

Identification of the molecular mechanisms underlying dilated cardiomyopathy via bioinformatic analysis of gene expression profiles

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Abstract. In the present study, gene expression profiles of patients with dilated cardiomyopathy (DCM) were re-analyzed with bioinformatics tools to investigate the molecular mechanisms underlying DCM. Gene expression dataset GSE3585 was downloaded from Gene Expression Omnibus, which included seven heart biopsy samples obtained from patients with DCM and five healthy controls. Differential analysis was performed using a Limma package in R to screen for differentially expressed genes (DEGs). Functional enrichment analysis was subsequently conducted for DEGs using the Database for Annotation, Visualization and Integration Discovery. A protein-protein interaction (PPI) network was constructed using information from Search Tool for the Retrieval of Interacting Genes software. A total of 89 DEGs were identified in the patients with DCM, including 67 upregulated and 22 downregulated genes. Functional enrichment analysis demonstrated that the downregulated genes predominantly encoded chromosomal proteins and transport-related proteins, which were significantly associated with the biological processes of ‘nucleosome assembly’, ‘chromatin assembly’, ‘protein-DNA complex assembly’, ‘nucleosome organization’ and ‘DNA packaging’ (H1 histone family member 0, histone cluster 1 H1c, histone cluster 1 H2bd and H2A histone family member Z). The upregulated genes detected in the present study encoded secreted proteins or phosphotransferase, which were associated with biological processes including ‘cell adhesion’ [connective tissue growth factor (CTGF)], ‘skeletal system development’ [CTGF and insulin-like growth factor

binding protein 3 (IGFBP3)], ‘muscle organ development’ (SMAD7) and ‘regulation of cell migration’ [SMAD7, IGFBP3 and insulin receptor (INSR)]. Notably, signal transducer and activator of transcription 3, SMAD7, INSR, CTGF, exportin 1, IGFBP3 and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha were hub nodes with the higher degree in the PPI network. Therefore, the results of the present study suggested that DEGs may alter the biological processes of ‘nucleosome formation’, ‘cell adhesion’, ‘skeletal system development’, ‘muscle organ development’ and ‘regulation of cell migration’ in the development of DCM.

Introduction

Dilated cardiomyopathy (DCM) is the most frequent type of non-ischemic cardiomyopathy worldwide, with an estimated prevalence of 1 in 2,500 people and an incidence of 7/100,000 people annually (1,2). DCM is characterized by dilatation and reduced contractile function of the left and right ventricles, which may lead to progressive heart failure and sudden cardiac-associated mortality in 4.5-79.3% of sufferers at 3 years (3). Therefore, the early diagnosis and treatment of DCM is important in order to prevent a poor prognosis.

The molecular mechanisms underlying DCM have been extensively explored in an attempt to provide effective diagnostic and therapeutic methods for DCM. These previous studies have demonstrated that DCM is associated with mutations in genes encoding cytoskeletal, contractile or inner-nuclear membrane proteins (4,5), including actin, desmin (6) and lamins A and C (7). Furthermore, it has been demonstrated that mutations in sarcomere protein genes account for ~10% of cases of familial DCM (8,9). Mitochondrial DNA mutations and mitochondrial abnormalities have also been implicated in the pathogenesis of DCM by altering myocardial ATP generation (10), including mitochondrial Hsp40 which has a crucial role in preventing DCM (11). As a result, various potential biomarkers have been elucidated, including high-sensitivity cardiac troponin T (12), serum matrix metalloproteinase-3 (13) and serum Tenascin-C (14). However, the mechanisms and pathogenesis underlying DCM remain poorly understood.

Microarray technology enables the measurement of global gene expression levels, and thus facilitates the identification of

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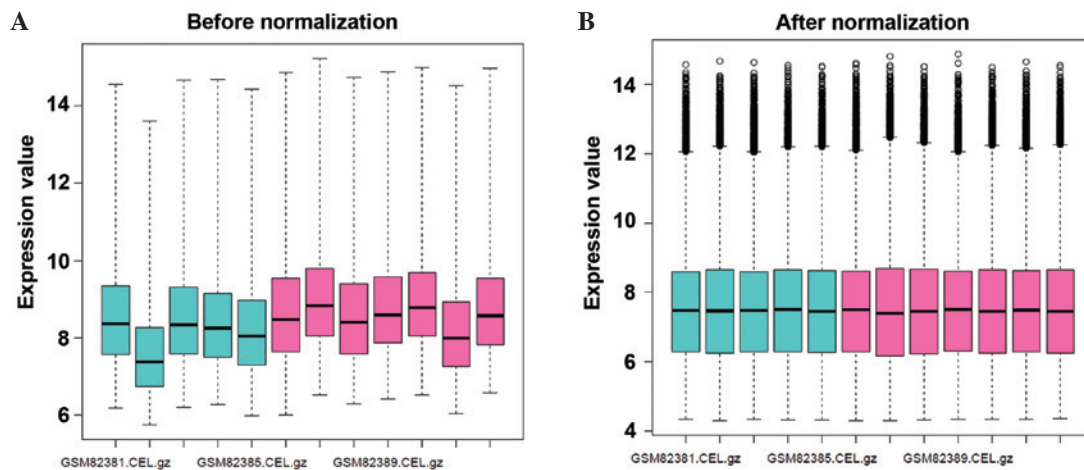


Figure 1. Box plots of gene expression data (A) before and (B) after normalization. Dilated cardiomyopathy samples are presented in blue, whereas normal controls are pink. Medians are represented as black horizontal lines in the boxes.

crucial genes and altered biological processes in DCM (15-17). Although Barth *et al* (16) have previously identified a common gene expression signature in DCM via microarray data, deeper analysis of the massive gene expression dataset is required via bioinformatics. Therefore, the present study reanalyzed gene expression data (16) to identify differentially expressed genes (DEGs) which may contribute to the development of DCM. Following this, functional enrichment and protein-protein interaction (PPI) analyses of the DEGs were performed using various bioinformatics tools. These findings may advance understanding of the molecular mechanisms underlying DCM.

Materials and methods

Gene expression data. Gene expression dataset (accession number, GSE3585) (16) was downloaded from Gene Expression Omnibus (<http://ncbi.nlm.nih.gov/geo/>). The GSE3585 dataset included seven heart biopsy samples obtained from patients with DCM at time of transplantation (GSM82386-GSM82392) and five non-failing (NF) heart biopsies from NF donor hearts (GSM82381-GSM82386). Gene expression levels were measured using Affymetrix Human Genome U133A Array (Affymetrix Inc., Santa Clara, CA, USA).

Data pre-processing and differential analysis. Raw data (CEL file) were read by an Affy package (<http://bioconductor.org/packages/release/bioc/html/affy.html>) (18) of *R* (version 3.1.2; <http://r-project.org/>) (19). Probes detected in >50% of samples were retained. Background correction, data normalization and determination of expression levels was conducted using a Robust Multi-array Average analysis method (20).

Differential analysis was performed using the linear model of the *lmFit* and empirical Bayes moderated *t* test provided by the *Limma* package (<http://bioconductor.org/packages/release/bioc/html/limma.html>) (21). \log_2 Fold Change (FC) >0.3 and false discovery rate (FDR) <0.05 were set as the cut-offs to screen DEGs between DCM and NF hearts.

Functional enrichment analysis. Functional enrichment analysis of the DEGs was conducted using the Database

for Annotation, Visualization and Integration Discovery (version 6.7; <http://david.abcc.ncifcrf.gov/>) (22), with a two-tailed Fisher exact test based on the hypergeometric distribution. Benjamini corrected P-values of <0.05 were set as the cut-off to screen out significant Gene Ontology biological process (GOBP) terms (23) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (24).

Construction of PPI network. A PPI network was constructed for the DEGs using the Search Tool for the Retrieval of Interacting Genes (STRING; version 9.1; <http://string-db.org/>) (25). Interacting pairs with a combined score >0.4 were selected for the construction of the PPI network. Proteins serve as the 'nodes' in the network and each pairwise protein interaction is represented by an undirected link. Degree was calculated for each node, which corresponds to the number of interactions of a protein with other proteins. Hub genes were subsequently selected according to the degree.

Results

Microarray data analysis identified DEGs between the DCM and control groups. Following data normalization, the mean expression levels of the gene expression profiles were presented in one consistent line (Fig. 1). Subsequently, according to the criteria of \log_2 FCI >0.3 and FDR <0.05 , 89 DEGs were detected between the DCM and NF hearts, including 22 downregulated and 67 upregulated genes. A greater number of upregulated genes were detected, as compared with the downregulated genes, which demonstrated that the DEGs had a tendency to upregulate in DCM. Based on the DEGs, the DCM samples were predominantly separated from the NF controls, implying the reliability of the DEGs (Fig. 2).

GO and KEGG pathway analysis revealed the functions of DEGs involved in DCM. Functional enrichment analysis was performed for the downregulated and upregulated genes, respectively. For the downregulated genes, two clusters were obtained using the Protein Information Resource. The first cluster was demonstrated to be associated with 'chromosomal proteins', including H1 histone family member 0

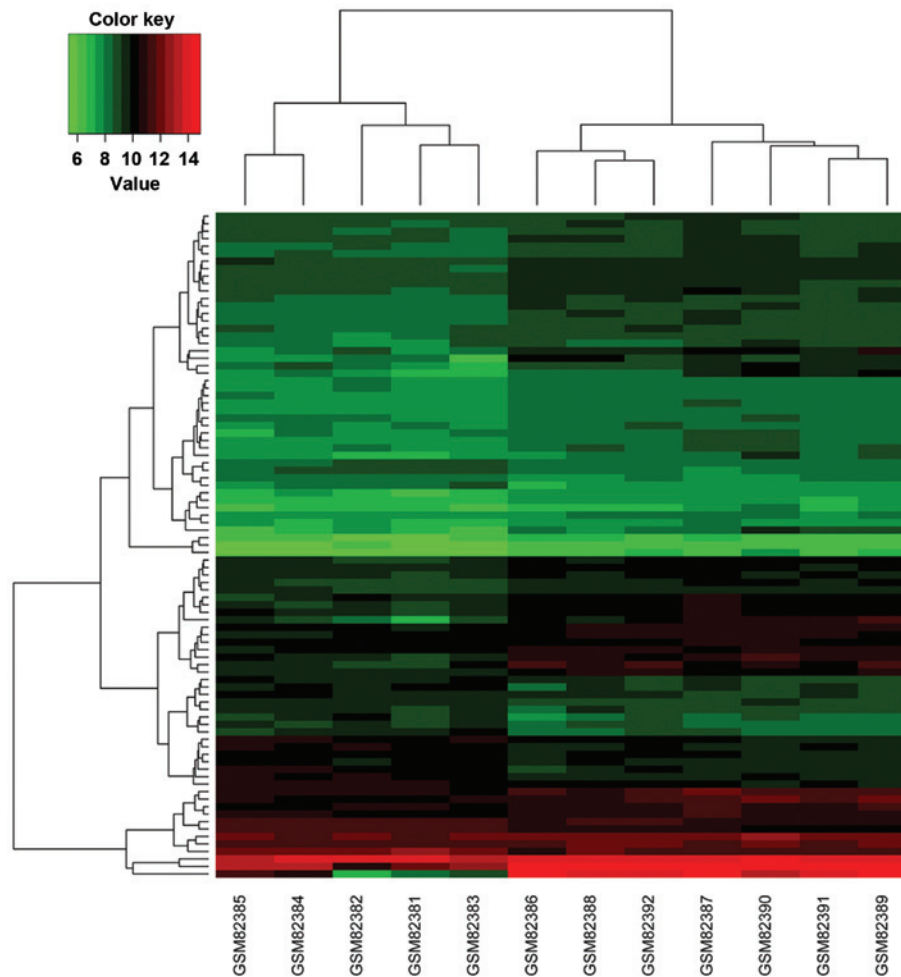


Figure 2. Heat map of hierarchical clustering analysis of the differentially expressed genes.

(H1F0; logFC=-0.61; FDR=1.91E-02), H2A histone family member Z (H2AFZ; logFC=-0.47; FDR=1.94E-02), histone cluster 1 H1c (HIST1H1C; logFC=-0.71; FDR=3.69E-02) and histone cluster 1 H2bd (HIST1H2BD; logFC=-0.69; FDR=2.89E-02). The second cluster was associated with transmembrane transport-related proteins, including STEAP family member 3 (logFC=-0.92; FDR=4.39E-02), potassium voltage-gated channel subfamily D member 3 (logFC=-0.54; FDR=4.48E-02) and insulin-like growth factor 2 receptor (logFC=-0.44; FDR=4.76E-02). Furthermore, downregulated genes (H1F0, HIST1H2BD, HIST1H1C and H2AFZ) were significantly enriched in the biological processes of ‘nucleosome assembly’ (P=1.22E-04), ‘chromatin assembly’ (P=1.35E-04), ‘protein-DNA complex assembly’ (P=1.55E-04), ‘nucleosome organization’ (P=1.65E-04) and ‘DNA packaging’ (P=3.25E-04; Table I).

For the upregulated genes, two clusters were also acquired using the Protein Information Resource, including ‘secreted proteins’ such as connective tissue growth factor (CTGF; logFC=0.84; FDR=3.04E-02) and insulin-like growth factor binding protein 3 (IGFBP3; logFC=0.84; FDR=3.04E-02) in cluster 1, and ‘phosphotransferase’ such as phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA; logFC=0.59; FDR=4.44E-02) and insulin receptor (INSR; logFC=0.61; FDR=2.27E-02) in cluster 2. Additionally,

six GOBP terms were detected in the upregulated genes (P<0.05), including ‘cell adhesion’ (CTGF), ‘skeletal system development’ (CTGF and IGFBP3), ‘muscle organ development’ (SMAD7; logFC=0.56; FDR=4.39E-02), and ‘regulation of cell migration’ (SMAD7, IGFBP3 and INSR) (Table II). No significant pathways were enriched for the DEGs.

PPI network analysis identifies crucial genes for DCM. A PPI network containing 22 DEGs was constructed using the protein interaction information obtained from STRING (Fig. 3). By calculating the degree of each gene in the network, seven DEGs were considered to be crucial for DCM with a degree of >3, including signal transducer and activator of transcription 3 (STAT3; degree, 7; logFC=-0.86; FDR= 2.27E-02), SMAD7 (degree, 6), INSR (degree, 5), exportin 1 (XPO1; degree, 5; logFC=0.62; FDR=2.74E-02), CTGF (degree, 4), IGFBP3 (degree, 4) and PIK3CA (degree, 4).

Discussion

In the present study, by reanalyzing the gene expression profiles of DCM and NF samples (16), 89 DEGs were identified in DCM patients, including 22 downregulated and 67 upregulated genes. Downregulated genes were demonstrated to encode chromosomal proteins and transmembrane

Table I. Significantly enriched functional terms of downregulated genes.

Category	Enrichment score	Terms	P-value	Genes
SP_PIR_KEYWORDS				
Annotation Cluster 1	1.689911619479253	Chromosomal protein	3.72E-04	H1F0, HIST1H2BD, HIST1H1C, H2AFZ
Annotation Cluster 2	0.32418860902901725	Transport	2.25E-01	STEAP3, KCND3, IGF2R, SELENBP1
GO_BP_FAT				
Annotation Cluster 1	2.7555946833424456			
GO:0006334		Nucleosome assembly	1.22E-04	H1F0, HIST1H2BD, HIST1H1C, H2AFZ
GO:0031497		Chromatin assembly	1.35E-04	H1F0, HIST1H2BD, HIST1H1C, H2AFZ
GO:0065004		Protein-DNA complex assembly	1.55E-04	H1F0, HIST1H2BD, HIST1H1C, H2AFZ
GO:0034728		Nucleosome organization	1.65E-04	H1F0, HIST1H2BD, HIST1H1C, H2AFZ
GO:0006323		DNA packaging	3.25E-04	H1F0, HIST1H2BD, HIST1H1C, H2AFZ

The functional enrichment analysis was performed using the Database for Annotation, Visualization and Integration Discovery. Gene symbols are listed in accordance with the National Center for Biotechnology Information database. SP, swiss-prot; PIR, protein information resource; GO, gene ontology; BP, biological process; FAT, functional annotation tool; H1F0, H1 histone family member 0; HIST1H2BD, histone cluster 1 H2bd; HIST1H1C, histone cluster 1 H1c; H2AFZ, H2A histone family member Z; STEAP3, STEAP family member 3; KCND3, potassium channel voltage gated shal related subfamily D member 3; IGF2R, insulin-like growth factor 2 receptor; SELENBP1, selenium binding protein 1.

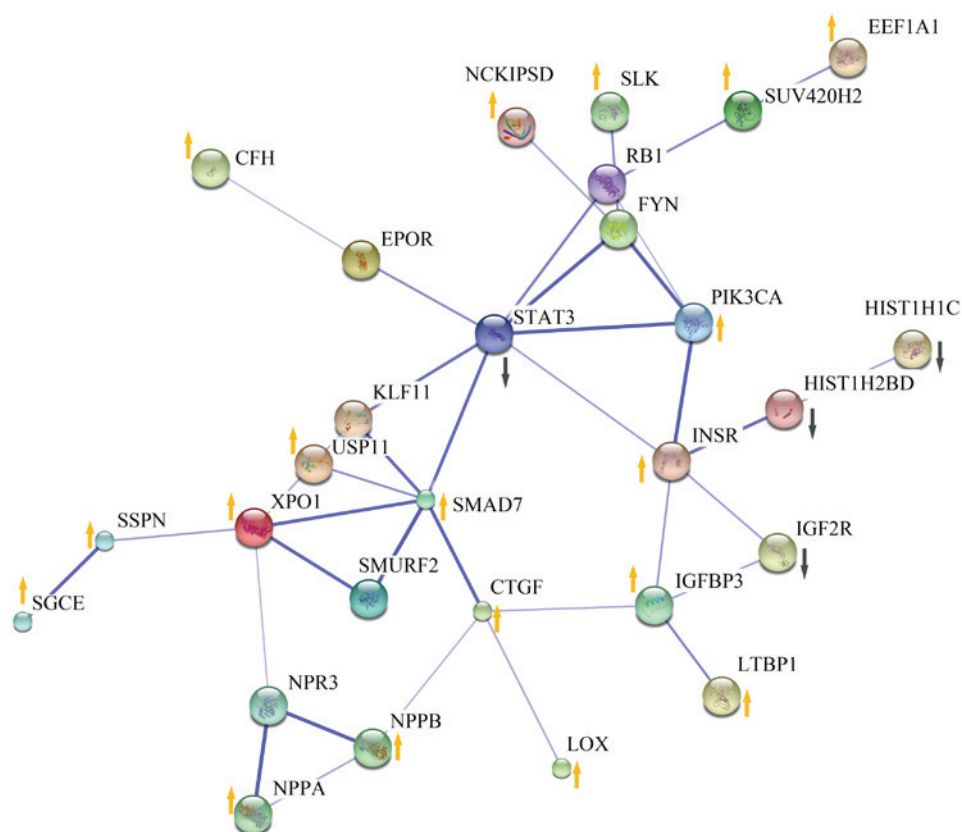


Figure 3. Protein-protein interaction network for the differentially expressed genes. Each node represents the protein and each line represents the interaction between paired proteins.

Table II. Significantly enriched functional terms of upregulated genes.

Category	Enrichment score	Terms	P-value	Genes
SP_PIR_KEYWORDS				
Annotation cluster 1	1.1489102585231292	Secreted	9.05E-03	AEBP1, LTBP1, SPOCK1, FRZB, OMD, CTGF, CFH, NPPB, LOX, PRSS23, LAMB1, IGFBP3, NPPA
Annotation cluster 2	1.1141850825108304	Phosphotransferase	2.94E-02	ROR1, PIK3CA, CLK1, INSR
GOTERM_BP_FAT				
Annotation cluster 1 GO:0007155	2.187528735182074	Cell adhesion	6.23E-03	OMD, AEBP1, CTGF, PKD2, SPOCK1, SGCE, DLG5, LAMB1, SSPN
Annotation cluster 2 GO:0001501	1.9708171852370016	Skeletal development	8.69E-03	AEBP1, CTGF, EXT1, FRZB, IGFBP3, CBF3
Annotation cluster 3 GO:0007517	1.5566908357862366	Muscle organ development	9.90E-03	AEBP1, SMAD7, SGCE, TPM1, MYH10
Annotation cluster 4 GO:0030334	1.3188719375499292	Regulation of cell migration	4.57E-03	SMAD7, LAMB1, IGFBP3, INSR, TPM1
Annotation cluster 5 GO:0007507	1.2806966535392923	Heart development	1.06E-02	SMAD7, PKD2, INSR, TPM1, MYH10
Annotation cluster 6 GO:0006163	1.1685982822293663	Purine nucleotide metabolic process	3.80E-02	MGEA5, ATP10D, ATP13A3, NPPA

Functional enrichment analysis was performed using the Database for Annotation, Visualization and Integration Discovery. Only the highest significantly enriched term for each annotation cluster are presented. Gene symbols are listed in accordance with the National Center for Biotechnology Information database. SP, swiss-prot; PIR, protein information resource; GO, gene ontology; BP, biological process.

transport-related proteins, which were associated with ‘nucleosome assembly’, ‘chromatin assembly’, ‘protein-DNA complex assembly’, ‘nucleosome organization’ and ‘DNA packaging’. Upregulated genes were enriched into two annotation clusters, ‘secreted proteins’ and ‘phosphotransferase’, which were associated with ‘cell adhesion’, ‘skeletal system development’, ‘muscle organ development’ and ‘regulation of cell migration’. Notably, upregulated *SMAD7*, *STAT3*, *INSR*, *XPO1*, *CTGF*, *IGFBP3* and *PIK3CA* were hub nodes in the PPI network.

Several histone family members were demonstrated to be downregulated in DCM, including *H1FO*, *H2AFZ*, *HIST1H2BD* and *HIST1H1C*. To the best of our knowledge, this is the first report of the association between these genes and DCM. Histones are basic nuclear proteins that are responsible for the nucleosome structure of chromosomal fiber in eukaryotes (26). Previous studies have indicated that histone acetylation/deacetylation regulates cardiac morphogenesis, growth and contractility (27,28). The present results demonstrated that these downregulated histone family member genes were significantly enriched in GO terms including ‘nucleosome assembly’, ‘chromatin assembly’, ‘protein-DNA complex

assembly’, ‘nucleosome organization’ and ‘DNA packaging’. Therefore, the downregulation of these genes may contribute to the blockade of nucleosome formation. As previously described, the untranscribed regions of eukaryotic genomes are packaged into nucleosomes, which may repress gene expression in general (29). Depletion of nucleosomal histones may lead to the silencing of specific genes (30). Therefore, we hypothesize that the downregulation of histones may induce the overexpression of numerous genes associated with the development of DCM via nucleosome formation, which may explain the tendency for upregulation of DEGs observed in the present study.

In the present study, 67 upregulated genes were detected in DCM. CTGF, which is a profibrotic cytokine, was demonstrated to be associated with ‘cell adhesion’ and ‘skeletal system development’. Previous studies have indicated that CTGF has a key role in the deleterious process of cardiac fibrosis, which is a hallmark of DCM (31,32). Furthermore, it has been demonstrated that CTGF is highly induced in viral myocarditis and its silencing may counteract cardiac fibrosis and heart muscle

dysfunction (33). CTGF was also demonstrated to be consistently upregulated in DCM patients in the present study, which may contribute to abnormal cardiac fibrosis. Moreover, it has been demonstrated that IGFBP3, which is a member of the insulin-like growth factor binding protein family, is upregulated in the failing hearts of DCM patients (34,35). Hassfeld *et al* (36) regard IGFBP3 as an independent predictors of a poor prognosis in patients with DCM. Notably, the results of the present study indicated that upregulated IGFBP3 was related to 'skeletal system development' and 'regulation of cell migration'. Similarly, INSR was upregulated in DCM and was associated with 'regulation of cell migration'. The binding of insulin to INSR stimulates glucose uptake which affects muscle function *in vitro* (37), thus the upregulated INSR may be associated with muscle contractility. Furthermore, SMAD7 was upregulated in DCM patients, which is one feature of dysregulated extracellular matrix degradation which may lead to cardiac fibrosis in patients with diabetic cardiomyopathy (38). DCM is characterized by abnormal contractile function (39,40). The DEGs mentioned above (CTGF, SMAD7, IGFBP3 and INSR) were found to be enriched in 'cell adhesion', 'skeletal system development', 'muscle organ development' and 'regulation of cell migration', and abnormalities in these processes may contribute to weakened contractile function. Notably, CTGF, SMAD7, IGFBP3 and INSR were also demonstrated to be hub nodes in the PPI network, suggesting their predominant roles among the DEGs. These four DEGs may be future therapeutic targets for the treatment of DCM.

Although the STAT3, XPO1 and PIK3CA GO terms were not enriched in the present study, they did form hub nodes in the PPI network, thus suggesting that these genes are also important in DCM. This is supported by previous studies. Podewski *et al* (41) observed that the protein expression levels of STAT3 were significantly decreased in the cardiomyocytes of patients with end-stage DCM. Furthermore, using the knockout technique, Hilfiker-Kleiner *et al* (42) demonstrated reduced myocardial capillary density and increased interstitial fibrosis in STAT3-deficient mice, leading to DCM with impaired cardiac function and premature mortality. PIK3CA, a stress-associated lipid kinase, has been shown to be important for maintaining cardiac structure and function, and its upregulation ultimately prolonged the survival of a mouse model of DCM (43,44). In addition, XPO1 has been reported to be a key nucleocytoplasmic transport-related gene in DCM (45,46), and upregulation of XPO1 may be involved in DCM by mediating the nuclear export of several molecules that regulate cardiac growth and development, including a member of the Rho family of GTPases, Ras homolog family member U (RhoU), and a transcriptional coactivator and cytoskeleton regulator, four and a half LIM domains 3 (FHL3) (46). It has been reported that the loss of RhoU results in the mis-localization of cell adhesion molecules, including Alcama and N-cadherin, to the cytoplasm and the apical and basal cell membrane, instead of the atrioventricular cardiomyocyte cell junctions, resulting in failure to form the atrioventricular canal and loop the linear heart tube and thus influencing cardiac function (47). Furthermore, downregulated FHL3 expression has been associated with the systolic dysfunction of DCM patients by inhibiting the expression of myosin heavy chain isoform 2a (48,49).

In conclusion, the results of the present study suggested that downregulated histones may lead to the overexpression of DCM-specific genes via damage to the nucleosome. Furthermore, upregulated genes, including CTGF, SMAD7, IGFBP3 and INSR, may lead to weakened contractile function via various biological pathways associated with muscle development. STAT3, XPO1 and PIK3CA may also be important for maintaining heart function. There were some limitations to the results of the present study. The DEGs identified by microarray data were not validated using real-time polymerase chain reaction and there was no intervention or patient follow-up performed. Despite these limitations, the present findings may advance the understanding of the pathogenesis of DCM and provide novel targets for clinical treatment and diagnosis.

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