Expression profiling of circular RNAs and their potential role in early-stage diabetic cardiomyopathy

SHENGZHONG DONG1, CHUNYAN TU1, XING YE1,2, LILIANG LI1, MINGCHANG ZHANG1, AIMIN XUE1, SHANGHENG CHEN1, ZIQIN ZHAO1, BIN CONG3, JUNYI LIN1 and YIWEN SHEN1

1Department of Forensic Medicine, School of Basic Medical Sciences, Fudan University, Shanghai 200032; 2Department of Forensic Science, Gannan Medical College, Ganzhou, Jiangxi 341000; 3Department of Forensic Medicine, Hebei Medical University, Hebei Key Laboratory of Forensic Medicine, Shijiazhuang, Hebei 050017, P.R. China

Received October 29, 2019; Accepted May 19, 2020

DOI: 10.3892/mmr.2020.11248

Abstract. Diabetic cardiomyopathy (DCM) is a severe cardiovascular complication of diabetes mellitus (DM). Detecting DCM during the early stages of the disease remains a challenge, as the molecular mechanisms underlying early-stage DCM are not clearly understood. Circular RNA (circRNA), a type of non-coding RNA, has been confirmed to be associated with numerous diseases. However, it is still unclear how circRNAs are involved in early-stage DCM. In the present study, heart tissues harvested from BKS-db/db knock-out mice were identified through high-throughput RNA sequencing technology. A total of 58 significantly differentially expressed circRNAs were identified in the db/db sample. Among these, six upregulated circRNAs and seven downregulated circRNAs were detected by reverse transcription-quantitative PCR and analyzed using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes. Furthermore, based on the predicted binding site with microRNAs (miRNAs) involved in DCM, five circRNAs (mmu_circ_0000652, mmu_circ_0000547, mmu_circ_0001058, mmu_circ_0000680 and novel_circ_0004285) were shown to serve as competing endogenous (ce)RNAs. The corresponding miRNAs and mRNAs of the ceRNAs were also verified, and two promising circRNA-miRNA-mRNA regulatory networks were determined. Finally, internal ribosome entry site prediction combined with open reading frame prediction indicated that it was highly possible that mmu_circ_0001160 encoded a protein. In the present study, a comprehensive analysis of the circRNA expression profile during the early phase of DCM was performed, and two promising circRNA-miRNA-mRNA regulatory networks were identified. These results lay the foundation for unravelling the underlying pathogenesis of DCM, and highlight potential biomarkers and therapeutic targets for the treatment of DCM at an early stage.

Introduction

Diabetes mellitus (DM) is a chronic disease worldwide, and is a major risk factor for cardiovascular disease. The incidence of diabetes is increasing; according to the International Diabetes Federation Diabetes Atlas (8th edition, 2017), >425 million individuals are living with diabetes, 50% of whom are undiagnosed (1). Among the complications of DM, cardiovascular disease has been confirmed to be the leading cause of death (2). DM can result in both structural and functional changes in the myocardium, thus leading to diabetic cardiomyopathy (DCM) (3).

In the past four decades, despite extensive molecular and cellular based research, which have unraveled the mechanisms underlying DM and DCM, the pathogenesis of DCM remains controversial. DCM is a lifelong and progressive disease, the progress of which can be divided into three stages: Early, advanced and late stage (4). During the early stage, the majority of the patients with DCM typically exhibit changes in the structure of the heart, without any noticeable symptoms, such as diastolic and contractile dysfunction. Therefore, these patients remain asymptomatic during the early stages. As the disease advances, it progresses to irreversible heart failure, for which there is no proven effective treatment (5). Hence, unveiling the underlying pathogenesis and identifying early and specific diagnostic indicators of DCM is paramount for preventing heart failure. Recently, numerous imaging techniques and biochemical markers have been used to address this issue. In type 2 diabetes mellitus (T2DM) mice, 2D-Echo-Doppler was used to detect early changes in diastolic function and myocardial hypertrophy (6). Shaver et al (7) identified a panel of biomarkers to detect modifications in cardiac structure and function; although none of these were conclusive, and only served as a compensatory index. Thus, there is an urgent need for accurate early detection and
Effective therapeutics for the diagnosis and treatment of DCM. Therefore, an improved understanding of the pathogenesis of DCM is required, particularly for early-stage DCM.

Circular RNAs (circRNAs) are a unique type of endogenous non-coding RNAs, which were initially considered to be abnormal splicing byproducts with limited functional potential (8). However, in recent years, increasing evidence has demonstrated that circRNAs play important roles in a range of diverse physiological and pathological processes (9). Although the functions of the majority of circRNAs remain to be fully elucidated, the established functions include acting as a sponge to sequester certain microRNAs (miRNAs), modulating transcription and interfering with splicing, and even translation into polypeptides (10-12). Characterized by their stability and tissue-specificity, circRNAs may not only serve as an ideal biomarker, but may also assist in elucidating the underlying mechanisms in various disease, including DM. For example, Zhao et al (13) detected circRNAs in the peripheral blood of patients with T2DM and found that hsa_circ_0054633 may be used as a diagnostic biomarker of pre-diabetes. Fang et al (14) showed that circANKRD36 was associated with inflammation in patients with T2DM. Nevertheless, relatively less is known on the expression profile and potential role of circRNAs in early-stage DCM. Therefore, in the present study, secondary sequencing technology was used to examine the expression of circRNAs in early-stage DCM mice. Key circRNAs were screened for further analysis in the pathogenesis of early-stage DCM, as well as their potential as biomarkers, for early diagnosis and treatment of DCM.

Materials and methods

Animals. A total of 5 male C57BL/BKS-Leprdb (db/db) mice (8 weeks old; weight, 42-47 g) were obtained from Gempharmatech Co., Ltd. A total of 5 monogenic Lepr mutated mice (db/-db; 8 weeks old; weight, 20-22 g) from the same company were used as control mice. Mice were maintained under controlled conditions at 20-22° C with 12-h light/dark cycles and 40-60% humidity, with free access to food and water. Body weight was measured and the fasting blood glucose levels were determined using the glucose oxidase-peroxidase method (15). All experiments were approved by the Institutional Animal Care and Use Committee of Fudan University (China).

Myocardium sample collection and RNAseq. Mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium solution at 55 mg/kg, and then sacrificed by cervical dislocation and the myocardium samples were harvested for RNAseq. A total of 5 µg RNA in each db/db and control sample was extracted for preparation of RNA samples. Epicentre Ribo-Zero™ tRNA Removal kit (Epicentre; Illumina, Inc.) was used to remove ribosomal RNA. To obtain enriched pure circRNA, linear RNA was digested with 3 U of RNase R (Epicentre; Illumina, Inc.) per µg of RNA. According to the manufacturer's protocol, the sequencing libraries were constructed using a NEBNext® Ultra™ Directional RNA Library Prep kit for Illumina® (New England Biolabs, Inc.). Briefly, circRNAs were interrupted randomly by adding a fragment reagent. Subsequently, the first strand cDNA was synthesized using random hexamer primers and M-MuLV reverse transcriptase (RNaseH), and subsequently the second strand of cDNA was synthesized. After the repair of the end of the DNA strand and adenylation of 3'ends of DNA fragments, AMPure XP beads were used to select cDNA fragments with the appropriate length. Subsequently, 3 µl USER Enzyme (New England Biolabs, Inc.) was used to degrade the second strand containing cDNA. Finally, PCR was performed to amplify the circRNA library using the same materials and methods as below, and products were purified using the AMPure XP system.

Reverse transcription-quantitative (RT-q)PCR. Total RNAs in heart tissue were extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Using a PrimeScript RT-PCR kit (cat. no. RR014; Takara Bio, Inc.) extracted RNA was reverse transcribed to cDNA following the 20 µl system protocol, the reaction condition were as follows: 37°C for 5 min, 85°C for 15 sec and 4°C for preservation. The expression levels of circRNAs and related miRNAs, brain natriuretic peptide (BNP), atrial natriuretic peptide (ANP) and interleukin (IL)-6 were detected using a SYBR Green PCR kit (Qiagen, Inc.) in a 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: Initial denaturation at 95°C for 2 min; followed by 40 cycles of 95°C for 5 sec and annealing at 60°C for 34 sec; then 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. The miRNA levels were detected using miRNA First Strand cDNA Synthesis kit (cat. no. B532451; Sangon Biotech Co., Ltd.), according to manufacturer's protocols. U6 and β-actin were used as internal controls. The 2-ΔΔCT method (16) was used for quantitative analysis of gene expression. The primer sequences are presented in Table I.

TUNEL assay. Cardiomyocyte apoptosis was detected using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) technique. Mice were sacrificed by cervical dislocation following anesthesia, then heart tissue was isolated and immediately fixed in 4% formalin solution for 24 h at 4°C and then embedded in paraffin. Next, the specimens were sectioned into 4-µm thick slides and stained with a DeadEnd™ Fluorometric TUNEL system kit at 37°C for 1 h (cat. no. G3250; Promega Corporation), following the manufacturer's instructions.

Prediction of circRNA-miRNA interactions and identification of key circRNAs. The bioinformatics platform miRanda version 3.3a (microrna.org/) was used to predict putative miRNAs that exhibited a potential association with one of the 13 differential circRNAs identified by RT-qPCR. The major parameters were as follows: i) -sc, 140; ii) -en, -10; iii) -scale, 4; iv) -strict. The circRNA-miRNA interaction network was constructed using Cytoscape version 3.01 (17). Subsequently, Gene Ontology (GO) enrichment analysis (18-20) was used to identify the processes with the highest degree of enrichment, in which the miRNA targets of the miRNAs were involved. Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to identify the significant pathways associated with these miRNAs (genome.jp/kegg/) (21). To
## Table I. Sequences of the primers used in the present study.

| Gene                  | Primer sequence (5'→3')                                                                 |
|-----------------------|----------------------------------------------------------------------------------------|
|                       | F, forward; R, reverse; BnP, brain natriuretic peptide; ANP, atrial natriuretic peptide; IL-, interleukin; circRNA, circular RNA; IGF-1, insulin-like growth factor 1; FOXO3A, forkhead box protein O3; CAB39, calcium-binding protein 39; SPRY1, sprouty homolog 1; miR, microRNA. |
| **BNP**               | F: GAGGTCACTCTATCTTCTCTGG<br>R: GCCATTCTCTCCGACTCTTCTC<br>                          |
| **ANP**               | F: GTCGGGTGTCACACACAGAT<br>R: TCCATCTGTAATCTCTACCC<br>                              |
| **IL-6**              | F: CCAAGAGTGAGTGGTTCCTCC<br>R: CTGTGGTGACACTCTCTCCTCT<br>                          |
| **mmu_circ_0001697**  | F: AGATGCTCCTAGCGTTCTTT<br>R: TAGCTTTCTGGAGGGTAG<br>                                |
| **mmu_circ_0001160**  | F: TGTTGAAAGGTCTCCATCC<br>R: CTTCCGACTGAGAGGAGGA<br>                                |
| **novel_circ_0008273**| F: CTCAGAGATCTGGGAGAGG<br>R: CAGCTTCTCTCCGAGGCT<br>                                |
| **novel_circ_0009344**| F: TGATGCTGCTTCTTTCCCTAG<br>R: TCCAAACCCCGACTGAGG<br>                              |
| **mmu_circ_0001625**  | F: CATCCGACATTGGTCTTC<br>R: GCCAAGAAAGAGGAGGA<br>                                  |
| **mmu_circ_0001697**  | F: ACTCTGAAGGCGAGATCCT<br>R: GTGCATCTCTTAAACACATC<br>                              |
| **mmu_circ_0001058**  | F: AGAGCTGCTGAGAAGACTGA<br>R: TGTTGAAAGGGAGGAGGA<br>                               |
| **mmu_circ_0000652**  | F: CAGAGAGGAGGAGCTACA<br>R: AGTTGCTGTTGAGAGG<br>                                  |
| **mmu_circ_0000058**  | F: GGTACCTGACAGTGCTCCA<br>R: TAATGTAATGTTGCC<br>                                  |
| **mmu_circ_0001058**  | F: AGAGCTGCTGAGAAGACTGA<br>R: TGTTGAAAGGGAGGAGGA<br>                               |
| **mmu_circ_0000680**  | F: ATCCAAACACGGTCCCTCCA<br>R: TCTGAGGAAAAAAGGAGA<br>                              |
| **novel_circ_000824** | F: CTCAGAGACCTGGAGGG<br>R: AAACCTGGCAGCCTGAGG<br>                                 |
| **mmu_circ_0000547**  | F: GCGCACGGCGAGATGAAAACA<br>R: GTCAAGACCTGCTGAGG<br>                              |
| **novel_circ_0004285**| F: GTGGAAAGAATGGAGAGG<br>R: GCAGATACCTGAGAGGAG<br>                                 |
| **IGF-1**             | F: GAATGGCCTCTCCAGGG<br>R: AGTGCTGCTTCTCTTTGCT<br>                                |
| **FOXO3A**            | F: GGAGAACCTGCTTCTATGCC<br>R: TCATTGCAAGCGCATGAAG<br>                              |
| **CAB39**             | F: TGCTGTGGACAGACACA<br>R: GGAGGTTCATCTTAGCTTGAGG<br>                              |
| **BCL2**              | F: GCTACGCCATGGGACTTC<br>R: CCCAACGAGACTAAAGAG<br>                                 |
| **SPRY1**             | F: GTGCTATAGTGGCTGAGG<br>R: GTTCCTTATTCACCAGAT<br>                                 |
| **miR-195**           | F: TAGCAGCAGAAATATTGGC<br>R: Universal PCR Primer R                               |
| **miR-21**            | F: TAGCTTATCAGACTGATGTTGA<br>R: Universal PCR Primer R                              |
| **miR-320**           | F: AAAAGCTGGTGGAGGGAGG<br>R: Universal PCR Primer R                                 |
| **miR-451**           | F: AAACCTGGACTTACAGTT<br>R: Universal PCR Primer R                                 |
| **miR-30d**           | F: TGTTAACAATCCAGCACTGGAAG<br>R: Universal PCR Primer R                             |
identify the key circRNAs, known DCM-related miRNAs were searched by reviewing the relevant literature in pubmed (pubmed.ncbi.nlm.nih.gov/), and the common miRNAs between the known DCM-related miRNAs and the predicted miRNAs were extrapolated to identify key miRNAs. The circRNAs that were associated with these key miRNAs were used to construct an integral circRNA-miRNA-mRNA regulatory network using Cytoscape version 3.0. Finally, these key miRNAs and their target mRNAs were verified by RT-qPCR, following the aforementioned protocol.

**Results**

**Early changes in the diabetic myocardium.** A series of biochemical biomarkers have been determined to crudely indicate functional changes in early-stage DCM (22,23). BNP, ANP and IL-6 expression levels were assessed to detect early modifications in db/db myocardial tissue. The body weight of the five db/db mice was measured and the fasting blood-glucose levels were measured three times on different days. The average weight of the five db/db mice was 44.34 g, whereas the control mice were 22.8 g (Fig. 1A). The fasting blood-glucose level of db/db mice ranged from 22.2-26.4 mmol/l with a mean of 23.8 mmol/l (Fig. 1B). Furthermore, the qPCR results exhibited a significant increase in the expression levels of BNP, ANP and IL-6 (P<0.05). In addition, TUNEL staining also exhibited an increasing trend of cardiomyocyte apoptosis in the db/db model (Fig. 1F), suggesting successful establishment of a model of early-stage DCM.

**Establishment of the circRNA-miRNA network.** Increasing evidence has suggested that circRNAs may regulate the function of miRNAs by acting as competing endogenous RNAs (ceRNAs) (24). In an attempt to identify the functions of these differentially-expressed circRNAs in DCM, circRNA-miRNA co-expression networks were constructed based on the miranda algorithm. The results showed there were 610 related miRNAs that exhibited close binding competency to these circRNAs (Fig. 5), the detailed data are listed in Table SII. Total scores of the targeting relationship for all binding sites were predicted and displayed on a color scale, where higher values indicated an increased likelihood of targeting. To further probe the function of these target genes related to these differentially-expressed circRNAs, GO enrichment analysis was performed. The results showed that these target genes participated in various biological processes, such as ‘metabolic process’, ‘binding’ and ‘negative regulation of cellular process’ (Fig. S1). KEGG analysis showed these target genes may have effects on several vital signaling pathways, such as ‘glycero-phospholipid metabolism’, ‘glycolysis/gluconeogenesis’, ‘either lipid metabolism’ and ‘fructose and mannose metabolism’ (Fig. S2). The results suggested that the detected circRNAs may be related to glucose and lipid metabolism, which is consistent with the fasting blood-glucose result. However, whether these circRNAs function in DCM and the underlying mechanisms need to be further explored.

**circuitRNA expression in the myocardial tissue of db/db mice.** To detect circRNA expression in the myocardial tissues of db/db mice, RNA-seq analysis was performed. Then, a volcano plot and heatmap were constructed using R software version 3.5 (r-project.org/). The length of the detected circRNAs was primarily in the 100-500 bp range (Fig. 2A), and a majority of the reads were covered in the exon of the genome (Fig. 2B). Overall, 58 circRNAs were determined to be significantly differentially expressed (P<0.05; Table S1), including 29 upregulated circRNAs and 29 downregulated circRNAs (Fig. 2C and D). Of these, three-quarters were newly identified circRNAs. In addition, all candidate circRNAs were found to be distributed among all the chromosomes (Fig. 3).

To validate the RNA-seq results, RT-qPCR was performed to analyze circRNA expression. According to the RT-qPCR results, six upregulated circRNAs (mmu_circ_0001697, mmu_circ_0001160, novel_circ_0008273, novel_circ_0009344, mmu_circ_0001625 and mmu_circ_0000431) and seven downregulated circRNAs (mmu_circ_0000652, mmu_circ_0000058, mmu_circ_0001058, novel_circ_0000824, mmu_circ_0000547 and novel_circ_0004285) were verified to be differentially expressed in the tissue samples (Fig. 4).
Mmu_circ_0001160 exhibits high protein-coding potential. Recent studies have reported that a fraction of circRNAs exhibit protein-coding potential based on the presence of IRES and ORF elements (32-34). IRES and ORF are both important regulatory elements for circRNA translation without the need for a 5' cap structure. To extensively explore the function of these circRNAs, IRES-finder software and circAtlas 2.0 were used to predict whether a circRNA sequence possessed potential IRES and ORF elements. Among the differentially expressed circRNAs, there were 9 circRNAs that had a potential IRES element. The closer the score value was to 1, the higher the credibility of the IRES in the circRNA was (Fig. 8). Based on the results, mmu_circ_0001160 had a relatively high IRES and ORF score, suggesting that this circRNA may function in a novel way by producing a polypeptide, the potential regulatory mechanisms of which are worthy of future studies.

Table II. The five potential ceRNA regulatory networks and functions in DCM.

| circRNA         | miRNA | mRNA   | Function                      | Refs. |
|-----------------|-------|--------|--------------------------------|-------|
| mmu_circ_0000680| miR-30d| FOXO3A | Cardiomyocyte pyroptosis      | (26)  |
| mmu_circ_0000547| miR-320| IGF-1  | Impaired angiogenesis          | (28)  |
| novel_circ_0004285| miR-451| CAB39  | Cardiomyocyte lipotoxicity     | (25)  |
| mmu_circ_0000652| miR-195| BCL2   | Cardiomyocyte apoptosis        | (27)  |
| mmu_circ_0001058| miR-21 | SPRY1  | Interstitial fibrosis          | (29)  |

miRNA/miR, microRNA; circRNA, circular RNA; ceRNAs, competing endogenous RNAs; IGF-1, insulin-like growth factor 1; FOXO3A, forkhead box protein O3; CAB39, calcium-binding protein 39; SPRY1, sprouty homolog 1.
Discussion

circRNAs can regulate the expression of genes at the transcriptional or post-transcriptional levels (24,35). In recent years, expression of circRNAs have been shown to be dysregulated in several types of diseases, including DM (1,11,36-39). However, their function and the underlying mechanisms in DCM remains largely unknown. In the present study, the circRNA expression profile was analyzed in a mouse model of early-stage DCM to improve our understanding of the pathogenesis of early-stage DCM and to find potential circRNA biomarkers. To the best of our knowledge, the present study was the first to explore the significance of circRNAs in early-stage DCM. Two promising circRNAs and their regulatory networks were identified, which may serve an important role in early-stage DCM.

An increasing number of studies have suggested that circRNAs serve a significant role in DM (13,14,36,40-42). For example, Zhao et al (13) found that hsa_circ_0054633 may serve as a diagnostic biomarker for pre-diabetes. However, relatively less is known about circRNAs in early-stage DCM. In the present study, 58 circRNAs were shown to be differentially expressed in a mouse model of early-stage DCM. RT-qPCR analysis results were consistent with the results of RNA-seq analysis, confirming the reliability of the RNA-seq data. KEGG analyses indicated that the related mRNAs were primarily involved in glucose metabolism and lipid metabolism pathways, both of which serve a significant role in triggering further alterations of the myocardial tissue. For example, lipid metabolism disorders may contribute to cardiomyocyte lipotoxicity, which is an initial deleterious response in the DM heart through the formation of fatty acid metabolites, as well as the release of mitochondrial and cytosolic ROS (43,44). Although the alterations in the KEGG pathway may be caused by high levels of blood-glucose in our model, the results still preliminarily suggested that these circRNAs may have an association with early stage DCM and the function of these circRNAs needs further investigation.
Figure 3. Differentially expressed circRNAs on mouse chromosomes. The yellow triangle represents the upregulated and downregulated circRNAs between the db/db and normal samples. circRNA, circular RNA.

Figure 4. Reverse transcription-quantitative PCR validation of differentially-expressed circRNAs. Relative expression levels of the six upregulated and seven downregulated circRNAs between the control group and db/db group. Data are presented as the mean ± standard deviation. n=3 per group. *P<0.05, **P<0.01, ***P<0.001 vs. control. circRNA, circular RNA.
Figure 5. Network map of circRNA-miRNA interactions. The six significantly upregulated circRNAs are represented by red nodes and the seven downregulated circRNAs are represented by blue nodes. Target miRNAs are represented by green and red nodes with the binding score ranging from 140-170. miRNA, microRNA; circRNA, circular RNA.

Figure 6. Key circRNA identification. (A) Predicted binding sites of key circRNAs and miRNAs. The dots between the bases represent the wobble base pairing between the purines and pyrimidines. (B) Reverse transcription-quantitative PCR validation of DCM-related miRNAs and the target mRNAs. Data are presented as the mean ± standard deviation. n=3 per group. *P<0.05, **P<0.01, ***P<0.001 vs. control. circRNA, circular RNA; miRNA, microRNA; DCM, diabetic cardiomyopathy; SPRY1, sprouty homolog 1; CAB39, calcium-binding protein 39; FOXO3A, forkhead box protein O3.
Numerous studies have shown that circRNAs may negatively regulate the inhibitory effects of miRNAs on their target mRNAs by directly binding to miRNAs, thereby acting as miRNA sponges (24,35,40,45-47). In the present study, mmu_circ_0000652 and mmu_circ_0001058 were found to potentially interact with miR-195 and miR-21, both of which have been shown to serve roles in the metabolism of dCM. miR-195 is associated with a number of pathological myocardial conditions, including hypertrophy, fibrosis, apoptosis, arrhythmia and heart failure (27). Zheng et al (48) showed that silencing miR-195 attenuated myocardial hypertrophy, promoted coronary circulation and reduced myocardial dysfunction in streptozotocin-induced diabetic mice. In addition, further experiments showed that these protective effects were associated with decreased apoptosis, generated by the induction of the direct targets of miR-195, BCL2 and sirtuin 1. In the present study, upregulation of miR-195 and downregulation of BCL2 were observed, suggesting that downregulation of mmu_circ_0000652 may promote apoptosis by indirectly inhibiting BCL2 in the db/db model. Apoptosis is a relatively early event in DCM, when apoptotic cardiomyocytes are lost, collagen is deposited to replace dead cardiomyocytes, which contributes to cardiac fibrosis and dysfunction (49). The function of miR-21 differs from miR-195. miR-21 regulates the ERK-MAP kinase signaling pathway by affecting SPRY1, ultimately resulting in fibrosis, hypertrophy and cardiac dysfunction (29). Similarly, mmu_circ_0001058 serves an important role in the progress of DCM by interfering with miR-21. Further studies on mmu_circ_0000652 and mmu_circ_0001058 may highlight the roles of these circRNAs in the underlying pathogenesis of early-stage DCM, thus potentially improving early diagnosis and treatment of early-stage DCM and minimizing its complications.

In addition to acting as an miRNA sponge, studies have reported that circRNAs exhibit protein/peptide coding capacity. To exhibit protein-coding potential, an IRES and ORF are required. The present results showed that mmu_circ_0001160 possessed a relatively high coding possibility amongst the identified circRNAs. Zn²⁺ transporter 7 (ZNT7), the linear parental gene of mmu_circ_0001160, has been shown to be associated with DM by interfering with insulin secretion (50,51). Furthermore, Tuncay et al (52) demonstrated that the novel role of ZNT7 in hyperglycemic cardiomyocytes by affecting sarco(endoplasmic reticulum-mitochondria coupling. Based on the hypothesis that circRNAs may affect its linearly expressed...
gene, it was hypothesized that mmu_circ_0001160 may interfere with early-stage DCM by producing a ZnT7-related protein. However, to validate whether this circRNA could encode a protein, in-depth studies are required. As the function of circRNA still remains to be fully elucidated, further functional experiments on mmu_circ_0001160 are required to extensively explore the role of circRNAs in various diseases.

There are several limitations of the present study. First, since the metabolic disturbances could cause a series of cardiac structural and functional alterations in the early stage of DCM (53), some additional structure and function parameters, such as echocardiographic examination and extra cardiac function biomarkers, are also important to confirm the successful establishment of our model (54), it will be applied in our future experiments. Second, although mice exhibit a high degree of homology with humans, the expression profile of its homologous sequences in humans have yet to be determined. Therefore, whether these results can be applied to humans requires further confirmation. In addition, due to the low validation rate of circRNA RNA-seq data compared with the RT-qPCR experiments, it is not possible to exclude the possibility of other dysregulated circRNAs that participate in the development of DCM. Thus, additional studies on these issues are required. As circRNAs are stably expressed in the blood, identifying circulating circRNAs in patients with DCM may provide a promising avenue for research, and should thus be an integral part of future studies.

In conclusion, the present study provided a comprehensive analysis of the circRNA expression profile during early phase DCM, and identified two promising circRNA-miRNA-mRNA axes. Further functional studies are required to determine the underlying pathogenesis of early-stage DCM, and the potential role of these axes in diagnosis and treatment of DCM.

Acknowledgements

Not applicable.

Funding

This work was supported by grants from the Key Projects of National Natural Science Foundation of China (grant no. 814300047) and National Key Research and Development Program (grant no. 2018 YFC0807202).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SD was a major contributor in writing the manuscript and performing the experiments. JL and YS designed the study and were in charge of reviewing the manuscript. SD, CT, XY and SC performed the experiments and analyzed the data. LL, MZ, AX, ZZ and BC interpreted the data and edited the important intellectual content of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care and Use Committee of Fudan University (approval no. 20190221-124).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW and Malanda B: IDF diabetes atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res Clin Pract 138: 271-281, 2018.
2. Marwick TH, Ritchie R, Shaw JE and Kaye D: Implications of underlying mechanisms for the recognition and management of diabetic cardiomyopathy. J Am Coll Cardiol 71: 339-351, 2018.
3. Jia G, Hill MA and Sowers JR: Diabetic cardiomyopathy: An update of mechanisms contributing to this clinical entity. Circ Res 122: 624-638, 2018.
4. Murtaza G, Virk AU, Khalid M, Lavie CJ, Ventura H, Mukherjee D, Ramu V, Bhogal S, Kumar G, Shanmugasundaram M and Paul TK: Diabetic cardiomyopathy: A comprehensive update. Prog Cardiovasc Dis 62: 315-326, 2019.
5. Lam CS: Diabetic cardiomyopathy: An expression of stage B heart failure with preserved ejection fraction. Diab Vasc Dis Res 12: 234-238, 2015.
6. Tadic M, Celvic V, Cuspidi C, Illic S, Pencic B, Radojkovic J, Ivanovic B, Stanisavljevic D, Kocabay G and Marjanovic T: Right heart mechanics in untreated normotensive patients with prediabetes and type 2 diabetes mellitus: A Two- and three-dimensional echocardiographic study. J Am Soc Echocardiogr 28: 317-327, 2015.
7. Shaver A, Nichols A, Thompson E, Mallick A, Payne K, Jones C, Manne ND, Sundaram S, Shapiro JI and Sodhi K: Role of serum biomarkers in early detection of diabetic cardiomyopathy in the west virginian population. Int J Med Sci 13: 161-168, 2016.
8. Li X, Yang L and Chen LL: The biogenesis, functions, and challenges of circular RNAs. Mol Cell 71: 428-442, 2018.
9. Zong HD, Jiang LH, Sun DW, Hou JC and Ji ZL: CircRNA: A novel type of biomarker for cancer. Breast Cancer 25: 1-7, 2018.
10. Pamudurti NR, Bartok O, Jens M, Ashwal-Fluss R, Stottmeister C, Ruhe L, Hanan M, Wyler E, Perez-Hernandez D, Ramberger E, et al: Translation of CircRNAs. Mol Cell 66: 9-21, 2017.
11. Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P and Wu M: Functions and properties of a novel potential biomarker for cancer. Mol Cancer 16: 94, 2017.
12. Huang C and Shan G: What happens at or after transcription: Insights into circRNA biogenesis and function. Transcription 6: 61-64, 2015.
13. Zhao Z, Li X, Jian D, Hao P, Rao L and Li M: Hsa_circ_0054633 in peripheral blood can be used as a diagnostic biomarker of pre-diabetes and type 2 diabetes mellitus. Acta Diabetol 54: 237-245, 2017.
14. Fang Y, Wang X, Li W, Han J, Jin J, Su F, Zhang J, Huang W, Xiao F, Pan Q and Zou L: Screening of circular RNAs and validation of circANKRD36 associated with inflammation in patients with type 2 diabetes mellitus. Int J Mol Med 42: 1865-1874, 2018.
15. Karkalas J: An improved enzymic method for the determination of native and modified starch. J Sci Food Agr 36: 1019-1027, 1985.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
17. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T: CytoScape: A software environment for integrated models of biomolecular interaction networks. Genome Res 13: 2498-2504, 2003.
18. Mi H, Muruganjan A, Ebert D, Huang X and Thomas PD: PANTHER version 14: More genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res 47: D419-D426, 2019.

19. The Gene Ontology Consortium: The gene ontology resource: 20 years and still going strong. Nucleic Acids Res 47: D330-D338, 2019.

20. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al: Gene ontology: Tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 25-29, 2000.

21. Kanehisa M, Goto S, Yamanishi Y, Matozaki M, Tanabe M: Data, information, knowledge and principle: Back to metabolism in KEGG. Nucleic Acids Res 42 (Database Issue): D199-D205, 2014.

22. Jellis CL, Sacre JW, Wright J, Jenkins C, Haluska B, Jeffriess L, Martin J and Marwick TH: Biomarker and imaging responses to spironolactone in subclinical diabetic cardiomyopathy. Eur Heart J Cardiovasc Imaging 15: 776-786, 2014.

23. Nunes S, Soares E, Fernandes J, Viana S, Carvalho E, Pereira FC and Reis F: Early cardiac changes in a rat model of prediabetes: Brain natriuretic peptide overexpression seems to be the best marker. Cardiovasc Diabetol 12: 44, 2013.

24. Cai J, Chen Z, Wang J, Wang J, Chen X, Liang L, Huang M, Zhang Z and Zuo X: circHECTD1 facilitates glutaminolysis to promote gastric cancer progression by targeting miR-1256 and activating the LKB1/aMPK pathway. Circ Res 116: 279-288, 2015.

25. Li X, Du N, Zhang Q, Li J, Chen X, Liu X, Hu Y, Qin W, Shen N, Xu C, et al: MicroRNA-30d regulates cardiomyocyte pyroptosis by directly targeting Foxo3a in diabetic cardiomyopathy. Cell Death Dis 5: e1479, 2014.

26. Zhu H, Yang Y, Wang Y, Li J, Schiller PW and Peng T: MicroRNA-30d inhibits the proliferation of gastric cancer cells by regulating the miR-376-Sp5/p27Kip1 axis. Mol Cancer 17: 151, 2019.

27. Zhang Z and Zuo X: circHecT1 facilitates glutaminolysis to promote gastric cancer progression by targeting miR-1256 and activating the LKB1/aMPK pathway. Circ Res 116: 279-288, 2015.

28. Wang XH, Qian RZ, Zhang W, Chen SF, Jin HM and Hu RM: MicroRNA-320 expression in myocardial microvascular endothelial cells and its relationship with insulin-like growth factor-1 in type 2 diabetic rats. Clin Exp Pharmacol Physiol 36: 181-188, 2009.

29. Thum T, Gross C, Fiedler J, Fischer T, Kessler S, Bussmann M, Galuppo P, Just S, Rotbauer W, Frantz S, et al: MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 456: 980-984, 2008.

30. Liu J, Liu W, Lu Y, Tian H, Duan C, Lu L, Gao G, Wu X, Wang X and Yang H: Piperlongumine restores the balance of autophagy and apoptosis by increasing Bcl2 phosphorylation in rotenone-induced Parkinson disease models. Autophagy 14: 845-861, 2018.

31. Chai C, Song LJ, Han SY, Li XQ and Li M: MicroRNA-21 promotes glioma cell proliferation and inhibits senescence and apoptosis by targeting SPRY1 via the PTEN/PI3K/AKT signaling pathway. Cns Neurosci Ther 24: 369-380, 2018.

32. Li Li, Leng RX, Fan YG, Pan HF and Ye DQ: Translation of noncoding RNAs: Focus on IncRNAs, pri-miRNAs, and circRNAs. Exp Cell Res 361: 1-8, 2017.

33. Liu H, Liu Y, Bian Z, Zhang J, Zhang R, Chen X, Huang Y, Wang Y and Zhu J: Circular RNA YAP1 inhibits the proliferation and invasion of gastric cancer cells by regulating the miR-376-Sp5/p27Kip1 axis. Mol Cancer 17: 151, 2019.

34. Altesha MA, Ni T, Khan A, Liu K and Zhong X: Circular RNA in cardiovascular disease. J Cell Physiol 234: 5588-5600, 2019.

35. Lin F, Zhao G, Chen Z, Wang X, Lv F, Zhang Y, Yang X, Liang W, Cai R, Li J, et al: circRNA-miRNA association for coronary heart disease. Mol Med Rep 19: 2527-2536, 2019.

36. Patop IL and Kadenier S: circRNAs in cancer. Curr Opin Genet Dev 48: 121-127, 2018.

37. Fischer JW and Leung AK: circRNAs: A regulator of cellular stress. Crit Rev Biochem Mol Biol 52: 220-233, 2017.

38. Huang M, Li X, Sun, Zhang Q, Chen X, Liu X, Hu Y, Qin W, Shen N, Xu C, et al: MicroRNA-30d regulates cardiomyocyte pyroptosis by directly targeting Foxo3a in diabetic cardiomyopathy. Cell Death Dis 5: e1479, 2014.

39. Fischer JW and Leung AK: circRNAs: A regulator of cellular stress. Crit Rev Biochem Mol Biol 52: 220-233, 2017.

40. Patop IL and Kadenier S: circRNAs in cancer. Curr Opin Genet Dev 48: 121-127, 2018.

41. Fischer JW and Leung AK: circRNAs: A regulator of cellular stress. Crit Rev Biochem Mol Biol 52: 220-233, 2017.

42. Wu H, Wu S, Zhi Y, Shun J, Liu Y, Zhang Y and Bu S: Hsa_circRNA_0054633 is highly expressed in gestational diabetes mellitus and closely related to glycosylation index. Clin Epigenetics 11: 22, 2019.

43. Nunes S, Soares E, Fernandes J, Viana S, Carvalho E, Pereira FC and Reis F: Early cardiac changes in a rat model of prediabetes: Brain natriuretic peptide overexpression seems to be the best marker. Cardiovasc Diabetol 12: 44, 2013.

44. Duncan JG: Mitochondrial dysfunction in diabetic cardiomyopathy. Biochim Biophys Acta 1813: 1351-1359, 2011.

45. Patop IL and Leung AK: circRNAs in cancer. Curr Opin Genet Dev 48: 121-127, 2018.

46. Patop IL and Leung AK: circRNAs in cancer. Curr Opin Genet Dev 48: 121-127, 2018.