Korean Circ J. 2024 Aug;54(8):468-481
https://doi.org/10.4070/kcj.2024.0033
pISSN 1738-5520·eISSN 1738-5555

kcj
Korean Circulation Journal

Original Research

Check for updates

Proteome-wide Characterization and Pathophysiology Correlation in Non-ischemic Cardiomyopathies

Seonhwa Lee, MD[1,*], Dong-Gi Jang, BS[2,3,*], Yeon Ju Kyoung, BS[2,3], Jeesoo Kim, BS[2,3], Eui-Soon Kim, MD[4], Ilseon Hwang, MD, PhD[5], Jong-Chan Youn, MD, PhD[6], Jong-Seo Kim, PhD[2,3], and In-Cheol Kim, MD, PhD[1]

[1]Division of Cardiology, Department of Internal Medicine, Cardiovascular Center, Keimyung University Dongsan Hospital, Keimyung University School of Medicine, Daegu, Korea
[2]Center for RNA Research, Institute for Basic Science, Seoul, Korea
[3]School of Biological Sciences, Seoul National University, Seoul, Korea
[4]Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Korea
[5]Department of Pathology, Keimyung University Dongsan Hospital, Keimyung University School of Medicine, Daegu, Korea
[6]Division of Cardiology, Department of Internal Medicine, Seoul St. Mary's Hospital, Catholic Research Institute for Intractable Cardiovascular Disease, College of Medicine, The Catholic University of Korea, Seoul, Korea

OPEN ACCESS

Received: Jan 23, 2024
Revised: Apr 10, 2024
Accepted: May 8, 2024
Published online: Jun 3, 2024

Correspondence to
Jong-Chan Youn, MD, PhD
Division of Cardiology, Department of Internal Medicine, Seoul St. Mary's Hospital, Catholic Research Institute for Intractable Cardiovascular Disease, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul 06591, Korea.
Email: jong.chan.youn@gmail.com

Jong-Seo Kim, PhD
Center for RNA Research, Institute for Basic Science and School of Biological Sciences, Seoul National University, 1, Gwanak-ro, Gwanak-gu, Seoul 08826, Korea.
Email: jongseokim@snu.ac.kr

In-Cheol Kim, MD, PhD
Division of Cardiology, Department of Internal Medicine, Cardiovascular Center, Keimyung University Dongsan Hospital, Keimyung University School of Medicine, 1035, Dalgubeol-daero, Dalseo-gu, Daegu 42601, Korea.
Email: kimic@dsmc.or.kr

*Seonhwa Lee and Dong-Gi Jang contributed equally to the study.

AUTHOR'S SUMMARY

Distinct proteomic expression profiles of non-ischemic cardiomyopathies were identified, showing a correlation with histopathology. The molecular pathophysiology identified by extensive proteomic analysis effectively represented the clinical and pathological properties of each cardiomyopathy with abundant proteomes. Precise diagnosis of underlying etiology according to multiple modalities, including proteomic analysis, could guide targeted treatment with advanced heart failure.

ABSTRACT

Background and Objectives: Although the clinical consequences of advanced heart failure (HF) may be similar across different etiologies of cardiomyopathies, their proteomic expression may show substantial differences in relation to underlying pathophysiology. We aimed to identify myocardial tissue–based proteomic characteristics and the underlying molecular pathophysiology in non-ischemic cardiomyopathy with different etiologies.
Methods: Comparative extensive proteomic analysis of the myocardium was performed in nine patients with biopsy-proven non-ischemic cardiomyopathies (3 dilated cardiomyopathy [DCM], 2 hypertrophic cardiomyopathy [HCM], and 4 myocarditis) as well as five controls using tandem mass tags combined with liquid chromatography–mass spectrometry. Differential protein expression analysis, Gene Ontology (GO) analysis, and Ingenuity Pathway Analysis (IPA) were performed to identify proteomic differences and molecular mechanisms in each cardiomyopathy type compared to the control. Proteomic characteristics were further evaluated in accordance with clinical and pathological findings.
Results: The principal component analysis score plot showed that the controls, DCM, and HCM clustered well. However, myocarditis samples exhibited scattered distribution. IPA

ORCID iDs

Seonhwa Lee
https://orcid.org/0000-0002-1620-795X
Dong-Gi Jang
https://orcid.org/0009-0005-0861-188X
Yeon Ju Kyoung
https://orcid.org/0009-0007-3607-5361
Jeesoo Kim
https://orcid.org/0009-0006-0528-8308
Eui-Soon Kim
https://orcid.org/0000-0003-2313-8646
Ilseon Hwang
https://orcid.org/0000-0002-6122-4417
Jong-Chan Youn
https://orcid.org/0000-0003-0998-503X
Jong-Seo Kim
https://orcid.org/0000-0002-8909-8440
In-Cheol Kim
https://orcid.org/0000-0002-5751-2328

Funding

This study was supported by the National Research Foundation of Korea (NRF-2020R1C1C1014161 to Kim IC, NRF-2021R1F1A1063430 to Youn JC), and Comparative Medicine Disease Research Center (NRF-2021R1A5A1033157 to Kim JS); by the grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI22C0198) to Kim IC; by the fund from the Korean Cardiac Research Foundation (202202-01) and by the Research Fund of Seoul St. Mary's Hospital, Catholic University of Korea (2022) to Youn JC. Kim JS thanks the Institute for Basic Science (IBS-R008-D1), funded by the Ministry of Science and ICT of Korea, for support.

Conflict of Interest

The authors have no financial conflicts of interest.

Data Sharing Statement

The data generated in this study is available from the corresponding authors upon reasonable request.

Author Contributions

Conceptualization: Lee S, Jang DG, Kim ES, Youn JC, Kim JS, Kim IC; Data curation: Lee S, Jang DG, Kyoung YJ, Kim J, Kim ES, Youn JC, Kim JS, Kim IC; Formal analysis: Lee S, Jang DG, Kim ES, Youn JC, Kim JS, Kim IC; Funding acquisition: Youn JC, Kim JS, Kim

revealed the downregulation of oxidative phosphorylation and upregulation of the sirtuin signaling pathway in both DCM and HCM. Various inflammatory pathways were upregulated in myocarditis with the downregulation of Rho GDP dissociation inhibitors. The molecular pathophysiology identified by extensive proteomic analysis represented the clinical and pathological properties of each cardiomyopathy with abundant proteomes.

Conclusions: Different etiologies of non-ischemic cardiomyopathies in advanced HF exhibit distinct proteomic expression despite shared pathologic findings. The benefit of tailored management strategies considering the different proteomic expressions in non-ischemic advanced HF requires further investigation.

Keywords: Cardiomyopathy; Heart failure; Proteomics; Pathology

INTRODUCTION

Heart failure (HF) prevalence is continuously increasing and is associated with substantial medical costs and social burdens. Despite ongoing developments in HF treatment,[1] an increasing number of patients suffer from advanced HF, which can be fatal or require heart transplantation or mechanical circulatory support.[2][3] Pathologic findings of advanced HF are characterized by extensive fibrosis and/or severe inflammation of the myocardium. Unlike ischemic cardiomyopathy from definite coronary artery obstruction, diseases that cause non-ischemic cardiomyopathy, namely idiopathic dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM), are often difficult to distinguish at advanced stages because of the similarity of their clinical course and pathological characteristics. Both DCM and HCM result in severe systolic dysfunction caused by extensive interstitial fibrosis and replacement fibrosis in the late stage. Myocarditis presenting with advanced HF demonstrates diffuse inflammatory cell infiltration with myocyte damage and oedema. However, in subacute or chronic conditions, they can also exhibit myocardial fibrosis resembling DCM.[4]

Treatment of advanced HF is mainly based on guideline-directed medical therapies according to the phenotype of HF classified as left ventricular ejection fraction rather than the etiology of HF. Recently, there has been a growing awareness of etiology-based medical treatment in some cardiomyopathies, such as HCM and cardiac amyloidosis.[5][6] With the complexity of diagnosis and recent advances in the treatment of non-ischemic cardiomyopathies in the era of precision medicine, understanding the molecular pathophysiology of advanced HF in relation to genetics has become critical.

Quantitative proteomics is an emerging and promising method for exploring the pathogenesis of various diseases and contributing to the development of potential diagnostic biomarkers and therapeutic targets. Such techniques allow for the profiling of the cardiac tissue proteome and may thereby enable the characterization of disease-specific molecular features to discriminate disease etiology in advanced HF patients, which can be critical for developing management strategies. Proteome-wide assessment of differentially expressed proteins (DEPs) in different cardiomyopathies may reveal previously unrecognized pathophysiological pathways in HF.[7] Mass spectrometry-based quantitative proteomics can accurately analyze protein mass and chemical structure to facilitate the evaluation of disease progression and prognosis. Previous studies have reported the possibility of large-scale cardiac proteomic analysis in various etiologies of HF to provide a better understanding of its mechanisms.[8][9]

IC; Investigation: Lee S, Jang DG, Kyoung YJ, Kim J, Hwang I, Youn JC, Kim JS, Kim IC; Methodology: Lee S, Jang DG, Kim ES, Youn JC, Kim JS, Kim IC; Project administration: Youn JC, Kim JS, Kim IC; Resources: Jang DG, Youn JC, Kim JS, Kim IC; Software: Lee S, Jang DG, Youn JC, Kim JS, Kim IC; Supervision: Kim ES, Youn JC, Kim JS, Kim IC; Validation: Lee S, Jang DG, Youn JC, Kim JS, Kim IC; Visualization: Lee S, Jang DG, Hwang I, Youn JC, Kim JS, Kim IC; Writing - original draft: Lee S, Jang DG, Youn JC, Kim JS, Kim IC; Writing - review & editing: Lee S, Jang DG, Youn JC, Kim IC.

However, differences in proteomic expression according to the different etiologies of cardiomyopathies have not been investigated.[10] Thus, we aimed to identify myocardial tissue–based proteomic characteristics and molecular pathophysiology in different types of cardiomyopathy using a highly multiplexed isobaric tagging method and state-of-the-art mass spectrometry with high quantification accuracy and detection sensitivity. In addition, we attempted to demonstrate the correlation between pathological findings and distinct proteomic characteristics in different types of cardiomyopathy.

METHODS

Ethical statement

The research protocol was approved by the Ethics Committee of the Keimyung University Dongsan Hospital (ID: DSMC 2022-11-041-003). This study was performed in accordance with the principles of the Declaration of Helsinki (2013). Written informed consent was obtained from all participants.

Study design

This study prospectively enrolled patients with non-ischemic cardiomyopathies who have received a heart transplant or undergone endomyocardial biopsy between 2017 and 2019. Diagnosis of DCM, HCM, and myocarditis was based on a multimodality approach, including clinical symptom evaluation, laboratory findings, echocardiography, cardiac computed tomography, coronary angiography, cardiac magnetic resonance imaging, and pathologic findings from endomyocardial biopsy or explanted heart specimen. Finally, this study included nine patients with cardiomyopathies (3 with DCM, 2 with HCM, and 4 with myocarditis) presenting with advanced HF. Cardiac tissues were obtained from diseased hearts extracted during heart transplantation in patients with DCM and HCM. For 1 patient with myocarditis, cardiac tissues were collected during a heart transplant was used. Cardiac tissues from 3 out of 4 patients with myocarditis were obtained from endomyocardial biopsy. Endomyocardial biopsy was performed at the time of highest troponin-I level and continued clinical deterioration.

As normal controls, endomyocardial biopsy samples from surveillance of heart transplant recipients with normal biopsy findings over 1 year were included. Detailed methods are described in the **Supplementary Method 1**.

RESULTS

Baseline clinical characteristics, genetic variants, and diagnostic test results, including pathological results, are summarized in **Supplementary Table 1**. Compared to the other groups, patients with myocarditis were younger and had no clinical risk factors or genetic variation. Patients with myocarditis showed various clinical characteristics. In one patient with myocarditis, high troponin-I levels (59.80 mg/dL) were observed 4 days after symptoms appeared. As clinical deterioration continued, heart transplantation was performed. Two patients with myocarditis showed high level of troponin-I 3 and 5 days after symptom onset, respectively, and their cardiac function recovered without sequale. The remaining myocarditis patient's cardiac function partially recovered with sequale.

https://doi.org/10.4070/kcj.2024.0033

According to the pathological findings, DCM showed extensive interstitial or replacement fibrosis with nuclear pleomorphism. Less prominent but extensive fibrosis with nuclear pleomorphism, myocyte hypertrophy, and fiber disarray were noted in HCM. Myocarditis showed typical findings of myocyte damage and oedema, accompanied by various degrees of inflammatory cell infiltration (**Supplementary Figure 1**). Fibrosis was not detected in myocarditis samples, suggesting an acute stage of the disease.

Clustering of cardiomyopathies with different etiologies and normal control

We employed a high-throughput proteomics workflow based on the tandem mass tag mass spectrometry approach (**Supplementary Figure 2**) for the in-depth proteomic profiling of myocardial tissue obtained from diseased individuals against normal controls. Whole-cell proteins extracted from cardiac tissue were processed using the filter-aided sample preparation approach for efficient and unbiased preparation of digest media. To maximize proteome profiling depth, TMTpro-labeled peptides were subjected to prefractionation based on the multiple-fraction concatenation strategy into 24 fractions using off-line mid-pH reversed-phase liquid chromatography (RPLC), which is orthogonal to low-pH RPLC. All fractions were analyzed using the synchronous precursor selection (SPS)-mass spectrometry to the third (MS3) method to measure reporter ions with high accuracy.

Proteins in the sample were identified using the SEQUEST HT algorithm by matching experimental MS2 spectra to theoretical spectra from the input sequence database. Protein abundances were quantified based on the signal-to-noise ratios of TMTpro reporter ion peaks in MS3 spectra. A total of 6,870 proteins were identified with high confidence (maximum 1% false discovery rate [FDR] at the protein level) across all heart samples, and 5,775 proteins were reproducibly quantified in at least one experimental group. The total-sum normalized protein abundance values obtained from Proteome Discoverer (PD) were further normalized to enable more accurate quantitative analysis, using the cyclic loess approach. To inspect the similarities and differences between all samples in an unsupervised manner, a score plot was obtained after principal component analysis (PCA). All cardiomyopathy and control groups were well-clustered and separated in 2D PCA, except for the myocarditis group (**Figure 1A**). The myocarditis group showed a highly dispersed distribution in the PCA space, which may reflect the fact that the diagnosis of myocarditis comprises several different diseases with diverse etiologies. Interestingly, patient 1 (mc_1) near the DCM and HCM cluster had the worst prognosis (heart transplantation), patients 2 (mc_2) and 3 (mc_3) near the normal control cluster completely recovered, and patient 4 (mc_4) recovered with myocardial sequelae. Pairwise correlation analysis was performed to quantitatively measure intra- and inter-group variations (**Figure 1B**). Consistent with the PCA results, intra-group correlations of all experimental groups were high (Pearson's $r>0.95$) with the exception of the myocarditis group (Pearson's $r=0.83–0.89$).

Differentially expressed proteins in cardiomyopathies compared to normal control

To elucidate changes in the abundance of specific proteins in each disease group, DEP analysis was conducted using pairwise 2-sided 2-sample t-test against the control group. Proteins with adjusted p value <0.05 and |fold change [FC]| >1.5 were considered DEPs. Compared to the controls, there were 528 (9.1%) DEPs in DCM (239 upregulated and 289 downregulated DEPs), 121 (2.1%) DEPs in myocarditis (83 upregulated and 38 downregulated DEPs), and 925 (16.5%) DEPs in HCM (460 upregulated and 490 downregulated DEPs). According to the DEP analysis results, there was a higher proportion of significantly

https://doi.org/10.4070/kcj.2024.0033

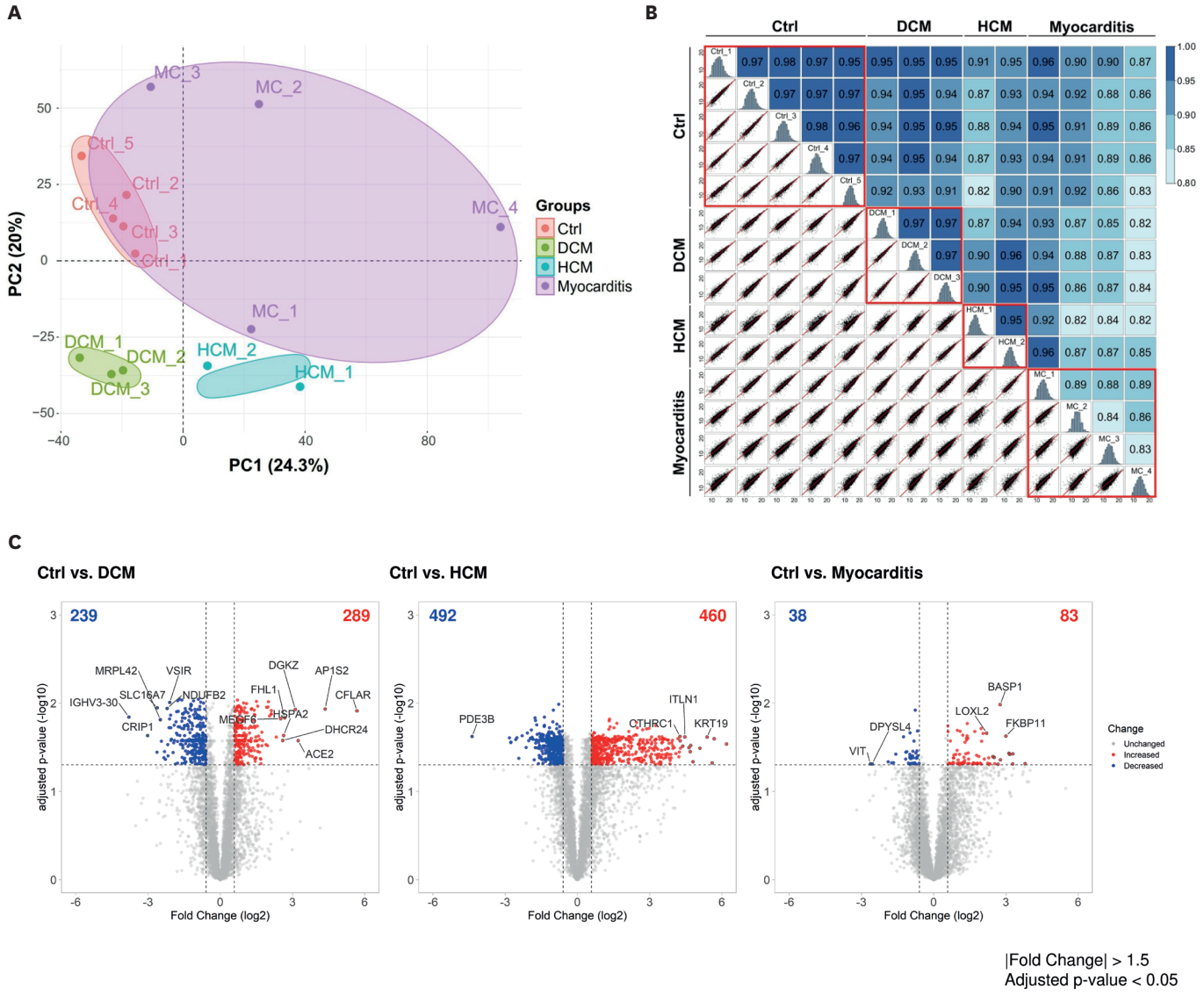

Figure 1. Proteomic analysis of cardiomyopathies and control group. (A) Principal component analysis score plot of proteomics data from cardiomyopathies and control group showing good clustering of controls (Ctrl 1, 2, 3, 4, and 5), dilated cardiomyopathy (DCM 1, 2, and 3), and hypertrophic cardiomyopathy (HCM 1 and 2). Myocarditis (MC 1, 2, 3, and 4) samples exhibit scattered distribution. Each dot represents one patient. Each group is delineated by ellipses that were estimated using the Khachiyan algorithm. (B) Scatter matrix showing the pairwise Pearson correlation values of proteomic expression between individuals (top right), histograms of log2 intensity distributions (diagonal), and corresponding scatter plots (bottom left) with linear fit line (red line). Each group is highlighted by a red border. (C) Volcano plots showing results from the 2-sided Student's t-test of the 5,775 quantified proteins. Each disease group was individually compared with the control group. In DCM, increased and decreased proteins were noted similarly, whereas increased proteins were predominant in HCM. Protein upregulation was more pronounced in myocarditis, with few proteins being downregulated. The x-axis shows the log2 FC of each identified protein, and the y-axis shows the corresponding −log10 p value. Significantly upregulated proteins in the disease group (FC >1.5 and adjusted p value <0.05) are shown in red; significantly downregulated proteins in the disease group (FC <−1.5 and adjusted p value <0.05) are shown in blue. Non-significant proteins that did not pass the threshold are shown in grey. The top 10 significant proteins in each comparison were labelled with corresponding protein names. All volcano plots were generated using the VolcaNoseR program.
Ctrl = control; DCM = dilated cardiomyopathy; FC = fold change; HCM = hypertrophic cardiomyopathy; MC = myocarditis; PC = principal components.

upregulated proteins (FC ≥1.5) in myocarditis, whereas there was a higher proportion of significantly downregulated proteins (FC ≤−1.5) in DCM when compared to the controls (**Figure 1C**). The most upregulated protein in DCM was CFLAR (CASP8 and FADD like apoptosis regulator), which functions as a regulator of apoptosis. The 2 most upregulated proteins in HCM were complement factor B (CFB) and fibromodulin (FMOD). The family

https://doi.org/10.4070/kcj.2024.0033

of interstitial proteoglycans, FMOD, plays a role in extracellular matrix (ECM) assembly. In myocarditis, RANSE 2 and 3 proteins, which affect antimicrobial activity against pathogens, were the most upregulated.

Acetylation-related proteins were differentially regulated in DCM and HCM when compared with those in the controls. In both DCM and HCM, four and a half LIM domains 2 (FHL2), myosin heavy chain 7 (MYH7), and troponin I3 (TNNI3) were downregulated. The ATP synthase F1 subunit α (ATP5F1A) was downregulated in HCM.

Functional annotation of differentially expressed proteins in cardiomyopathies compared to control

A hierarchical cluster analysis was performed to determine proteins that exhibit similar expression patterns among experimental groups. The proteins with significant differences between groups (analysis of variance [ANOVA], p<0.05) were well-separated into 4 clusters

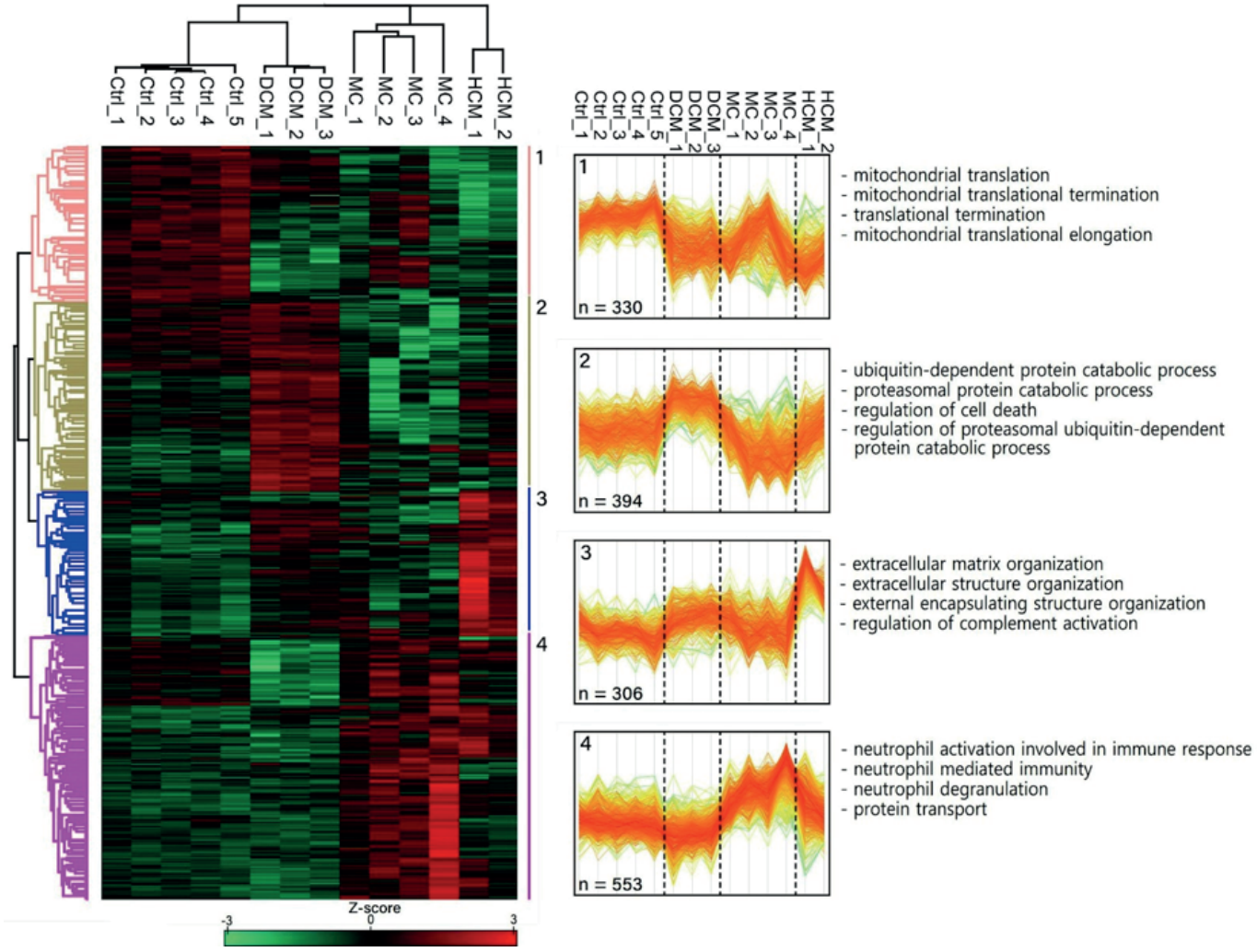

Figure 2. Hierarchical clustering of proteins that passed the cut-off of one-way ANOVA (q-value <0.05) was performed in Perseus on Z-scored logarithmized abundances using Euclidean distance and average linkage. The top 4 GO:BP terms in each cluster are represented. DEPs in the control group and in each cardiomyopathy group were clustered into 4 groups according to functional annotation.
ANOVA = analysis of variance; BP = Biological Process; Ctrl = control; DCM = dilated cardiomyopathy; GO = Gene Ontology; HCM = hypertrophic cardiomyopathy; MC = myocarditis.

https://doi.org/10.4070/kcj.2024.0033

according to their expression profiles (**Figure 2**). Cluster 1 exhibited decreases in DCM and HCM; cluster 2 exhibited an increase in DCM; cluster 3 exhibited a considerable increase in HCM; and cluster 4 exhibited an increase in myocarditis. Proteins in each cluster were selectively enriched with several Gene Ontology (GO):Biological Process (BP) terms. Cluster 1 was enriched with terms related to mitochondrial translation of its own genome. In cluster 2, proteins upregulated exclusively in DCM were largely associated with proteasomal protein catabolic processes. In cluster 3, proteins with high expression exclusively in HCM were associated with ECM organization. In cluster 4, proteins upregulated in myocarditis participate in neutrophil-mediated immune responses.

GO enrichment analyses were performed to determine the biological functions of DEPs in cardiomyopathy compared with the control group (**Figure 3**). Both upregulation and downregulation of proteins were observed in DCM, with more prominent upregulation in HCM, and predominant upregulation with limited downregulation of proteins noted in myocarditis. In DCM, neutrophil degranulation, neutrophil activation involved in the immune response, and muscle contraction were significantly upregulated. Regarding molecular function, telethonin binding, calcium ion binding, and protease binding were significantly upregulated in DCM. Mitochondrial translation and aerobic electron transport chain were downregulated in DCM. In HCM, the neutrophil-mediated immune response, ECM organization, and platelet degranulation were upregulated. Similar to DCM, mitochondrial ATP synthesis-coupled electron transport and aerobic electron transport chains are downregulated in HCM. In myocarditis, the neutrophil mediated immunity is upregulated.

Signaling pathway

Ingenuity Pathway Analysis was conducted to investigate the biological significance of DEPs in each cardiomyopathy. **Supplementary Figure 3** shows major canonical pathways that were significantly associated with cardiomyopathies. In both DCM and HCM, the oxidative phosphorylation pathway, a key factor influencing mitochondrial function, was significantly inactivated compared to that in the control group. In patients with HCM, the activation of the liver X receptor/retinoid X receptor (LXR/RXR) pathway was increased significantly compared with that in the control group. Fc receptor–mediated phagocytosis in macrophages and the monocyte pathway were upregulated in patients with myocarditis. Additionally, several pathways related to inflammatory changes were activated (**Table 1**).

Organellar level proteomic analysis

Organellar protein proportions in each cardiomyopathy were compared with the control group (**Supplementary Figure 4**). In the control group, mitochondria constituted 28.1% and myofibril constituted 29.4% of the total cellular protein mass, which were comparable with the previous normal myocardial specimen. Relatively low proportions of mitochondrial protein were observed in the DCM and HCM subjects. In myocarditis, the proportion of each organelle between samples was varied.

Proteomic characterization with pathologic correlation by cardiomyopathy type

Table 1 showed important ingenuity pathway pathways in the pathogenesis of each cardiomyopathy and the pathophysiology corresponding to those pathways. Proteomic characterization was compared with the pathological findings in each cardiomyopathy (**Table 1**). Decreased protein expression related to the Coordinated Lysosomal Expression and Regulation (CLEAR) and wound healing signaling pathways in DCM was correlated with diffuse fibrosis and

https://doi.org/10.4070/kcj.2024.0033

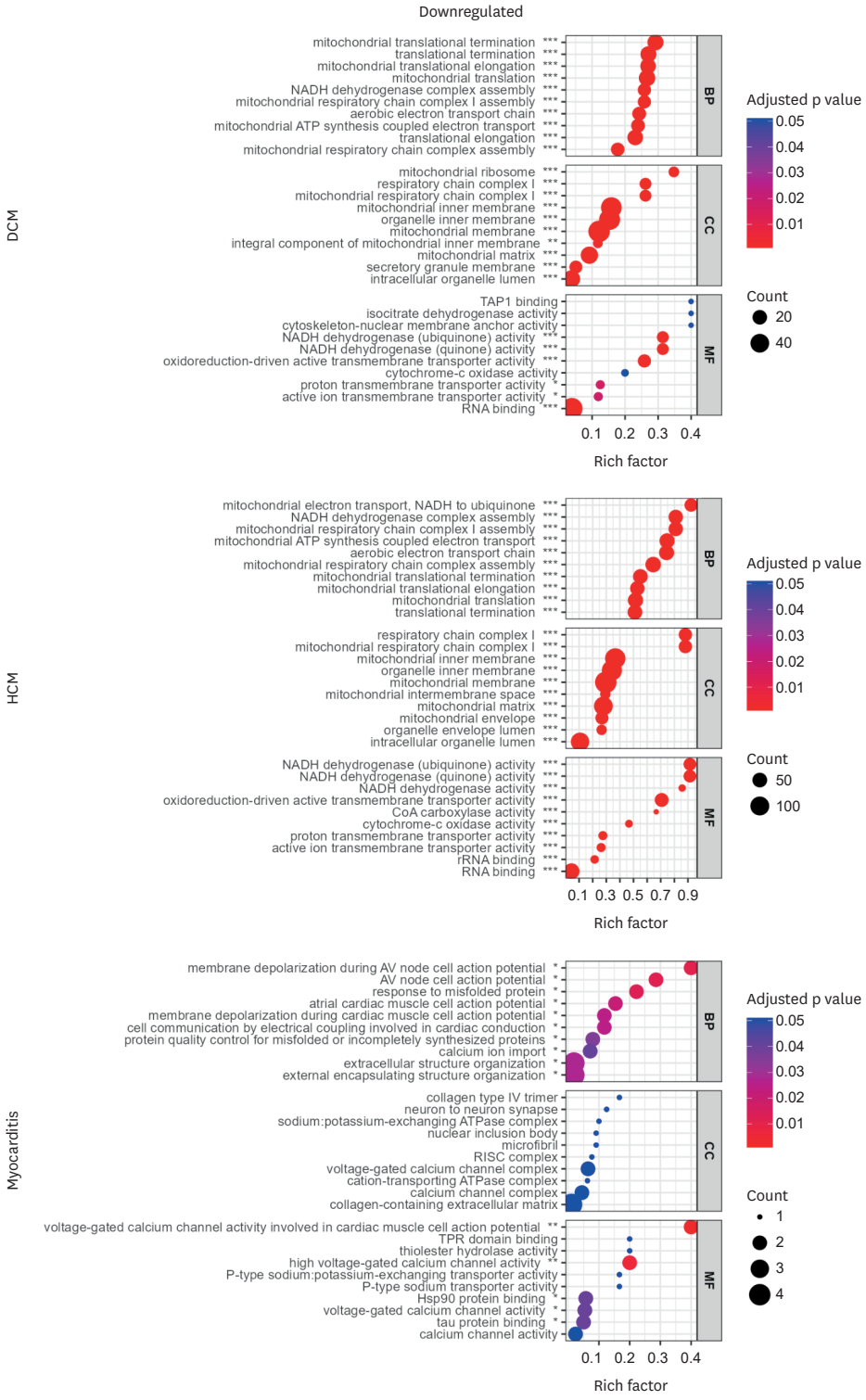

Figure 3. Dot plots showing the results of GO enrichment analysis in three categories (BP, MF, CC) in differentially expressed proteins from EnrichR webtool. Protein upregulation and downregulation were observed in DCM; protein upregulation was predominant in HCM; and upregulation with limited downregulation of protein was noted in myocarditis. Dots are color-coded from blue to red based on the adjusted p value. Dot size is proportional to the number of proteins in each GO term. The Rich factor shown in the x-axis indicates the ratio of the number of enriched proteins to the number of total annotated proteins.
BP = Biological Process; CC = Cellular Compartment; DCM = dilated cardiomyopathy; GO = Gene Ontology; HCM = hypertrophic cardiomyopathy; MF = Molecular Function.
The asterisks (*) indicate the statistical significance of each GO term (adjusted p values *<0.05, **< 0.01, ***<0.001). (continued to the next page)

https://doi.org/10.4070/kcj.2024.0033

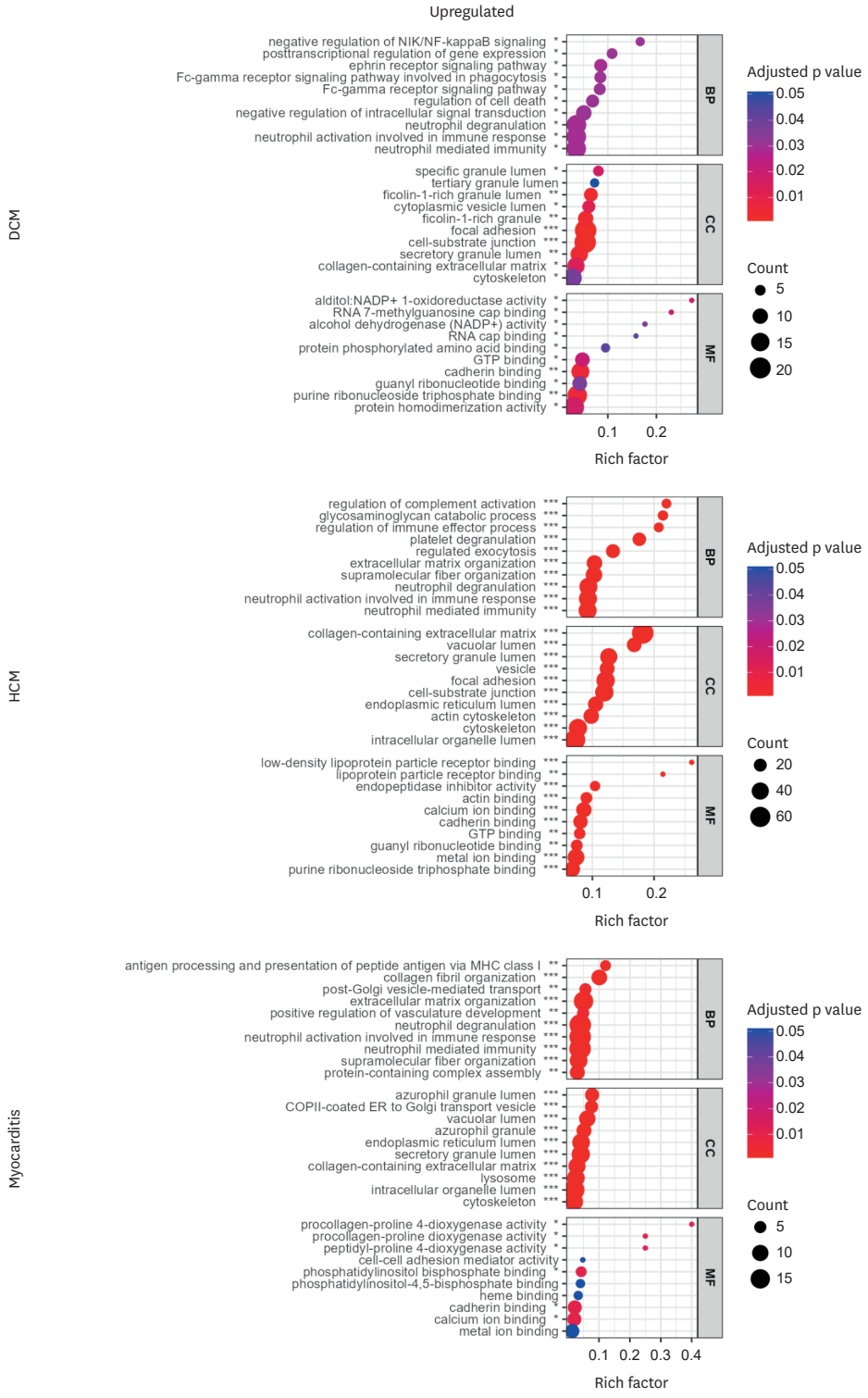

Figure 3. (Continued) Dot plots showing the results of GO enrichment analysis in three categories (BP, MF, CC) in differentially expressed proteins from EnrichR webtool. Protein upregulation and downregulation were observed in DCM; protein upregulation was predominant in HCM; and upregulation with limited downregulation of protein was noted in myocarditis. Dots are color-coded from blue to red based on the adjusted p value. Dot size is proportional to the number of proteins in each GO term. The Rich factor shown in the x-axis indicates the ratio of the number of enriched proteins to the number of total annotated proteins.
BP = Biological Process; CC = Cellular Compartment; DCM = dilated cardiomyopathy; GO = Gene Ontology; HCM = hypertrophic cardiomyopathy; MF = Molecular Function.
The asterisks (*) indicate the statistical significance of each GO term (adjusted p values *<0.05, **< 0.01, ***<0.001).

https://doi.org/10.4070/kcj.2024.0033

kcj
Korean Circulation Journal

Table 1. Proteomic characterization and pathophysiology

Proteomics (IPA)	Pathophysiology	DCM	HCM	Myocarditis
Oxidative phosphorylation	Cardiac mitochondrial dysfunction	↓	↓↓	-
Sirtuin signaling pathway	Cardiac hypertrophy and apoptosis	↑	↑↑	-
LXR/RXR pathway	Adaptive metabolic response to hypertrophic stress	↑	↑↑	-
Phagosome formation	Immune and inflammatory response against pathogen	-	-	↑↑
CLEAR signaling pathway	Autophagy	↓↓	-	-
Wound healing signaling pathway	Cardiac fibroblasts after cardiac injury	↓	↑↑	↑
	Pathology			
	Interstitial or replacement fibrosis	+++	++	–
	Nuclear pleomorphism	+	++	–
	Myocyte atypia	+	–	–
	Myocyte hypertrophy or fiber disarray	–	++	–
	Myocyte damage or oedema	–	–	++
	Inflammatory cells infiltration*	–	–	+++

↑, upregulation; ↓, downregulation; +, expression. The number of symbols indicates the degree of regulation or expression.
CLEAR = Coordinated Lysosomal Expression and Regulation; DCM = dilated cardiomyopathy; HCM = hypertrophic cardiomyopathy; IPA = Ingenuity Pathway Analysis; LXR = liver X receptor; RXR = retinoid X receptor.
*Lymphocyte, neutrophil, eosinophil, and macrophage detected either by hematoxylin-eosin or immunostaining.

myocyte atypia in DCM. Prominently decreased protein expression of oxidative phosphorylation along with increased protein expression of the sirtuin, LXR/RLX, and wound healing signaling pathways were observed in HCM, which was characterized by diffuse fibrosis combined with myocyte hypertrophy, fiber disarray, and ischemic injury. In myocarditis, increased protein expression related to phagosome formation and the wound healing signaling pathway was correlated with active inflammation and varying degrees of myocyte damage.

DISCUSSION

We performed an in-depth proteomic analysis of cardiac tissue samples for the identification of proteins and pathways associated with each type of cardiomyopathy and a control group.

To date, molecular variations in the human heart associated with cardiomyopathy have mostly been studied at the gene expression level.[11] Previous studies have investigated proteomics to evaluate HF pathophysiology[12-14] and shown that the expression of certain proteins is associated with pathogenesis and influences prognosis. A comparison of proteomic expression and pathological correlation in advanced HF with different etiologies of non-ischemic CMP has not yet been performed.

Changes in heart energy metabolism, both in terms of alterations in energy substrate preference and decreased mitochondrial oxidative metabolism, are the main factors influencing HF development.[15] Most of the energy required for myocardial contraction is obtained from aerobic metabolism. The normal myocardium mainly uses oxygen for phosphate production by oxidative phosphorylation. Impaired aerobic metabolism of the myocardium may arise due to defects in mitochondrial oxidative phosphorylation or fatty acid oxidation.[16] In the present study, acetylation-related proteins were downregulated in both DCM and HCM. Notably, ATP5F1A, which is related to sirtuin 3 (SIRT3), was downregulated in HCM. Downregulated SIRT3 expression has been observed in human hearts near failure, which induces increased protein acetylation.[17] In addition, SIRT3 inhibition has been shown to induce mitochondrial oxidative stress and hypertrophy.[18]

https://doi.org/10.4070/kcj.2024.0033

Our study showed that the main pathophysiological mechanism is impaired oxidative phosphorylation in DCM and HCM but not in myocarditis. Mitochondria in advanced HF exhibit membrane disruption and matrix depletion and a decreased capacity for oxidative phosphorylation.[19] Kalsi et al.[20] reported that despite varying associated mechanisms of cardiac failure, impaired oxidative capacity is observed in both DCM and HCM.

Sirtuins play diverse roles in heart diseases. Each sirtuin regulates autophagy and provides protection against ageing, oxidative stress, ischemia–reperfusion injury, and cardiac hypertrophy.[21] In the present study, the sirtuin signaling pathway was upregulated in DCM and HCM. Several studies have reported the beneficial and protective roles of Sirt 1 in end-stage HF.[22] However, a recent study reported that Sirt1 upregulation is not necessarily protective against heart disease. In a mouse model, mild-to-moderate Sirt1 expression slowed heart ageing, whereas high Sirt1 expression induced cardiomyopathy.[23] Considering these findings, the mechanism and role of sirtuin signaling pathway upregulation requires further investigation.

LXR and RXR are nuclear receptors that bind to LXR response elements in the nucleus. LXR has been involved in adaptation to hypertrophic stress.[24][25] In the present study, the LXR and RXR pathways were upregulated in both HCM and DCM, with a more pronounced effect in HCM. LXR regulates the hypertrophic cardiac remodeling process. LXR agonists have been demonstrated to decrease cellular hypertrophy. In a previous study,[24] only human myectomy tissue showed LXR/RXR pathway upregulation, and this was not observed in mouse cardiac tissue of sarcomeric HCM. The differences between human HCM and mouse HCM in cardiac physiology could imply the activation of the compensatory LXR/RXR pathway in human HCM. The finding is consistent with the pathway findings observed in our study.

Autophagy is essential for homeostasis in the heart and is associated with several cardiac diseases.[26] Transcription factor EB (TFEB) modulates the expression of genes harboring the CLEAR motif, thereby regulating lysosomal biogenesis and autophagy.[27] Several studies have reported that TFEB protects the myocardium against proteotoxicity and cell death by promoting autophagy and lysosomal functions. Moreover, recent evidence has suggested that decreased TFEB function is associated with cardiomyopathy and cardiotoxicity. Our results showed that the CLEAR signaling pathway is impaired in patients with DCM. Recently, the significance of autophagy in failing DCM has been reported, and the authors showed that autophagy is related to positive remodeling in DCM.[28] Patients with DCM included in our study also had failing DCM, and impaired autophagy could have played a role in its pathophysiology.

Phagocytosis is a protective mechanism that increases inflammatory and immune responses against pathogens. Activated immune cells, including monocytes and macrophages, have been found in human and experimental myocarditis.[29] Corresponding to the pathogenesis of inflammatory cardiomyopathy, our study demonstrates that phagosome formation is upregulated in myocarditis when compared with normal controls. Similarly, the upregulation of the wound-healing signaling pathway in myocarditis regulates the pathophysiological responses of cardiac fibroblasts. Monocytes and macrophages release growth factors and cytokines that activate cardiac fibroblasts in damaged myocytes.

Conventional diagnostic methods, including biomarkers, imaging modalities, genetic testing, and biopsy results, can guide physicians in accurate diagnosis and better treatment planning. However, there are limitations associated with characterizing different etiologies, especially in advanced HF, which may show similar clinical and histopathologic features.[30] Despite the

predominant myocardial fibrosis in histopathology and severe ventricular systolic dysfunction observed in both DCM and HCM, their proteomic expression profiles were distinct.

In patients with myocarditis, a scattered score plot pattern was observed, unlike the good clustering results obtained for DCM and HCM. Furthermore, the proportions of organellar proteins exhibited high variability in myocarditis cases. This suggests that various degrees of inflammation and myocyte damage were reflected in proteome analysis. These findings suggest the possible utility of proteome-based treatment planning and prognosis.

In clinical practice, differentiating the etiology of HF is crucial yet often challenging. Precise diagnosis of the underlying etiology according to multiple modalities, including proteomic analysis, could be useful for patients with advanced HF in therapeutic preventive measure. The therapeutic and prognostic implications of proteomic analyses require further verification.

The present study had several limitations. First, the number of samples was too small to generalize our observations. Future studies with larger populations may validate the results and reinforce the clinical implications of quantitative proteomics. Second, proteomic analysis requires complex processes that inhibit adoption in everyday practice. However, with advancements in technologies and evidence accumulation, the application of proteomics could be expanded. Third, the heterogeneity of disease status in myocarditis impeded precise characterization, unlike other etiologies of non-ischemic cardiomyopathies. Proteomic analysis focusing on different stages of myocarditis needs to be performed to identify the molecular pathophysiology based on the full clinical spectrum of myocarditis. Fourth, using heart transplantation recipients as control groups constitutes a notable limitation given the difference in transplantation-specific factors across patients.

In conclusion, our study suggests myocardial proteome analysis is an effective approach for distinguishing etiologies of cardiomyopathy despite shared clinical features and pathologic findings. Moreover, we showed that detailed quantitative proteomic analysis is feasible with clinical endomyocardial biopsy samples. Further tailored management and prognostication should be investigated according to proteomic expression in different cardiomyopathies.

SUPPLEMENTARY MATERIALS

Supplementary Method 1
Detailed study methods

Supplementary Table 1
Population characteristics

Supplementary Figure 1
Representative pathologic findings of DCM, HCM, and myocarditis. Hematoxylin and eosin stain and Masson's trichrome stain of the heart showed interstitial fibrosis with myocyte atypia. Masson's trichrome stain of the HCM displayed marked myocyte fibre disarray. In myocarditis, hematoxylin and eosin-stained heart tissue showed inflammatory cell infiltration.

https://doi.org/10.4070/kcj.2024.0033

Supplementary Figure 2

Schematic representation of proteomic experimental workflow. Cardiac biopsies from patients with cardiomyopathy and control groups were cryo-pulverized and processed the filter-aided sample preparation protocol. Peptide samples from each patient were differentially labelled with TMTpro reagents and pooled equivalently. The labelled sample mixture was fractionated by offline mid-pH RPLC into 24 fractions. Whole fractions were subjected to TMTpro-based protein quantification by LC-SPS-MS3 analysis.

Supplementary Figure 3

The top canonical pathways in each disease group were analysed using IPA. The y-axis shows the -log10 p value of each pathway bar, and the orange points represent the ratio of the number of proteins in a given pathway that satisfy the criteria (|FC| >1.5, p value <0.05) to the total number of proteins annotated to that pathway. Bars are colour-coded from blue to orange based on the Z-score, as predicted by IPA based on the gene expression changes within each pathway. Pathways with Z-scores close to zero are indicated in white, and those with unpredictable Z-scores are indicated in grey.

Supplementary Figure 4

Organellar analysis. (A) A cartoon diagram showing the organellar protein mass composition averaged from the control group. (B) Organelles in each subject were sized based on their proportion to the total cellular protein mass.

REFERENCES

1. Greenberg B. Medical management of patients with heart failure and reduced ejection fraction. *Korean Circ J* 2022;52:173-97. PUBMED | CROSSREF
2. Dunlay SM, Roger VL, Killian JM, et al. Advanced heart failure epidemiology and outcomes: a population-based study. *JACC Heart Fail* 2021;9:722-32. PUBMED | CROSSREF
3. Choi HM, Park MS, Youn JC. Update on heart failure management and future directions. *Korean J Intern Med* 2019;34:11-43. PUBMED | CROSSREF
4. Cooper LT Jr. Myocarditis. *N Engl J Med* 2009;360:1526-38. PUBMED | CROSSREF
5. Olivotto I, Oreziak A, Barriales-Villa R, et al. Mavacamten for treatment of symptomatic obstructive hypertrophic cardiomyopathy (EXPLORER-HCM): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2020;396:759-69. PUBMED | CROSSREF
6. Maurer MS, Schwartz JH, Gundapaneni B, et al. Tafamidis treatment for patients with transthyretin amyloid cardiomyopathy. *N Engl J Med* 2018;379:1007-16. PUBMED | CROSSREF
7. Cao TH, Jones DJ, Voors AA, et al. Plasma proteomic approach in patients with heart failure: insights into pathogenesis of disease progression and potential novel treatment targets. *Eur J Heart Fail* 2020;22:70-80. PUBMED | CROSSREF
8. Aye TT, Scholten A, Taouatas N, et al. Proteome-wide protein concentrations in the human heart. *Mol Biosyst* 2010;6:1917-27. PUBMED | CROSSREF
9. Colak D, Alaiya AA, Kaya N, et al. Integrated left ventricular global transcriptome and proteome profiling in human end-stage dilated cardiomyopathy. *PLoS One* 2016;11:e0162669. PUBMED | CROSSREF
10. Li W, Rong R, Zhao S, et al. Proteomic analysis of metabolic, cytoskeletal and stress response proteins in human heart failure. *J Cell Mol Med* 2012;16:59-71. PUBMED | CROSSREF
11. Lee JH, Lee SE, Cho MC. Clinical Implication of Genetic Testing in Dilated Cardiomyopathy. *Int J Heart Fail* 2021;4:1-11. PUBMED | CROSSREF
12. Rueda F, Borràs E, García-García C, et al. Protein-based cardiogenic shock patient classifier. *Eur Heart J* 2019;40:2684-94. PUBMED | CROSSREF
13. Cao TH, Jones DJ, Quinn PA, et al. Using matrix assisted laser desorption ionisation mass spectrometry

(MALDI-MS) profiling in order to predict clinical outcomes of patients with heart failure. *Clin Proteomics* 2018;15:35. PUBMED | CROSSREF

14. Garmany R, Bos JM, Tester DJ, et al. Multi-Omic architecture of obstructive hypertrophic cardiomyopathy. *Circ Genom Precis Med* 2023;16:e003756. PUBMED | CROSSREF
15. Ketema EB, Lopaschuk GD. Post-translational acetylation control of cardiac energy metabolism. *Front Cardiovasc Med* 2021;8:723996. PUBMED | CROSSREF
16. Antozzi C, Zeviani M. Cardiomyopathies in disorders of oxidative metabolism. *Cardiovasc Res* 1997;35:184-99. PUBMED | CROSSREF
17. Zhang X, Ji R, Liao X, et al. MicroRNA-195 regulates metabolism in failing myocardium via alterations in sirtuin 3 expression and mitochondrial protein acetylation. *Circulation* 2018;137:2052-67. PUBMED | CROSSREF
18. Peugnet V, Chwastyniak M, Mulder P, et al. Mitochondrial-targeted therapies require mitophagy to prevent oxidative stress induced by SOD2 inactivation in hypertrophied cardiomyocytes. *Antioxidants* 2022;11:723. PUBMED | CROSSREF
19. Schaper J, Froede R, Hein S, et al. Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. *Circulation* 1991;83:504-14. PUBMED | CROSSREF
20. Kalsi KK, Smolenski RT, Pritchard RD, Khaghani A, Seymour AM, Yacoub MH. Energetics and function of the failing human heart with dilated or hypertrophic cardiomyopathy. *Eur J Clin Invest* 1999;29:469-77. PUBMED | CROSSREF
21. Matsushima S, Sadoshima J. The role of sirtuins in cardiac disease. *Am J Physiol Heart Circ Physiol* 2015;309:H1375-89. PUBMED | CROSSREF
22. Tanno M, Kuno A, Yano T, et al. Induction of manganese superoxide dismutase by nuclear translocation and activation of SIRT1 promotes cell survival in chronic heart failure. *J Biol Chem* 2010;285:8375-82. PUBMED | CROSSREF
23. Alcendor RR, Gao S, Zhai P, et al. Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* 2007;100:1512-21. PUBMED | CROSSREF
24. Liu Y, Afzal J, Vakrou S, et al. Differences in microRNA-29 and pro-fibrotic gene expression in mouse and human hypertrophic cardiomyopathy. *Front Cardiovasc Med* 2019;6:170. PUBMED | CROSSREF
25. Cannon MV, van Gilst WH, de Boer RA. Emerging role of liver X receptors in cardiac pathophysiology and heart failure. *Basic Res Cardiol* 2016;111:3. PUBMED | CROSSREF
26. Lu H, Sun J, Hamblin MH, Chen YE, Fan Y. Transcription factor EB regulates cardiovascular homeostasis. *EBioMedicine* 2021;63:103207. PUBMED | CROSSREF
27. Sardiello M, Palmieri M, di Ronza A, et al. A gene network regulating lysosomal biogenesis and function. *Science* 2009;325:473-7. PUBMED | CROSSREF
28. Kanamori H, Yoshida A, Naruse G, et al. Impact of autophagy on prognosis of patients with dilated cardiomyopathy. *J Am Coll Cardiol* 2022;79:789-801. PUBMED | CROSSREF
29. Heymans S, Eriksson U, Lehtonen J, Cooper LT Jr. The quest for new approaches in myocarditis and inflammatory cardiomyopathy. *J Am Coll Cardiol* 2016;68:2348-64. PUBMED | CROSSREF
30. Melacini P, Basso C, Angelini A, et al. Clinicopathological profiles of progressive heart failure in hypertrophic cardiomyopathy. *Eur Heart J* 2010;31:2111-23. PUBMED | CROSSREF