ORIGINAL ARTICLE

An altered left ventricle protein profile in human ischemic cardiomyopathy revealed in comparative quantitative proteomics

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KEY WORDS
comparative quantitative proteomics, ischemic cardiomyopathy, left ventricle

ABSTRACT

BACKGROUND Ischemic cardiomyopathy (ICM) resulting from coronary artery disease is a major cause of heart failure. The identification and quantification of differentially expressed proteins in patients with ICM may potentially lead to more effective diagnostic workup and treatment.

AIMS Liquid chromatography coupled to tandem mass spectrometry analysis was applied to identify differentially expressed proteins in individuals with ICM.

METHODS To identify proteins involved in the molecular mechanisms of ICM, we quantitatively analyzed the left ventricular proteome profiles of patients with ICM who had undergone heart transplantation. Liquid chromatography coupled to tandem mass spectrometry, which presents better comprehensiveness and accuracy of quantification than 2-dimensional electrophoresis, in combination with bioinformatics was applied to analyze cardiac samples and identify proteins that were differentially expressed in the left ventricles of 6 patients with ICM compared with 7 normal heart donors.

RESULTS A total of 1723 proteins was successfully quantified in 2 repeated experiments. Out of those, 104 proteins were upregulated and 63 proteins were downregulated in the left ventricles of individuals with ICM. For all these altered proteins, gene ontology (GO) analysis, the Kyoto Encyclopedia of Genes and Genomes pathway mapping, and protein interaction analysis were performed, which showed that most of the proteins were related to the extracellular matrix, metabolism, 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.

CONCLUSIONS Collectively, these differentially expressed proteins represent potential novel diagnostic and therapeutic targets for the treatment of patients with ICM.

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Received: May 27, 2019.
Revision accepted: August 20, 2019.
Published online: August 20, 2019.
Kardiol Pol. 2019; 77 (10): 951-959
doi:10.33963/KP.14936

INTRODUCTION With the ageing of the population, ischemic cardiomyopathy (ICM) is becoming an increasing social and economic burden to society. The best treatment strategy for ICM is heart transplant; however, the donors are currently scarce.[1] Thus, an enormous amount of clinical research has been undertaken to identify diagnostic markers and therapeutic targets for better management of ICM.[2-5] Studies that have led to an increased understanding of the ICM process at the molecular level have also helped in combating ICM to a certain extent.[4,6] For example, several proteins related to inflammation and ventricular remodeling, such as C-reactive

WHAT'S NEW?

Aiming to complement previous analyses using a deeper approach, liquid chromatography coupled to double mass spectrometry analysis was applied. A total of 1723 proteins were successfully quantified, among which 104 proteins were upregulated and 63 proteins were downregulated in the left ventricles of individuals with ischemic cardiomyopathy compared with 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. The identified proteins represent novel diagnostic and therapeutic targets for the treatment of ischemic cardiomyopathy.

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 in examining biological processes through identification of unknown biological functions.[9-11] The identification and quantification of differentially expressed proteins in human samples using proteomics may potentially lead to more effective diagnostic workup and treatment of ICM. Classic 2-dimensional electrophoresis (2-DE) followed by mass spectrometry assessment of the left ventricles in patients with ICM was first performed in 2012.[12] The results showed that 35 proteins were differentially regulated (20 upregulated and 15 downregulated) in the left ventricular tissue in patients with ICM as compared with controls, and the differentially expressed proteins were mainly implicated in metabolism, the respiratory chain as well as immune and stress responses.[12] However, an inherent limitation of classic 2-DE is that very hydrophobic proteins cannot be identified or characterized, which can cause some differentially expressed proteins in patients with ICM as compared with healthy controls to be overlooked.[13] Here, we compared human left ventricular tissue from patients with ICM with that from healthy controls using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis, which presents better comprehensiveness and accuracy of quantification than 2-DE, to complement previous analyses using a more refined 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.

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 were conducted in accordance with the principles outlined in the Declaration of Helsinki. All patients were volunteers and provided informed consent prior to participation. Left ventricular tissue samples (peri-infarct zones) were collected from 6 patients with ICM while they were undergoing heart transplantation due to irreversible heart failure caused by myocardial infarction. Control samples were obtained from 7 normal heart donors who died of accidents but whose hearts were deemed unsuitable for transplantation due to noncardiac reasons.[14-16] The tissue samples were washed with phosphate-buffered saline (PBS) immediately after the removal and then cut into small pieces, directly frozen in liquid nitrogen, and processed for proteomic and Western blot analyses.

Extraction and digestion of left ventricle proteins Extraction and digestion of left ventricle proteins were performed as described elsewhere.[17,18] Briefly, left ventricular tissues from 6 patients with ICM and 7 healthy controls were used for proteomic analysis. The tissues were homogenized in HNTG lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol) with a pooled approach to minimize biological variations. The resulting samples were then vortexed and left ventricle proteins were collected and divided into aliquots for 2 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 the Bradford assay, the proteins were digested with trypsin at a ratio of 1:50 (trypsin / protein, weight / weitgh) desalted by using a Sep-Pak C18 cartridge (Waters, Milford, Massachusetts, United States) and dried with a SpeedVac (Thermo Scientific, Waltham, Massachusetts, United States).

Stable-isotope dimethyl labeling and strong cation-exchange chromatographic fractionation Stable-isotope dimethyl labeling and strong cation-exchange fractionation of the peptides were performed as described in our previous study.[17] The desalted peptides were resuspended in sodium acetate. Next, 4% formaldehyde (CH_2O, a light label) was added to the peptides from the study group, and 4% deuterated formaldehyde (CD_2O, a heavy label) was added to the peptides extracted from the control group. After labeling, the peptides were mixed and desalted again prior to separation via strong cation-exchange chromatography. During gradient elution, nearly 12 fractions were collected and desalted prior to MS analysis.

Liquid chromatography coupled to tandem mass spectrometry and data processing All LC-MS/MS experiments were performed as described elsewhere.[17] Dried peptides were dissolved, loaded onto a C18 trap column, and subsequently eluted. The MS/MS data were acquired by the information-dependent acquisition mode of a TripleTOF 5600+ System coupled with an Ultra 1D Plus nano-liquid chromatography device (SCIEX, Boston, Massachusetts, United States). The generated raw spectra were analyzed with ProteinPilot 4.5 software (SCIEX) by using the Paragon algorithm. The UniProt database (*Homo sapiens*, 2016-03-29, UP000000589) was used. We used the same parameters for data analysis as in our previous study.[17] As described previously, proteins were considered to be successfully identified when at least 2 correctly assigned peptides (95% confidence) were obtained. The false discovery rates of the peptide-spectra matches determined by a decoy database search were set to 1.0%.

Bioinformatics analysis of the identified proteins Based on our previous study, for quantification of the identified proteins, 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) tool, 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 categories of biological processes. Significance was determined as described in our previous study.[17,19] Intracellular pathway analysis was performed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database.[20] For the analysis of protein interaction network 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 The Western blot analysis was performed as described elsewhere.[14,17,21-23] Briefly, protein was extracted with RIPA lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4). The protein concentration was determined with a BCA Protein Assay Kit (Bio-Rad, Shanghai, China). The denatured protein was separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), 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 the ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, California, United States), and then the gray values of the proteins were analyzed with the Image Lab software (version 5.2.1, Bio-Rad).

Statistical analysis The data are expressed as mean and standard error (SE). Two-tailed *t* tests were performed to compare the means of the 2 groups. *P* values of less than 0.05 were considered significant. The SPSS 13.0 statistical software (IBM. Armonk, New York, United States) was used to perform all statistical analyses in the present study.

RESULTS

Clinical characteristics of the patients In the present study, 13 human samples were included: 6 samples from patients with ICM and 7 from normal controls. The clinical and echocardiographic characteristics of the participants are summarized in Supplementary material, *Table S1*. Patients with ICM were all men at a mean age of 59 years. Mean heart rate was 78.7 bpm, with mean systolic and diastolic blood pressure of 106.5 mm Hg and 58.5 mm Hg, respectively. An increased left ventricular end-diastolic dimension and decreased left ventricular ejection fraction were found in the study group compared with the control group.

Differentially expressed proteins 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 CD_2O) and patients with ICM (light-labeled with CH_2O) was performed by 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 samples from ventricles from 7 controls and 6 patients with ICM, and a total of 1723 ventricle proteins were quantified in both experiments, as shown in Supplementary material, *Figure S2A*. The Pearson correlation coefficient analysis was described in Supplementary material, *Figure S2B*. It showed good reproducibility between the 2 experiments, as evidenced by an r value of 0.73. Gaussian distribution of the shared quantitative data (as

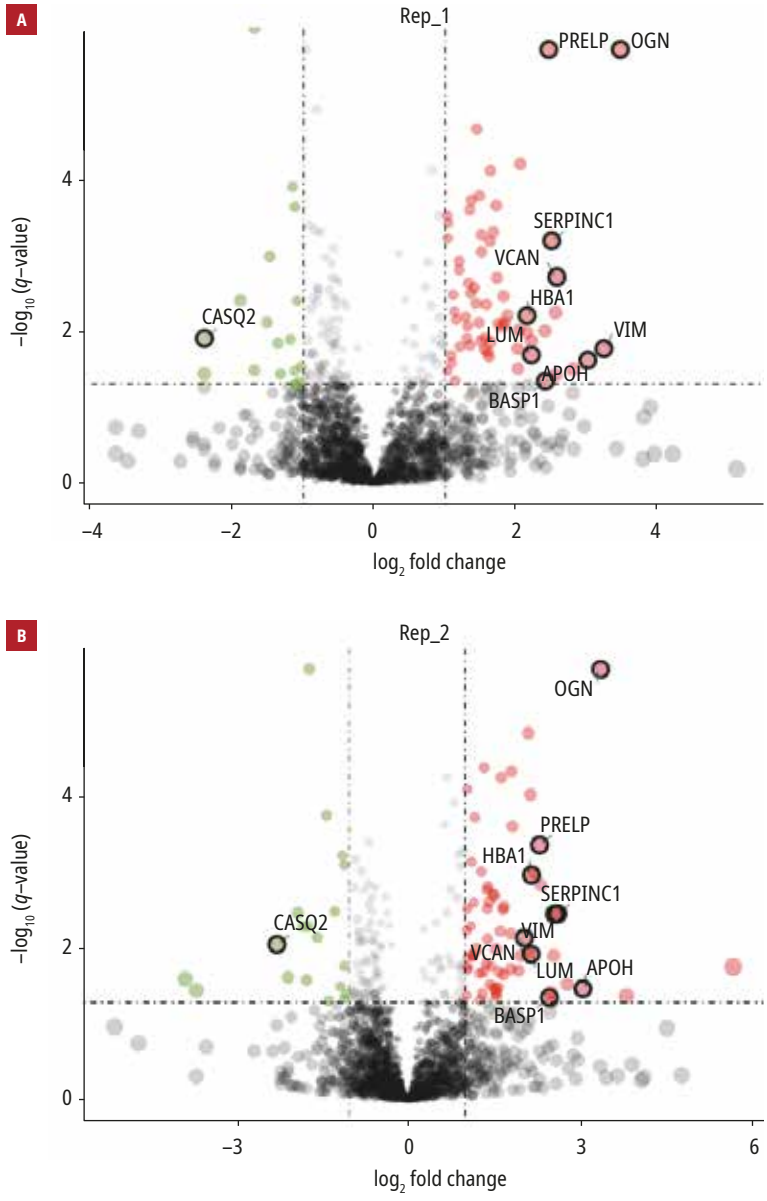

FIGURE 1 **A**, **B** – volcano plots of the quantified proteins in the 2 experimental replicates, respectively. The red dots represent the proteins that were significantly upregulated, the green dots represent the proteins that were significantly downregulated, and the black dots represent the proteins that changed insignificantly. The dots with black circles represent proteins whose absolute values of $\log_2$ fold change were greater than 2 and P <0.05 in both replicate experiments.

the $\log_2$ [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 data set, we identified 167 differentially expressed proteins in the left ventricles of patients with ICM compared with controls, which are summarized in Supplementary material, *Table S3*. A total of 63 proteins were found to be significantly downregulated (average ratio of the 2 repetitions <0.8 and P <0.05, or ratio <0.67 in the both repetitions) in the ventricles of patients with ICM, whereas 104 proteins were upregulated (average ratio of the 2 repetitions >1.2 and P <0.05, or ratio >1.5 in the both 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 2 repetitions, and most of the P values calculated with ProteinPilot software were significant. To verify the reliability of the quantitative proteomics results, we applied the 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, the Western blot analysis demonstrated that the protein level of *SERPINC1* was abnormally expressed in the ventricles of patients with ICM compared with their normal counterparts, while *TGM2* was markedly downregulated. The Western blot data are 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 was 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 $\log_2$ fold change >2 and <0.05 in both replicate experiments) (FIGURE 1A and 1B).

Alterations of multiple biological processes in the left ventricles of patients with ischemic cardiomyopathy After identifying the differentially expressed proteins in the samples from patients with ICM compared with those from controls, the associated molecular functions and biological processes were further explored. A functional annotation tool was used to generate clusters of overrepresented GO terms associated with the development of ICM. The upregulated proteins in the left ventricles of patients with ICM 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 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

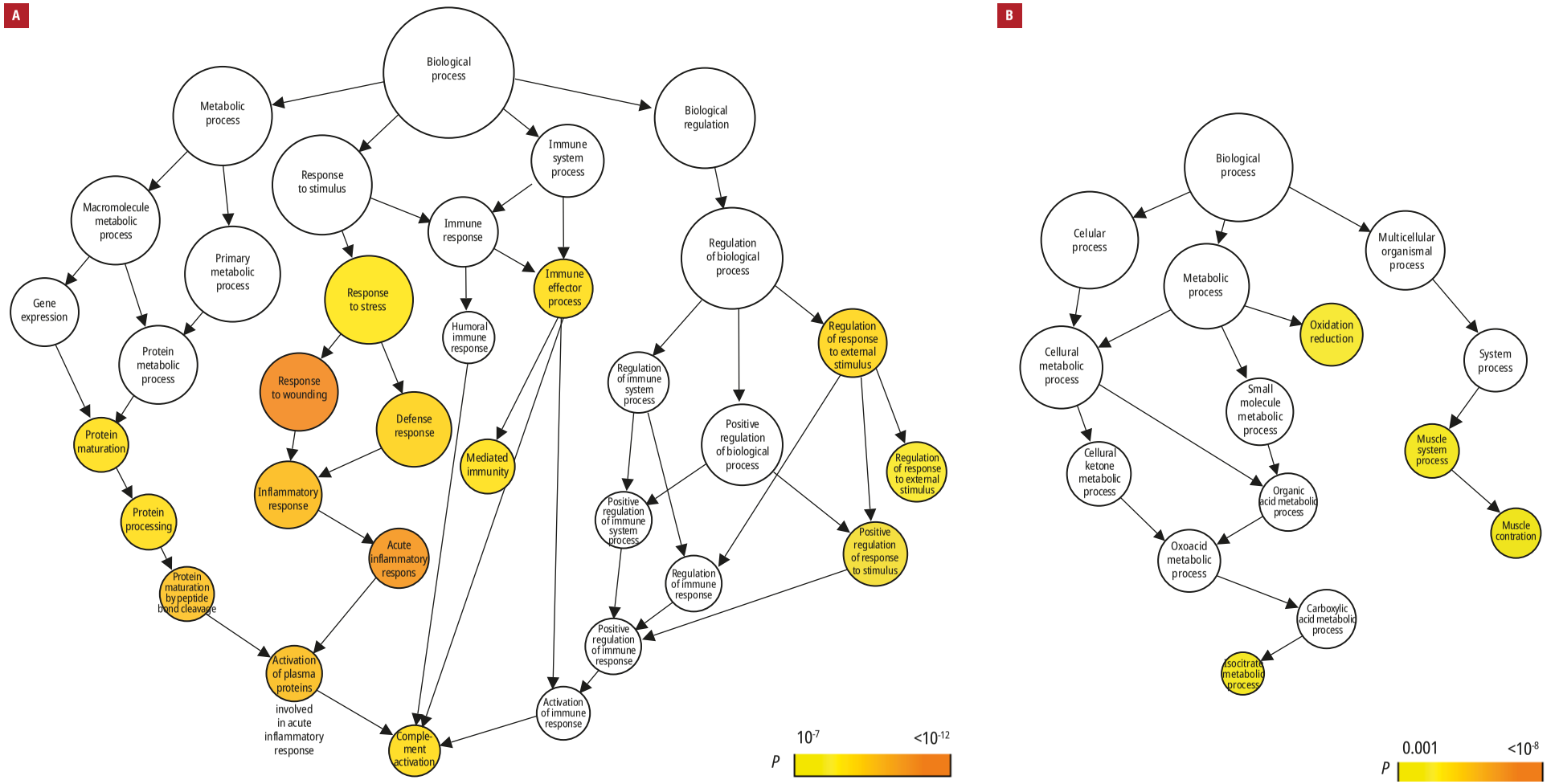

FIGURE 2 Gene ontology analysis of the differentially expressed proteins. **A** – 15 terms, including the response to wounding, acute inflammation response, and immune effector process terms, were overrepresented in the biological process category for the upregulated proteins. **B** – 4 terms, including the oxidation reduction, muscle system process, muscle contraction, and isocitrate metabolic process, were overrepresented in the biological process category for the downregulated proteins. The terms are depicted as nodes and are connected by arrows that 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.

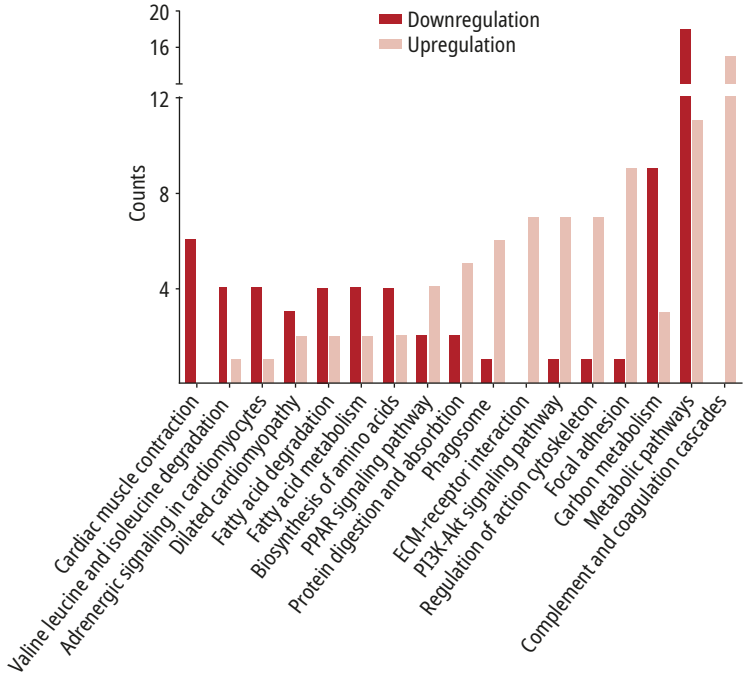

FIGURE 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed proteins. Pathway enrichment analysis was performed by submitting the differentially expressed proteins to the KEGG database.

in metabolic pathways, carbon metabolism, and cardiac muscle contraction were primarily downregulated (FIGURE 3).

Interaction network in the left ventricles of patients with ischemic cardiomyopathy To further systematically analyze the possible regulated signaling network associated with the altered left ventricle proteomes of patients with ICM, 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, immune response, extracellular matrix, transcription / translation, cytoskeleton organization, cardiac muscle contraction, and intracellular signal transduction was significantly altered in the left ventricles of individuals with ICM (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 patients with ICM, while most of the altered proteins involved in metabolism, muscle contraction, and signal transduction were markedly decreased in the samples from patients with ICM. With regard to the extracellular matrix, the vast majority of changed proteins associated with collagen were upregulated (eg, *COL6A3*, *COL6A1*, *COL14A1*, and *COL6A2*) in the left ventricles of patients with ICM. A similar situation was also observed for complement-related proteins (eg, *C1R*, *C1QC*, *C3*, *C7*, *C8B*, and *C9*), while the opposite situation was observed for proteins associated with amino acid metabolism (eg, *SLC3A2*, *TGM2*, *GPT*, and *ALDH6A1*) and the TCA cycle (eg, *NDUFB4*, *NDUFA6*, *COX5B*, and *COX6B1*). All proteins involved in the network analysis were summarized in Supplementary material, *Table S4*.

DISCUSSION Previous studies involving proteomic analysis of ventricular tissues from patients with ICM using 2-DE have been narrow in scope.[12] Therefore, in this study, LC-MS/MS was performed on a number of samples from ventricles of patients with ICM undergoing cardiac transplantation. 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 ICM.

We found 167 proteins to be differentially regulated in the left ventricular tissue in the ICM and control groups (104 upregulated and 63 downregulated). In the dysregulated proteins shown by the volcano plot, *CASQ2* was significantly downregulated, and the proteins including *SERPINC1*, *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 show the overrepresented pathways. In the ICM-specific biological process analysis of this study, the top 4 significant biological process terms included the response to wounding, inflammatory response, regulation of response to stimulus and immune effector process terms, whereas the top 4 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 4 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 with the control group (7 downregulated, including *ABHD10*, *TSTA*, *IDH2*, *IDH3B*, *ME2*, *IDH3G*, and *GBAS*; 4 upregulated, including *GUSB*, *SLC2A1*, *ENO2*,

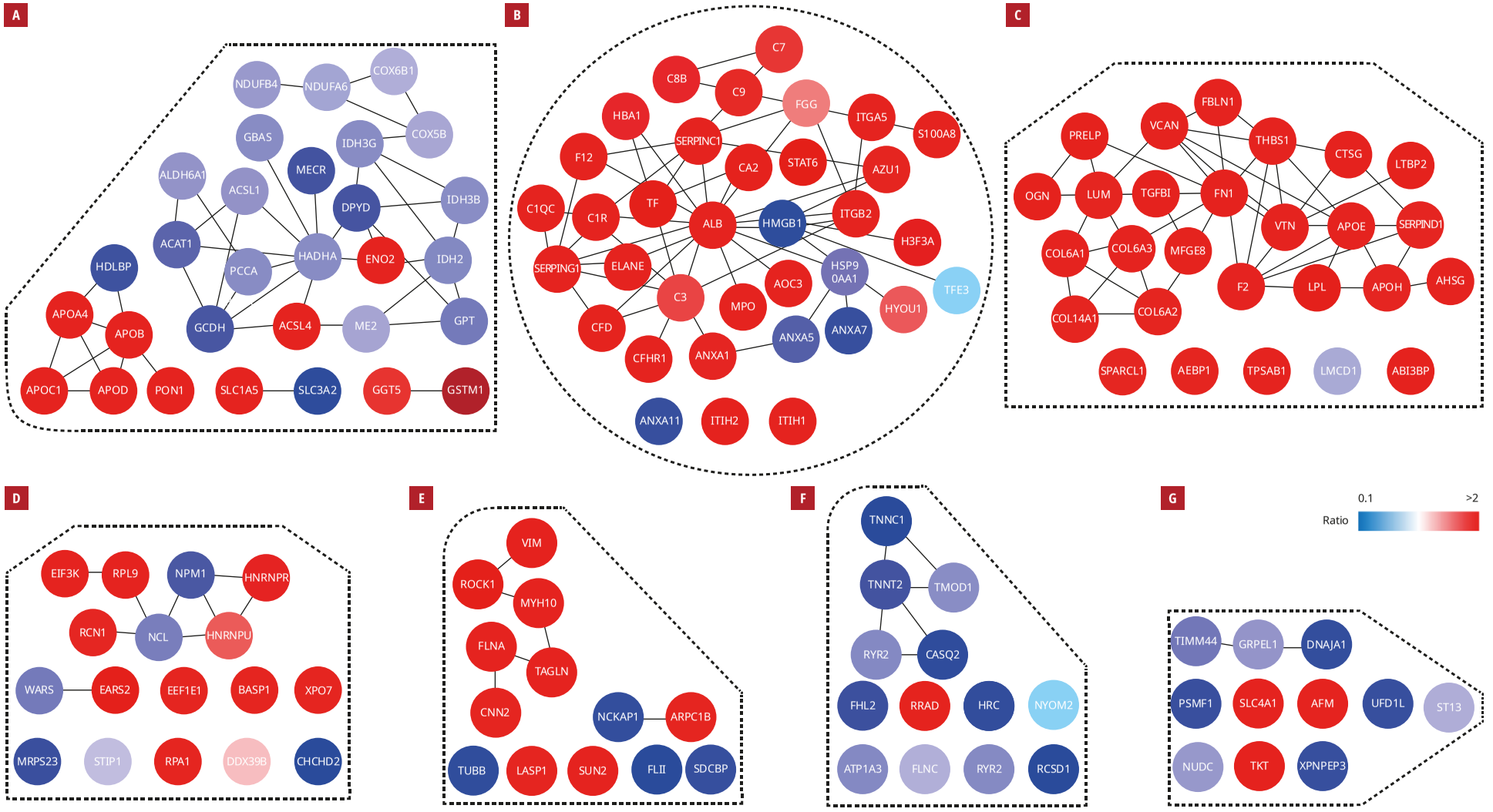

FIGURE 4 Protein interaction analysis. Eight functional subnetworks, including metabolism (**A**), immune response (**B**), extracellular matrix (**C**), transcription / translation (**D**), cytoskeleton organization (**E**), cardiac muscle contraction (**F**), and intracellular signal transduction (**G**) subnetworks, were identified according to UniProt Gene Ontology annotations. The node color represents the quantitative ratio.

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 the ventricle samples from patients with ICM compared with controls. It has been shown that the deleterious effects of complement activation can be ameliorated by complement inhibition in animal studies.[28,29] A *C1* inhibitor has been reported 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, Emmens et al[33] 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.

In summary, quantitative proteomic analysis identified differential protein expression in the left ventricle that can distinguish between patients with ICM and healthy individuals. Many of these proteins are involved in biological pathways pertinent to the processes of cardiac metabolism, as well as 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.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/kardiologiapolska.

ARTICLE INFORMATION

ACKNOWLEDGMENTS This work was partially supported by grants from the National Natural Science Foundation of China (No. 81700249; to D-SJ.) and the Youth Talents Project of Hubei Health Commission (WJ2019Q043; to XY).

CONFLICT OF INTEREST None declared.

HOW TO CITE Yi X, Jiang D-S, Feng G, et al. An altered left ventricle protein profile in human ischemic cardiomyopathy revealed in comparative quantitative proteomics. Kardiol Pol. 2019; 77: 951-959. doi:10.33963/KP.14936

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