Expression of Bcl-2 and microRNAs in cardiac tissues of patients with dilated cardiomyopathy

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Received October 26, 2015; Accepted November 1, 2016

DOI: 10.3892/mmr.2016.5977

Abstract. Dilated cardiomyopathy (DCM) is associated with sudden cardiac death and heart failure, resulting in a significant medical burden. The mechanisms underlying the pathogenesis of DCM remain elusive. In the present study, human cardiac tissues from patients with DCM and healthy donors were collected and their pathology was examined. The expression levels of apoptosis regulator Bcl-2 and fibrosis-associated microRNAs were also evaluated. Extensive myocardial fibrosis and apoptosis in DCM cardiac tissues was observed. As demonstrated by western blotting, reverse transcription-quantitative polymerase chain reaction and immunohistochemistry, the expression of Bcl-2 was significantly increased in the apex, and the left and right ventricle of the heart in patients with DCM. In the specified locations, it was identified that miR-21 was upregulated, while members of miR-29 family (miR-29a, miR-29b and miR-29c) and miR-133 family (miR-133a and miR-133b) were downregulated. The present study suggested that Bcl-2 and specific microRNAs may be involved in DCM pathogenesis, with a potential implication as therapeutic targets.

Introduction

Dilated cardiomyopathy (DCM) is characterized by left ventricular dilation and systolic dysfunction with an ejection fraction of <50% (1). DCM is associated with sudden cardiac death and heart failure, resulting in a significant medical burden due to the high rate of hospital admissions and the potential requirement for heart transplantation (2). Despite therapeutic advances, the prevalence of DCM was 1 in 2,500-3,000 and the 5-year mortality remained ~20% in 2010 (2,3). Patients with DCM may also develop diastolic dysfunction and impaired right ventricular function. Genetic inheritance, myocarditis, alcohol intake and toxic effects from medications may contribute to the development and progression of DCM (2).

Cardiomyocyte apoptosis and fibrosis are the major pathological changes that occur in DCM. Apoptosis is an actively regulated process that includes DNA fragmentation, membrane blebbing, cell shrinking and chromatin condensation (4). Fibrosis is the formation of excess fibrous tissues or scar tissue, which is characterized by the net accumulation of extracellular matrix proteins in the cardiac interstitium (5). In the hearts of patients with DCM, apoptosis of cardiomyocytes leads to the increase of the intercellular space, where it is filled by granulation tissue, composed of macrophages, endothelial cells and fibroblasts (6,7). In addition, cardiac fibroblasts excessively produce extracellular matrix (ECM), leading to fibrosis and increasing tissue stiffness.

B-cell lymphoma-2 (Bcl-2) is an important antiapoptotic protein (8,9). It was isolated from the t(14;18) chromosomal breakpoint in follicular B-cell lymphoma (10). The Bcl-2 protein has been demonstrated to bind to Bak, Bax, Bcl-xL, Bcl-xS, mcl-1 and a variety of other intracellular proteins (11). The relative levels and competing dimerizations between Bcl-2 and its binding proteins determine the potential of a cell to undergo apoptosis (12). Overexpression of Bcl-2 in transgenic models leads to accumulation of cells, due to evasion of cell death mechanisms (13). Induction of apoptosis by diverse stimuli, including radiation, hyperthermia, growth factor withdrawal and multiple classes of chemotherapeutic agents, is inhibited by Bcl-2 in vitro (14). The inhibitory effects are proportional to the level of Bcl-2 expression (14). In addition, an increase in the expression levels of the Bcl-2 family proteins was observed in the end stage of heart failure (15). However, the role of Bcl-2 in DCM remains to be thoroughly studied.

Micro (mi)RNAs are 22 nucleotides in length and serve important regulatory roles by targeting messenger RNAs for...
cleavage or translational repression via RNA silencing (16). They are the most abundant class of regulatory molecules for the expression of protein-coding genes in response to pathological stimuli (17). Genome-wide profiling of miRNAs demonstrated that 43 miRNAs were differentially expressed during human heart failure (18). The level of miRNAs in blood circulation may function as markers for DCM (19). Specific miRNAs, including miR-21, may regulate Bcl-2 and mediate fibrosis (20). Therefore, the expression of miRNAs in cardiac tissues may be associated with the pathogenesis of DCM.

In the present study, human hearts from three DCM patients and three healthy donors were collected. Myocardial fibrosis and apoptosis of the cardiac tissues was evaluated, and the mRNA and protein expression levels of Bcl-2 were examined. In addition, the expression of three miRNAs and their family members (miR-21; miR-29a, miR-29b and miR-29c; miR-133a and miR-133b) were examined in normal and DCM cardiac tissues. The results of the present study suggested that Bcl-2 and specific miRNAs are involved in human DCM.

Materials and methods

Human cardiac tissue samples. Experiments involving human tissues complied with the Declaration of Helsinki and were approved by the Shandong Provincial Qianfoshan Hospital Ethical Review Board (Jinan, China; no. S074). Human hearts were collected from three patients with DCM-caused heart failure (46.57±9.06 years) who underwent a heart transplantation. The normal cardiac tissue samples were from three healthy donors with no history of cardiac disease. All patients were functionally classified according to the New York Heart Association criteria. The diagnosis of DCM was based on the World Health Organization/International Society and Federation Cardiology definition (21). Clinical history, electrocardiogram, echocardiography, hemodynamic studies and coronary angiography data were measured for all patients (Table I). Transmural samples were taken from near the apex, septum, and left and right ventricle, and were stored in frozen liquid nitrogen or fixed in 10% buffered formalin for analysis.

Masson's trichrome staining. Masson's trichrome staining was performed as previously described (22). The frozen cardiac tissues were cut into 6 µm transverse sections, stained with Weigert's iron hematoxylin for 10 min at room temperature, then washed and stained in 1% ponceau-acetic acid solution (equal volumes of 0.5% ponceau 2R in 1% acetic acid and 0.5% acid fuchsin in 1% acetic acid) for 5 min at room temperature. Following washing, the sections were incubated with 1% phosphomolybdic acid for 5 min and counterstained with light green. The images were captured under a FSX100 microscope (Olympus Corporation, Tokyo, Japan) and analyzed using Image-Pro Plus Software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Terminal deoxynucleotidyl-mediated dUTP nick end labeling (TUNEL) assay. TUNEL (EMD Millipore, Billerica, MA, USA) was used to detect the apoptosis of cardiac tissues. The frozen tissue sections were pretreated with 20 µg/ml proteinase K (EMD Millipore) for 15 min at room temperature, and the endogenous peroxidase activity was eliminated using 3% hydrogen peroxide in phosphate-buffered saline (PBS; 5 min at room temperature). Following washing with equilibration buffer from the kit, the tissue sections were incubated with the reaction mixture from the kit for 50 min at 37˚C. Following the anti-digoxigenin-peroxidase reaction for 30 min, the positive cells were visualized by diaminobenzidine (Dako, Glostrup, Denmark) and the slides were counterstained with hematoxylin. The number of positively stained cells and total cells were calculated under a FSX100 microscope (Olympus Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA isolation was performed using the RNasy Mini kit (Qiagen GmbH, Hilden, Germany). The cDNA was synthesized with the High Capacity RNA-to-cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). qPCR was performed using a Viia7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Reactions were performed at 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative Bcl-2 expression was calculated using GAPDH as an endogenous internal control. The primer sequences were as follows: Bcl-2, forward: 5'-ATGGGGCTGGGCGATAGAC-3' and reverse: 5'-TTGAGGCATGTTGTCACTC-3'; GAPDH, forward: 5'-AGCTGAAGGGGAAGTCACT-3' and reverse: 5'-TGGTCCACGTGACAGTTG-3'.

Additionally, RT-qPCR was performed with primers specific for human miR-21 (MIMAT0004494), miR-29a (MIMAT0000868), miR-29b (MIMAT000100), miR-29c (M1 MAT0000681), miR-133a (MIMAT000427) and miR-133b (MIMAT000770). All primers were designed by Tiangen Biotech Co., Ltd. (Beijing, China). Each reaction was repeated in triplicate, and the experiments were repeated to confirm reproducibility.

Immunohistochemical analysis. Tissue sections were deparaffinized in xylene and rehydrated through graded alcohol washes, prior to incubating with 1% H2O2 in methanol for 10 min at room temperature to inhibit endogenous peroxidase activity. Following washing with PBS, the tissue sections were incubated with a mouse anti-Bcl-2 antibody (1:100; catalog no. ab692; Abcam, Cambridge, MA, USA) in a humid chamber at 4˚C overnight, followed by incubation with a biotinylated anti-mouse secondary antibody (1:200; catalog no. ZDR-5307; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 30 min at room temperature. Diaminobenzidine tetrahydrochloride was used to visualize the antibody binding, and hematoxylin counterstain was performed prior to the addition of coverslips. Omission of the primary antibody resulted in a lack of specific staining, therefore, serving as the negative control. Specimens were examined with an FSX100 microscope (Olympus Corporation). A total of 20 images of each section were captured at a magnification of x20. The expression of Bcl-2 was analyzed using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.). The positive expression of Bcl-2 was determined by the ratio of the positive area to the total area.
Western blot analysis. Tissue samples were lysed in ice-cold RIPA buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1% NP-40, 0.1% deoxycholate, 0.1% SDS and 1 mM EDTA, supplemented with 1 mM PMSF and 1 µg/ml leupeptin]. The protein concentration was determined using Bio-Rad DC Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). An equal quantity (20 mg) of the samples were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Chalfont, UK). The membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween, and were subsequently incubated overnight at 4°C with a polyclonal rabbit anti-Bcl-2 antibody (1:1,000; catalog no. ab59348; Abcam) or a rabbit GAPDH polyclonal antibody (1:3,000, catalog no. 10494-1-AP; ProteinTech Group, Inc., Chicago, IL, USA). Following washing three times, the membranes were incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG (1:5,000; catalog no. BA1054; Boster Systems, Inc., Pleasanton, CA, USA), and the immunoblots were visualized using enhanced chemiluminescence Western Blotting Luminol reagent (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The intensity of the bands from each protein under investigation was quantified using ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The data are presented as the mean ± standard error of the mean (n=3; n.s., non-significant; *P<0.05; **P<0.01 compared with the Normal group). The data are presented as the mean ± standard error of the mean (n=3; n.s., non-significant; *P<0.05; **P<0.01 compared with the Normal group). LV, left ventricle; RV, right ventricle; DCM, dilated cardiomyopathy.

Results

Fibrosis and apoptosis is increased in cardiac tissues of patients with DCM. Pathological changes in myocardial tissue of DCM patients were examined using Masson's trichrome staining and TUNEL. Masson's trichrome staining demonstrated that collagen was appropriately arranged among cardiomyocytes in the normal group, while it was increased and disorganized in the DCM group (data not shown). The size of the nucleus was uniform in the normal cardiomyocytes, but irregular in the cardiomyocytes from patients with DCM (data not shown). In the DCM group, the collagen volume fractions, a morphometric measure of the percentage of interstitial collagen, were significantly higher when compared with the normal group in the apex, and the left and right ventricles (Fig. 1A; 4.23±1.28 vs. 9.84±1.39%, P<0.05; 3.89±0.99 vs. 10.84±1.65%, P<0.05; 5.19±1.36 vs. 11.09±1.07%, P<0.05, respectively). No significant difference was observed in either the mRNA or protein expression level of collagen in the septum (Fig. 1A; 3.48±1.11 vs. 4.21±1.31, P=0.69). TUNEL was used to label apoptotic cells. The results presented in the present study indicated a greater TUNEL staining in the tissue sections of apex, and left and right ventricles of the DCM group when compared with the normal group (Fig. 1B). The myocardial apoptosis index, denoted as the percentage of TUNEL positive cells, was significantly higher in the apex, and the left and right ventricle of the DCM group when compared with the normal group (Fig. 1B; 8.25±1.93 vs. 43.6±7.31%, P<0.01; 9.70±2.13 vs. 49.02±9.08%, P<0.05; 8.92±1.56 vs. 50.89±8.99%, P<0.05, respectively). The myocardial apoptosis index in the septum presented no statistical difference between the two groups (Fig. 1B; 7.92±1.83 vs. 13.35±1.76%, P=0.09). Therefore, cardiac fibrosis and apoptosis were significantly increased in apex, and the left and right ventricle of patients with DCM when compared with the normal control group.

Expression of Bcl-2 is upregulated in cardiac tissues from patients with DCM. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. The present study analyzed the expression levels of Bcl-2 in cardiac tissue from human samples. Western blot analysis demonstrated that Bcl-2 was significantly increased in the apex, and the left and right ventricle of DCM samples when compared with the normal group (Fig. 2A), which was confirmed by densitometry analysis (Fig. 2B; 0.6623±0.0824 vs. 1.1991±0.1623, P<0.05; 0.6540±0.1029 vs. 1.0994±0.1243, P<0.05, respectively). The mRNA expression of Bcl-2 was determined by RT-qPCR. This indicated that Bcl-2 was significantly upregulated in the apex, and the left and right ventricle of the patients with DCM compared with the normal group (Fig. 2C; 0.0007±0.0002 vs. 0.0018±0.0003, P<0.05; 0.0007±0.0003 vs. 0.0021±0.0002, P<0.01; 0.0006±0.0001 vs. 0.0019±0.0004, P<0.05, respectively). No significant difference was observed in the eDNA or protein expression level of Bcl-2 in the septum (Fig. 2B, P=0.95; Fig. 2C, P=0.60, respectively). Immunohistochemistry of Bcl-2 was performed on the cardiac tissues (Fig. 3A) and the staining was quantified by the intensity of cytoplasmic staining and percentage of expression. Each analysis indicated that the expression of Bcl-2 was increased in the apex, and the left and right ventricle
Expression of miRNAs is differentially regulated in cardiac tissues from patients with DCM. The expression levels of miR-21, and members of the miR-29 and miR-133 families were examined in the apex, septum, and left and right ventricles using RT-qPCR. miR-21 was significantly upregulated in the apex, and the left and right ventricles of patients with DCM when compared with the normal group (Fig. 4A; P<0.05, P<0.01, P<0.05, respectively). In these locations, the expression levels of miR-29a, miR-29b, miR-29c, miR-133a and miR-133b were significantly downregulated in comparison with the normal group (Fig. 4B-F; miR-29a: P<0.05, P<0.01, P<0.01, respectively; miR-29b: P<0.05, P<0.05, P<0.05, respectively; miR-29c: P<0.05, P<0.05, P<0.05, respectively; miR-133a: P<0.05, P<0.01, P<0.05, respectively; miR-133b: P<0.01, P<0.01, P<0.05, respectively). In the septum, no significant difference was identified for the expression of all miRNAs between normal and DCM groups (Fig. 4A-F; miR-21, P=0.86; miR-29a, P=0.09; miR-29b, P=0.59; miR-29c, P=0.55; miR-133a, P=0.99; miR-133b, P=0.96). Thus, the expression of miRNAs is differentially regulated in DCM.

Discussion

DCM is associated with significant morbidity and premature mortality (23). The mechanisms of DCM pathogenesis remain unclear. In the present study, fibrosis and apoptosis in cardiac tissues of healthy donors and patients with DCM were examined. It was demonstrated that the expression of Bcl-2 was upregulated in the apex, and the left and right ventricles of the heart in patients with DCM. Furthermore, these results demonstrated that miRNAs are differentially regulated in the cardiac tissues of DCM patients, which are associated with the expression of Bcl-2. Taken together, the present study suggested that dysregulation of Bcl-2 and specific miRNAs may involve in the pathogenesis of DCM.

Cardiovascular fibrosis alters myocardial stiffness and leads to ventricular dysfunction (5). Biopsy studies have demonstrated that DCM is associated with diffuse myocardial fibrosis in the ventricles (24). Unverferth et al (25) observed extensive left ventricle tissue fibrosis in patients with DCM. In the present study, it was identified that fibrosis, which is calculated by collagen content, significantly increased in the apex, and the left and right ventricles of patients with DCM when compared with the normal group. In these locations of the heart, an elevated number of TUNEL-positive cells were observed in the tissues from patients with DCM. Based on the observation of dead myocytes and replacement fibrosis, myocardial cell loss was likely attributed to tissue necrosis. However, apoptosis also occurs in human hearts under various pathological conditions, including acute myocardial infarction, arrhythmogenic right ventricular dysplasia and restenosis (4,26,27). These results support that apoptosis serves a critical role in DCM. No significant change was demonstrated in fibrosis and apoptosis in the septum, suggesting that septum may be affected in a later stage compared with the ventricles and apex.
Bcl-2 is one of the primary regulators of apoptosis and the Bcl-2 proto-oncogene suppresses apoptosis (28). Using immunochemistry, western blotting and RT-qPCR, significant increases in the expression of Bcl-2 were observed in the apex, and the left and right ventricles in patients with DCM compared with the normal donors. These locations also
demonstrated intensive fibrosis and apoptosis, suggesting that upregulation of Bcl-2 may be associated with cardiac fibrosis and apoptosis. Upregulation of this antiapoptotic protein raises the apoptotic threshold, which increases apoptosis resistance under cellular abnormalities. Although the detailed mechanisms of Bcl-2 protein function remain to be fully understood, it binds to proapoptotic proteins and inhibits channels crossing mitochondria (29). Since apoptosis is responsible for the progression of heart failure and the chronic loss of ventricular function, the increased expression of Bcl-2 may implicate the presence of a compensatory mechanism in patients with DCM.

miRNAs are endogenous, non-coding, 21-23 nucleotide RNAs that regulate gene expression by binding to the 3'untranslated region (3'UTR) of the target gene mRNAs to repress translation or induce mRNA cleavage. Increasing evidence suggests that certain miRNAs contribute to cardiac fibrosis (30). The present study identified that six miRNAs (miR-21; miR-29a, miR-29b and miR-29c; miR-133a and miR-133b) were significantly associated with the pathogenesis of DCM. miR-21 has been increasingly drawing the interests of cardiovascular research. Thum et al (31) demonstrated that upregulated expression of miR-21 enhances the signal transduction of the EPK-MAPK pathway, thereby leading to the proliferation of fibroblasts and cardiac fibrosis. In addition, silencing of the miR-21 gene inhibits the transduction of EPK-MAPK signals, thus inhibiting the fibrosis and improving cardiac functions (31). In a mouse model of ischemia-reperfusion, miR-21 regulates MMP-2 expression via a PTEN pathway during cardiac fibrosis and remodeling (32). This previous study revealed that the expression of miR-21 was higher in highly fibrotic sections of DCM tissue, which is consistent with its role in fibrosis.

miR-29 and miR-133 are also positive regulators of fibrosis (32-35). The miR-29 family of miRNAs has been demonstrated to target gene transcripts that encode ECM proteins involved in fibrotic responses, including different collagen isoforms (COL1A1, COL1A2, and COL3A1), fibrillin-1 and elastin (33). Downregulation of miR-29 induces the expression of collagens, whereas overexpression of miR-29 in fibroblasts reduces collagen expression (33). miR-29 acts as a positive regulator of fibrosis and represents a potential therapeutic target. Additionally, miR-133 is a novel molecular player in the heart. Decreased miR-133 in myocytes induces an upregulation of TGF-β1 and TGF-βRII proteins, which leads to enhanced collagen production and fibrosis generation (34). In addition, miR-133 positively regulates the β1AR pathway at multiple levels, which causes pathological cardiac hypertrophy and fibrosis (35,36). In the present study, the expression levels of miR-29 and miR-133 family members were lower in DCM tissues compared with in the corresponding locations in normal heart tissue. This suggested that mechanisms of negative regulation on fibrosis through miRNAs may exist during the progression of DCM.

In conclusion, extensive myocardial fibrosis and apoptosis in human DCM cardiac tissues was observed. The expression of the apoptosis regulator Bcl-2 was increased in the apex, and the left and right ventricles in patients with DCM compared with that in normal controls. In these locations, miR-21 was upregulated, while members of miR-29 family and miR-133 family were downregulated. The present study suggested that miRNAs are differentially regulated during cardiac fibrosis, thus they may represent a novel class of therapeutic targets for DCM.

Acknowledgements

The present study was supported by grants from The Natural Science Foundation of Shandong Province (nos. Z2006C10 and ZR2013HM028) and The National Natural Science Foundation of China (no. 81370269). The authors are grateful for the support from Shandong Taishan Scholarship awarded to Ju Liu.

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