Identification of potential hub genes for the diagnosis and therapy of dilated cardiomyopathy with heart failure through bioinformatics analysis

Xinghui Zhuang, Mengyue Tian, Liming Li, Shurong Xu, Meiling Cai, Xiaojie Yang, Zhihuang Qiu, Tianci Chai, and Liangwan Chen

1 Department of Cardiovascular Surgery, Fujian Medical University Union Hospital, Fuzhou, China
2 Key Laboratory of Cardio-Thoracic Surgery (Fujian Medical University), Fujian Province University, Fuzhou, China
3 Key Laboratory of Ministry of Education for Gastrointestinal Carcinoma, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, China
4 Department of Thoracic Surgery, Fujian Medical University Union Hospital, Fuzhou, China
5 Department of Nursing, Fujian Medical University Union Hospital, Fuzhou, China
6 Department of Anesthesiology, Xinyi People’s Hospital, Xuzhou, China

Abstract

Dilated cardiomyopathy (DCM) is a common cause of heart failure. However, genetic-level treatments are not available for this condition. In this study, we searched for biological markers and therapeutic targets for DCM from a genetic perspective. We chose microarray datasets of idiopathic DCM with heart failure tissues and normal function (NF) heart tissues, which were downloaded from the Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) were analyzed by the GEO2R tool. Gene ontology (GO) and gene set enrichment analysis were used to analyze the functions of DEGs and the pathways in which they are involved. Next, protein-protein interaction networks were built to filter out the hub genes from DEGs. The expression of hub gene was validated by other GEO datasets. Receiver operating characteristic (ROC) curves were plotted to verify the accuracy of the genetic diagnosis. In the end, the mRNA-miRNA-lncRNA network was built to find potentially correlative genes. Twenty-eight common DEGs in total were screened, and GO analysis showed that DEGs were mainly associated with neutrophil degranulation and activation, regulation of Wnt signaling pathway and the development of cardiac cell and tissue. Five hub genes (asporin [ASPN], osteoglycin [OGN], secreted frizzled-related protein 4 [SFRP4], membrane metalloendopeptidase [MME], and natriuretic peptide gene [NPPA]) were shown to be highly expressed in the validation sets and accurate in distinguishing between DCM and NF by ROC curves. miRNA prediction of the hub genes revealed that hsa-mir-28b-5p was associated with SFRP4, ASPN, and MME. All of them may serve as biological diagnostic indicators and provide direction for treatment at the genetic level.

Keywords: Dilated cardiomyopathy; Heart failure; Bioinformatics analysis; Differentially expressed genes; Hub genes
1. Introduction

Dilated cardiomyopathy (DCM) is a disease of heart muscle. The enlargement of the ventricle and reduced systolic function without the abnormal loads is some of the manifestations of DCM\[1\]. Most DCM cases will eventually develop into chronic heart failure. Approximately 1 in 250 – 2500 people suffers from DCM\[2\]. The study shows that about 67% of patients eventually die of heart failure and the rest die of sudden cardiac death\[3\]. Child mortality attributed to DCM is significantly higher than that of adults\[3\]. The manifestations, such as right heart failure, pulmonary hypertension, and enlargement of both ventricles, are often indicative of a poor outcome\[4\].

The etiology of DCM is either hereditary, mixed (mainly non-hereditary), or acquired. Some causes such as infectious myocarditis, drug, and alcohol abuse often lead to DCM\[4\]. But in a large percentage of cases, the etiology of DCM has not been fully identified and is classified as idiopathic. Idiopathic DCM is a primary heart muscle disease of unknown cause\[5\]. About 30 – 50% of patients with idiopathic DCM have a family history, so DCM with family heredity is also known as familial DCM. The diagnosis of familial DCM depends on the medical history, clinical manifestations of DCM or heart failure, and features of echocardiography, chest X-ray, cardiac magnetic resonance imaging (MRI), and endomyocardial biopsy. Echocardiography of idiopathic DCM would show dilated left ventricle and systolic dysfunction, while enlarged cardiac shadow with >50% cardiothoracic ratio is a typical finding on X-ray. Cardiac MRI helps to identify diseases such as myocarditis\[6\]. Endomyocardial myocardial biopsy helps to clarify the diagnosis. The accuracy of genetic testing of DCM is only 15 – 40% due to the complex pathogenesis\[5\]. The most widely used markers are B-type natriuretic peptide and N-terminal pro-brain natriuretic peptide, but they are only used to reflect the level of heart failure and have poor diagnostic specificity\[7\]. The primary objectives of the DCM treatment are to treat the cause and control fluid levels. Some of the therapeutic agents for DCM include beta adrenoreceptor blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and vasodilators. Cardiac resynchronization therapy is available for patients with symptoms of heart failure who have failed to receive optimal drug therapy; eventually, heart transplantation may be considered if the heart failure treatment fails\[8\]. Nevertheless, genetic diagnosis and treatment are lacking among the existing clinical diagnostic and therapeutic tools for DCM.

Bioinformatics is a discipline involving processes that obtain, reserve, and visualize biological data using certain tools and methods\[9\]. By using bioinformatics, we may be able to learn about mechanisms and pathways of disease development or cancer metastasis, and can identify new potential therapeutic targets and discover mechanisms of resistance to drugs\[10\]. The Gene Expression Omnibus (GEO) is a database for reserving and searching publicly available data, such as high-throughput gene expression data\[11\]. Through bioinformatics analysis, we can analyze gene expression profiles from the GEO database to obtain differentially expressed genes (DEGs) between DCM with heart failure tissues and normal function (NF) heart tissues. Then, the gene ontology (GO) analysis and gene set enrichment analysis (GSEA) can be used to reveal the role of the genes and the pathway they participate. Hub genes are important in biological processes (BPs) as they often play a dominant role in the pathway by regulating other genes and are significant research hotspots and therapeutic targets. Protein-protein interaction (PPI) network, a network of physical contact between two or more proteins, is involved in biological signaling, gene expression regulation, energy and substance metabolism, and cell cycle regulation\[12\]. The mRNA-miRNA-lncRNA network, a network formed by mRNAs, miRNAs, and lncRNAs with interacting sites, plays a post-transcriptional regulatory role\[13\]. In this study, the hub genes in DCM with heart failure tissue were screened with the aid of the PPI networks. Finally, the predicted mRNA-miRNA-lncRNA network was established to help uncover more potential therapeutic targets. The genes identified in this study may have potential value in the diagnosis and therapy of DCM at genetic level.

2. Materials and methods

2.1. Data source

All the gene expression datasets (GSE29819, GSE57338, GSE5406, and GSE79962) of idiopathic DCM with heart failure tissues and NF heart tissues were downloaded from the GEO database. GSE29819 and GSE57338 were used as test sets. GSE29819 from GPL570: Affymetrix Human Genome U133 Plus 2.0 Array includes 14 DCM samples and 12 NF samples; GSE57338 from GPL11532: Affymetrix Human Gene 1.0 ST Array includes 14 DCM samples and 13 NF samples. GSE5406 and GSE79962 served as validation sets in this study. GSE5406 from GPL96: Affymetrix Human Genome U133 Plus 2.0 Array includes 86 DCM samples and 136 NF samples. GSE5406 and GSE79962 were used as test sets. GSE5406 from GPL570: Affymetrix Human Genome U133 Plus 2.0 Array includes 14 DCM samples and 12 NF samples; GSE79962 from GPL6244: Affymetrix Human Gene 1.0 ST Array includes 9 DCM samples and 11 NF samples. All the data can be obtained online, and no experiments involving animals and humans were performed in this study.

2.2. Identification of DEGs

The DEGs from each datasets were analyzed by the GEO2R online tool\[14\], setting the screening condition as
logFC ≥1 and adjusted $P < 0.05$ to determine the DEGs. Using ggplot2 package in R (version 3.6.3), the volcano plot and heat map of DEGs were plotted and the common DEGs were obtained by Venn Diagram.

### 2.3. Enrichment analysis

The clusterProfiler package (version 3.6.3) in R was applied to analyze and visualize the results of GO, Kyoto Encyclopedia of Genes and Genomes (KEGG), and GSEA. Elements of the enrichment analysis include BPs, cell components, and molecular functions (MFs) of the DEGs. If adjusted $P < 0.05$, the findings were considered to be significant. The reactome pathway was analyzed by David database\(^\text{[10]}\).

### 2.4. PPI network construction and hub gene identification

The online database of STRING was used for analyzing the reaction pathways and PPI network of the common DEGs\(^\text{[14]}\). The results were loaded into Cytoscape (version 3.7.2) for visualization. The top 5 hub genes were determined by maximal clique centrality (MCC) algorithm and Degree through CytoHubba plugin of Cytoscape software.

### 2.5. Validation of hub gene expression

The ggplot2 package of R software was applied to visualize gene expression in different datasets.

### 2.6. mRNA-miRNA-IncRNA network construction

Gene-miRNA interaction analysis of DEGs was performed by Network analyst using the comprehensive, experimentally validated miRNA-gene interaction data in miRTarBase\(^\text{[17]}\). Then, the miRNA-IncRNA network (Clip Date with medium stringency) was predicted by ENCORI\(^\text{[18]}\). Finally, all predicted outcome was plotted into a network using Cytoscape software.

### 2.7. Statistical analysis

The R software pROC package and ggplot2 package were used to analyze the data and plot the receiver operating characteristics (ROC) curve. Sensitivity and specificity were determined by the area under the curve (AUC). The higher is the AUC, the higher, and more consistent are the sensitivity and specificity\(^\text{[19]}\).

### 3. Results

#### 3.1. Identification of DEGs

Using the GEO2R tool, 697 DEGs were screened from GSE29819 and 62 DEGs were obtained from GSE57338. Volcano plots (Figure 1A and B) and heat maps (Figure 2A and B) were plotted using R software. Venn diagram of DCM tissues and NF tissues indicates that 28 genes were in common, including 17 upregulated genes and 11 downregulated genes (Figure 2C).

#### 3.2. Enrichment analysis

BP analysis showed that the DEGs mainly participate in neutrophil degranulation and activation, neutrophil-mediated immune response, extracellular structural organization, cell-matrix adhesion processes, positive regulation of external stimuli, positive regulation of inflammatory response, regulation of blood pressure, cardiac cell and tissue development, and regulation of Wnt signaling pathway and serine/threonine kinase signaling pathway (Table 1 and Figure 3C). Cellular component analysis revealed that the DEGs are associated with collagen-containing extracellular matrix, vacuolar lumen, azurophil granule, and primary lysosome (Table 2 and Figure 3A). MF analysis showed that the DEGs mainly take part in glycosaminoglycan binding, extracellular matrix structural constituent,
organic acid binding, carboxylic acid binding, Wnt-protein binding, and extracellular matrix structural constituent conferring compression resistance (Table 3 and Figure 3B). It was probable that the number of DEGs was too few to obtain the relevant KEGG pathway. A reactome pathway was obtained from the David database: R-HSA-390522, which was associated with striated muscle contraction. The result of GSEA enrichment indicated the close association of DEGs from GSE29819 with associated matrisome, innate immune system, neutrophil degranulation, cytokine receptor interaction, and lung fibrosis (false discovery rate [FDR] < 0.25 and \( P_{\text{adj}} < 0.05 \) (Figure 4). GSEA enrichment analysis results concerning the DEGs from GSE57338 were inconclusive under existing conditions.

3.3. PPI network construction and hub gene identification

The PPI network was constructed through String online database and the data were imported into Cytoscape.
software for visualization (Figure 5); upregulated genes were emphasized in red and downregulated genes in green. The top 5 hub genes were determined by the MCC algorithm and Degree of CytoHubba plugin in Cytoscape.
software, and the results are shown in Figure 6. The deeper the color is, the more points it scored. Asporin (ASPN), secreted frizzled-related protein 4 (SFRP4), membrane metalloendopeptidase (MME), natriuretic peptide A gene (NPPA), and osteoglycin (OGN) were found to be the hub genes, and they were all highly expressed in DCM with heart failure.

### 3.4. Validation of hub gene expression

GSE5406 and GSE79962 were used as the validation sets in the present study. The results of visualization using R software showed that all 5 hub genes were highly expressed in DCM (Figure 7).

### 3.5. ROC curve plotting

In the ROC curve of GSE29819, all five hub genes had high value in diagnosing DCM relative to NF samples; OGN (AUC = 0.958) had the highest diagnostic value, followed by NPPA (AUC = 0.917), SFRP4 (AUC = 0.881), ASPN (AUC = 0.863), and MME (AUC = 0.863), as shown in Figure 8A. On the other hand, in the ROC curve of GSE57338, SFRP4 (AUC = 0.923) had the highest diagnostic value, followed by ASPN (AUC = 0.914), MME (AUC = 0.894), OGN (AUC = 0.891), and NPPA (AUC = 0.747), as shown in Figure 8B.

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**Table 1.** Remarkably enriched biological processes of the differentially expressed genes.

<table>
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<tr>
<th>ID</th>
<th>Term</th>
<th>Gene counts</th>
<th>P value</th>
</tr>
</thead>
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<td>6.90e-05</td>
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<td>6</td>
<td>7.14e-05</td>
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<td>GO:0050729</td>
<td>Positive regulation of inflammatory response</td>
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<td>7.62e-05</td>
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<td>GO:0042119</td>
<td>Neutrophil activation</td>
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<td>7.99e-05</td>
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<td>GO:0002446</td>
<td>Neutrophil mediated immunity</td>
<td>6</td>
<td>8.08e-05</td>
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<tr>
<td>GO:0032103</td>
<td>Positive regulation of response to external stimulus</td>
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<td>GO:0008217</td>
<td>Regulation of blood pressure</td>
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<td>GO:0031589</td>
<td>Cell-substrate adhesion</td>
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<td>2.25e-04</td>
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<td>GO:0007160</td>
<td>Cell-matrix adhesion</td>
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<td>GO:0090287</td>
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<td>GO:0033687</td>
<td>Osteoblast proliferation</td>
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<td>GO:0043304</td>
<td>Regulation of mast cell degranulation</td>
<td>2</td>
<td>9.82e-04</td>
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</table>

**Table 2.** Remarkably enriched cellular components of the differentially expressed genes.

<table>
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</tr>
<tr>
<td>GO:0033006</td>
<td>Regulation of mast cell activation involved in immune response</td>
<td>2</td>
<td>0.001</td>
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<tr>
<td>GO:0035567</td>
<td>Non-canonical Wnt signaling pathway</td>
<td>3</td>
<td>0.001</td>
</tr>
<tr>
<td>GO:0060070</td>
<td>Canonical Wnt signaling pathway</td>
<td>4</td>
<td>0.001</td>
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<tr>
<td>GO:0030049</td>
<td>Muscle filament sliding</td>
<td>2</td>
<td>0.002</td>
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<tr>
<td>GO:0033275</td>
<td>Actin-myosin filament sliding</td>
<td>2</td>
<td>0.002</td>
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**Table 1. (Continued).**

<table>
<thead>
<tr>
<th>ID</th>
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<td>GO:0033275</td>
<td>Actin-myosin filament sliding</td>
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(Contd...)
Thus, ASPN, MME, OGN, SFRP4, and NPPA could serve as potential biological markers for the diagnosis of DCM with heart failure on the grounds of their high diagnostic value.

3.6. mRNA-miRNA-lncRNA network construction

mRNA-miRNA-lncRNA networks were built by Sytoscape software (Figures 9 and 10). Unfortunately, no miRNA

Figure 4. GSEA plot showing the most relevant gene sets of DEGs in GSE29819. (A) Significant enrichment in associated matrisome gene set. (B) Significant enrichment in neutrophil degranulation gene set. (C) Significant enrichment in lung fibrosis gene set. (D) Significant enrichment in innate immune system gene set. (E) Significant enrichment in cytokine receptor interaction gene set. The screening criteria for significant genomes are FDR < 0.25 and P_adjust < 0.05. DEGs: Differentially expressed genes, FDR: False discovery rate, NES: Normalized enrichment score.
and lncRNA that target NPPA were predicted from the databases. From the results, it can be seen that hsa-miR-26b-5p can interact with SFRP4, ASPN, and MEE, and may be able to silence their mRNA expression. miRNA silences mRNA expression through sponge function, while lncRNA promotes target gene expression by binding to miRNA\(^{[13]}\). Hence, the predicted miRNAs and lncRNAs could be the potential therapeutic targets.

**4. Discussion**

The proteins encoded by the genes of DCM have a wide range of biological functions; therefore, there are a diverse array of mechanisms underlying the pathogenesis of DCM, including mutations in cytoskeletal proteins, mitochondrial proteins, and bridging proteins\(^{[20]}\). DCM with an inherited trait is called familial DCM and accounts for approximately 20 – 50% of patients with DCM\(^{[21]}\). Autosomal dominant inheritance usually occurs in the vast majority of familial DCM patients, so nearly half of the children will be impacted\(^{[22]}\). More than a quarter of patients with familial DCM are due to mutations in the TTN\(^{[23]}\). The mutations of LMNA also cause DCM, contributing a high rate of sudden death and necessitating pacemaker implantation in the patients\(^{[24]}\). So far, gene therapy approaches for DCM under study mainly include CRISPR/Cas9, gene replacement, and treatment of BPs caused by mutations\(^{[25]}\). The method of CRISPR/Cas9, which knocked out the PLN gene, has been found to delay death in mice with heart failure, but there are no reports of such approach being applied in patients with DCM up to now\(^{[26]}\). The treatment of injecting adenovirus-associated virus 1/SERCA2a to treat heart failure has been reported, but it was not effective in clinical trials\(^{[27]}\). It has also been shown that p38 is involved in the pathogenic process orchestrated by LMNA in DCM, and the inhibitor of p38 is effective in rats with heart failure, which is being tested in clinical trials\(^{[25,28]}\). Hence, heart transplantation is still the only method with higher curative effect on DCM at the present.

In this study, we obtained five hub genes (ASPN, OGN, MME, NPPA, and SFRP4) and a miRNA (hsa-miR-26b-5p) that was closely linked to the identified genes. ASPN, one of the members of small leucine-rich repeat proteoglycan (SLRP) family, facilitates cardiomyocyte apoptosis and fibrillation through the regulation of Bax and TGF-β1 expression\(^{[29,30]}\). The GO analysis showed that ASPN was primarily involved in the formation of collagen-containing extracellular matrix, regulation of cellular responses to growth factors, and regulation of the serine/threonine kinase signaling pathway. Therefore, we hypothesize that ASPN regulates cardiomyocyte apoptosis and fibrosis through the growth factor-mediated serine/threonine kinase signaling pathway.

OGN is also a member of the SLRP family, which negatively affects the proliferation and migration of myocardial fibroblasts, thereby inhibiting myocardial fibrosis\(^{[31]}\). The expression of OGN increases during cardiac pressure overload; however, its absence leads to increased cardiac inflammation and fibrosis, which ultimately leads to increased diastolic dysfunction\(^{[32]}\). Overexpression of microRNA-22 downregulates OGN, which promotes the...
migratory activity of cardiac myofibroblasts\textsuperscript{[33]}. Hence, OGN correlates with the degree of fibrosis in DCM.

MME is one of the transmembrane glycoproteins. High expression of MME can suppress stress from oxidation and
inflammation caused by high glucose\textsuperscript{34}. This correlates with neutrophil activation and neutrophil-associated immune responses in the enrichment analysis. We speculate that an inflammatory response is involved in the pathogenesis of DCM.

The \textit{NPPA} encodes the atrial natriuretic peptide (ANP), and increased expression of \textit{NPPA} leads to ANP secretion when cardiac load is increased\textsuperscript{35}. Mutations of \textit{NPPA} induce cardiomyocyte fibrosis by encoding mutant ANP,
and the methylation of NPPA promoter may be associated with hypertension\cite{36,37}. Our results showed that NPPA was involved in cardiomyocyte growth, differentiation, and hypertrophy. In particular, NPPA was involved in mast cell activation, degranulation, and mast cell-mediated immune responses. We speculate that mast cells may lead to DCM by increasing the secretion of ANP.

Low expression of SFRP4 inhibits apoptosis and attenuates post-ischemic cardiomyocytes injury of mice\cite{38}. Our results revealed that SFRP4 was engaged in the regulation of serine/threonine kinase signaling pathway, Wnt signaling pathway, BMP signaling pathway, and growth factor stimulation responses. These pathways and responses may be linked to the pathogenic mechanisms of DCM.

Hsa-miR-26b-5p has been reported to have antifibrotic effects in mouse cardiac fibroblasts\cite{39}. Its low expression promotes the procession of hypertrophy in rat cardiomyocytes through exercising\cite{40}. In this study, hsa-miR-26b-5p interacted with ASPN, MME, and SFRP4 in the mRNA-miRNA-lncRNA network and may be a gene therapy target for DCM.

The background and hypothesis postulated in the above may be helpful for researchers in exploring the pathogenic mechanism of DCM. By knocking out these highly expressed genes in DCM models, researchers may observe the disease development, which might be insightful for the development of disease treatment. According to the ROC curves, these five hub genes are better at diagnosing DCM, and when combined together for diagnosing DCM, they showed the best diagnostic efficacy. Therefore, genetic tests can be performed on patients to detect the expression levels of these genes, which could be used to guide the differential diagnosis of DCM from NF condition, although conventional diagnostic indicators should still be employed.

In conclusion, DCM is a type of myocardial disease that seriously threatens human health, and more in-depth studies concerning the causes and mechanisms of DCM are required. Next-generation gene sequencing can identify...
many specific pathogenic genes, providing many clues to gain insight into the pathogenic mechanisms. However, DCM is a complex disease with multi-gene involvement and diverse clinical manifestations. There is an urgent need for in-depth genotypic and phenotypic studies in DCM to uncover new pathogenic genes in this disease. The translation of these genetic data into clinical practice would effectively help clinical practitioners improve the efficiency of diagnosis and treatment of DCM and improve patient prognosis.

However, there were some limitations in our study. Due to the limitations of gene expression profiles, we were only able to analyze the expression profiles of DCM with heart failure and NF heart tissues, so the DEGs or even hub genes may include co-expressed genes of heart failure which is caused by factors that are not specific to DCM with heart failure. Since the sample size of DCM patients is very small and the fact that the samples are difficult to obtain, we were unable to experimentally validate the hub genes and the predicted miRNAs and lncRNAs. Another limitation of this study is that no clinical information is available for survival and prognosis analysis of the patients. It is expected that other
researchers will continue to explore the mechanisms of DCM at the genetic level.

5. Conclusion
Our study found that five genes (ASPN, OGN, MME, NPPA, and SFRP4) are potential hub genes. The associated mRNA-miRNA-lncRNA network was also established in the study. Our findings may also provide clues for exploring the mechanisms, diagnostic biomarkers and genetic-level treatments of DCM with heart failure.

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Conflict of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contribution
Conceptualization: Xiaojie Yang, Tianci Chai, Liangwan Chen
Data curation: Liming Li, Meiling Cai, Zhihuang Qiu
Formal analysis: Xinghui Zhuang, Mengyue Tian
Methodology: Xinghui Zhuang, Mengyue Tian, Shurong Xu
Writing – original draft: Xinghui Zhuang, Mengyue Tian
Writing – review and editing: Xiaojie Yang, Tianci Chai
All authors have read and approved the original manuscript.

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