

Research Article

Bioinformatics-Based Identification of lncRNA-miRNA-mRNA Network in Dilated Cardiomyopathy and Drug Prediction

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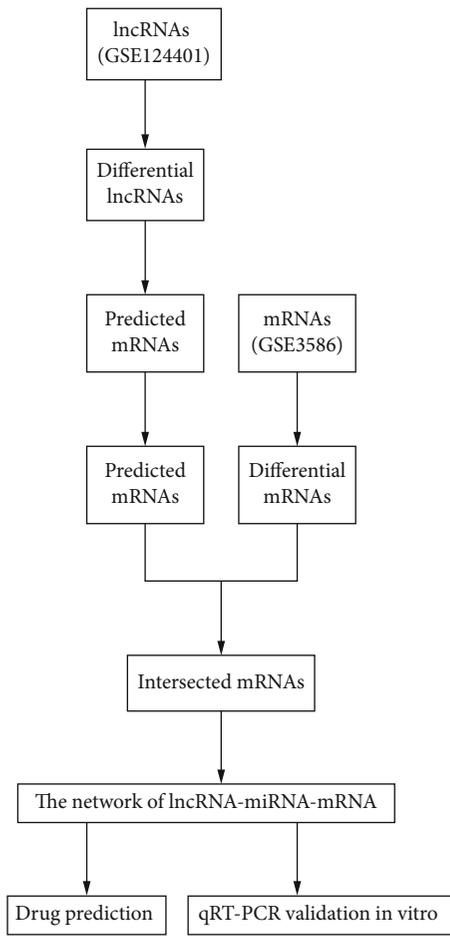
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Background. Dilated cardiomyopathy (DCM) is a cardiovascular disease of unknown etiology with progressive aggravation. More and more studies have shown that long noncoding RNAs (lncRNAs) play an essential role in dilated cardiomyopathy formation and development. The mechanism of action of competitive endogenous RNA (ceRNA) networks formed based on the principle that lncRNAs affect mRNAs' expression level by competitively binding microRNAs (miRNAs) in dilated cardiomyopathy has rarely been reported. **Objective.** This study is aimed at constructing a lncRNA-miRNA-mRNA ceRNA network by bioinformatics analysis methods, discovering, and validating potential biomarkers of DCM in the ceRNA network and determining possible therapeutic targets from them for drug prediction. **Methods.** A lncRNA dataset and a mRNA microarray dataset were downloaded from the Gene Expression Omnibus Database (GEO). Gene expression was compared between blood samples from patients with dilated cardiomyopathy and blood samples from normal subjects to identify differential expression of lncRNAs and mRNAs. The lncRNA-miRNA-mRNA network was constructed using bioinformatics tools, and functional and pathway enrichment analysis and protein-protein interactions were performed. The mRNAs in the network and the proteins they encode are then used as targets for predicting drugs. Besides, the expression of lncRNAs in the ceRNA network was validated by real-time quantitative PCR (qRT-PCR) experiments in vitro. **Results.** The differentially expressed lncRNA-miRNA-mRNA ceRNA network in dilated cardiomyopathy was successfully established. Two differentially overexpressed key lncRNAs were found from the network: AC093817 and AC091062, and qRT-PCR experiments further validated the overexpression of AC093817 and AC091062. The mRNAs in the network and the proteins encoded by the mRNAs were used for drug prediction to get related drugs. **Conclusion.** This study supports a possible mechanism and drug development of dilated cardiomyopathy, AC093817 and AC091062 being potential biomarkers of dilated cardiomyopathy.

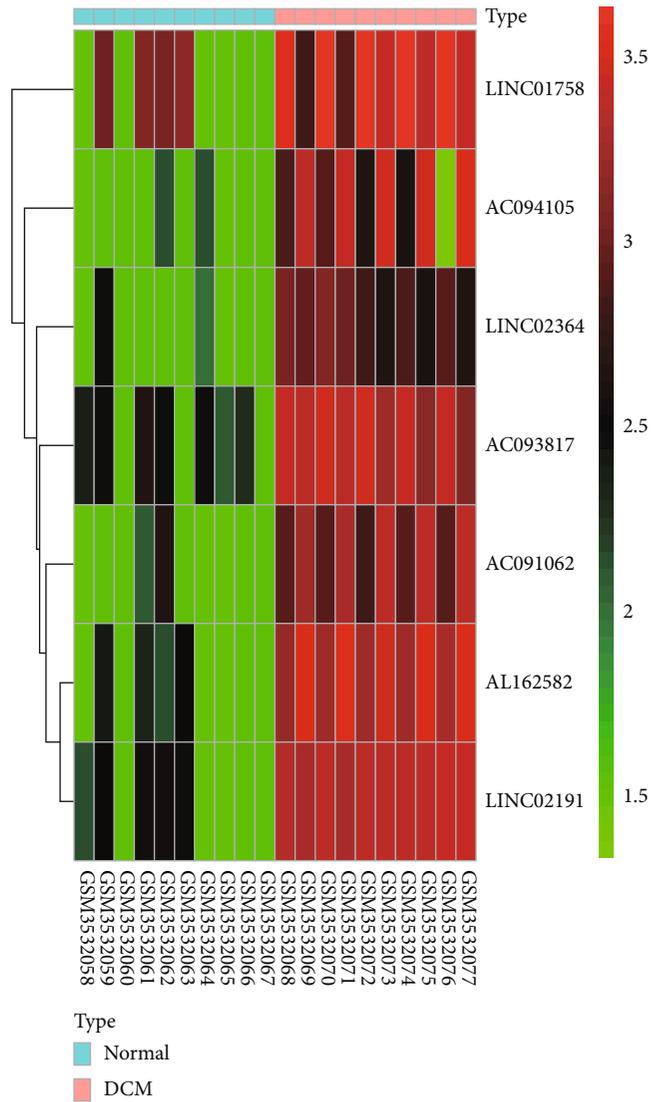
1. Introduction

Dilated cardiomyopathy is defined as left ventricular dilation and left ventricular systolic dysfunction in the absence of abnormal load (hypertension, valvular disease) or coronary artery disease sufficient to cause global systolic function impairment [1]. Risk factors for dilated cardiomyopathy include genetic and environmental factors. Dilated cardiomyopathy may present with autosomal dominant, monogenic features with X chromosome, autosomal recessive inheritance, or with the effects of environmental factors such

as nutritional deficiency, endocrine dysfunction, and taking cardiotoxic drugs such as Adriamycin [1, 2]. The early symptoms of dilated cardiomyopathy are not obvious, and many patients have progressed to the terminal stage when they are diagnosed with the disease, which can lead to heart failure or even death [3]. Therefore, new biomarkers are essential for the early diagnosis and prognosis of dilated cardiomyopathy. Although previous studies have found that genetic factors play a crucial role in dilated cardiomyopathy, the pathogenesis of dilated cardiomyopathy is still unclear, and new treatment strategies are lacking.



(a)



(b)

FIGURE 1: Continued.

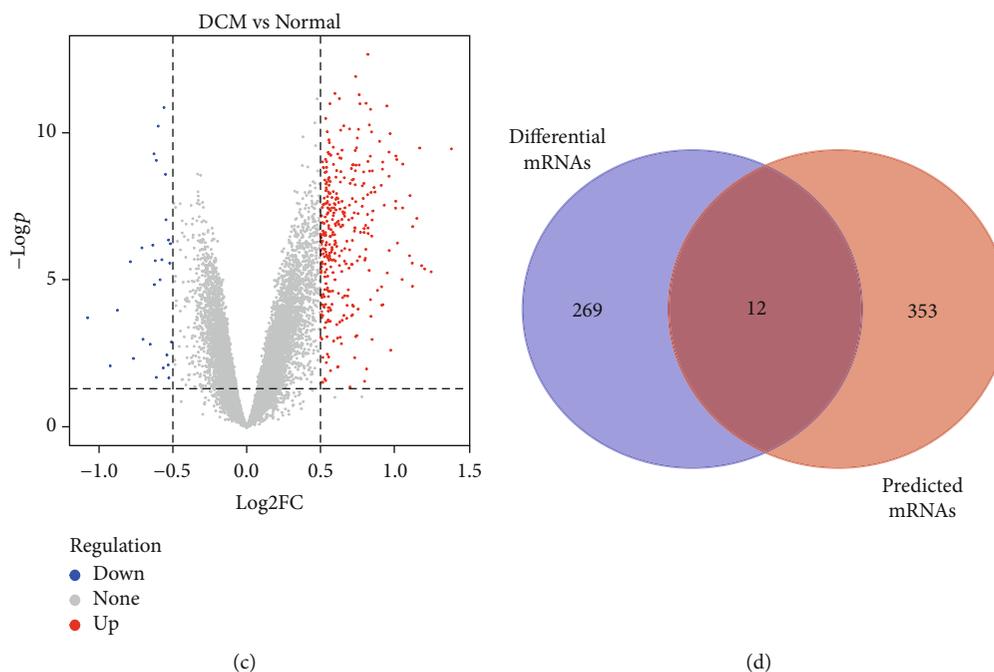


FIGURE 1: Flow chart of the approach and identification of intersected mRNAs in DCM (dilated cardiomyopathy). (a) Flow chart of the approach utilized in this study. (b) The heatmap of differentially expressed lncRNAs from the GSE124401 dataset. (c) The volcano plot of differentially expressed mRNAs from GSE3586 dataset. (d) The intersection analysis of predicted mRNAs and differential mRNAs.

lncRNAs are a group of RNAs > 200 nucleotides in length, without protein-coding function, regulating gene expression at the epigenetic, transcriptional, and posttranscriptional levels participating actively in various physiological and pathological processes [4, 5]. In recent years, more and more evidence has shown that lncRNAs play a vital role in the occurrence and development of dilated cardiomyopathy and are very critical biomarkers and therapeutic targets. Li et al. found the most upregulated lncRNA RP11-544D21.2 in DCM patients, and this human-specific lncRNA significantly regulated DCM-related genes in cardiomyocytes and affected the ductal formation and cell migration in endothelial cells [6]. Lin et al. found that differentially expressed lncRNAs are involved in some specific biological processes and regulate some signaling pathways in the plasma of heart failure patients with dilated cardiomyopathy and ischemic cardiomyopathy [7]. Wang et al. predicted a novel function of an annotated lncRNA-H19 that may regulate apoptotic signal-regulated kinases through pathway analysis of 39 key lncRNAs that regulate key pathways in myocardial infarction [8]. Cheng and Jiang found that lncRNA HAND2-AS1 may be involved in end-stage dilated cardiomyopathy [3].

Luo et al. identified IDI2-AS1 and XIST two lncRNAs and their associated pathways in the pathogenesis of DCM, providing potential targets for the diagnosis and treatment of DCM [9]. Zhang et al. found that circulating lncRNA ENST00000507296 is a biomarker for the prognosis of DCM patients [10]. Qiu et al. found that AC061961.2, LING01-AS1, and RP11-13E1.5 were downregulated in DCM patients' myocardial tissue, and these lncRNAs could be used as critical diagnostic biomarkers and therapeutic targets for DCM [11]. Zhang et al. found that the H19/miR-675 axis was involved in the promoting effect of cardiomyocyte

apoptosis by targeting PA2G4, providing a new therapeutic strategy for treating doxorubicin-induced DCM [5]. Tao et al. identified four lncRNA-miRNA pairs associated with DCM, which can be used as candidate diagnostic biomarkers or potential therapeutic targets for DCM [12]. However, the mechanism of action of lncRNA-associated ceRNA networks in dilated cardiomyopathy is unknown. Therefore, it is necessary to construct a lncRNA-miRNA-mRNA ceRNA network to obtain more information about the treatment and diagnosis of dilated cardiomyopathy from the ceRNA network.

The aim of this study was to identify differentially expressed lncRNAs and mRNAs using the limma package in R software. miRNAs were predicted using the miRcode database by differentially expressed lncRNAs, mRNAs were predicted by the combination of Targetscan, miRTarBase, and miRDB databases, and finally, differentially expressed mRNAs and predicted mRNAs were intersected to construct a lncRNA-miRNA-mRNA ceRNA network by Cytoscape software. mRNAs in the network were subjected to GO enrichment analysis, KEGG enrichment analysis, and construction of protein-protein interaction network. The lncRNAs in the network were validated by qRT-PCR in vitro. The mRNA-encoded proteins in the network were used for drug prediction through the Drugbank database, and mRNA-protein-drug networks were constructed. Drug-Gene interaction database and Connectivity map predicted the mRNAs in the network to find linked chemical drugs. The flow chart is illustrated in Figure 1(a).

2. Materials and Methods

2.1. Microarray Data Sources. From the Gene Expression Omnibus Database (GEO) (<https://www.ncbi.nlm.nih.gov/>)

TABLE 1: Basic information of the two microarray datasets from GEO.

Data source	Platform	Series	Sample size (N/T)
lncRNA	GPL16956	GSE124401	10/10
mRNA	GPL3050	GSE3586	15/13

N: normal; T: DCM.

geo/), two DCM microarray expression datasets (GSE124401 and GSE3586) were downloaded. The lncRNA dataset included lncRNA expression data in the plasma of 10 normal subjects and ten heart failure (HF) patients diagnosed with dilated cardiomyopathy (DCM). The samples were tested according to GPL16956Agilent-045997 ArraystarhumanlncRNA-microarrayV3 (ProbeNameVersion) data. The mRNA dataset consisted of mRNA expression data from septal myocardial tissue samples from 13 dilated cardiomyopathy hearts at transplantation and 15 NF donor hearts not transplanted due to visible coronary calcification, tested against data GPL3050HumanUnigene3.1cDNAArray37.5Kv1.0. Table 1 summarizes the necessary information of two DCM microarray expression datasets (GSE124401 and GSE3586).

2.2. Identification of Differentially Expressed Genes. Differential expression analysis was performed on two DCM microarray expression datasets (GSE124401 and GSE3586) using the “limma” package of the R language. The screening criteria for differentially expressed lncRNAs in GSE124401 were $|\log 2FC| > 2$ (p value < 0.05), and the screening criteria for differentially expressed mRNAs in GSE3586 were $|\log 2FC| > 0.5$ (p value < 0.05).

2.3. Acquisition of Predicted miRNAs and Predicted mRNAs. The differentially expressed lncRNAs described above were predicted by the miRCode database to obtain the corresponding predicted miRNAs, and the predicted miRNAs were further predicted by Targetscan (http://www.targetscan.org/vert_72/), miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/index.html>), and miRDB (<http://www.mirdb.org/>), three databases in combination to get the corresponding predicted mRNAs.

2.4. Cross-Analysis of Differentially Expressed mRNA and Predicted mRNA. The intersection of the differentially expressed mRNA with the predicted mRNA described previously was taken to obtain the intersection mRNA, through the Calculate and draw custom Venn diagrams online website (http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.html), for visualization.

2.5. Construction of Network. The intersected mRNAs and the corresponding predicted miRNAs and differentially expressed lncRNAs together were used to construct the lncRNA-miRNA-mRNA ceRNA network by Cytoscape software. The mRNAs in the ceRNA network were then analyzed using the STRING online website (<https://string-db.org/>), identified the encoded proteins and constructed protein-protein interaction (PPI) networks.

2.6. Functional Enrichment Analysis of Differentially Expressed Genes. Using the DAVID online website (<https://david.ncicrf.gov/home.jsp>), GO analysis of differentially expressed mRNAs in the network was performed to assess the cellular component (CC), biological process (BP), and molecular function (MF) of each mRNA. KEGG analysis of differentially expressed mRNAs in the network was performed using the R language clusterProfiler package to enrich the related pathways. The links between differentially expressed mRNAs and corresponding pathways were visualized with Cytoscape software to construct mRNA-pathway networks.

2.7. Acquisition of Drug Molecules. The proteins encoded by the mRNAs in the ceRNA network mentioned above were accessed through the Drugbank online website (<https://go.drugbank.com/>), for drug prediction, and an mRNA-protein-drug network was constructed by Cytoscape software. The mRNAs in the ceRNA network were analyzed by Drug-Gene interaction database (<https://dgidb.genome.wustl.edu/>); the drug was predicted directly and visualized with Cytoscape software. The mRNAs in the ceRNA network passed through Connectivity map (<https://portals.broadinstitute.org/cmap/>), predicted to select drugs with “up score” below -0.645 (i.e., drugs most likely to reverse gene differential expression for DCM), summarized the specific connectivity score of each drug for each mRNA in the specific cell line, and made a heatmap with the R language pack.

2.8. Cell Culture. The establishment of a cellular model of DCM was performed as previously described [13]. H9c2 cardiomyocytes (Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. H9c2 cardiomyocytes were treated as follows: control cells, in which cells were treated with medium only; doxorubicin treatment group, in which cells were treated with 5 μM doxorubicin for 24 hours.

2.9. Real-Time Quantitative PCR. Total RNA was isolated from H9c2 cells using Trizol reagent (TaKaRa, Japan) according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). Quantitative RT-PCR was then performed with Pro Taq HS Premix Probe qPCR Kit (Accurate, Hunan, China). The amplification program consisted of one cycle of pre-denaturation at 95°C for 5 min, 37 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 10 min. The GAPDH gene was used as an endogenous control gene for normalizing the expression of target genes. Each sample was analyzed in triplicate. Primer sequences are shown in Table 2.

3. Results

3.1. Differential Expression Analysis and Prediction of Genes. Seven differentially expressed lncRNAs were obtained from GSE124401 by the screening criteria of $|\log 2FC| > 2$ (p value < 0.05), and a heatmap was made with the R language pack

TABLE 2: The sequences of primers used for qRT-PCR.

Gene name	Primer sequences (5'-3')	Annealing temperature
AC093817	F: GCAGGAGAACGAAATTAAGAGACAAG R: GGCTAGAGGATTATTTGAGACCAGGAT	60°C
AC091062	F: TATTGCCCATGCCCCTAACTC R: CTGAAGCCCCCAGACAGTGA	60°C
Gapdh	F: ACAGCAACAGGGTGGTGGAC R: TTTGAGGGTGCAGCGAACTT	60°C

F: forward primer; R: reverse primer.

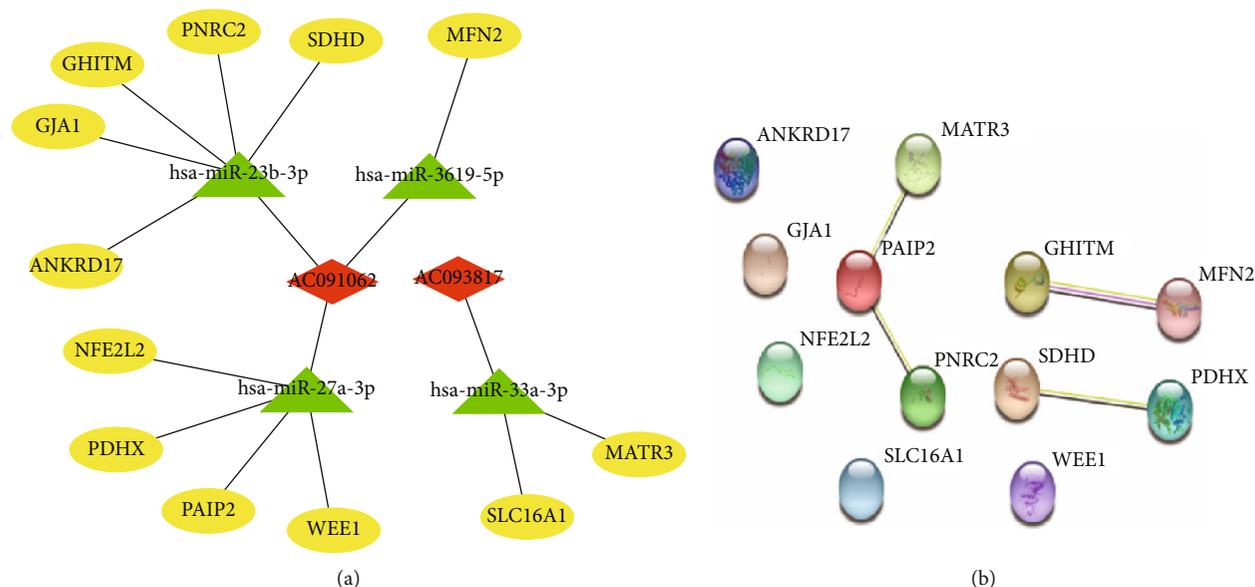


FIGURE 2: Molecular regulatory interaction network. (a) lncRNA-miRNA-mRNA biomolecular network. Red indicates lncRNA, green indicates miRNA, and yellow indicates mRNA. (b) Protein-protein interaction network of mRNA.

(Figure 1(b)). A screening criterion of $|\log 2FC| > 0.5$ (p value < 0.05) yielded 365 differentially expressed mRNAs from GSE3586, and a volcano map was made with the R language pack (Figure 1(c)). The seven differentially expressed lncRNAs resulted in 47 predicted miRNAs by miRcode prediction, and these 47 predicted miRNAs were further predicted by a combination of three databases: Targetscan, miRTarBase, and miRDB resulting in 281 predicted mRNAs.

3.2. Acquisition of Intersection mRNA and Network Construction. From GSE3586, 365 differentially expressed mRNAs were obtained intersecting with 281 predicted mRNAs to take the intersection resulting in 12 intersection mRNAs, visualized with a Wayne diagram (Figure 1(d)). These 12 intersection mRNAs and the corresponding predicted miRNAs and differentially expressed lncRNAs constructed the lncRNA-miRNA-mRNA ceRNA network by Cytoscape software (Figure 2(a)). Twelve mRNAs in the ceRNA network were identified to encode proteins and create a protein interaction (PPI) network through the STRING online website (Figure 2(b)). In the PPI network, protein PAIP2 was connected to the other two proteins, indicating its central regulatory role.

3.3. Enrichment Analysis of mRNAs in the ceRNA Network. GO and KEGG enrichment analyses were performed on the mRNAs in the ceRNA network (Figure 3). GO enrichment analysis showed that “pyruvate metabolic process,” “neuronal projection morphogenesis,” and “apoptotic process” were enriched in the biological process (BP) classification and “protein binding” was enriched according to the molecular function (MF) classification. Enrichment of “mitochondria,” “chromatin,” and “mitochondrial outer membrane” was shown in the cellular component (CC) classification (Figures 3(a) and 3(b)); KEGG enrichment analysis showed that differentially expressed mRNAs in the network were mainly involved in signaling pathways such as “Parkinson’s disease,” “citric acid cycle (TCA cycle),” “amyotrophic lateral sclerosis,” and “neurodegeneration” (Figures 3(c) and 3(d)). The mRNA-pathway network was visualized and constructed with Cytoscape software (Figure 4(a)).

3.4. Drug Prediction of mRNA in the ceRNA Network. Twelve mRNA encoded proteins in the ceRNA network predicted drugs through the online website of Drugbank, and four of them got related drugs and constructed the mRNA-protein-drug network (Figure 4(b)). Twelve mRNAs in the ceRNA network predicted drugs directly through the drug-gene

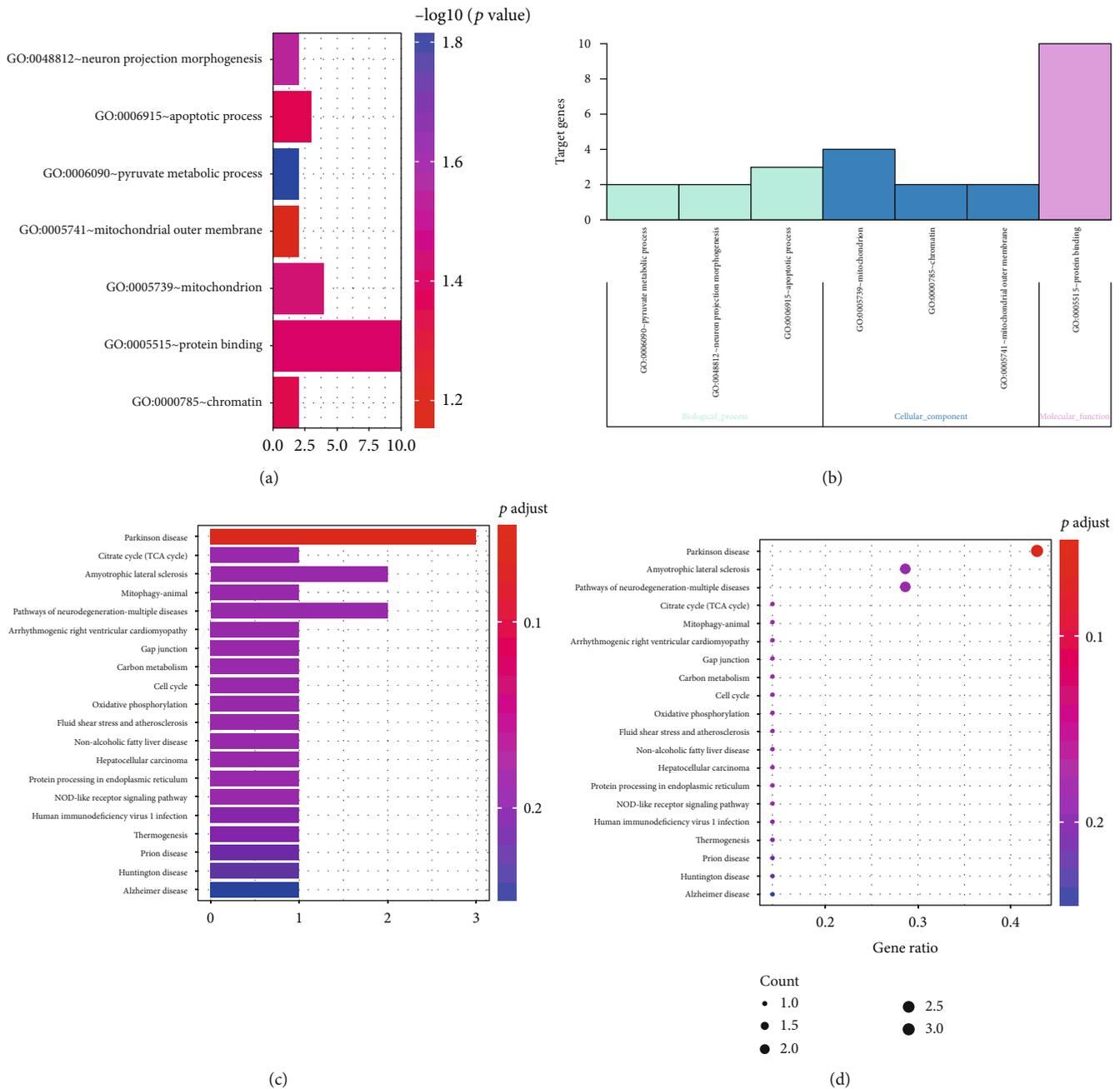


FIGURE 3: Enrichment analysis of the intersected mRNAs. (a) Bar plot of the GO analysis. (b) Histogram of GO cluster analysis. Target genes are intersected mRNAs. (c) Bar plot of the KEGG analysis. (d) Bubble plot of the KEGG analysis.

interaction database, and five of them got related drugs and constructed the mRNA-drug network (Figure 4(c)). Twelve upregulated mRNAs in the ceRNA network were added to the up tag list. Two downregulated mRNAs were added to the down tag list from GSE3586 differentially expressed mRNAs (setting down downregulated list is only necessary for uploading files, which does not affect drug prediction of the up tag list). The 16 drugs with “up scores” below -0.645 were predicted by the connectivity map. The specific connectivity score of these 16 drugs regarding the expression of 12 upregulated mRNAs was summarized, and the heatmap (Figure 4(d)) was made with the R language package.

3.5. Expression of Two lncRNAs in DCM Cells. To validate the expression levels of these two lncRNAs (AC093817 and AC091062) in DCM cells, we validated them by qRT-PCR. The results showed that both AC093817 and AC091062 were significantly overexpressed in DCM cells compared with the normal group (Figure 5). These results are consistent with the results of bioinformatics analysis.

4. Discussion

Dilated cardiomyopathy (DCM) is a cardiovascular disease of unknown pathogenesis, one of the leading causes of heart

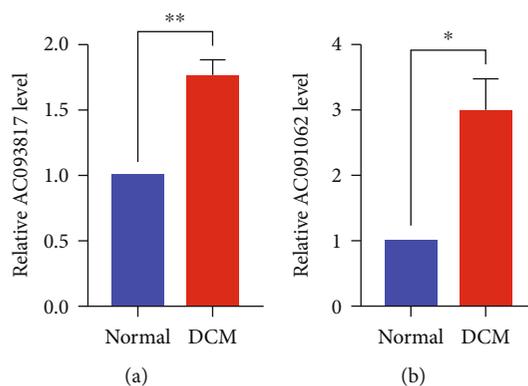


FIGURE 5: The expression levels of two lncRNAs involved in DCM were measured by qRT-PCR. $n = 3$; $*p < 0.05$ and $**p < 0.01$. (a) AC093817 was upregulated in DCM cells. (b) AC091062 was upregulated in DCM cells.

failure. Patients at any stage of the disease are at risk of sudden death. The etiology of dilated cardiomyopathy is very complex and can be regarded as a complex interaction between environmental factors and genetic background. Dilated cardiomyopathy is more common in men than in women, and its prevalence in the general population is estimated at 36 cases per 100,000 [14]. A large number of patients with dilated cardiomyopathy may have an incubation period, clinical manifestations are asymptomatic, the condition is progressively aggravated, severe myocardial failure has developed at the time of diagnosis, which will lead to heart transplantation or even death, and almost 50% of patients die within five years, which is extremely harmful [14]. Therefore, there is an urgent need to clarify the role of ceRNA networks in the course of DCM and find serum biomarkers and related potential therapeutic agents to provide further support for the diagnosis and treatment of DCM.

Many studies have investigated the mechanism and clinical significance of cardiac lncRNA or even lncRNA-miRNA pairs in DCM in recent years. However, the impact of serum ceRNA networks on DCM in patients with DCM has not been addressed. This study constructed a complete lncRNA-miRNA-mRNA ceRNA network based on lncRNA and mRNA expression profiles for targeted prediction and taking the intersection, providing new ideas for the posttranscriptional level of gene regulation. Drug prediction of mRNAs and their encoded proteins in the ceRNA networks by multiple pharmacogenomics databases may be useful therapeutic molecules in the future. Connectivity map's prediction results showed that all 16 drugs were effective in downregulating the expression of some of these 12 overexpressed mRNAs. However, these 16 drugs also upregulated the expression of some mRNAs to a greater or lesser extent. This may provide support for the active drug structural design of DCM.

The two lncRNAs and 12 mRNAs overexpressed in the network were found for the first time, which are very different from the differentially expressed lncRNAs and mRNAs obtained from previous studies, such as LMNA, FLNC, and TTN, which have been intensely studied in precision medicine for DCM [15]. This may be because previous studies have directly studied differential gene expression from heart samples of DCM patients and controls. Differently, this study newly added the

differential gene expression of plasma samples from DCM patients and controls as a starting point on this basis. The new biomarker lncRNAs obtained from the new starting point: AC093817 and AC091062, may provide new ideas for the therapeutic diagnosis of DCM. More importantly, for these two lncRNAs in the ceRNA network, the comparison of qRT-PCR results between doxorubicin-induced derived DCM cells and control further validated that both AC093817 and AC091062 were significantly overexpressed in DCM cells.

The "mitochondria," "pyruvate metabolic process," and "apoptotic process" involved in the overexpressed mRNA in the network may indicate that the cardiac cell metabolism is abnormally active in DCM patients, corresponding to the clinical characteristics of ventricular enlargement in DCM, which is a compensatory mechanism for weakened myocardial contraction and reduced score. The pathway pathways involving overexpressed mRNA not only show cellular metabolic pathways such as citric acid cycle (TCA cycle) and oxidative phosphorylation but also are related to atherosclerosis, viral infection, and even a variety of neurodegenerative diseases (Parkinson's disease, Huntington's disease, Alzheimer's disease, etc.). The diversity of pathways in which it is involved corresponds to its various pathogenic factors such as cardiotoxic compounds, metabolism, rheumatological and endocrine diseases, and cellular infiltration and viral infection [16]. Due to multiple heterogeneous etiologies, dilated cardiomyopathy is an "umbrella" term that describes the final common pathway of different pathogenic processes and gene-environment interactions [17]. A further accurate understanding of the molecular mechanisms and signaling pathways of DCM caused by various etiologies facilitates further subdivision of DCM, which is of great significance for targeted therapy and prognosis of DCM.

In most cases, dilated cardiomyopathy treatment refers to heart failure treatment based on medical treatment with angiotensin-converting enzyme inhibitors (angiotensin receptor blockers or angiotensin receptor/neprilysin inhibitors, if applicable), beta-blockers, and mineralocorticoid receptor antagonists according to current guidelines for heart failure [16]. Different from the previous ideas of symptomatic treatment with drugs to delay disease progression, this study selects the corresponding drugs through multiple

pharmacogenomics databases based on the new thinking of targeted therapy for specific disease mechanisms to reverse the disease process, targeting the 12 mRNAs overexpressed in the network and their encoded proteins, and the mechanistic analysis of the downregulation of mRNA expression and inhibition of proteins by these drugs in the future will further reveal the common pathways of their effects, which can be used as the starting point for subsequent studies.

Validating effective RNA treatment strategies in a number of cancer treatment studies [18–24] can also be used as an adjuvant treatment for DCM. In the future, we can construct a nanotargeted therapeutic system by designing miRNA overexpression vector gene drugs together with chemical drugs through rational encapsulation of nanomaterials such as cationic polymers and liposomes and connecting cardiac-targeting ligands in the outer layer [25–27]. With the rapid development of RNA nanotechnology, we can also construct RNAs with specific folded structures and spatial conformations to achieve downregulation of overexpressed mRNAs, such as miRNAs and siRNAs with specific structures and functions. Of course, this requires a lot of basic analysis and experimental validation [28–30].

5. Conclusion

In conclusion, in this study, we constructed a complete DCM differentially expressed lncRNA-miRNA-mRNA ceRNA network, found that AC093817 and AC091062 are potential biomarkers of dilated cardiomyopathy, and proposed potential therapeutic agents that can be used for this disease by integrating and analyzing microarray gene expression data, providing support for possible mechanisms and drug development of dilated cardiomyopathy.

Data Availability

The data used to support the findings of this study are available in the Gene Expression Omnibus (GEO) repository (<https://www.ncbi.nlm.nih.gov/gds>).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Supplementary 1. Table S1: the gene name corresponding to the entrezID of the mRNA on Figure 4(a).

Supplementary 2. Table S2: the drug name corresponding to the Drugbank-ID of the drug on Figure 4(b).

Supplementary 3. Table S3: all 821 drug names corresponding to NFE2L2 on Figure 4(c).

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