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Comparative Quantitative Proteomics Revealed an Altered Left Ventricle Protein Profile in Human Ischemic Cardiomyopathy

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Running Title: Proteomics study of left ventricle in ischemic cardiomyopathy

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What is new?

Aiming to complement previous analyses using a deeper approach, liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis, which presents better comprehensiveness and accuracy of quantification than two-dimensional electrophoresis (2DE), was applied. A total of 1,723 proteins were successfully quantified, among which 104 proteins were upregulated and 63 proteins were downregulated in the left ventricles of ICM (ischemic cardiomyopathy) individuals compared to normal heart donors. All of these altered proteins were related to the extracellular matrix, metabolism, the immune response, muscle contraction, cytoskeleton organization, transcription/translation and signal transduction. Most importantly, in response to an ischemic stimulus, the C1 inhibitor SERPING1 helped to compensate for increases in complement activation through complement inhibition. The identified proteins represent novel diagnostic and therapeutic targets for the treatment of ICM.
Abstract

Background Ischemic cardiomyopathy (ICM) resulting from coronary artery disease is a major cause of heart failure (HF). The identification and quantification of differentially expressed proteins in ICM patients may provide the potential to diagnose and treat ICM more effectively.

Aim Liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis was applied to identify differentially expressed proteins in ICM individuals.

Methods To identify proteins involved in the molecular mechanisms of ICM, we quantitatively analyzed the left ventricular proteome profiles of ICM patients who had undergone heart transplantation. LC-MS/MS, which presents better comprehensiveness and accuracy of quantification than two-dimensional electrophoresis (2DE), in combination with bioinformatics was applied to analyze cardiac samples and identify proteins that were differentially expressed in the left ventricles of six ICM patients compared to seven normal heart donors.

Results In the present study, a total of 1,723 proteins were successfully quantified in two repeated experiments, among which 104 proteins were upregulated and 63 proteins were downregulated in the left ventricles of ICM individuals. For all these altered proteins, Gene Ontology (GO) analysis, KEGG pathway mapping and protein interaction analysis were performed, which showed that most of the proteins were related to the extracellular matrix, metabolism, the immune response, muscle contraction, cytoskeleton organization, transcription/translation and signal transduction.

Conclusions Collectively, these differentially expressed proteins represent potential novel diagnostic and therapeutic targets for the treatment of ICM.
**Key Words:** Ischemic cardiomyopathy; Comparative quantitative proteomics; Left ventricle
**Introduction**

With the increasing age of the population, ischemic cardiomyopathy (ICM) is becoming an increasingly heavy social and economic burden to society. And the best treatment strategy for ICM is heart transplant, however, the donors are currently scarce [1]. Thus, an enormous amount of clinical and basic research has been undertaken to identify diagnostic markers and therapeutic targets for better management of ICM [2-5]. Studies that have led to increased molecular understanding of the ICM process have helped in combating ICM to a certain extent [4, 6]. For example, several proteins related to inflammation and ventricular remodeling, such as C-reactive protein and natriuretic peptides, might contribute to the molecular pathogenesis of ICM [7, 8]. However, the pathogenic mechanism of ICM remains incompletely understood. Thus, a holistic understanding of the disease process holds the key to providing accurate, reliable and cost-effective information about ICM to aid in diagnosis, prognosis, or monitoring of therapy.

In recent years, proteomics has become a powerful approach for examination of biological processes through identification of unknown biological functions [9-11]. The identification and quantification of differentially expressed proteins in human samples using proteomics may provide the potential to diagnose and treat ICM more effectively. Classic two-dimensional electrophoresis (2DE) followed by mass spectrometry analysis of the left ventricles of ICM patients was first performed in 2012 [12]. The results showed that 35 proteins were differentially regulated (20 upregulated and 15 downregulated) in left ventricular tissue between ICM patients and control subjects, and the differentially expressed proteins were mainly implicated in metabolism, the
respiratory chain, the immune response and the stress response [12]. However, an inherent limitation of classic 2DE is that very hydrophobic proteins cannot be identified or characterized, which can cause some differentially expressed proteins between ICM patients and healthy subjects to be overlooked [13]. Here, we compared human left ventricular tissue from patients with ICM with that from healthy subjects using liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis, which presents better comprehensiveness and accuracy of quantification than 2DE, to complement previous analyses using a deeper approach. The proteins discovered and the molecular signatures analyzed in the present study will provide insights into ICM and identify biomarkers useful for diagnostic, prognostic, and therapeutic purposes.

Materials and methods

Sample Collection

Approval was granted by the Renmin Hospital of Wuhan University Review Board (Wuhan, China), and the procedures involving human samples in this study abided by the principles outlined in the Declaration of Helsinki. All patients were volunteers and provided informed consent prior to participation in all cases. Left ventricular tissues (peri-infarct zones) were collected from six ICM patients while they were undergoing heart transplantation due to irreversible heart failure caused by myocardial infarction. Control samples were obtained from seven normal heart donors who died of accidents but whose hearts were deemed unsuitable for transplantation due to noncardiac reasons [14-16]. The tissues were washed with PBS immediately after removal and then cut into small pieces, directly frozen in liquid nitrogen and processed for proteomic and western blot analysis.
Extraction and digestion of left ventricle proteins

Extraction and digestion of left ventricle proteins were performed as previously described [17, 18]. Briefly, left ventricular tissues from six patients with ICM and seven healthy controls were used for proteomic analysis. The tissues were homogenized in HNTG lysis buffer with a pooled approach to minimize biological variations. The resulting samples were then vortexed, and the left ventricle proteins were collected and divided into aliquots for two independent experimental replicates. In-solution digestion of the proteins was performed as described in a previous study [18]. The proteins were precipitated and then centrifuged. The protein pellets were resuspended and then alkylated with iodoacetamide in the dark. After measuring the protein concentrations via Bradford assay, the proteins were digested with trypsin at a ratio of 1:50 (trypsin/protein w/w), desalted by using a Sep-Pak C18 cartridge (Waters) and dried with a SpeedVac.

Stable isotope dimethyl labeling and strong cation exchange (SCX) chromatographic fractionation

Stable isotope dimethyl labeling and SCX fractionation of the peptides were performed as described in our previous study[17]. The desalted peptides were resuspended in sodium acetate. Next, 4% formaldehyde (CH2O, a light label) was added to the peptides from the ICM group, while 4% deuterated formaldehyde (CD2O, a heavy label) was added to the peptides extracted from the normal group. After labeling, the peptides were mixed and desalted again prior to separation via strong cation exchange (SCX) chromatography. During gradient elution, nearly twelve fractions were collected and desalted prior to MS analysis.

LC-MS/MS and data processing
All LC-MS/MS experiments were performed as previously described [17]. Dried peptides were dissolved, loaded onto a C18 trap column, and subsequently eluted. The MS/MS data were acquired by using the information-dependent acquisition (IDA) mode of a TripleTOF 5600+ System coupled with an Ultra 1D Plus nano-liquid chromatography device (SCIEX, USA). The generated raw MS spectra were analyzed with ProteinPilot 4.5 software (SCIEX, USA) by using the Paragon algorithm. The UniProt database (Homo sapiens, 2016-03-29, UP000000589) was used. The data analysis parameters were those used in our previous study [17]. As described previously, proteins were considered to be successfully identified when at least two correctly assigned peptides (95% confidence) were obtained. The false discovery rates (FDRs) of the peptide-spectra matches determined by a decoy database search were set to 1.0%.

**Bioinformatics analysis of the identified proteins**

According to our previous study, for quantification of the identified proteins, Gene Ontology (GO) term enrichment was performed for significantly up- or downregulated proteins and significantly under- and overrepresented functional GO categories were identified with the Biological Networks Gene Ontology (BiNGO) 3.03 plugin [17]. To further illustrate the qualified and dysregulated proteins, the volcano plot was also applied. The Cytoscape network visualization platform with the latest release of the BiNGO plug-in was used to identify proteins that were annotated on the basis of biological process categories. Statistical significance was determined as described in our previous study [17, 19]. Intracellular pathway analysis was performed by using the KEGG PATHWAY Database [20]. For protein interaction network analysis of the regulated proteins involved in various pathways, UniProt functional annotations were
used to classify the proteins into several clusters. Based on the quantified MS results, proteins matched in any clusters were extracted and submitted to STRING 9.0 to qualify the physical and functional interactions of these proteins.

**Western blot analysis**

Western blot analysis was performed as previously described [14, 17, 21-23]. Briefly, protein was extracted with RIPA lysis buffer. The protein concentration was determined with a BCA Protein Assay Kit. The denatured protein was separated by SDS-PAGE, and the separated proteins were then transferred to a polyvinylidene fluoride membrane. After being blocked with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with primary antibodies overnight at 4°C. On the following day, the membrane was incubated with secondary antibodies for 2 h at room temperature. The protein signals were detected by using a ChemiDoc XRS+ Imaging System (Bio-Rad), and then the gray values of the proteins were analyzed with Image Lab software (version 5.2.1, Bio-Rad).

**Statistical analysis**

The data are expressed as the mean ± standard error (SE). Student’s two-tailed t-tests were performed to compare the means of the two groups. A value of p<0.05 was considered to indicate statistical significance. SPSS 13.0 statistical software was used to perform all the statistical analyses in the present study.

**Results**

**Clinical characteristics of the patients**

In the present study, thirteen human samples were included: six ICM samples and seven normal controls. The clinical and echocardiographic characteristics of the participants are
summarized in Supplementary material, **Table S1.** The ICM patients were all men with a mean age of 59 years. Their mean heart rates were 78.7 bpm, with the mean systolic and diastolic blood pressures of 106.5 mmHg and 58.5 mmHg, respectively. A significantly dilated left ventricular end-diastolic dimension (LVEDD) and a significantly decreased left ventricular ejection fraction (LVEF) were found in the ICM group compared to the normal group.

**A total of 167 proteins were differentially expressed in the left ventricles of ICM patients compared to healthy subjects**

To understand the pathological changes associated with ICM and to gain insight into the consequences of ICM, it was critical to comprehensively investigate the molecular mechanisms of ICM at the protein level. Therefore, comparative quantitative proteomic analysis of left ventricles obtained from normal heart donors (heavy-labeled with CD2O) and ICM patients (light-labeled with CH3) was performed by liquid chromatography coupled to mass spectrometry (LC-MS/MS). The schematic representation of quantitative proteomics experiment was described in Supplementary material, **Figure S1.** Two independent repeated experiments were performed, which included seven normal and six ICM ventricles, and a total of 1,723 ventricle proteins were quantified in both experiments, as shown in Supplementary material, **Figure S2A.** Pearson correlation coefficient (PCC) analysis was described in Supplementary material, **Figure S2B,** showed good reproducibility between the two experiments, as evidenced by an $r$ value of 0.73. A Gaussian distribution of the shared quantitative data (as the log2 (ratio)) was analyzed and showed a reasonable ratio of distribution, as shown in Supplementary material, **Figure S2C.** All the quantified proteins were summarized in Supplementary
material, Table S2. From the whole quantitative dataset, we identified 167 differentially expressed proteins in the left ventricles of ICM patients compared with the normal samples, were summarized in Supplementary material, Table S3. Among which 63 proteins were found to be significantly downregulated (average ratio of <0.8 and p<0.05 in both repetitions or ratio of <0.67 in the two repetitions) in ICM ventricles, whereas 104 proteins were upregulated (average ratio of >1.2 and p<0.05 in both repetitions or ratio of >1.5 in the two repetitions). A heatmap of the ratios and p values (variation significance) for these differentially expressed proteins is shown in Supplementary material, Figure S2D. The results showed good quantitative correlation for the two repetitions, and most of the p values calculated with ProteinPilot software were less than 0.05. To verify the reliability of the quantitative proteomics results, we applied conventional western blot analysis to further assess the expression levels of some proteins in the same specimens. In accordance with the results of proteomic analysis, western blot analysis demonstrated that the protein level of SERPINC1 was abnormally expressed in ICM ventricles compared with their normal counterparts, while TGM2 was markedly downregulated. The western blot data were shown in Supplementary material, Figure S3. Collectively, the findings indicated that the data from the comparative quantitative proteomic analysis in the present study were very reliable. To further illustrate the qualified and dysregulated proteins, the volcano plot was applied. It could be found that CASQ2 was significantly downregulated, and the proteins including SERPINC1, OGN, PRELP, VCAN, HBA1, LUM, BASP1, APOH and VIM were significantly upregulated (absolute values of log2(fold change) >2 and p<0.05 in both replicate experiments) (Figure 1).
Multiple biological processes were associated with the differential expression in the left ventricles of ICM patients

After identifying the differentially expressed proteins in the ICM samples compared with the normal samples, the associated molecular functions and biological processes were further explored. A functional annotation tool was used to generate clusters of overrepresented Gene Ontology (GO) terms associated with the development of ICM. The upregulated proteins in the left ventricles of ICM patients were remarkably enriched for biological process terms including the wound response, inflammatory response, regulation of response to stimulus, complement activation, and protein maturation by peptide bond cleavage terms (Figure 2A), while the downregulated proteins were mostly enriched for the oxidation reduction, isocitrate metabolic process, muscle system process and muscle contraction terms (Figure 2B).

To determine the intracellular pathways associated with these differentially expressed proteins, KEGG PATHWAY Database mapping analysis was performed. Our results revealed that a total of 17 pathways were involved (Figure 3); the proteins involved in complement and coagulation cascades, metabolic pathways, focal adhesion, regulation of the actin cytoskeleton, the PI3K-Akt signaling pathway and ECM-receptor interaction were mainly upregulated, while those involved in metabolic pathways, carbon metabolism and cardiac muscle contraction were primarily downregulated (Figure 3).

Interaction network of the differentially expressed proteins in the left ventricles of ICM patients

To further systematically investigate the possible regulated signaling network associated with the altered left ventricle proteomes of ICM patients, the STRING online database
and UniProt functional annotation were used to generate protein-protein interaction networks for all the differentially expressed proteins. Our results indicated that the protein interaction network related to terms including metabolism, the immune response, the extracellular matrix, transcription/translation, cytoskeleton organization, cardiac muscle contraction, and intracellular signal transduction was significantly altered in the left ventricles of ICM individuals (Figure 4A-4G). The majority of changed proteins associated with the extracellular matrix, immune response, cytoskeleton organization and transcription/translation terms were markedly increased in the left ventricles of ICM patients, while most of the altered proteins involved in metabolism, muscle contraction and signal transduction were markedly decreased in the ICM samples. With regard to the extracellular matrix, the vast majority of changed proteins associated with collagen were upregulated (e.g., COL6A3, COL6A1, COL14A1, and COL6A2) in the left ventricles of ICM patients. A similar situation was also observed for complement-related proteins (e.g., C1R, C1QC, C3, C7, C8B and C9), while the opposite situation was observed for proteins associated with amino acid metabolism (e.g., SLC3A2, TGM2, GPT and ALDH6A1) and the TCA cycle (e.g., NDUFB4, NDUFA6, COX5B and COX6B1). All the proteins involved in the network analysis were summarized in Supplementary material, Table S4.

Discussion

Previous proteomic analysis studies on ventricular tissues from ischemic cardiomyopathy patients using 2DE have been narrow in scope [12]. Therefore, in this study, LC-MS/MS was performed on a number of human ventricle samples from ICM patients undergoing
cardiac transplantation, which allowed us to collect that region of heart tissue. Comprehensive proteomic analysis of the human left ventricles provided quantitative information on thousands of proteins, revealed an array of coordinated changes in the heart proteome, and provided insight into the cellular mechanisms related to the development of ICM.

We found 167 proteins to be differentially regulated in left ventricular tissue between the ICM and control groups (104 upregulated and 63 downregulated). In the dysregulated proteins shown by volcano plot, CASQ2 was significantly downregulated, and the proteins including SERPING1, OGN, PRELP, VCAN, HBA1, LUM, BASP1, APOH and VIM were significantly upregulated. GO analysis was performed to reveal the common and disease-specific biological processes; furthermore, KEGG pathway analysis was performed to reveal the overrepresented pathways. In the ICM-specific biological process analysis of this study, the top four significant biological process terms included the response to wounding, inflammatory response, regulation of response to stimulus and immune effector process terms, whereas the top four significant KEGG pathways were the metabolic, complement and coagulation cascades, carbon metabolism and focal adhesion pathways. These results suggest that abnormal metabolism and inflammatory and immune responses might play important roles in the pathogenesis of ICM. To acquire deeper insight into the interactions of the differentially expressed proteins, the proteins were mapped to protein-protein interaction networks. The top four clusters were associated with metabolism, the immune response, the extracellular matrix, and transcription/translation.
Altered expression of various proteins involved in metabolism has been detected by cardiovascular proteomics, and the results of this study are in accordance with those of previous studies [24, 25]. Moreover, proteins involved in carbohydrate metabolism were markedly changed in the ICM group compared to the control group (7 downregulated, including ABHD10, TSTA, IDH2, IDH3B, ME2, IDH3G and GBAS; 4 upregulated, including GUSB, SLC2A1, ENO2 and TMX2). The immune system process is common in ICM. Complement activation contributes significantly to inflammation-related damage in the heart after myocardial infarction [26, 27]. However, understanding of the factors that regulate complement activation is incomplete. In the present study, complement activation-related factors were found to play pivotal roles in the process of ICM. Factors including C3, C7, C9, SERPING1, CFHR1, C1R, C1QC, CFD and C8B were all increased in ICM ventricles compared to normal samples. It has been found that the deleterious effects of complement activation can be ameliorated by complement inhibition in animal studies [28, 29]. A C1 inhibitor has been found to inhibit complement activation and reduce myocardial injury in patients with acute myocardial infarction [30]. Among the altered complement-related factors in the present study, SERPING1, a member of a serine proteinase inhibitor (SERPIN) family, is a C1 inhibitor that regulates both complement activation and blood coagulation [31, 32]. Consistent with our findings, Reindert W. Emmens et al found that endogenous SERPING1 likely plays an important role in the regulation of complement activity following acute myocardial infarction, indicating that SERPING1 might be a novel biomarker and therapeutic target in ischemic heart disease [33].
In summary, quantitative proteomic analysis identified differential protein expression in the left ventricle that can distinguish between patients with ICM and healthy subjects. Many of these proteins are involved in biological pathways pertinent to the processes of cardiac metabolism and inflammatory and immune responses. In addition, our experiment demonstrated that in response to an ischemic stimulus, the C1 inhibitor SERPING1 helps compensate for increases in complement activation through complement inhibition. The identified proteins represent novel diagnostic and therapeutic targets for the treatment of ICM. However, additional in-depth studies on these novel potential biomarkers are warranted in the future.

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**Conflict of interest statement**

The authors declare that they have no conflict of interest.

**References**


Figure 1. The volcano plots of the quantified proteins. A-B. Volcano plots of the quantified proteins in the two experimental replicates, respectively. The red dots represent the proteins which significantly up-regulated, the green dots represent the proteins which significantly down-regulated, and the black dots represent that the
difference fold of the proteins do not reach statistical difference. The dots with black circles represent proteins whose absolute values of log₂(fold change) were greater than 2 and p<0.05 in both replicate experiments.
Figure 2. Gene Ontology (GO) analysis of the differentially expressed proteins. A. Fifty-five terms, including the response to wounding, acute inflammation response and immune effector process terms, were overrepresented in the biological process category for the upregulated proteins. B. Four terms, including the oxidation reduction, muscle system process, muscle contraction and isocitrate metabolic process terms, were overrepresented in the biological process category for the downregulated proteins. The terms are depicted as nodes and are connected by arrows, which represent the hierarchies and relationships between terms. The node size is proportional to the number of proteins assigned to a given ontology term, whereas the node color represents the corrected p-value corresponding to the enrichment of the term.
Figure 3. KEGG pathway analysis of the differentially expressed proteins. Pathway enrichment analysis was performed by submitting the differentially expressed proteins to the KEGG database. A total of 17 KEGG pathways were associated with the proteins, among which cardiovascular disease-related pathways and metabolic pathways were principally enriched among the downregulated and upregulated proteins, respectively. The quantity of the regulated proteins in each pathway is also given.
Cytoskeleton organization

Transcription/Translation

Cytoskeleton organization
Figure 4. Protein interaction analysis. The interactions among all 95 differentially expressed proteins were obtained with STRING 9.0. A-G. Eight functional subnetworks, including metabolism (A), immune response (B), extracellular matrix (C), transcription/translation (D), cytoskeleton organization (E), cardiac muscle contraction (F) and intracellular signal transduction (G) subnetworks, were identified according to UniProt Gene Ontology annotations. The node color represents the quantitative ratio.
Supplementary materials Legends

**Table S1.** The information of normal donors and patients with severe ischemic cardiomyopathy (ICM).

**Table S2.** A total of 1,723 ventricle proteins were quantified in both experiments.

**Table S3.** A total of 167 differentially expressed proteins were found in the left ventricles of ICM patients compared with the normal samples.

**Table S4.** All the proteins involved in the network analysis were summarized.

**Figure S1. Schematic representation of the quantitative proteomics experiment.**
Proteins were extracted from the left ventricles of 7 normal heart donors and 6 ICM patients. After pooling the protein extracts, the normal and ICM samples were reduced, alkylated and tryptically digested. Then, by using a stable isotope dimethyl-labeling strategy, we performed comparative quantitative proteomic analysis of the left ventricles from normal heart donors (heavy-labeled with CD2O) and ICM patients (light-labeled with CH2O).

**Figure S2. Quantitative proteomics data overview. A.** A total of 2201 proteins were successfully quantified in the two experimental replicates, among which 1,723 proteins overlapped. **B.** Pearson correlation analysis of the overlapped proteins showed a good correlation (Pearson correlation coefficient (PCC) = 0.73) for the two replicates. **C.** Gaussian distribution of the average quantitative data (Log2 (ratio)) shared by the two
replicates. D. Heatmap of the 167 differentially expressed proteins (average ratio of <0.8 or >1.2 and p<0.05 in both repetitions or ratio of <0.67 or >1.5 in both repetitions) in the left ventricles of ICM patients.

**Figure S3. Western blot analysis of TGM2 and SERPINC1.** The downregulation of the protein TGM2 and the upregulation of the protein SERPINC1 were confirmed by western blot analysis (ICM group: n=6; normal group: n=7). GAPDH served as a loading control.