Research Article

Screening of Serum Protein Markers for Avascular Osteonecrosis of Femoral Head Differentially Expressed after Treatment with Yuanshi Shengmai Chenggu Tablets

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Avascular necrosis of the femoral head (ANFH) is an often occurring orthopaedic disease with high morbidity. Traditional Chinese Medicine (TCM) Yuanshi Shengmai Chenggu Tablet is a valid prescription for treating steroid-induced femoral head necrosis. However, there are rare investigations about the serum protein marker expression after the acting of drugs on hormone and TCM. In the present study, we aimed to systematically discover and validate the serum biomarker expression difference in patients with steroid-induced avascular necrosis of femoral head (SANFH) after taking Yuanshi Shengmai Chenggu Tablets (SANFH-TCM), so as to reveal the action mechanism of TCM from the molecular level by using isobaric tags for relative and absolute quantification (iTRAQ) with multiple reaction monitoring quantification. Significant differences in fibrinogen alpha, fibrinogen beta, fibrinogen gamma, fibronectin, C-reactive protein, apolipoprotein A, apolipoprotein D, and apolipoprotein E were found among SANFH, SANFH-TCM, and healthy controls. Therefore, our study proposes potential biomarkers for SANFH diagnosis and for the prognosis of femoral head necrosis after Traditional Chinese Medicine treatment.

1. Introduction

Avascular necrosis of the femoral head (ANFH) is a clinical common orthopaedic disease [1–3] with very high morbidity. It is difficult to cure and is one of the major medical problems that have not yet been overcome [4, 5]. Primary osteonecrosis of the femoral head is due to gene or gene mutation of patients. Secondary osteonecrosis of the femoral head could divide into traumatic and nontraumatic osteonecrosis of femoral head [6–8], in which traumatic osteonecrosis of the femoral head is the avascular necrosis of osteocyte caused by interruption of blood flow in the blood vessels in femoral head, which was due to trauma [9, 10]. Its etiology is still unclear. It was demonstrated that long-term and large dosage usage of hormone and drinking are two important factors that cause ANFN [4, 11]. In recent years, with the wide use of corticosteroids clinically, the cases with ANFN have also increased greatly [12, 13]. However, the pathogenesis of steroid-induced ANFN is still unknown. For developing new methods to prevent and treat ANFN, study on the pathogenesis of steroid-induced ANFN is particularly urgent [12, 14, 15].

Recent reports have shown that the occurrence of ANFN could be greatly decreased by an early intervention on high-risk crowds of ANFN who use hormones such as steroid and alcohol [16]. However, the composition of the serum is very complex [17]. It contains high-abundance proteins like albumin and immunoglobulins (mainly IgG), as well as low abundance proteins that are secreted by tissue or cells [18, 19]. Some of them are key proteins involved in signal transduction and regulation [20]. Tan et al. [21] adopted two-dimensional electrophoresis technology and separated 7 differentially expressed proteins between patients with primary femoral head necrosis and normal subjects from 10 pairs serum samples. They found that four important proteins including tissue-type plasminogen activator (t-PA), plasminogen activator...
inhibitor type 1 (PAI-1), Crosslaps, and anti-p53 antibody were significantly changed and that all of them can be used as the diagnosis serum markers of nontraumatic femoral head necrosis. Although the pathogenesis of ANFN is still unclear and the relevance of this finding with the further clinical application was not reported, analysis of the differentially expressed proteins in the serum could provide useful information.

Traditional Chinese Medicine (TCM) Yuanshi Shengmai Chenggu Tablet is valid and specialty drug for steroid-induced ANFN treatment. Yuanshi Shengmai Chenggu Tablet has obtained the certificate of new medicine in China and has been applied in clinical [22]. Its active ingredients are mainly flavonoids such as vitexin. By clinical studies, it was demonstrated the application of Yuanshi Shengmai Chenggu Tablet can significantly relieve the patients' pain and accelerate the absorption of dead bone and formation of new bone, showing a relatively strong osteogenic activity [22].

Liu et al. [23] extracted proteins in bone tissue from the femur and humerus bone in rat osteonecrosis model with or without Yuanshi Shengmai Chenggu Tablet TCM treatment and performed proteomics research. They reported that anticoagulating proteins heavy chain II B, phospholipid hydroperoxide glutathione peroxidase, and ubiquitin enzymes E2 (MW: 17 kD) are closely associated with steroid-induced bone necrosis, as well as the therapeutic efficacy of TCM.

In this study we aimed to investigate the differentially expressed protein in serum between steroid-induced ANFN patients with or without TCM treatment (Yuanshi Shengmai Chenggu Tablets). For this purpose, the proteomics method isobaric tags for relative and absolute quantification (iTRAQ) with multiple reaction monitoring (MRM) quantification was adopted in this study, so as to reveal the molecular mechanism of TCM treated the SANFN in the molecular level.

2. Material and Methods

2.1. Participants. Patients diagnosed as ANFN in the First Affiliated Hospital of Traditional Chinese Medicine University of Guangzhou from February 2014 to February 2015 were included. The ANFN diagnosis was established by referring to standard of adult femoral head necrosis diagnosis expert consensus (2012 edition) and the diagnosis and treatment of avascular necrosis of the expert advice of diagnostic criteria. Patients in active period of ANFN, alcoholics who are simultaneously treated by long-term high dose of glucocorticoids (taken steroid > 10 mg/d longer than 3 years), or patients with combining chronic disease which needs prolonged treatment were excluded in present study. All participants gave written informed consent before being enrolled in the study (AE-2013012011).

2.2. Specimens and Groups. Patients with ANFN who have used long-term and high-dosage of steroid (SANFN) were further treated with TCM Yuanshi Shengmai Chenggu Tablet (6 tablets each time, 3 times per day, total 3 months; prepared by the First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine, Guangzhou, China). Serum samples (n = 5) from patients with or without TCM were prospectively collected after obtaining written informed consent. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine. Five healthy subjects were collected during the same period who were sex- and age-matched. Thus, the verification population was divided into 3 groups: steroid-induced avascular necrosis of femoral head (SANFH), SANFH-TCM treatment, and healthy controls.

All serum samples were centrifuged at 1250 g for 5 min and then 13500 g for 15 min at 4 °C within 1 h of collection. All samples were then stored at −80 °C until use.

2.3. iTRAQ Analysis of Serum Samples. iTRAQ labeling and mass spectrometry analysis were performed as previously described [24]. Then, six iTRAQ labeled sample wells were generated (steroid-induced avascular necrosis of femoral head, SANFH-TCM treatment, and health controls, each for two subgroups). Briefly, high-abundance serum proteins such as albumin, IgG, and haptoglobin were removed by using the Human 14 Multiple Affinity Removal System (Agilent Technologies, Santa Clara, CA, USA). Then, 50 µg protein of each sample was concentrated and desalted, followed by digestion using trypsin before iTRAQ labeling. Six groups were labeled, including steroid-induced avascular necrosis of femoral head, iTRAQ reagent 113, 116; SANFH-TCM treatment 114, 117; and health controls 115, 118. The six sample groups were mixed, desalted, and dried.

The iTRAQ labeled peptides were separated by Strong Cation Exchange (SCX) chromatography (Bonna-Agela Technologies, Tianjin, China). SCX was carried out on a Polysulfoethyl 4.6 × 100 mm column (5 µm, 200 Å, PolyLC Inc., Maryland, USA). The peptides were eluted at the 45 min gradient from 100% buffer A (10 mM KH₂PO₄, pH 3.0, 25% acetonitrile) to 45% buffer B (10 mM KH₂PO₄, pH 3.0, 500 mM KCl, 25% acetonitrile) at the flow rate of 800 µL/min on Agilent 1210 LC system. All the fractions were analyzed by MALDI-TOF/TOF 5800 mass spectrometer (AB SCIEX, California, USA). Protein quantification and identification were performed with the Proteome Discoverer (version 1.3, thermos). The default bias correction was used and all quantitative variables were analyzed by the Proteome Discoverer 1.3. Peptide abundances were calculated based on the areas of the monoisotopic peaks. Protein ratios were the average ratios of all quantified peptides. Proteins with quantification P value < 0.05 in at least two pairs (113:114, 113:115, 114:115; 116:117, 116:118, 117:118) and with the ratio > 1.2 (the average ratio of two repeat experiments) or ratio < 0.83 were considered as differentially expressed proteins, using a cutoff of 2 times standard deviation [25].

2.4. Bioinformatics Analysis. Biomarker candidates were then prioritized using scoring a system based on iTRAQ values from Proteome Discoverer analysis. The cellular component,
molecular function, and biological process were analyzed through Gene Ontology (GO) database. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping was performed by KEGG Mapper (http://www.genome.jp/kegg/mapper.html), and the enrichment analysis was performed by Blast2GO PRO software (https://www.blast2go.com/, version 2.8).

2.5. Validation of Differential Expressed Protein by Multiple Reaction Monitoring (MRM) Quantification. To validate the expression of biomarker candidates, MRM quantifications were performed as previously described [26]. Briefly, 30 µg protein of each sample was digested using trypsin before being desalted. Then, desalted peptide mixtures were loaded onto an Acclaim PePmap C18-reversed phase column (100 Å, Thermo Scientific, Massachusetts, USA) and separated with reversed phase C18 column (300 Å, Bonn-Agela Technologies) mounted on a Dionex ultimate 3000 nano-LC system. Peptides were eluted using a gradient of 5–80% (v/v) acetonitrile in 0.1% formic acid over 45 min at a flow rate of 300 nL/min combined with a Q Exactive mass spectrometer (Thermo Scientific, Massachusetts, USA), at a flow rate of 300 nL/min combined with a Q Exactive mass spectrometer (Thermo Scientific, Massachusetts, USA), and then the eluates were directly entered in Q Exactive MS (Thermo Scientific, Massachusetts, USA), setting in positive ion mode and data-dependent manner with full MS scan within 350–2000 m/z, full scan resolution at 70000, MS/MS scan resolution at 17500, and MS/MS scan with minimum signal threshold 1E+5, isolation width at 2 Da. To evaluate the performance of this mass spectrometry on the iTRAQ labeled samples, two MS/MS acquisition modes and higher collision energy dissociation (HCD) were employed. And to optimize the MS/MS acquisition efficiency of HCD, normalized collision energy (NCE) was systemically examined, stepped 20%. Each MS/MS spectrum was searched against a mascot database (Uniprot, version 2.8). The search parameters were as follows: sample type, iTRAQ 8-plex (Peptide Labeled); cysteine modification by methyl methane-thiosulfonate; digestion, trypsin enzyme; proteins with, at least, two peptides with a high confidence score (>95%) and a low FDR (estimated local FDR of 5%) were considered positively identified.

2.6. Statistical Analysis. All studies to identify biomarkers by iTRAQ/MRM LC-MS/MS were performed on three separate occasions. Statistical analysis was performed using R (version 3.4.2, Bell Laboratories, USA). Analysis of variance (ANOVA) was performed for groups comparison. A P value < 0.05 was considered as statistical significantly.

3. Results

3.1. Populations. A total of 26 patients were included in the present study. Demographic characteristics of present population were summarized in Table 1. All of them were diagnosed as Association Research Circulation Osseous (ARCO) II stage SONFH, and the time windows of being illness were from 6 to 34 months. The mean age was 39.5 years old and 11 (42.3%) of them were males, suggesting that the patients SONFH were younger. Primary cause of 50% of patients was systemic lupus erythematosus, indicating a high risk of long-term high dose of steroid in systemic lupus erythematosus.

3.2. Protein Identification and Differentially Abundant Proteins. Serum proteins of steroid-induced ANSF (SANFH) patients, SANFH-TCM treatment patients, and health subjects were screened using iTRAQ. The experiment was repeated twice and detected 399 proteins. Among them, 61 proteins were differentially expressed between SANFH and healthy controls (Table 2), including 35 significantly upregulated proteins (>1.21-fold, P < 0.05) and 26 significantly downregulated proteins (<0.83-fold, P < 0.05). The top four upregulated proteins in SANFH compared to healthy controls were serum amyloid A-2 (SAA2), Ig lambda, sodium/potassium-transporting ATPase subunit alpha-3 (ATPIA3), and calcium-binding mitochondrial carrier protein Aralar I (SLC25A12) with fold change values of 4.57, 2.64, 2.07, and 1.95. The top four downregulated proteins were properdin, keratin type I cytoskeletal 9 (KRT9), apolipoprotein (a) (LPA), and tropomysin alpha-4 (TPM4) with fold change values of −1.77, −1.57, −1.65, and −1.61, respectively.

A total of 74 proteins were differentially expressed between SANFH-TCM and healthy controls (Table 3), including 45 significantly upregulated proteins (>1.21-fold, P < 0.05), and 29 significantly downregulated proteins (<0.83-fold, P < 0.05). The top four upregulated proteins in SANFH-TCM compared to healthy controls were ATP-binding cassette subfamily B member 9 (ABCB9), fibrinogen alpha, fibrinogen gamma, and fibrinogen beta with fold change values of 17.47, 13.05, 12.67, and 12.11. The top four downregulated proteins were C-reactive protein (CRP), Tubulin alpha-1A (TUBA1A), fibronectin, and LPA with fold change values of −1.73, −1.73, −1.52, and −1.48, respectively.

A total of 81 proteins were differentially expressed between SANFH-TCM and SANFH (Table 4), including significantly 44 upregulated proteins (>1.21-fold, P < 0.05) and 37 significantly downregulated proteins.

| Table 1: Demographic characteristics. |
|----------------------------------------|
| In Total | n |
| Male, n (%) | 11 (42.3) |
| Age, yr, mean ± SD | 39.5 ± 5.3 |
| History of SONFH, months, median (min, max) | 23.1 (6, 34) |
| Primary disease, n (%) | |
| Systemic lupus erythematosus | 13 (50.0) |
| Anaphylactoid purpura | 5 (19.2) |
| Eczema | 4 (15.4) |
| Psoriasis | 2 (7.7) |
| Thrombocytopenic purpura | 1 (3.8) |
| Fever of unknown origin | 1 (3.8) |

**Table 1: Demographic characteristics.**
Table 2: Differentially expressed protein between SANFH and healthy controls.

| Accession | Description                                                                 | P value      | Ratio (mean) |
|-----------|------------------------------------------------------------------------------|--------------|--------------|
| **Upregulated protein**                                                                 |
| P0DJI9    | Serum amyloid A-2 protein                                                    | 0.0000233    | 4.5740273    |
| P01714    | Ig lambda chain V-III region SH                                              | 0.000116405  | 2.64233333   |
| P13637    | Sodium/potassium-transporting ATPase subunit alpha-3                        | 0.010160416  | 2.06833333   |
| O75746    | Calcium-binding mitochondrial carrier protein Aralar1                        | 0.008612823  | 1.95325      |
| P01613    | Ig kappa chain V-1 region Ni                                                | 0.005619256  | 1.9485167    |
| P01743    | Ig heavy chain V-I region HG3                                                | 0.00017147   | 1.93190909   |
| P01742    | Ig heavy chain V-I region EU                                                 | 1.61E – 18   | 1.8255       |
| P0DJI8    | Serum amyloid A-1 protein                                                    | 0.00000019   | 1.72607353   |
| P01857    | Ig gamma-1 chain C region                                                    | 2.12E – 208  | 1.71611466   |
| P01861    | Ig gamma-4 chain C region                                                    | 0.00000135   | 1.6678889    |
| P06889    | Ig lambda chain V-IV region MOL                                              | 0.0000383    | 1.59966667   |
| P01860    | Ig gamma-3 chain C region                                                    | 6.59E – 46   | 1.59404098   |
| P02741    | C-reactive protein                                                           | 6.38E – 146  | 1.58455880   |
| Q8NBJ4    | Golgi membrane protein 1                                                     | 0.000564362  | 1.4622       |
| P0COL4    | Complement C4-A                                                              | 1.33E – 10   | 1.45864286   |
| P12335    | ADP/ATP translocase 1                                                        | 0.016291795  | 1.4941       |
| P06576    | ATP synthase subunit beta, mitochondrial                                    | 0.002154497  | 1.43466667   |
| P13645    | Keratin, type I cytoskeletal 10                                              | 4.63E – 13   | 1.41348333   |
| P04433    | Ig kappa chain V-III region VG (Fragment)                                    | 0.000000732  | 1.412        |
| Q08380    | Galectin-3-binding protein                                                   | 3.33E – 45   | 1.38927444   |
| Q14624    | Inter-alpha-trypsin inhibitor heavy chain H4                                | 6.37E – 87   | 1.37619099   |
| P22692    | Insulin-like growth factor-binding protein 4                                 | 0.025918263  | 1.362        |
| P01620    | Ig kappa chain V-III region SIE                                              | 8.22E – 09   | 1.3591875    |
| P02452    | Collagen alpha-(I) chain                                                    | 1.95E – 09   | 1.3503       |
| O75636    | Ficolin-3                                                                    | 2.26E – 53   | 1.341312     |
| P01717    | Ig lambda chain V-IV region Hil                                              | 0.003213417  | 1.3341       |
| P14625    | Endoplasmnin                                                                | 0.00000523   | 1.3105       |
| P01621    | Ig kappa chain V-III region NG9 (Fragment)                                   | 0.000190716  | 1.2999       |
| P51884    | Lumican                                                                      | 0.002279512  | 1.29618421   |
| P02747    | Complement C1q subcomponent subunit C                                       | 1.85E – 11   | 1.290775     |
| P25705    | ATP synthase subunit alpha, mitochondrial                                    | 0.000000675  | 1.28091667   |
| P22792    | Carboxypeptidase N subunit 2                                                 | 7.1E – 09    | 1.27185227   |
| P09543    | 2',3'-Cyclic-nucleotide 3'-phosphodiesterase                                | 0.034218978  | 1.2625       |
| P18428    | Lipopolysaccharide-binding protein                                           | 2.71E – 47   | 1.2541706    |
| P01009    | Alpha-1-antitrypsin                                                          | 6.68E – 34   | 1.21782482   |
| **Downregulated protein**                                                                 |
| P01781    | Ig heavy chain V-III region GAL                                              | 0.00000597   | 0.82966667   |
| O43852    | Calumenin                                                                    | 0.000374747  | 0.82916667   |
| Q9UK55    | Protein Z-dependent protease inhibitor                                       | 2.62E – 11   | 0.82908108   |
| O43866    | CD5 antigen-like                                                             | 1.19E – 23   | 0.82744578   |
| Q04756    | Hepatocyte growth factor activator                                           | 0.000530289  | 0.821        |
| P63104    | I4-3-3 protein zeta/delta                                                    | 0.009022339  | 0.8194       |
| Q68CQ4    | Digestive organ expansion factor homolog                                     | 0.000458581  | 0.81775      |
| P68366    | Tubulin alpha-4A chain                                                       | 0.00331478   | 0.8143       |
| P0421I    | Ig lambda chain V region 4A                                                  | 0.021633191  | 0.812        |
| Q6Q788    | Apolipoprotein A-V                                                           | 0.00000051    | 0.8107381    |
| P59665    | Neutrophil defensin 1                                                        | 0.01081692   | 0.7993       |
| P05164    | Myeloperoxidase                                                              | 0.001604817  | 0.7984       |
| P02649    | Apolipoprotein E                                                             | 5.01E – 267   | 0.7911827    |
| P04070    | Vitamin K-dependent protein C                                                | 1.41E – 22   | 0.78128922   |
| Q13103    | Secreted phosphoprotein 24                                                   | 9.56E – 09   | 0.76858333   |
(<0.83-fold, \( P < 0.05 \)). The top four upregulated proteins in SANFH-TCM compared to SANFH were ABCB9, IQ, and AAA domain-containing protein 1 (IQCA1), fibrinogen alpha, and fibrinogen beta, which showed the fold change values of 21.82, 13.86, 13.66, and 13.64, respectively. The top four downregulated proteins were serum amyloid A-2 (SAA2), Ig lambda, CRP, and collagen alpha-1 with fold change values of 2.24, −2.26, −2.24, and −2.04, respectively.

### 3.3. Biomarkers Prediction and Validation

MRM was performed to verify the results obtained from iTRAQ proteomics (Figure 1). The upregulation of fibrinogen alpha, fibrinogen beta, and fibrinogen gamma and apolipoprotein A (LPA) and apolipoprotein D (LPD) in SANFH-TCM versus healthy controls and SANFH-TCM versus SANFH was confirmed by MRM, respectively (\( P < 0.05 \)). Meanwhile, MRM also verified the decreased expression of fibronectin and CRP in SANFH-TCM versus healthy controls and SANFH-TCM versus SANFH identified by iTRAQ, respectively (\( P < 0.05 \)) (Table 5). In SANFH versus healthy controls, CRP and LPD were confirmed to upregulate, and LPD and apolipoprotein E (LPE) were confirmed to downregulate. Using MRM, fibrinogen alpha, fibrinogen beta, and fibrinogen gamma were significantly increased in SANFH compared with healthy controls. However, iTRAQ did not detect significant changes in the expression of fibrinogen alpha, fibrinogen beta, and fibrinogen gamma between SANFH and healthy controls. Although some difference existed between iTRAQ and MRM, all these data added confidence to the results obtained from iTRAQ.

### 3.4. Go Analysis of Differentially Expressed Proteins

The differentially expressed proteins (SANFH versus healthy controls, SANFH-TCM versus healthy controls, and SANFH-TCM versus SANFH) were classified by Gene Ontology (GO) based on their cellular component, molecular function, and biological process. For SANFH versus healthy controls (Figure 2), the top five significantly enriched GO terms concerning biological process were mainly associated with purine ribonucleotide biosynthetic process, nucleoside phosphate biosynthetic process, negative regulation of endothelial cell proliferation, mitochondrial transport and immune response-regulating signaling pathway, and cellular component, in which the top listed five GO terms were proton-transporting two-sector ATPase complex, catalytic domain, proton-transporting two-sector ATPase complex, proton-transporting ATP synthase complex, pigment granule, and organelle inner membrane. With respect to molecular function, transmembrane transporter activity, substrate-specific transmembrane transporter activity, primary active transmembrane transporter activity, p-p-bond-hydrolysis-driven transmembrane transporter activity, and monovalent inorganic cation transmembrane transporter activity were the top five GO terms.

For SANFH-TCM versus healthy controls (Figure 3), transport, response to organic substance, response to chemical, response to calcium ion, and regulation of triglyceride metabolic process were the top five GO terms concerning biological process; vesicle lumen, site of polarized growth, secretory granule lumen, secretory granule, and platelet alpha granule lumen were the top five GO terms concerning cellular component; and sulfur compound binding, small molecule binding, serine-type endopeptidase inhibitor activity, quaternary ammonium group binding, and peptidase regulator activity were the top five GO terms concerning molecular function.

For SANFH-TCM versus SANFH (Figure 4), most enriched GO terms were response to metal ion, response to inorganic substance, response to calcium ion, regulation of lipoprotein oxidation, and protein polymerization in biological process, site of polarized growth, secretory granule lumen, secretory granule, ribosome, and platelet alpha granule in cellular component, and sulfur compound binding, serine-type endopeptidase inhibitor activity, scavenger receptor activity, ribonuclease activity, and ribonuclease A activity in molecular function.

### 3.5. Pathway Enrichment Analysis of Differentially Expressed Proteins

The differentially expressed proteins (SANFH versus healthy controls, SANFH-TCM versus healthy controls, and SANFH-TCM versus SANFH) were mapped to the
| Accession | Description                                          | P value      | Ratio (mean) |
|-----------|------------------------------------------------------|--------------|--------------|
| **Upregulated protein**                                                                                                           |
| Q9NP78    | ATP-binding cassette subfamily B member 9            | 8.47E − 09   | 17.47338     |
| P02671    | Fibrinogen alpha chain                               | 0            | 13.049       |
| P02679    | Fibrinogen gamma chain                               | 0            | 12.66544     |
| P02675    | Fibrinogen beta chain                                | 0            | 12.11164     |
| Q86XH1    | IQ and AAA domain-containing protein 1               | 0.000199     | 8.9745       |
| P01019    | Angiotensinogen                                       | 9.49E − 09   | 2.256        |
| Q68CQ4    | Digestive organ expansion factor homolog             | 0.000267     | 1.882        |
| P03950    | Angiogenin                                           | 4.8E − 10    | 1.7776       |
| P13637    | Sodium/potassium-transporting ATPase subunit alpha-3 | 0.017893     | 1.742833     |
| Q8VXD2    | Secretogranin-3                                      | 1.34E − 07   | 1.71675      |
| P06396    | Gelsolin                                             | 3.3E − 46    | 1.659898     |
| Q8NBJ4    | Golgi membrane protein 1                             | 7.61E − 08   | 1.6227       |
| Q96KN2    | Beta-Ala-His dipeptidase                             | 1.18E − 06   | 1.588538     |
| P02775    | Platelet basic protein                               | 1.22E − 17   | 1.544688     |
| P06727    | Apolipoprotein A-IV                                  | 0            | 1.529316     |
| P01023    | Alpha-2-macroglobulin                                | 1.85E − 68   | 1.506488     |
| Q14624    | Inter-alpha-trypsin inhibitor heavy chain H4         | 4.2E − 152   | 1.503549     |
| P02776    | Platelet factor 4                                    | 1.49E − 56   | 1.487591     |
| Q722Y8    | Interferon-induced very large GTPase 1               | 0.047372     | 1.4735       |
| P29122    | Proprotein convertase subtilisin/kexin type 6        | 0.002827     | 1.47275      |
| P05154    | Plasma serine protease inhibitor                     | 1.09E − 06   | 1.446654     |
| P14618    | Pyruvate kinase PKM                                   | 0.005908     | 1.444167     |
| P06889    | Ig lambda chain V-I region MOL                       | 9.86E − 05   | 1.4295       |
| P01857    | Ig gamma-1 chain C region                            | 4E − 180     | 1.427066     |
| P22792    | Carboxypeptidase N subunit 2                         | 3.39E − 15   | 1.348591     |
| P01742    | Ig heavy chain V-I region EU                         | 3.44E − 19   | 1.336141     |
| P05090    | Apolipoprotein D                                      | 1.43E − 66   | 1.330211     |
| P1588     | Lumican                                              | 6.82E − 07   | 1.323868     |
| P01613    | Ig kappa chain V-I region Ni                         | 6.17E − 05   | 1.319958     |
| P01860    | Ig gamma-3 chain C region                            | 4.37E − 19   | 1.318262     |
| P14625    | Endoplasmnin                                         | 0.000738     | 1.3125       |
| Q04756    | Hepatocyte growth factor activator                   | 4.34E − 05   | 1.312333     |
| P80108    | Phosphatidylinositol-glycan-specific phospholipase D | 6.28E − 16   | 1.309714     |
| P02787    | Serotransferrin                                       | 0.03443      | 1.2721       |
| P00450    | Ceruloplasmin                                         | 1.43E − 27   | 1.269935     |
| P10720    | Platelet factor 4 variant                            | 1.65E − 05   | 1.255714     |
| P80748    | Ig lambda chain V-III region LOI                     | 0.003        | 1.24625      |
| P01009    | Alpha-1-antitrypsin                                  | 6.2E − 51    | 1.245105     |
| Q9NP79    | Coagulation factor XII                               | 0.001987     | 1.24225      |
| P02671    | Retinoic acid receptor responder protein 2           | 0.008513     | 1.234838     |
| P02679    | Ig kappa chain V-II region TEW                       | 0.024594     | 1.226038     |
| P02675    | Kallistatin                                          | 0.016269     | 1.223857     |
| Q86XH2    | Complement C1q subcomponent subunit A                | 8.72E − 05   | 1.222116     |
| P01020    | FERM and PDZ domain-containing protein 1             | 2.36E − 06   | 1.212885     |
| Q68CQ5    | Retinol-binding protein 4                            | 4.81E − 08   | 1.2112       |
| **Downregulated protein**                                                                                                          |
| Q02818    | Nucleobindin-1                                       | 0.002012     | 0.8295       |
| Q9UK55    | Protein Z-dependent protease inhibitor               | 2.62E − 11   | 0.829081     |
| P02656    | Apolipoprotein C-III                                 | 3.32E − 34   | 0.823015     |
| P62258    | 14-3-3 protein epsilon                               | 0.04376      | 0.81975      |
Table 3: Continued.

| Accession | Description | P value | Ratio (mean) |
|-----------|-------------|---------|--------------|
| P01877    | Ig alpha-2 chain C region | 0.024488 | 0.817692 |
| Q6Q788    | Apolipoprotein A-V | 5.25 × 10⁻⁸ | 0.817692 |
| P36980    | Complement factor H-related protein 2 | 0.001599 | 0.813253 |
| P35542    | Serum amyloid A-4 protein | 2.65 × 10⁻²⁹ | 0.800375 |
| P01620    | Ig kappa chain V-III region SIE | 1.42 × 10⁻⁵ | 0.794833 |
| O14818    | Proteasome subunit alpha type-7 | 0.024316 | 0.794833 |
| P18428    | Lipopolysaccharide-binding protein | 4.26 × 10⁻⁷⁵ | 0.789371 |
| P04438    | Ig heavy chain V-II region SESS | 0.044412 | 0.78775 |
| P49721    | Proteasome subunit beta type-2 | 0.026571 | 0.7765 |
| P04196    | Histidine-rich glycoprotein | 6.01 × 10⁻²⁸ | 0.775008 |
| P02649    | Apolipoprotein E | 0 | 0.75853 |
| Q86UD1    | Out at first protein homolog | 7.29 × 10⁻⁸ | 0.755267 |
| P04211    | Ig lambda chain V region 4A | 1.19 × 10⁻⁰⁵ | 0.751667 |
| P0DJ9     | Serum amyloid A-2 protein | 7.71 × 10⁻⁰⁵ | 0.746467 |
| P67936    | Tropomyosin alpha-4 chain | 0.0005 | 0.74375 |
| Q8NDV3    | Structural maintenance of chromosomes protein 1B | 0.000726 | 0.741833 |
| P62158    | Calmodulin | 0.005327 | 0.733677 |
| Q16610    | Extracellular matrix protein 1 | 5.58 × 10⁻¹⁰ | 0.731882 |
| Q92954    | Proteoglycan 4 | 7.15 × 10⁻¹² | 0.731882 |
| P59665    | Neutrophil defensin 1 | 0.000726 | 0.728857 |
| P20742    | Pregnancy zone protein | 4.88 × 10⁻⁷ | 0.7177 |
| P02741    | C-reactive protein | 2.7 × 10⁻⁷³ | 0.696948 |
| Q7IU36    | Tubulin alpha-1A chain | 0.000833 | 0.677154 |
| P02751    | Fibronectin | 0 | 0.657333 |
| P08519    | Apolipoprotein(a) | 7.96 × 10⁻⁰⁹ | 0.56335 |

4. Discussion

This is the first study to reveal proteins associated with steroid-induced avascular necrosis of the femoral head with or without Traditional Chinese Medicine treatment on the proteome level. MRM was used to add confidence to the results obtained by iTRAQ and was attempted for validating 8 proteins (fibrinogen alpha, fibrinogen beta, fibrinogen gamma, fibronectin, C-reactive protein, apolipoprotein A, apolipoprotein D, and apolipoprotein E).

Currently, the pathogenesis theories on femoral head necrosis mainly include [27, 28]: theory of osteoporosis; theory of vascular wall damage or compression and theory of blood lipid disorder [29]; theory of high intraosseous pressure; theory of intravascular coagulation; theory of secondary collision; and so forth [30]. Secondary collision theory [31] considers that osteonecrosis of the femoral head

reference pathways in KEGG database to identify significantly enriched metabolic pathways or signal transduction pathways. In total, 47, 58, and 20 significantly enriched pathways were obtained in SANFH versus healthy controls (Table 6), SANFH-TCM versus healthy controls (Table 7), and SANFH-TCM versus SANFH (Table 8) (P < 0.05), respectively. The top listed five pathways were Alzheimer’s disease, salivary secretion, Huntington’s disease, Parkinson’s disease, and oxidative phosphorylation in SANFH versus healthy controls (Table 6); mineral absorption, PPAR signaling pathway, chemokine signaling pathway, adrenergic signaling in cardiomyocytes, and neurotrophin signaling pathway in SANFH-TCM versus healthy controls (Table 7); and chemokine signaling pathway, platelet activation, cytokine-cytokine receptor interaction, glycosylphosphatidylinositol-(GPI-) anchor biosynthesis, and beta-alanine metabolism in SANFH-TCM versus SANFH (Table 8) (P < 0.03), respectively. The predicted biomarker LPE was involved in the enriched pathway of Alzheimer’s disease in both SANFH versus healthy and SANFH-TCM versus healthy controls. In SANFH-TCM versus healthy controls, LPA was involved in the enriched pathway of PPAR signaling pathway; fibronectin was involved in the enriched pathway of pathways in cancer, small-cell lung cancer, and bacterial invasion of epithelial cells. Fibrinogen alpha, fibrinogen beta, and fibrinogen gamma were involved in the enriched pathway of platelet activation in both SANFH-TCM versus healthy controls and SANFH-TCM versus SANFH. In SANFH-TCM versus SANFH, fibronectin was involved in the enriched pathway of regulation of actin cytoskeleton, and fibrinogen gamma was also involved in the enriched pathway of Staphylococcus aureus infection.
| Accession | Description                                                                 | P value   | Ratio (mean) |
|-----------|-----------------------------------------------------------------------------|-----------|--------------|
| **Upregulated protein** |                                                                              |           |              |
| Q9NP78    | ATP-binding cassette sub-family B member 9                                   | 7.38E-10  | 21.82029412  |
| Q86XH1    | IQ and AAA domain-containing protein 1                                       | 0.000343733 | 13.86725     |
| P02671    | Fibrinogen alpha chain                                                       | 0         | 13.6598665   |
| P02675    | Fibrinogen beta chain                                                        | 0         | 13.64325401  |
| P02679    | Fibrinogen gamma chain                                                       | 0         | 13.23321973  |
| P01019    | Angiotensinogen                                                              | 0.000000129 | 2.39730769   |
| Q68CQ4    | Digestive organ expansion factor homolog                                    | 0.0000351 | 2.26525      |
| P03950    | Angiogenin                                                                   | 0.0000196 | 2.0039       |
| P35527    | Keratin, type I cytoskeletal 9                                              | 3.95E-11  | 1.928576923  |
| Q8WXD2    | Secretogranin-3                                                             | 0.000728124 | 1.91025     |
| P27918    | Properdin                                                                    | 1.39E-40  | 1.763869048  |
| P14618    | Pyruvate kinase PKM                                                          | 0.002960903 | 1.607923077 |
| P05154    | Plasma serine protease inhibitor                                             | 2.03E-12  | 1.60933333   |
| Q04756    | Hepatocyte growth factor activator                                           | 2.81E-08  | 1.59033333   |
| P02775    | Platelet basic protein                                                       | 2.34E-18  | 1.56775      |
| P02776    | Platelet factor 4                                                            | 3.43E-84  | 1.559232955  |
| P02749    | Beta-2-glycoprotein 1                                                        | 0.0000322 | 1.548        |
| Q96KN2    | Beta-Ala-His dipeptidase                                                     | 0.0000169 | 1.53115385   |
| P01023    | Alpha-2-macroglobulin                                                        | 1.1E-62   | 1.51928916   |
| P06727    | Apolipoprotein A-IV                                                          | 0         | 1.4910721    |
| P06396    | Gelsolin                                                                    | 7.08E-33  | 1.485481481  |
| P01769    | Ig heavy chain V-III region GA                                               | 0.009729195 | 1.45675     |
| P01720    | Platelet factor 4 variant                                                    | 0.00000465 | 1.450190476 |
| P05090    | Apolipoprotein D                                                             | 5.92E-70  | 1.385849624  |
| P19823    | Inter-alpha-trypsin inhibitor heavy chain H2                                 | 1.59E-76  | 1.376936508  |
| P01871    | Ig mu chain C region                                                         | 1.12E-123 | 1.37344898   |
| P01616    | Ig kappa chain V-II region MIL                                               | 0.004694041 | 1.35375    |
| P34096    | Ribonuclease 4                                                               | 0.016807995 | 1.3485      |
| P29122    | Proprotein convertase subtilisin/kexin type 6                                | 0.007174928 | 1.3275     |
| P80108    | Phosphatidylinositol-glycan-specific phospholipase D                         | 1.54E-12  | 1.320666667  |
| P55056    | Apolipoprotein C-IV                                                          | 0.0000081 | 1.3169       |
| Q95445    | Apolipoprotein M                                                             | 0.0000275 | 1.314954545  |
| Q9UHG3    | Prenylcysteine oxidase 1                                                     | 0.0000266 | 1.305045455  |
| P08603    | Complement factor H                                                          | 8.17E-39  | 1.296578143  |
| P02787    | Serotransferrin                                                              | 0.002337342 | 1.2947      |
| P05164    | Myeloperoxidase                                                              | 0.020349711 | 1.282       |
| Q8WWA0    | Intelectin-1                                                                 | 0.0000227 | 1.2735       |
| P29622    | Kallistatin                                                                  | 0.0000069 | 1.267285714  |
| Q9NP79    | Immunoglobulin J chain                                                       | 4.06E-10  | 1.260973684  |
| Q86XH2    | CD5 antigen-like                                                             | 3E-31     | 1.248355422  |
| P02671    | Plasma kallikrein                                                            | 6.95E-08  | 1.236383333  |
| P02675    | Serum amyloid P-component                                                    | 1.03E-24  | 1.23613239   |
| P02679    | Inter-alpha-trypsin inhibitor heavy chain H1                                 | 1.7E-30   | 1.230389423  |
| P01019    | Alpha-actinin-4                                                              | 0.045621384 | 1.23        |
| **Downregulated protein** |                                                                              |           |              |
| P04217    | Alpha-1B-glycoprotein                                                        | 0.0000256 | 0.826346154  |
| P01613    | Ig kappa chain V-I region Ni                                                | 0.010289928 | 0.825583333 |
| Q16610    | Extracellular matrix protein 1                                               | 0.00000945 | 0.82129412   |
| Q92954    | Proteoglycan 4                                                               | 1.05E-09  | 0.8145       |
Table 4: Continued.

| Accession | Description                                      | P value    | Ratio (mean)   |
|-----------|-------------------------------------------------|------------|----------------|
| Q02818    | Nucleobindin-1                                  | 0.000247875| 0.813125       |
| P01742    | Ig heavy chain V-I region EU                     | 1.22E−08   | 0.803428571    |
| P04434    | Ig kappa chain V-III region VH (Fragment)        | 0.030092513| 0.802166667    |
| P55774    | C-C motif chemokine 18                           | 0.003307656| 0.79825        |
| Q9HDC9    | Adipocyte plasma membrane-associated protein     | 0.00008447 | 0.798033333    |
| P01011    | Alpha-1-antichymotrypsin                         | 7.56E−13   | 0.796217391    |
| P01621    | Ig kappa chain V-III region NG9 (Fragment)       | 0.0000675  | 0.795          |
| P01717    | Ig lambda chain V-IV region Hil                  | 0.0000608  | 0.78905        |
| P04196    | Histidine-rich glycoprotein                      | 2.81E−26   | 0.783991667    |
| P04433    | Ig kappa chain V-III region VG (Fragment)        | 0.0000651  | 0.783357143    |
| P35542    | Serum amyloid A-4 protein                        | 1.77E−40   | 0.752563218    |
| P0DJ18    | Serum amyloid A-1 protein                        | 4.33E−11   | 0.748036765    |
| P27824    | Calnexin                                         | 0.038086684| 0.73825        |
| P00740    | Coagulation factor IX                            | 6.16E−23   | 0.733622222    |
| Q9UGM5    | Fetuin-B                                         | 0.00451302 | 0.721833333    |
| P0Col4    | Complement C4-A                                  | 4.74E−14   | 0.71725        |
| Q08380    | Galectin-3-binding protein                       | 9.69E−59   | 0.71372805     |
| P04438    | Ig heavy chain V-III region SESS                 | 0.03934686 | 0.70425        |
| P20742    | Pregnancy zone protein                           | 9.73E−65   | 0.698695238    |
| P13645    | Keratin, type 1 cytoskeletal 10                  | 1.17E−19   | 0.69635        |
| Q86UD1    | Out at first protein homolog                     | 3.02E−09   | 0.694666667    |
| P02751    | Fibronecin                                       | 0           | 0.692870958    |
| P04208    | Ig lambda chain V-I region WAH                   | 0.00226793 | 0.6685         |
| P01861    | Ig gamma-4 chain C region                        | 2.47E−08   | 0.655777777    |
| P18428    | Lipopolysaccharide-binding protein               | 5.83E−130  | 0.640994118    |
| P49721    | Proteasome subunit beta type-2                   | 0.019809794| 0.6185         |
| P25789    | Proteasome subunit alpha type-4                  | 0.0061102  | 0.609          |
| P01743    | Ig heavy chain V-I region HG3                    | 0.0000275  | 0.607136364    |
| P01620    | Ig kappa chain V-III region SIE                  | 1.87E−11   | 0.5894375      |
| P02452    | Collagen alpha-1(I) chain                       | 0.000916641| 0.4901         |
| P02741    | C-reactive protein                               | 1.19E−243  | 0.445641414    |
| P01714    | Ig lambda chain V-III region SH                  | 1.91E−09   | 0.442666667    |
| P0DJ19    | Serum amyloid A-2 protein                        | 1.16E−08   | 0.424595238    |

is multifactor disease and it is related to genetic susceptibility factor and exposure to specific risk factors. The occurrence of femoral head necrosis is the collusion result of posterior acquired factors and genetic predisposing factor. Clinical studies also indicate that not all patients that had taken high dose hormone for a long time will suffer from femoral head necrosis and only 10% of patients will be attacked by femoral head necrosis. Though there are many clinical and basis studies about femoral head avascular necrosis, its specific pathophysiological mechanism is still not determined [10, 14]. The beginning of proteomic technology applying in femoral head necrosis is relatively late and there are rare reports. The proteomics study of femoral head necrosis will be helpful to explain the pathological physiology mechanism of femoral head necrosis.

By using meprednisone to induce chicken femoral head necrosis, Li et al. [32] found that there are adipose tissue proliferation and new bone formation through the histological examination; by two-dimensional electrophoresis, 13 protein expression differences were found. Among them, 9 kinds of proteins were downregulated 3 times after hormone treatment, which were serum amyloid P-component precursor, zinc finger protein 28, endothelial zinc finger protein 71, T-box transcription factor 3, cyclin-dependent kinase inhibitor 1, myosin ID, dimethylamine monoxygenase, and two kinds of unknown proteins. However, the animal species were
Table 5: MRM was performed to verify the results obtained from iTRAQ proteomics.

| Accession number | Description       | Relative protein abundance (MRM) | Relative protein abundance (iTRAQ) |
|------------------|------------------|----------------------------------|-----------------------------------|
|                  |                  | SANFH versus healthy controls    | SANFH-TCM versus healthy controls |
|                  |                  | SANFH-TCM versus SANFH           | SANFH versus healthy controls     |
|                  |                  | SANFH-TCM versus healthy controls|
| P02671           | Fibrinogen alpha | 1.8493                           | 0.95                             |
|                  |                  | 62.3799                          | 7.502                            |
| P02675           | Fibrinogen beta  | 3.2104                           | 0.936                            |
|                  |                  | 143.2262                         | 5.8                              |
| P02679           | Fibrinogen gamma | 1.7341                           | 0.953                            |
|                  |                  | 79.1554                          | 6.721                            |
| P02751           | Fibrinectin      | 0.8527                           | 0.999                            |
|                  |                  | 0.3081                           | 0.568                            |
| P02741           | C-reactive protein | 2.2723                        | 1.559                            |
|                  |                  | 0.522                            | 0.658                            |
| P06727           | Apolipoprotein A | 1.2723                           | 1.532                            |
|                  |                  | 1.8632                           | 1.45                             |
| P05090           | Apolipoprotein D | 0.9796                           | 1.302                            |
|                  |                  | 1.4369                           | 1.341                            |
| P02649           | Apolipoprotein E | 0.6693                           | 0.713                            |
|                  |                  | 0.6865                           | 0.934                            |
Figure 1: Continued.
Figure 1: MRM quantification of results obtained from iTRAQ proteomics. MRM was performed to verify the different expressions of selected proteins including fibrinogen alpha, fibrinogen beta, and fibrinogen gamma, fibronectin, apolipoprotein A (LPA), apolipoprotein D (LPD), and apolipoprotein E (LPD), and C-reaction protein in SANFH versus healthy controls, SANFH-TCM versus healthy controls, and SANFH-TCM versus SANFH.
Table 6: Differently enriched pathways were obtained in SANFH versus healthy controls.

| Pathway_acc | Pathway_Name                                      | P value     | Protein in Background | Protein in Diff Exp | Protein list                                           |
|-------------|--------------------------------------------------|-------------|-----------------------|---------------------|-------------------------------------------------------|
| hsa05010    | Alzheimer’s disease                              | 0.015362    | 7                     | 4                   | P25705, P102649, P62158, P06576                        |
| hsa04970    | Salivary secretion                               | 0.032411    | 5                     | 3                   | P62158, P04220, P13637                                |
| hsa05016    | Huntington’s disease                             | 0.057318    | 6                     | 3                   | P25705, P12235, P06576                                |
| hsa05012    | Parkinson’s disease                              | 0.057318    | 6                     | 3                   | P25705, P12235, P06576                                |
| hsa00190    | Oxidative phosphorylation                        | 0.070274    | 3                     | 2                   | P25705, P06576,                                     |
| hsa04261    | Adrenergic signaling in cardiomyocytes           | 0.088785    | 7                     | 3                   | P67936, P62158, P13637                                |
| hsa04915    | Estrogen signaling pathway                       | 0.125873    | 4                     | 2                   | P62158, P14625                                        |
| hsa04020    | Calcium signaling pathway                        | 0.125873    | 4                     | 2                   | P12235, P62158                                        |
| hsa04974    | Protein digestion and absorption                 | 0.125873    | 4                     | 2                   | P13637, P02452                                        |
| hsa04260    | Cardiac muscle contraction                       | 0.125873    | 4                     | 2                   | P67936, P13637,                                     |
| hsa04961    | Endocrine and other factor-regulated calcium reabsorption | 0.162907    | 1                     | 1                   | P13637                                                |
| hsa04964    | Proximal tubule bicarbonate reabsorption         | 0.162907    | 1                     | 1                   | P13637                                                |
| hsa04960    | Aldosterone-regulated sodium reabsorption        | 0.162907    | 1                     | 1                   | P13637                                                |
| hsa04070    | Phosphatidylinositol signaling system            | 0.162907    | 1                     | 1                   | P62158                                                |
| hsa04744    | Phototransduction                                | 0.162907    | 1                     | 1                   | P62158                                                |
| hsa04976    | Bile secretion                                  | 0.162907    | 1                     | 1                   | P13637                                                |
| hsa05130    | Pathogenic Escherichia coli infection            | 0.167478    | 9                     | 3                   | Q71U36, P63104, P68366,                               |
| hsa04918    | Thyroid hormone synthesis                        | 0.188181    | 5                     | 2                   | P14625, P13637                                        |
| hsa04971    | Gastric acid secretion                           | 0.188181    | 5                     | 2                   | P62158, P13637                                        |
| hsa04540    | Gap junction                                    | 0.253612    | 6                     | 2                   | Q71U36, P68366,                                       |
| hsa04972    | Pancreatic secretion                             | 0.299618    | 2                     | 1                   | P13637                                                |
| hsa04720    | Long-term potentiation                           | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa04911    | Insulin secretion                                | 0.299618    | 2                     | 1                   | P13637                                                |
| hsa05214    | Glioma                                           | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa04978    | Mineral absorption                               | 0.299618    | 2                     | 1                   | P13637                                                |
| hsa04973    | Carbohydrate digestion and absorption            | 0.299618    | 2                     | 1                   | P13637                                                |
| hsa04014    | Ras signaling pathway                            | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa04270    | Vascular smooth muscle contraction               | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa04910    | Insulin signaling pathway                        | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa05031    | Amphetamine addiction                           | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa04750    | Inflammatory mediator regulation of TRP channels | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa04912    | GnRH signaling pathway                           | 0.299618    | 2                     | 1                   | P62158                                                |
| hsa05152    | Tuberculosis                                     | 0.319543    | 7                     | 2                   | P18428, P62158, P00731, P0C0L4, P13645, P02747        |
| hsa05150    | Staphylococcus aureus infection                  | 0.334256    | 18                    | 4                   | P01742, P01781, P01743, P63104, P62158                |
| hsa04120    | Ubiquitin mediated proteolysis                   | 0.357215    | 13                    | 3                   | P01742, P01781, P01743, P63104, P62158                |
| hsa04114    | Oocyte meiosis                                   | 0.3841      | 8                     | 2                   | P13637,                                               |
| hsa04919    | Thyroid hormone signaling pathway                | 0.41429     | 3                     | 1                   | P13637,                                               |
| hsa04916    | Melanogenesis                                    | 0.41429     | 3                     | 1                   | P62158                                                |
| hsa04064    | NF-kappa B signaling pathway                     | 0.41429     | 3                     | 1                   | P18428,                                               |
| hsa04728    | Dopaminergic synapse                             | 0.41429     | 3                     | 1                   | P62158                                                |
| hsa04621    | NOD-like receptor signaling pathway              | 0.41429     | 3                     | 1                   | P14625,                                               |
| hsa05215    | Prostate cancer                                  | 0.41429     | 3                     | 1                   | P14625,                                               |
Table 6: Continued.

| Pathway_acc | Pathway_Name                        | P value | Protein in Background | Protein in Diff Exp | Protein list               |
|------------|------------------------------------|---------|-----------------------|---------------------|---------------------------|
| hsa04740   | Olfactory transduction             | 0.41429 | 3                     | 1                   | P62158,                  |
| hsa04620   | Toll-like receptor signaling pathway| 0.41429 | 3                     | 1                   | P18428,                  |
| hsa04713   | Circadian entrainment              | 0.41429 | 3                     | 1                   | P62158,                  |
| hsa03320   | PPAR signaling pathway             | 0.445992| 9                     | 2                   | P08519, Q6Q788,          |
| hsa05133   | Pertussis                          | 0.45325 | 15                    | 3                   | P0C0L4, P62158, P02747,  |

Note. Italic font indicated the candidate protein.

Figure 2: GO analysis of differentially expressed proteins between SANFH and healthy controls. The significantly enriched GO terms concerning biological process, cellular component, and molecular function were shown.
In the present study, we further suggested that the serum amyloid A-2 (SAA2), Ig lambda, sodium/potassium-transporting ATPase subunit alpha-3 (ATP1A3), calcium-binding mitochondrial carrier protein Aralar1 (SLC25A12), properdin, KRT9, LPA, TPM4, ABCB9, fibrinogen alpha,

Figure 3: GO analysis of differentially expressed proteins between SANFH-TCM and healthy controls. The significantly enriched GO terms concerning biological process, cellular component and molecular function were shown.

different, the cases in the clinical study were few, and the pathogenesis was different, so they lacked comparability and the study results were also different, without representativeness, so they were not sufficient to explain the pathogenesis of femoral head necrosis.

Considering the sampling of bone tissue is an invasive operation, which will bring regional trauma for patients increasing their suffering, the sampling of serum is easier and is also easy for patients to accept. There are few studies on femoral head necrosis. Researchers [3, 33] conducted serum proteomics study on 11 patients with drinking, hormone treatment, or specific femoral head necrosis (3 female and 8 male) and they found 8 protein differential points. Comparing with the serum of healthy volunteers, the serum of patients with femoral head had higher kininogen 1 variant, complement factor C3 precursor, and complement factor H. Besides, patients with femoral head necrosis had significant lower apolipoprotein A-IV precursor, antithrombin III chain B, and gelsolin isoform α precursor.

In the present study, we further suggested that the serum amyloid A-2 (SAA2), Ig lambda, sodium/potassium-transporting ATPase subunit alpha-3 (ATP1A3), calcium-binding mitochondrial carrier protein Aralar1 (SLC25A12), properdin, KRT9, LPA, TPM4, ABCB9, fibrinogen alpha,
fibrinogen gamma, fibrinogen beta, CRP, TUBA1A, fibronectin, IQCA1, AA2, and collagen alpha-1 were potential serum marker by iTRAQ and further confirmed the changes of fibrinogen alpha, fibrinogen beta, fibrinogen gamma, fibronectin, C-reactive protein, apolipoprotein A, apolipoprotein D, and apolipoprotein E. The predicted biomarker LPE was involved in the enriched pathway of Alzheimer’s disease; LPA was involved in the enriched pathway of PPAR signaling pathway; fibronectin was involved in the enriched pathway of pathways in cancer, small-cell lung cancer, and bacterial invasion of epithelial cells; fibrinogen alpha, fibrinogen beta, and fibrinogen gamma were involved in the enriched pathway of platelet activation; fibronectin was involved in the enriched pathway of regulation of actin cytoskeleton, and fibrinogen gamma was also involved in the enriched pathway of Staphylococcus aureus infection. Consistently, it has demonstrated that apolipoprotein A1 is potential risk for femoral head necrosis [34–36]. Fibronectin related to extracellular matrix integrity and adhesion is also an identified serum marker for broiler chickens with femoral head necrosis [35]. Fibrinogen beta was candidate biomarker of infection and inflammation [37] and femoral head necrosis [35]. CRP is an acute-phase protein, negatively correlated with adiponectin level in osteonecrosis of the femoral head [38].

In conclusion, our results identified 74 differentially expressed proteins between SANTH-TCM and healthy controls, 62 proteins between SANFH and healthy controls.
| Pathway, acc | Pathway Name                                      | P value   | Protein in background | Proteins in Diff Exp | Protein list                                                                 |
|------------|--------------------------------------------------|-----------|-----------------------|----------------------|----------------------------------------------------------------------------|
| hsa04978   | Mineral absorption                              | 0.034949  | 2                     | 2                    | P02787, P13637,                                                           |
| hsa03320   | PPAR signaling pathway                          | 0.068345  | 9                     | 4                    | P02656, P08589, Q6Q788, P06727, P01720, P02776, P02775,                   |
| hsa04062   | Chemokine signaling pathway                     | 0.083336  | 6                     | 3                    | P62158, P67936, P13637, P14625,                                           |
| hsa04261   | Adrenergic signaling in cardiomyocytes           | 0.126188  | 7                     | 3                    | P62158, P62258,                                                           |
| hsa04722   | Neurotrophin signaling pathway                   | 0.161789  | 4                     | 2                    | P62158, P62158,                                                           |
| hsa04915   | Estrogen signaling pathway                       | 0.161789  | 4                     | 2                    | P62158, P14625,                                                           |
| hsa04260   | Cardiac muscle contraction                       | 0.161789  | 4                     | 2                    | P67936, P13637,                                                           |
| hsa04114   | Oocyte meiosis                                   | 0.17539   | 8                     | 3                    | P62158, P6225, Q8NVD3,                                                  |
| hsa00410   | beta-Alanine metabolism                          | 0.18797   | 1                     | 1                    | Q96KN2,                                                                   |
| hsa04964   | Proximal tubule bicarbonate reclamation          | 0.18797   | 1                     | 1                    | P13637,                                                                   |
| hsa00340   | Histidine metabolism                             | 0.18797   | 1                     | 1                    | Q96KN2,                                                                   |
| hsa04070   | Phosphatidylinositol signaling system            | 0.18797   | 1                     | 1                    | P62158,                                                                   |
| hsa04961   | Endocrine and other factor-regulated calcium reabsorption | 0.18797   | 1                     | 1                    | P13637,                                                                   |
| hsa04976   | Bile secretion                                   | 0.18797   | 1                     | 1                    | P13637,                                                                   |
| hsa00860   | Porphyrin and chlorophyll metabolism             | 0.18797   | 1                     | 1                    | P00450,                                                                   |
| hsa00563   | Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | 0.18797   | 1                     | 1                    | P80108,                                                                   |
| hsa04960   | Aldosterone-regulated sodium reabsorption        | 0.18797   | 1                     | 1                    | P13637,                                                                   |
| hsa00230   | Purine metabolism                               | 0.18797   | 1                     | 1                    | P14618,                                                                   |
| hsa04666   | Fc gamma R-mediated phagocytosis                 | 0.18797   | 1                     | 1                    | P06196,                                                                   |
| hsa02010   | ABC transporters                                 | 0.18797   | 1                     | 1                    | Q9NP78,                                                                    |
| hsa04744   | Phototransduction                                | 0.18797   | 1                     | 1                    | P62158,                                                                   |
| hsa04611   | Platelet activation                              | 0.228912  | 9                     | 3                    | P02675, P02679, P02771,                                                  |
| hsa04060   | Cytokine-cytokine receptor interaction           | 0.228912  | 9                     | 3                    | P01720, P02776, P02775,                                                  |
| hsa04918   | Thyroid hormone synthesis                        | 0.23765   | 5                     | 2                    | P14625, P13637,                                                          |
| hsa04971   | Gastric acid secretion                           | 0.23765   | 5                     | 2                    | P62158, P13637,                                                          |
| hsa04970   | Salivary secretion                               | 0.23765   | 5                     | 2                    | P62158, P13637,                                                          |
| hsa05200   | Pathways in cancer                               | 0.34907   | 6                     | 2                    | P02751, P14625,                                                          |
| hsa04910   | Insulin signaling pathway                        | 0.340991  | 2                     | 1                    | P62158,                                                                   |
| hsa04912   | GnRH signaling pathway                           | 0.340991  | 2                     | 1                    | P62158,                                                                   |
| hsa04911   | Insulin secretion                                | 0.340991  | 2                     | 1                    | P13637,                                                                   |
| hsa04270   | Vascular smooth muscle contraction               | 0.340991  | 2                     | 1                    | P62158,                                                                   |
| hsa05214   | Glioma                                           | 0.340991  | 2                     | 1                    | P62158,                                                                   |
| hsa04114   | Ras signaling pathway                            | 0.340991  | 2                     | 1                    | P62158,                                                                   |
| hsa04614   | Renin-angiotensin system                         | 0.340991  | 2                     | 1                    | P08019,                                                                   |
| hsa05222   | Small cell lung cancer                           | 0.340991  | 2                     | 1                    | P02751,                                                                   |
| hsa05031   | Amphetamine addiction                           | 0.340991  | 2                     | 1                    | P62158,                                                                   |
| Pathway acc | Pathway Name                                      | P value | Protein in background | Proteins in Diff Exp | Protein list                                    |
|-------------|--------------------------------------------------|---------|-----------------------|----------------------|------------------------------------------------|
| hsa04750    | Inflammatory mediator regulation of TRP channels | 0.340991| 2                     | 1                    | P62158,                                         |
| hsa04720    | Long-term potentiation                           | 0.340991| 2                     | 1                    | P62158,                                         |
| hsa04973    | Carbohydrate digestion and absorption            | 0.340991| 2                     | 1                    | P13637,                                         |
| hsa04972    | Pancreatic secretion                             | 0.340991| 2                     | 1                    | P13637,                                         |
| hsa05203    | Viral carcinogenesis                             | 0.342177| 11                    | 3                    | P14618, P06396, P62258,                        |
| hsa05152    | Tuberculosis                                     | 0.390394| 7                     | 2                    | P18428, P62158,                                |
| hsa04110    | Cell cycle                                       | 0.390394| 7                     | 2                    | P62258, Q8NDV3,                                |
| hsa03050    | Proteasome                                       | 0.390394| 7                     | 2                    | P49721, O14818,                                |
| hsa05080    | Alzheimer's disease                              | 0.390394| 7                     | 2                    | P62158, P02649,                                |
| hsa04740    | Olfactory transduction                           | 0.465489| 3                     | 1                    | P62158,                                         |
| hsa04713    | Circadian entrainment                            | 0.465489| 3                     | 1                    | P62158,                                         |
| hsa04728    | Dopaminergic synapse                             | 0.465489| 3                     | 1                    | P62158,                                         |
| hsa04064    | NF-kappa B signaling pathway                     | 0.465489| 3                     | 1                    | P18428,                                         |
| hsa04930    | Type II diabetes mellitus                        | 0.465489| 3                     | 1                    | P14618,                                         |
| hsa04620    | Toll-like receptor signaling pathway             | 0.465489| 3                     | 1                    | P18428,                                         |
| hsa04916    | Melanogenesis                                    | 0.465489| 3                     | 1                    | P62158,                                         |
| hsa05100    | Bacterial invasion of epithelial cells           | 0.465489| 3                     | 1                    | P02751,                                         |
| hsa00620    | Pyruvate metabolism                              | 0.465489| 3                     | 1                    | P14618,                                         |
| hsa04919    | Thyroid hormone metabolism                       | 0.465489| 3                     | 1                    | P13637,                                         |
| hsa04621    | NOD-like receptor signaling pathway              | 0.465489| 3                     | 1                    | P14625,                                         |
| hsa00330    | Arginine and proline metabolism                  | 0.465489| 3                     | 1                    | Q96KN2,                                         |
| hsa05215    | Prostate cancer                                  | 0.465489| 3                     | 1                    | P14625,                                         |

Note. Italic font indicated the candidate protein.
Table 8: Differently enriched pathways were obtained in SANFH-TCM versus SANFH.

| Pathway_name | Pathway_Name | P value | Protein in background | Proteins in Diff Exp | Protein list |
|--------------|--------------|---------|-----------------------|----------------------|-------------|
| hsa04062     | Chemokine signaling pathway | 0.01871823 | 6 | 4 | P10720, P55774, P02776, P02775, P02675, P02679, P02671, P02452 |
| hsa04611     | Platelet activation | 0.0942118 | 9 | 4 | P06396, P14618, P02452, P13645, P08603, P0C0L4, P3645, P20731, O43707, P06396 |
| hsa04060     | Cytokine-cytokine receptor interaction | 0.0942118 | 9 | 4 | P10720, P55774, P02776, P02775, P14618, O43707, P02452 |
| hsa00563     | Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | 0.2080201 | 1 | 1 | P80108, P96KN2, P9NP78 |
| hsa00410     | beta-Alanine metabolism | 0.2080201 | 1 | 1 | Q96KN2, P06396, P14618, P9NP78 |
| hsa02010     | ABC transporters | 0.2080201 | 1 | 1 | Q96KN2, P06396, P14618, P9NP78 |
| hsa04666     | Fc gamma R-mediated phagocytosis | 0.2080201 | 1 | 1 | P06396, P14618, P02452, P13645, P08603, P0C0L4, P3645, P20731, O43707, P06396 |
| hsa00900     | Terpenoid backbone biosynthesis | 0.2080201 | 1 | 1 | P06396, P14618, P02452, P13645, P08603, P0C0L4, P3645, P20731, O43707, P06396 |
| hsa00230     | Purine metabolism | 0.2080201 | 1 | 1 | P06396, P14618, P02452, P13645, P08603, P0C0L4, P3645, P20731, O43707, P06396 |
| hsa03430     | Histidine metabolism | 0.2080201 | 1 | 1 | P06396, P14618, P02452, P13645, P08603, P0C0L4, P3645, P20731, O43707, P06396 |
| hsa04120     | Ubiquitin mediated proteolysis | 0.275615 | 13 | 4 | P01742, P04438, P01769, P01743, P08603, P0C0L4, P3645, P20731, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P02751, P02787, O43707 |
| hsa04830     | Regulation of actin cytoskeleton | 0.2820364 | 9 | 3 | P02751, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P06396 |
| hsa05150     | Staphylococcus aureus infection | 0.3119404 | 18 | 5 | P01742, P04438, P01769, P01743, P08603, P0C0L4, P3645, P20731, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P02751, P02787, O43707 |
| hsa05146     | Amoebiasis | 0.3460206 | 10 | 3 | P01742, P04438, P01769, P01743, P08603, P0C0L4, P3645, P20731, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P02751, P02787, O43707 |
| hsa05222     | Small cell lung cancer | 0.3731817 | 2 | 1 | P01742, P04438, P01769, P01743, P08603, P0C0L4, P3645, P20731, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P02751, P02787, O43707 |
| hsa04614     | Renin-angiotensin system | 0.3731817 | 2 | 1 | P01742, P04438, P01769, P01743, P08603, P0C0L4, P3645, P20731, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P02751, P02787, O43707 |
| hsa04978     | Mineral absorption | 0.3731817 | 2 | 1 | P01742, P04438, P01769, P01743, P08603, P0C0L4, P3645, P20731, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P02751, P02787, O43707 |
| hsa05412     | Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 0.3731817 | 2 | 1 | P01742, P04438, P01769, P01743, P08603, P0C0L4, P3645, P20731, O43707, P06396, P02679, P06681, P13645, P02452, P14618, O43707, P02751, P02787, O43707 |
| hsa05203     | Viral carcinogenesis | 0.4095525 | 11 | 3 | P14618, O43707, P06396, P02751, P02787, O43707 |
| hsa03050     | Proteasome | 0.4460931 | 7 | 2 | P49721, P25789, O43707, P02751, P02787, O43707 |

Note: Italic font indicated the candidate protein.
and 81 proteins between SANFH-TCM and SANFH. Those upregulated proteins including ABCB9, IQCA1, fibrinogen alpha, and fibrinogen beta and downregulated proteins including serum amyloid A-2 (SAA2), Ig lambda, CRP, and collagen alpha-1 are promising serum diagnosis markers of femoral head necrosis, and also the marker could be used for prognosis of femoral head necrosis after Traditional Chinese Medicine treatment. The key points of treating femoral head necrosis are early diagnosis, early treatment, and reserving prognosis of femoral head necrosis after Traditional Chinese Medicine femoral head necrosis, and also the marker could be used for screening of osteonecrosis.

Our findings on the screening of femoral head necrosis are early diagnosis, early treatment, and reserving prognosis of femoral head necrosis after Traditional Chinese Medicine. We found that the marker could be used for screening of osteonecrosis.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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