in mature heat (113 up-regulated and 50 down-regulated) were detected, indicating that the body has produced a series of feedback mediations on virus infection. Subsequently, comparing mature heat with immature heat samples, 30 proteins differentially expressed proteins of influenza patients (18 up-regulated and 12 down-regulated); 54 proteins differentially expressed proteins of measles patients (12 up-regulated and 42 down-regulated); 122 proteins differentially expressed proteins of mumps patients (90 up-regulated and 32 down-regulated) were screened out for further analyzes (Fig. 2A). Furthermore, in total of 178 differentially expressed proteins were found overlapping in those 3 groups. KEGG analysis results of the 178 proteins showed that 20 pathways were highly impacted (*P*-value >2.5) including cardiac muscle contraction, pathogenic *Escherichia coli* infection, retinol metabolism, dilated cardiomyopathy and adrenergic signaling in cardiomyocytes (Fig. 2B). According to the GO Biology Processes (BP) term results, 17 terms were found in multiple molecular functions, cellular component and biological processes, including molecular binding, extracellular region, enzyme regulator and catalytic activity, metabolic process and response to stimulus (Table 1).

3.3. PPI analysis of the union of the differentially expressed proteins

According to the PPI analysis of the union of the differentially expressed proteins in the three groups, the network connection diagram of the differentially expressed proteins was obtained in Figure 3A. Obviously, proteins associated with cell shape and motility, including actins (ACTN, ACTA, ACTB, ACTR2, TMSB4X, and VCL), Myosin (MYL and TPM) and intermediate filaments (VIM) were critical points in the PPI network. Besides, other genes which regulating biological processes, including intracellular signaling (FSCN1), peroxiredoxin (PRDX2 and P4HB) Transcription (HSPE1 and HSP90AB1), inflammation (PPIA), and apoptosis, also rendered significant roles. After functional enrichment, there were only 2 genes, namely *FBP2* and *Talin-1*, which had a difference of 1.5 times. The *FBP2* was associated with the insulin signaling pathway, which was down-regulated in the influenza group and measles group (Fig. 3B), but up-regulated in mumps group between mature heat and immature heat samples.

4. Discussion

In TMM, all febrile diseases were named as warm diseases, which can be divided into caused by pathogenic microorganisms, traumatism, toxication, and disorganized lifestyle. Among them, the fever caused by infectious diseases is the most common types of warm diseases. Recent studies found that FBPase, a rate-limiting enzyme in gluconeogenesis, play critical roles in tumor initiation and progression in several cancer types.[11]*Talin-1* may play a role in the reinforcement of cell proliferation, cell adhesion, and angiogenesis in colon cancer. Thus, the *Talin-1* protein activity may be a novel biomarker to detect colon cancer in humans.[12] FBPase and *Talin-1* are involved in numerous signaling pathways and is a vital protein marker in tumor screening.

Fever is a pathological process and can cause a series of metabolic and functional changes.[13] Firstly, as the patient's temperature rises, the sugar metabolism increases, and the oxygen supply is relatively insufficient. Between mature heat and immature heat samples of all the groups, the different expressions of *FBP2* and *Talin-1* were most obvious. FBPase is a key enzyme in gluconeogenesis, which catalyzes the reverse of the reaction of fructose bisphosphatase.[14] Three different groups of FBPases have been identified in eukaryotes and bacteria (FBPase1-3).[15] Of which, FBP1 is found in liver and kidney, and *FBP2* is found in muscle in human.[16] Thus, the differential expression of *FBP2* was consistent with the increase of muscle activity due to the trembling during fever, which leads to the up-regulated anaerobic glycolysis. And the different regulatory trends of *FBP2* in influenza, measles and mumps group (Fig. 3B) might be related to the pathologic changes of disease. However, the particular physiological role of *FBP2* in cell metabolism still remains unclear,[17] which needed further research to confirm. Moreover, *Talin-1* is a protein that ubiquitously expressed, and mostly is localized in cardiac and skeletal muscle cells,[18] which functions to mediate cell-cell adhesion via the linkage of integrins to the actin cytoskeleton and in the activation of integrins.[19,20] Previous researches showed that conditional knockout of *Talin-1* in cardiomyocytes was correlated with blunted ERK1/2, p38, Akt, and glycogen synthase kinase 3 responses, and suggested that up-regulation of *Talin-1* in cardiac hypertrophy might be detrimental to cardiomyocytes function.[21]

The functions of those 2 key differentially expressed proteins were both to affect glucose metabolism and muscle activity, and in particular, had a great effect on cardiomyocytes. It was consistent with the nominated KEGG pathways, including myocardial contraction, dilated cardiomyopathy and adrenergic signaling in cardiomyocytes. As is well-known, the activity of the sinus node and the sympathetic-adrenal medullary system is stimulated by high blood temperature during fever, which can

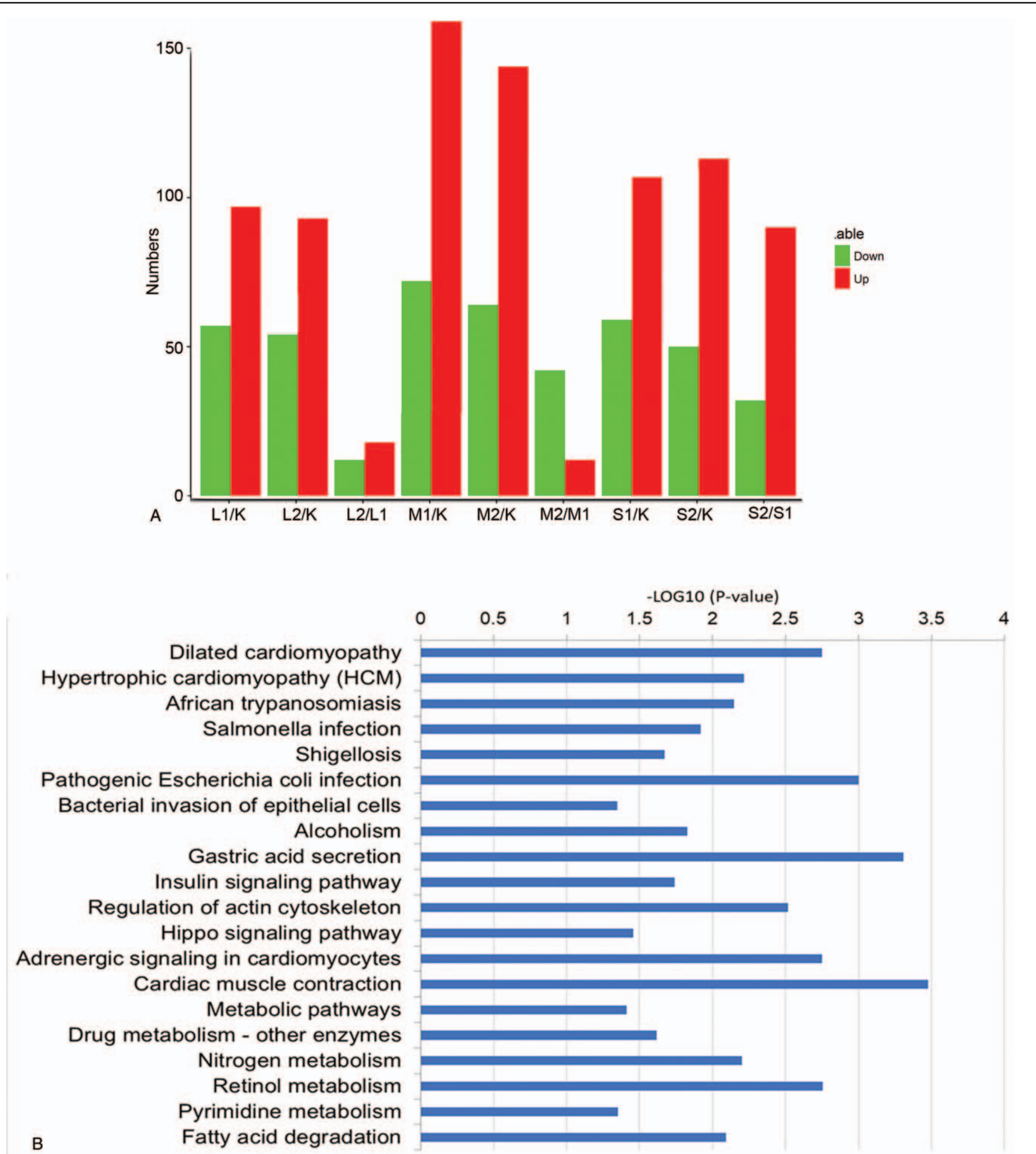

Figure 2. Analysis of differential proteins. A. The number of differentially expressed proteins. L1 was influenza patients in immature heat group; L2 was influenza patients in mature heat group; M1 was measles patients in immature heat group; M2 was measles patients in mature heat group; S1 was mumps patients in immature heat group; S2 was mumps patients in mature heat group; K was normal people group. B. KEGG enrichment result.

cause the heart rate to accelerate. The raised heart rate and myocardial contractility may also increase the burden of the heart, and even lead to myocardial damage and induced heart failure.[22] In addition to the infection pathway, the retinol metabolic pathway in the KEGG analysis results was also involved in the synthesis of glycoproteins and the mediation of immunoglobulin synthesis, whose metabolic abnormalities was able to decrease the cellular immunity.

It follows that the results of significant differential expression protein and signal pathway were consistent with the pathological changes of fever and were also in line with the theory of pathology in TMM theory. The appearance of abnormal glucose metabolism and potential heart injury could be used as a meaningful difference between mature and immature heat symptoms. And identified core differential proteins might be considered as potential biomarkers, which helps to evaluate the

Table 1

GO enrichment result of differential proteins.

Category	Term	Number	*P*-Value
Biological_process	Cellular process	127	0
Biological_process	Localization	84	0
Biological_process	Metabolic process	242	0
Biological_process	Multicellular organismal process	156	0
Biological_process	Pigmentation	277	0
Biological_process	Response to stimulus	234	0
Cellular_component	Cell part	304	0
Cellular_component	Extracellular region	39	0
Cellular_component	Extracellular region part	42	0
Cellular_component	Macromolecular complex	35	0
Cellular_component	Organelle	249	0
Cellular_component	Organelle part	148	0
Molecular_function	Binding	405	0
Molecular_function	Catalytic activity	117	0
Molecular_function	Structural molecule activity	30	3.14E-07
Biological_process	Biological regulation	31	9.51E-06
Molecular_function	Enzyme regulator activity	20	0.01614856

evolutionary tendency of TMM warm disease symptoms and make an accurate diagnose of immature and mature heat.

The immature heat period is the initial stage of the disease after pathogenic microorganisms' infection and it is the beginning to undergo pathophysiological changes. It is a common pathological process that all diseases characterized by fever may pass through and is a critical period of diagnosis and treatment. It is forbidden to use cold drugs or surgery during this period, otherwise, it may evolve into other syndromes of heat, and increase the difficulty of treatment, even worse. The mature heat period is the second stage of disease development. At this time, the heat is increased to the highest point and old medicine and surgery could also be used. Due to the distinct type of disease and the different driving factors, treatment strategies are different. It is of great value to understand the diagnosis and treatment strategies of different symptoms in TMM with scientific basis, and to promote the control of infectious diseases and the development of traditional medicine.

Mongolian medicine has its own uniqueness in the classification of warm diseases, and it also has its own characteristics in pathological analysis and treatment strategies. There are few studies on the correlation between the immature heat phase, the transition of the mature heat phase and the cytokines. At present, traditional Chinese medicine has achieved many successes in the mechanism of fever, while, Mongolian doctors have just started research on cytokines and fever mechanisms. In this study, 2 potential biomarkers which were identified as core differential proteins may benefit to evaluate the evolutionary tendency of TMM warm disease symptoms. It is important to give different treatment and care in different stage. The TMM is a complex and profound subject, and there are many worthy of exploration. In the future, Mongolian medicine could also achieve gratifying results for diagnosis, pathological analysis, and treatment strategies.

In this study, we used a high throughput mass spectrometry to identify potential protein markers of immature and mature heat

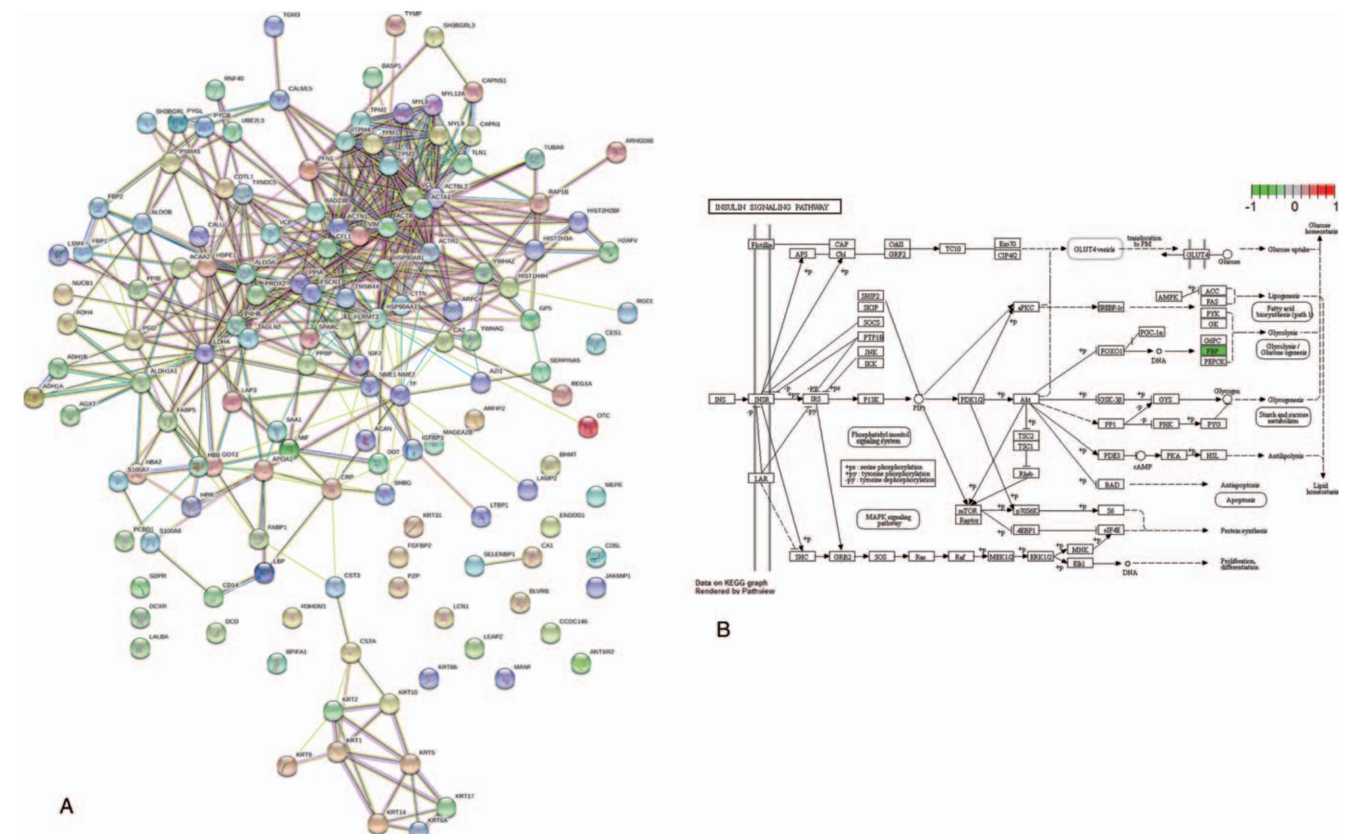

Figure 3. PPI analysis of the union of the differentially expressed proteins. A. Network diagram of differential proteins. B. *FBP2* associated signaling pathway in influenza group and measles group. PPI = protein-protein interactions.

symptom in influenza, measles, and mumps. 50 venous blood samples were isolated and prepared for differential protein analysis. 1500 proteins and 7619 polypeptides and 1323 differential expressions were identified between those 2 TMM symptom types. Among them, *FBP2* and *Talin-1* were critical points and have the potential to be evolution directions of TMM warm disease symptom.

Author contributions

Conceptualization: Xiaoying Wu, Li Li, and Eerdunchaolu.
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Writing – original draft: Xiaoying Wu.
Writing – review and editing: Li Li and Eerdunchaolu.

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