

Original Research

Comprehensive Proteomic Characterization of Articular Cartilage from Femoral Head Necrosis Patients

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Abstract

Background: Avascular necrosis of the femoral head (AVNHF) is a progressive, multifactorial, and challenging clinical disease that causes hip pain and loss of hip joint function. Till now, the pathogenesis of AVNHF was not fully understood. In this study, we characterized cartilage protein profiles of patients with AVNHF and identified the potential proteins involved in the progress of AVNHF using proteomics technique. **Methods:** Proteins from the cartilage of 6 patients (3 AVNHF patients and 3 fracture patients) were extracted and identified using label-free proteomics. AVNHF-responsive proteins were compared with those of the fracture patients and duly identified. Bioinformatics analyses including gene ontology (GO), KEGG, and STRING were performed to identify the functions of AVNHF-responsive proteins. **Results:** A total of 1512 proteins were identified from cartilage tissues of the patients. Compared to fracture patients, 255 significantly changed proteins were identified in cartilage tissues of patients with AVNHF. Functional categorization indicated that the significantly changed proteins were mainly involved in ECM-receptor interaction, focal adhesion, and glycolysis pathways. Interestingly, adipocyte enhancer-binding protein 1, cytoskeleton-associated protein 4, and ASPN protein were dramatically decreased, however, anti leukoproteinase, erythrocyte membrane protein, and lysozyme c were highly increased in patients with AVNHF. **Conclusions:** The current proteomic results suggest that ECM-receptor interaction and focal adhesion related proteins contribute to development of AVNHF. To our knowledge, this is firstly reported proteomic study on cartilage tissues of patients with AVNHF. The marker proteins including caveolae-associated protein 3 and procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 could help us to understand the pathogenesis of AVNHF.

Keywords: proteomics; avascular necrosis of femoral head (AVNHF); marker proteins

1. Introduction

Avascular necrosis of femoral head (AVNHF) is a progressive, multifactorial and challenging clinical disease that commonly affects 30- to 50-year-old individuals [1]. Once AVNHF occurs, patients suffer from hip pain and loss of the hip joint function [1–3]. It is a common and frequently-occurring clinical disease in orthopedics. Currently, there are more than 20 million patients in the world. Recently, with the indiscriminate use of hormonal drugs [4], increasing number of people who drink alcohol [5], and the increase of hip high-energy trauma (high-speed traffic accident injury, high fall injury of construction workers, high injury rate of sportive people, etc.) [6], the incidence of AVNHF seems to increase more rapidly than other musculoskeletal diseases. Pathological features of AVNHF comprise necrosis of the femoral head, collapse and secondary osteoarthritis of the hip joint [7], which lead to intractable pain, claudication and limb length differences in patients, ultimately resulting in high disability rates. Till now, the pathogenesis of AVNHF was not fully understood.

The common known treatments for AVNHF include

microwave therapy, magnetic therapy, physical therapy, far-infrared drilling decompression, and bone flap transplantation surgery [8]. Most of these known treatment methods are confined to the symptomatic management of AVNHF and are unable to block or reverse the underlying ischemic hypoxia of the pathological processes of necrotic bone cells. Patients eventually choose femoral head resection or artificial joint replacement. These are not only time-consuming processes but are usually accompanied by pain and even mental trauma. Conventional magnetic resonance imaging (MRI) is useful for diagnosing AVNHF [9,10], but it is not sensitive to biochemical changes which precede the morphological changes of AVNHF. Proteomics is defined as the high-throughput study of proteins [11,12]. Proteomics via state-of-the-art quantitative techniques and bioinformatics enables identification, quantitation, validation and characterization of a variety of proteins from specific organs, tissues or cells. The information obtained therein aside from aiding to determine protein structure, underlying enzymatic mechanisms and regulatory functions, provide invaluable links between protein levels and diseases.



Table 1. Baseline characteristics of patients enrolled in this study.

ID	Age	gender	Height (cm)	weight (kg)	BMI (kg/m ²)	Pathological feature
Patient 1	52	male	170	65	22.49	femoral head necrosis
Patient 2	54	female	165	60	22.04	femoral head necrosis
Patient 3	58	female	155	68	28.30	femoral head necrosis
Patient 4	78	female	163	55	20.70	fracture
Patient 5	62	male	172	70	23.66	fracture
Patient 6	67	male	168	72	25.51	fracture

BMI, Body mass index.

In this study, a comparative clinical proteomic analysis was performed to profile the proteome of patients with AVNFB. Through identification of AVNFB-marked proteins, we sought to at least in part, uncover the pathogenesis of AVNFB and contribute to determining potential novel targets for clinical intervention.

2. Materials and Methods

2.1 Patients' Samples

The review board of the First Affiliated Hospital of USTC, University of Science and Technology of China approved this study. Six fragments of articular cartilages from 6 patients (3 men, 3 women; mean age, 61.8 years) (Table 1) with AVNFB and fracture were used for this study. AVNFB was diagnosed based on its established diagnostic criteria [13,14].

2.2 Protein Extraction, Digestion, and Desalting

For extraction of proteins from the articular cartilages, the Tris-phenol and Acetone precipitation methods were used. Details of the Tris-phenol method is as follows: a portion (0.1 g) of each sample was ground to powder in liquid nitrogen using a mortar and pestle and then mixed with 1 mL extraction buffer (500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 2% β -mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF) and adjusted to pH 8.0 with HCl.). An equal volume of 50 mM Tris-buffered phenol (pH 8.0) was added to the mixture and kept at 4 °C for 30 min. After centrifugation at 5000 xg for 30 min at 4 °C, the phenol supernatant was collected. Five-fold volume of 0.1 M ammonium acetate in methanol was added to the collected supernatant and stored at -20 °C for 18 h. Then protein was precipitated through centrifugation at 12,000 xg for 10 min at 4 °C and the resulting precipitate collected. The crude precipitate (protein) was washed using five-fold volume of ice-cold methanol by centrifugation at 12,000 xg for 10 min at 4 °C. The wash step was repeated twice using ice-cold acetone to remove remaining methanol. The washed proteins were dried and dissolved in a lysis buffer which consisted of 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine. The dissolved protein solution was centrifuged again at 12,000 xg for 10 min at 4 °C and the supernatant was collected as the final protein sample.

The acetone precipitation method was performed as follows: the sample was ground in liquid nitrogen with a mortar and pestle and then mixed with extraction buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, phosphatase inhibitor mixture (Sigma, St. Louis, MO, USA), and protease inhibitor mixture (Roche, Werk Penzberg, Germany). After centrifugation at 12,000 xg for 20 min, the obtained supernatant was diluted with three volumes of cold acetone containing 0.07% 2-mercaptoethanol and then incubated at -20 °C for 2 h. The resulting precipitate was collected and washed three times with an acetone solution containing 0.07% 2-mercaptoethanol. Finally, the protein pellets were dried in a Speed-Vac and resuspended in a lysis buffer which consisted of 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine. The protein concentration was then measured using the Bradford method with bovine serum albumin as the standard [15].

The purified proteins were reduced and alkylated according to the manufacturer's instructions (Sigma, St. Louis, MO, USA) with some modifications. Briefly, the protein solution containing 100 μ g was re-precipitated with 5-fold ice-cold acetone and centrifuged at 12,000 xg for 10 min at 4 °C. The resulting precipitate (protein) was dried and re-dissolved in 50 μ L of a dissolution buffer. The reaction was initiated by adding 4 μ L of a reducing reagent and allowing the reaction to go on for 1 h at 60 °C. Afterward, 2 μ L of cysteine-blocking reagent was added and kept at room temperature for 10 min. The alkylated protein sample was then enriched with 10k ultrafiltration tube and digested using 50 μ L of 50 ng/ μ L trypsin at 37 °C for 12 h. The digested peptides were acidified with 2 μ L of 100% formic acid to pH <3 and desalted with a C18-pipette tip.

2.3 Nano-Liquid Chromatography-Tandem Mass Spectrometry Analysis

Peptides in 0.1% formic acid were loaded onto an EASY-nLC1000 nano-liquid chromatography (LC) system (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a C18 PepMap trap column (100 μ m ID \times 20 mm, Thermo Fisher Scientific). The peptides were eluted from the trap column and then separated using 0.1% formic acid in acetonitrile at a flow rate of 300 nL/min on a C18 Tip column (75 μ m ID \times 150 mm, Thermo Fisher Scientific).

with a spray voltage of 1.8 kV. Peptides were separated using a 0%–45% acetonitrile gradient (90 min) with 0.1% formic acid at a flow rate of 350 nL/min. The peptide ions in the spray were detected and analyzed on a nanospray LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), which was operated in the positive mode to measure full scan MS spectra (from m/z 350–1800 in the Orbitrap analyzer at resolution $R = 60,000$) followed by isolation and fragmentation of the 15 most intense ions (in the LTQ part) by collision-induced dissociation. The normalized collision energy was 35.0. Dynamic exclusion was employed within 30 s to prevent repetitive selection of peptides.

2.4 Protein Identification

Raw mass spectrometry (MS/MS) spectra search was conducted using MaxQuant-associated Andromeda search engine [16], and a uniprot-Homo sapiens database (82616 sequences). Initial maximum precursor and fragment mass deviations were set to 6 ppm and 0.5 Da, respectively. Variable modification (methionine oxidation and N-terminal acetylation) and fixed modification (cysteine carbamidomethylation) were set for the search and trypsin with a maximum of two missed cleavages was chosen for searching. The minimum peptide length was set to 7 amino acids and the false discovery rate (FDR) for peptide and protein identification was set to 0.01. The precursor ion mass accuracy was improved using the time- and mass-dependent recalibration option. Frequently observed laboratory contaminants were removed and the protein identification was considered valid only when at least 2 matched peptides and 1 unique peptide were present.

2.5 Analysis of Differentially Abundant Proteins Using Acquired Mass Spectrometry Data

The freely available software Perseus (version 1.4.1.3) (available online: http://141.61.102.17/perseus_doku/doku.php?id=start) was used to compare the peak intensities across the whole set of measurements to obtain quantitative data for all of the peptides in the sample. The label-free quantitation (LFQ) intensities of proteins from the MaxQuant analysis were imported and transformed to logarithmic scale with base 2. The missing values were replaced with the value of the lowest intensity. The protein quantification and calculation of statistical significance was carried out using Student-*t* test and error correction (p -value < 0.05) using the method of Benjamini–Hochberg. For visualization, PCA was performed. All the proteins that showed a fold-change of at least 1.5 and satisfied $p < 0.05$ were considered differentially changed. The differentially changed proteins were identified from the comparison of AVNFH with fracture patients.

2.6 Protein Function and Interaction Analyses

To perform the functional analysis, the identified proteins were analyzed using QuickGO (<http://www.ebi.ac.uk/QuickGO/>). Pathway mapping of identified proteins was performed using the KEGG database (<http://www.genome.jp/kegg/>). Protein-protein interaction was analyzed using the STRING database.

3. Results

3.1 Protein Profiles of Cartilage Tissues from AVNFH and Fracture Patients

To uncover the inherent mechanism of femoral head necrosis, comparative proteomics was performed using cartilage tissues from AVNFH and fracture patients (Fig. 1). The AVNFH and fracture patients were diagnosed by X-ray scans (Fig. 2). Proteins from cartilage tissues were extracted by both the Tris–phenol method and acetone precipitation method. SDS-PAGE analysis indicated that proteins extracted by Tris–phenol had more bands than those extracted by the acetone precipitation (Fig. 3). The proteins extracted using Tris–phenol method were then subjected to LC-MS/MS analysis. A total of 1510 proteins were identified from the cartilage tissues of AVNFH and fracture patients (Fig. 4A, **Supplementary Table 1**). Among them, 1419 proteins were common between the AVNFH and fracture patients (Fig. 4B). Also, 81 and 10 proteins were specifically identified in the AVNFH and fracture patients, respectively (Fig. 4B, **Supplementary Table 2**). For the common proteins, 88 were significantly increased while 74 proteins were decreased, respectively in the AVNFH patients compared to the fracture patients (Fig. 4B, **Supplementary Table 3**).

3.2 Functional Categorization of Proteins Involved in Femoral Head Necrosis

To understand the cellular processes and functions of the significantly changed proteins, GO and KEGG databases were used. Through these bioinformatic analyses, abundant common proteins (Fig. 5A) and unique proteins (Fig. 5B) in either the AVNFH patients or fracture patients were enriched in biological processes, cell components, molecular functions, and KEGG pathway. At the GO level, the significantly changed proteins were mainly involved in anatomical structure development, extracellular region/organelle, and cell adhesion molecule binding (Fig. 6A). The unique proteins in either the AVNFH or fracture patients significantly related to anatomical structure development, extracellular vesicle, and protein binding (Fig. 6B).

To further determine the biological pathways that are mediated by the identified proteins in cartilage tissue from AVNFH, the significantly changed proteins were analyzed using the KEGG database. These proteins were mainly involved in ECM-receptor interaction, focal adhesion, and complement and coagulation cascades (Fig. 7A).

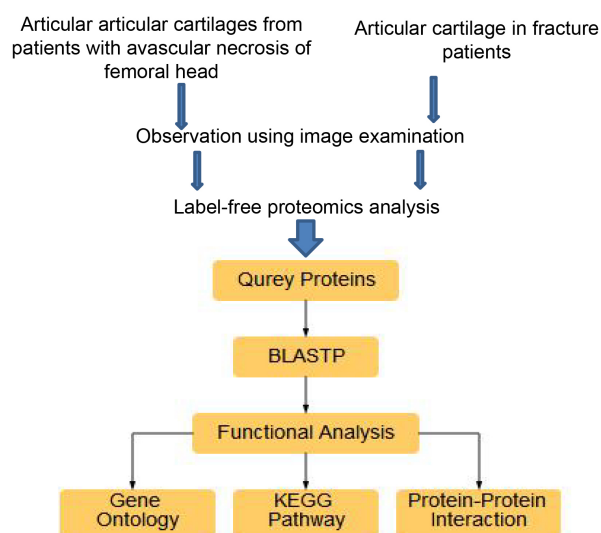


Fig. 1. Experimental design of the study. Images of articular cartilages from patients with avascular necrosis of femoral head and fracture were captured using nuclear magnetic resonance (NMR). The articular cartilages were comparatively analyzed using proteomics. The functions of identified marker proteins were analyzed using Gene Ontology (GO), KEGG pathway, and STRING database.

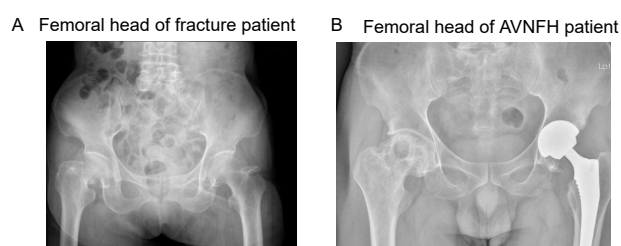


Fig. 2. X-ray images of femoral head from fracture and AVNFB patients. (A) X-ray image of femoral head from fracture patient. (B) X-ray image of AVNFB patient.

The unique proteins in either the AVNFB or fracture patients significantly related to complement and coagulation cascades, phagosome, and ECM-receptor interaction (Fig. 7B).

To determine the proteins that play central roles in pathological development of AVNFB, protein-protein interactions of AVNFB responsive proteins were analyzed with reference to the STRING database. As a result, increased proteins including L-lactate dehydrogenase B chain, hemopexin, and endoplasmic reticulum luminal Ca^{2+} binding protein grp78 interacted with many of the AVNFB-responsive proteins. AVNFB-unique proteins such as AP-2 complex subunit alpha-1, Rho GDP-dissociation inhibitor 1, 60S acidic ribosomal protein P2, and coatomer subunit interacted with one another and formed a network. Also, catalase, superoxide dismutase,

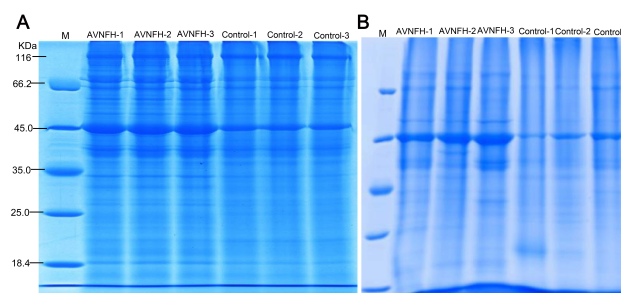


Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of extracted proteins from AVNFB patients and fracture patients (control). (A) Proteins were extracted from AVNFB patients and fracture patients (control) using Tris-phenol buffer. After extraction, protein concentration was determined using BCA kit and protein quality was analyzed by SDS-PAGE. (B) Proteins were extracted from AVNFB patients and fracture patients (control) using Acetone precipitation method. After extraction, protein concentration was determined using BCA kit and protein quality was analyzed by SDS-PAGE.

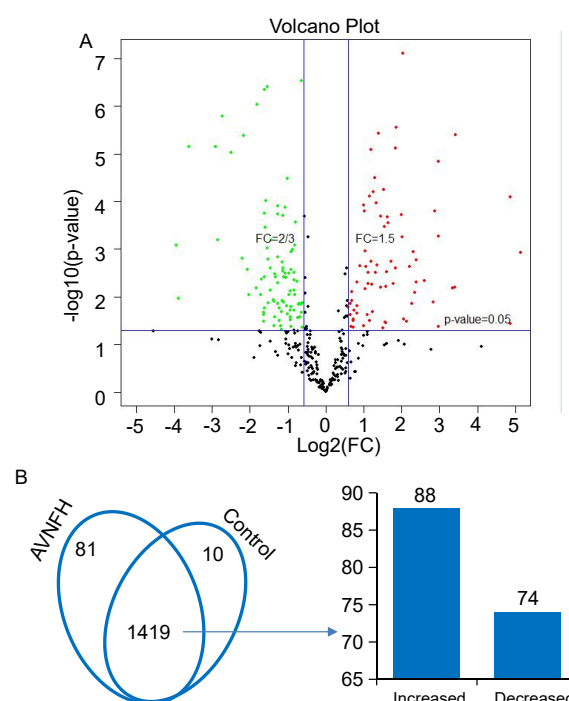


Fig. 4. Significantly changed proteins between AVNFB and fracture patients. Proteins extracted from articular cartilages were digested, analyzed using nano-LC-MS/MS, and compared through MaxQuant analysis. The comparison was performed using abundance of protein in articular cartilages of patients with AVNFB divided by those in patients with fracture. (A) Volcano plot showing the significantly changed proteins. (B) Venn diagram showing the distribution of identified proteins in fracture and AVNFB patients.

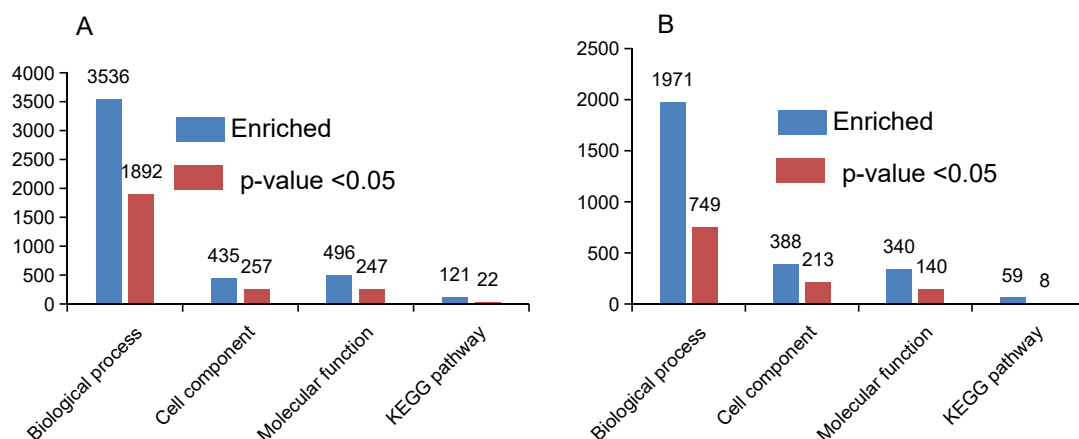


Fig. 5. Statistical analysis of GO and KEGG pathways of significantly changed proteins. (A) The number of KEGG pathways and GO categorization including biological process, cell component, and molecular function, the commonly identified proteins are involved. (B) The number of KEGG pathways and GO categorization including biological process, cell component, and molecular function for the fracture-specific and AVNFH-specific proteins. The orange color indicates significantly enriched proteins ($p < 0.05$).

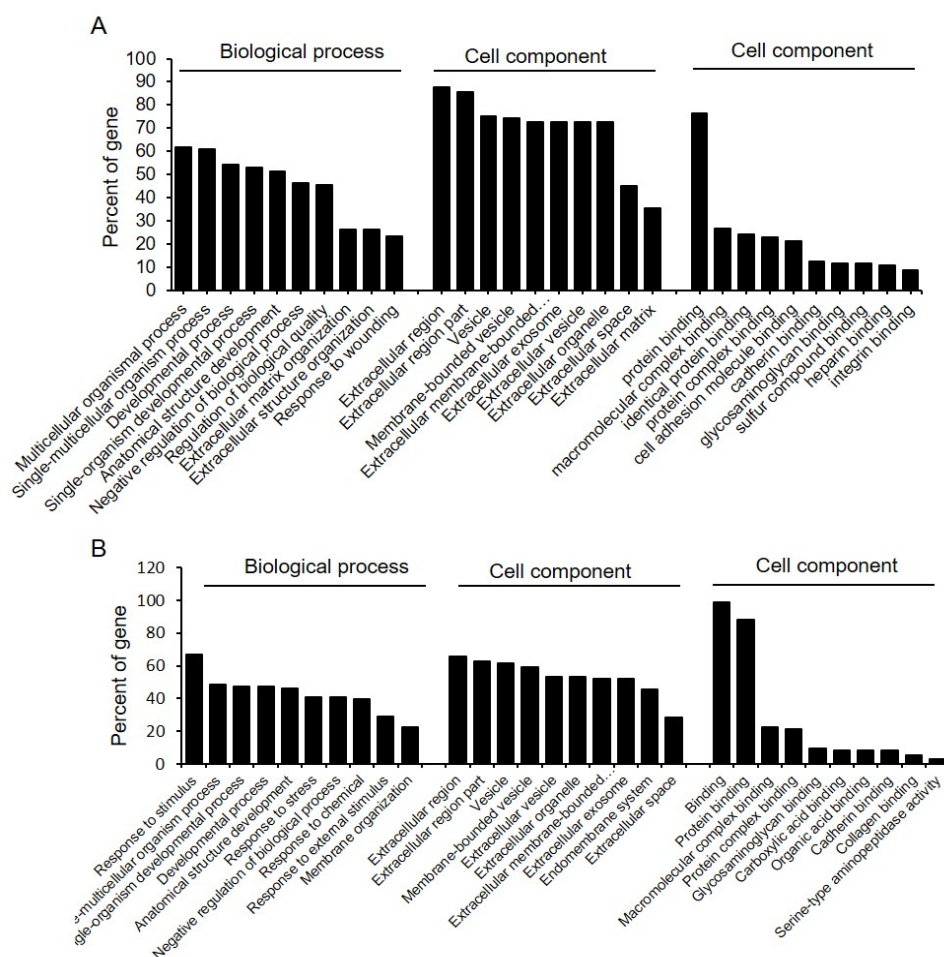


Fig. 6. Detailed information of functional categorization of significantly changed proteins. (A) Details of GO analysis of common proteins identified in both fracture and AVNFH patients. (B) Details of GO analysis of proteins specifically identified in only the fracture or AVNFH patients.

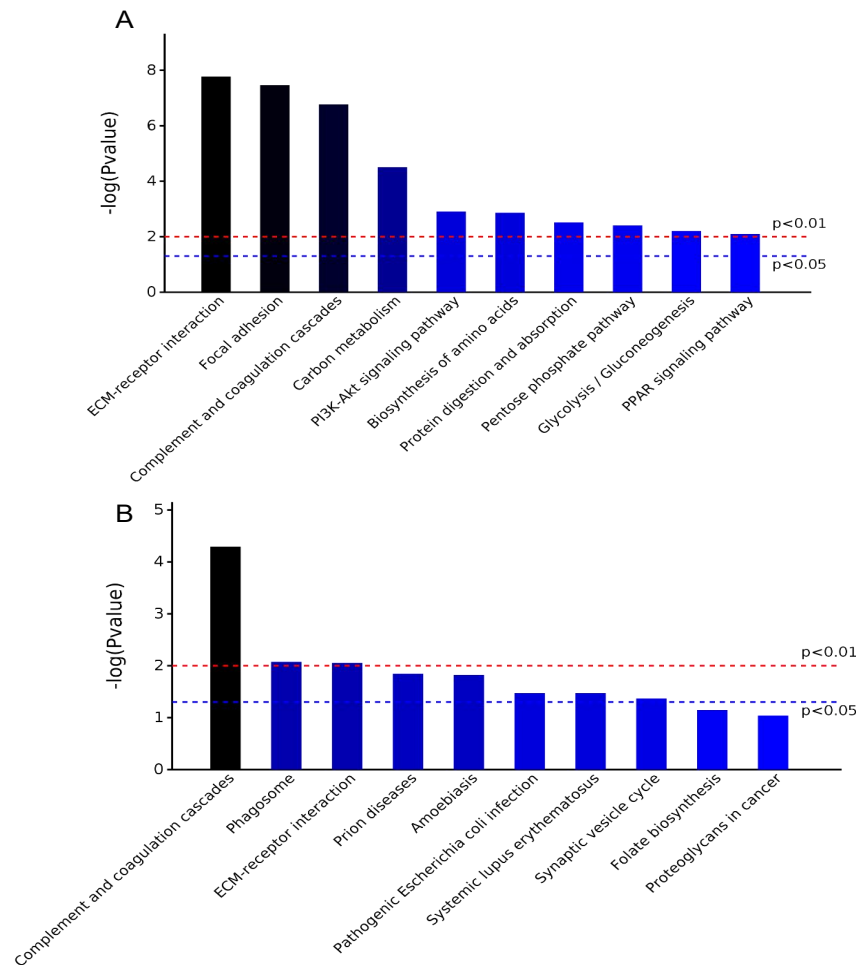


Fig. 7. Detailed information of pathway enrichment analysis. (A) Common proteins identified in both fracture and AVNFH patients were analyzed using KEGG database. (B) Pathways of proteins specifically identified in either the fracture or AVNFH patients involved were also analyzed using the KEGG database.

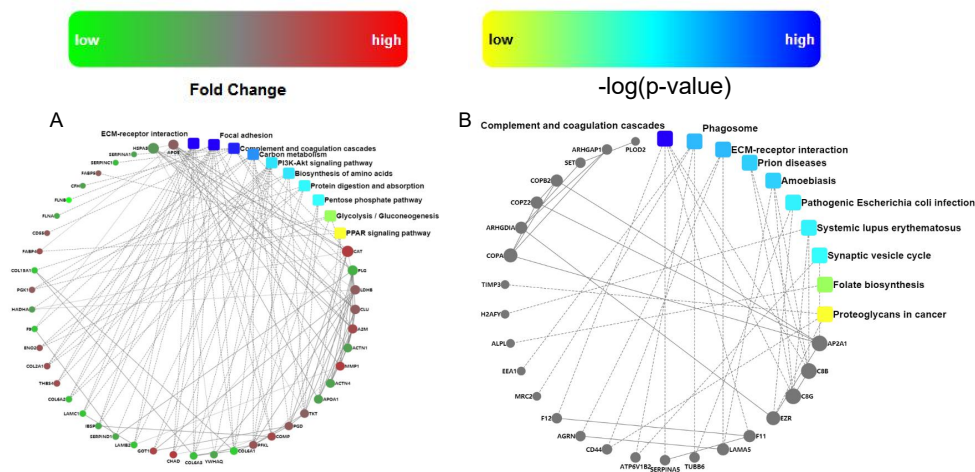


Fig. 8. Protein-protein interaction analyses of identified proteins. (A) Protein-protein interaction analysis of common proteins identified in both the fracture and AVNFH patients. (B) Protein-protein interaction analysis of proteins specifically identified in either the fracture or AVNFH patients. Colors from green to red indicate the change tendencies of proteins while colors from yellow to blue show significant values.

interstitial collagenase, plasminogen, protein disulfide-isomerase were decreased in the AVNFH patients and interacted with one another (Fig. 8).

4. Discussion

4.1 Phenol Extraction of Proteins was the Most Suitable Method for the Cartilage Tissues

In our study, proteins from the cartilage tissues were extracted by Tris-phenol buffer method and acetone precipitation method. SDS-PAGE analysis indicated that the Tris-phenol extracted proteins had more bands compared to the acetone extracted proteins (Fig. 1). Protein extraction using phenol was first reported by Hurkman and Tanaka in 1986 [17]. The advantages of phenol extraction included efficient protein recovery and nonprotein components removal. The use of phenol is known to be more efficient in extracting proteins from recalcitrant tissues [18]. Protein extraction from cartilage tissues is difficult compared to protein extraction from cell. Previous protein extraction from cartilage tissues using chaotropic buffer (4 M GdnHCl, 50 mM NaAc, 100 mM 6-aminocaproic acid, 5 mM benzamidine, 5 mM N-ethylmaleimide, pH 5.8) identified 653 proteins [19]. A total of 814 proteins were identified from the articular cartilage tissue from normal donors and patients with osteoarthritis through SDS-PAGE followed by in-gel digestion [20]. Here, a total of 1510 proteins were identified, indicating the suitability of phenol extraction for clinical cartilage tissues.

4.2 Protein Profile of the Cartilage Tissues from AVNFH Patients is Different from the Fracture Patients

In this study, 253 proteins were differentially changed between AVNFH and fracture patients. A total of 91 proteins were specifically detected in either the AVNFH patients or fracture patients (**Supplementary Tables 2,3**), further indicating the protein profile differences between them. From these 91 proteins, 81 proteins including caveolae-associated protein 3, tubulin polymerization-promoting protein 3, and Rho GTPase-activating protein 1, procollagen-lysine 2-oxoglutarate 5-dioxygenase 2, were specifically detected in the AVNFH patients; 10 proteins including hormone-sensitive lipase, perilipin-2, and metalloproteinase inhibitor 3 were reduced to undetectable levels in the AVNFH patients (**Supplementary Table 2**). Caveolae-associated protein is a principal component of caveolae membranes and functions as a scaffolding protein to organize and concentrate certain caveolin-interacting proteins within the caveolae membranes [21]. It is reported that caveolin-3 increases dramatically with age [22]. Tubulin polymerization promoting protein (TPPP) was identified as a disordered protein that affects microtubular system [23,24]. It was reported that TPPP promotes the formation of synuclein filament, which is probably a crucial pathological process in certain neurological diseases [25]. The Rho GTPase-activating proteins are one of the major classes of

regulators of Rho GTPases that are crucial in cell cytoskeletal organization, growth, differentiation, neuronal development and synaptic functions [26]. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD) genes are involved in fibrotic processes and tissue remodeling, and induced under hypoxic conditions [27]. These proteins were only detected in the AVNFH patients, indicating the abnormal expression of proteins associated with tissue or cytoskeletal remodeling, in the development of femoral head necrosis. Hormone-sensitive lipase is a key enzyme in the mobilization of fatty acids from acylglycerols in adipocytes as well as non-adipocytes [28]. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation [29]. The matrix metalloproteinases which degrade most of the components of the extracellular matrix, including fibrillar, non-fibrillar collagens, and fibronectin [30], and inhibitor of matrix metalloproteinases was drastically decreased in the AVNFH patients. These results indicate that accumulation of acylglycerols and degradation of extracellular matrix might happen alongside the development of AVNFH.

4.3 ECM-Receptor Interaction or Focal Adhesion-Related Pathways Involved in AVNFH

In this study, many ECM-receptor interaction or focal adhesion-related proteins including collagen alpha-1(VI) chain, thrombospondin-4, T-complex protein 1, chondroadherin, bone sialoprotein 2, and laminin subunit beta-2 were identified to be significantly changed between the AVNFH and fracture patients (Table 2). COL6A1 plays an important role in maintaining the integrity of various tissues, and alpha-1 subunit of type VI collagen is the encoded protein [31]. The thrombospondins are a family of extracellular calcium-binding proteins, of which, thrombospondin-4 binds specifically to collagenous extracellular matrix proteins via C-terminal domains [32]. T-complex protein 1 plays a role in the folding of actin and tubulin [33]. These proteins were significantly increased in AVNFH development, indicating the accumulation of extracellular matrix proteins and structural change of cartilage cells. Chondroadherin is a minor component of bone organic matrix and functions to promote attachment of osteoblastic cells to solid-state substrates [34]. Bone sialoprotein 2 functions to enhance fibroblast attachment *in vitro* [35]. Laminin subunit beta-2 is involved in sustaining the integrity of sarcolemmal basement membrane [36]. These proteins were significantly decreased in the AVNFH patients—an indication that, collapse of the microstructure is an inducer of AVNFH development. In future, the above identified key proteins which might be involved in AVNFH, need to be validated in a large sample size using other techniques such as western blot analysis and ELISA assays.

Table 2. The ECM-receptor interaction or focal adhesion pathways related proteins.

ECM-receptor interaction					
Protein ID	Abbreviation	Description	Peptides	Ratio	<i>p</i> -value
O15335	CHAD	Chondroadherin	9	0.183091241	0.0005828
P02458	COL2A1	Collagen alpha-1(II) chain	33	0.381662001	0.0025274
P11047	LAMC1	aminin subunit gamma-1	27	0.298013084	0.4224665
P12109	COL6A1	Collagen alpha-1(VI) chain	19	2.746900978	0.0004817
P12110	COL6A2	Collagen alpha-2(VI) chain	18	1.757307133	0.1391233
P12111	COL6A3	Collagen alpha-3(VI) chain	88	3.11349462	0.0000000628
P21815	IBSP	Bone sialoprotein 2	2	0.453476403	0.0120048
P35443	THBS4	Thrombospondin-4	14	3.035757644	0.0029566
P17987	COMP	T-complex protein 1 subunit alpha	6	1.645976288	0.0437059
P55268	LAMB2	Laminin subunit beta-2	13	0.32725274	0.0173353
Focal adhesion					
O15335	CHAD	Chondroadherin	9	0.183091241	0.0005828
O43707	ACTN4	Alpha-actinin-4	17	0.532413165	0.0027161
O75369	FLNB	Filamin-B	49	0.139170798	0.0006382
P02458	COL2A1	Collagen alpha-1(II) chain	33	0.381662001	0.0025274
P11047	LAMC1	aminin subunit gamma-1	27	0.298013084	0.4224665
P12109	COL6A1	Collagen alpha-1(VI) chain	19	2.746900978	0.0004817
P12110	COL6A2	Collagen alpha-2(VI) chain	18	1.757307133	0.1391233
P12111	COL6A3	Collagen alpha-3(VI) chain	88	3.11349462	0.0000000628
P12814	ACTN1	Alpha-actinin-1	17	0.547312925	0.0010154
P21333	FLNA	Filamin-A	58	0.467185659	0.0038697
P21815	IBSP	Bone sialoprotein 2	2	0.453476403	0.0120048
P35443	THBS4	Thrombospondin-4	14	3.035757644	0.0029566
P17987	COMP	T-complex protein 1 subunit alpha	6	1.645976288	0.0437059
P55268	LAMB2	Laminin subunit beta-2	13	0.32725274	0.0173353

5. Conclusions

In summary, a total of 1512 proteins were identified from cartilage tissues of the patients. Relative to the fracture patients, 255 significantly changed proteins were identified in cartilage tissues of patients with AVNFB. The results of functional categorization revealed that the significantly changed proteins were mainly involved in ECM-receptor interaction, focal adhesion, and glycolysis pathways. Of note, adipocyte enhancer-binding protein 1, cytoskeleton-associated protein 4, and ASPN protein were greatly decreased, however, antileukoproteinase, erythrocyte membrane protein, and lysozyme c were highly increased in patients with AVNFB.

The changed protein expression in the disease state is associated with many aspects of the pathogenesis of femoral head necrosis, such as increased proteolysis, lipid metabolism, immune response, degradation of extracellular matrix, and collapse of the microstructure. To our knowledge, this is the first time that a large portion of these proteins and their expression patterns have been identified in cartilage of femoral head necrosis patients. The findings of this study will help us to better understand the pathogenesis of AVNFB at the protein level and provide novel pathologic mediators and biomarkers for femoral head necrosis. This

study also provides at least a proof-of-concept of the applicability of label-free proteomics in unraveling the mechanisms that underlie various diseases. In future studies, we would increase the sample size of the participants, concentrate on selected proteins and their underlying mechanisms as well as validate and quantify their levels by other known methods such as using ELISA kits and western blot analysis.

Author Contributions

JH, FH, RNA, and XY made the major contributions to this study in the conception, design, drafting part of manuscript, and final revision. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The review board of the First Affiliated Hospital of USTC, University of Science and Technology of China approved this study (2020L L106) and participants provided signed consent forms.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2706181>.

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