MicroRNA-186-5p is expressed highly in ethanol-induced cardiomyocytes and regulates apoptosis via the target gene XIAP

YE LIU[1,2] and BO YU[1]

[1]Department of Cardiology, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001;
[2]Electrocardial Center, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning 121000, P.R. China

Received June 22, 2018; Accepted February 11, 2019

DOI: 10.3892/mmr.2019.9953

Abstract. Ethanol has a toxic effect on the heart, resulting in cardiomyocyte damage. Long-term high intake of ethanol leads to a non-ischemic dilated cardiomyopathy termed alcoholic cardiomyopathy (ACM). However, the pathogenesis of alcoholic cardiomyopathy remains unclear. The apoptosis of cardiomyocytes serves an important role in the pathogenesis of ACM. X-linked inhibitor of apoptosis protein (XIAP) is an important anti-apoptotic protein in human tissue cells. To the best of our knowledge, no studies have reported on its function in ethanol-induced cardiomyopathy. Previous works have screened the ACM-associated differentially expressed microRNAs (miRs), including miR-186-5p and miR-488-3p. TargetScan bioinformatics software was used to predict 949 target genes associated with miR-186-5p, and XIAP was demonstrated to be a target of miR-186-5p. The present study firstly analyzed the levels of apoptosis in ethanol-treated cardiomyocytes using flow cytometry. Alterations in the expression levels of miR-186-5p and XIAP were subsequently evaluated in ethanol-treated AC16 cardiomyocytes to assess the specific molecular mechanisms of ethanol-induced cardiomyocyte apoptosis. The levels of apoptosis in AC16 cardiomyocytes increased following ethanol treatment, and further increased with the rise in concentration and action time of ethanol. The expression levels of miR-186-5p were upregulated, and the expression levels of XIAP were downregulated in ethanol-treated cardiomyocytes. miR-186-5p may regulate ethanol-induced apoptosis in cardiomyocytes using XIAP as the direct target gene. This study provides a novel therapeutic target for the prevention and treatment of ACM.

Correspondence to: Professor Bo Yu, Department of Cardiology, The First Affiliated Hospital of China Medical University, 155 Nanjing North Street, Heping, Shenyang, Liaoning 110001, P.R. China
E-mail: ybdyybdy@163.com

Key words: cardiomyocyte, apoptosis, alcoholic cardiomyopathy, microRNA-186, X-linked inhibitor of apoptosis protein

Introduction

Ethanol has a toxic effect on the heart, resulting in cardiomyocyte damage. Long-term high intake of ethanol leads to a non-ischemic dilated cardiomyopathy termed alcoholic cardiomyopathy (ACM) (1), which is characterized by cardiac hypertrophy and compensatory systolic dysfunction (2). The apoptosis of cardiomyocytes serves an important role in the pathogenesis of ACM. X-linked inhibitor of apoptosis protein (XIAP) is a member of the inhibitor of apoptosis proteins (IAP) family and is the most potent known IAP protein in human tissues (3-5). MicroRNAs (miRs) are small RNA molecules whose length is 21-23 nucleotides. It is suggested that abnormalities in miR levels serve an important role in cardiac hypertrophy, pathological remodeling and the occurrence of heart failure (6). In response to ethanol-induced cardiomyopathy, another study revealed abnormalities in the expression of multiple miRs in hypertrophic myocardial tissue, including miR-133a, miR-125 and miR-195 (7), suggesting that ACM may be associated with the abnormal expression of certain miRs.

A previous study assessed ACM-associated miRs that are differentially expressed, which include miR-186-5p and miR-488-3p among others. TargetScan bioinformatics software predicted miR-186-5p-associated target genes (949 in total) and demonstrated that XIAP is a target gene of miR-186-5p. The study demonstrated that miR-186 binds to the XIAP 3' untranslated region (UTR) and decreases its expression, thus regulating the expression levels of downstream target proteins including caspase 3, BCL2-associated agonist of cell death, cyclin D1 and microtubule affinity regulating kinase 2 (8). XIAP may therefore be an important anti-apoptotic protein in human tissues. However, no studies have reported on its function in ethanol-induced cardiomyopathy, to the best of our knowledge.

In this experiment, the levels of apoptosis in ethanol-treated cardiomyocytes were first analyzed using flow cytometry. Modifications of the expression levels of miR-186-5p and XIAP were detected in ethanol-treated human AC16 cardiomyocytes, which were derived from left ventricular cells (9). These results elucidated the specific molecular mechanisms of ethanol-induced cardiomyocyte apoptosis.

Materials and methods

Materials. AC16 human cardiomyocytes, derived from left ventricular cardiomyocytes, were supplied by Guangzhou Shenglei Biological Technology Company (Guangzhou, China) and stored in the central laboratory of the First Affiliated Hospital of China Medical University (Liaoning, China). Anhydrous ethanol, which is the highly purified ethanol-water solution with an ethanol concentration of 99.5%, was obtained from Guoyao Group Industry Co., Ltd. (Beijing, China). F12/Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The MTT kit and RNA extraction reagent TRIzol® were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The reverse transcriptase reagent was obtained from Takara (Takara Bio, Inc., Otsu, Japan), Dharma FECT transfection reagent was obtained from Dharmacon (GE Healthcare Dharmacon, Inc., Lafayette, CO, USA), and the luciferase reporter gene plasmid vector (pmirGLO) was purchased from Shanghai gemma pharmaceutical technology Co., Ltd. miR-186-5p mimic/inhibitor was supplied by Guangzhou RiboBio Co., Ltd. (Guangzhou, China), miR-186 primer was supplied by Shanghai Bio-biology Company (Shanghai, China). The XIAP plasmid, the quantitative polymerase chain reaction (qPCR) reagent SYBR® Master Mix, Lipofectamine® 3000, primary antibodies against Actin (cat. no. 3700) and XIAP (cat. no. 2042), and enhanced chemiluminescence (ECL) color kits were all obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA), the luciferase reporter gene kit was supplied by Promega Corporation (Madison, WI, USA), and the Annexin V- fluorescein isothiocyanate (FITC)/Phycoerythrin (PE) Apoptosis Detection kit was supplied by BD Pharmingen (BD Biosciences, San Jose, CA, USA).

AC16 myocardial cell culture and maintenance. The AC16 myocardial cells were cultured at 37˚C in an incubator (Sanyo MCO-18AIC carbon dioxide incubator; Sanyo, Osaka, Japanese) containing 5% CO_2 to a density of ~90%, and the cells were rinsed with PBS. A solution of 0.25% trypsin in PBS was used to digest the cells and to examine the cell state. When the cells became round and bright, the cells were detached from the wall of the culture bottle using a dropper, and 5 ml DMEM was added to terminate the reaction. The cell mixture was collected in 15 ml sterile test tubes using a dropper. The solution was centrifuged at 1,000 x g and room temperature for 5 min. The supernatant was discarded, and the cells were counted. The cell culture bottles were placed under the microscope to observe cell morphology and density, and the best results were achieved when cell density reached 80-90%.

Flow cytometry. Previous studies have demonstrated that abnormal mitochondrial structures in cardiomyocytes may be observed in patients or animal models with alcoholic cardiomyopathy; these manifest as a reduction in the number of mitochondria, swelling of the mitochondria, along with disorganization and reduction or elimination of the cristae (10,11). Based on our preliminary experiments, 200, 400 and 800 mmol/l ethanol was applied to cardiomyocytes for different durations (24 or 48 h) in order to prepare the smears for electron microscopy. The ultrastructures of AC16 cardiomyocytes were observed under electron microscopy (Olympus CX41; Olympus Corporation, Tokyo, Japan) and compared with the characteristics of human cardiomyocytes of ACM from the literature, with the goal of determining the ideal culture time required for model establishment. The mitochondrial structures of cardiomyocytes from patients with ACM and animal models were observed under the electron microscope, and were revealed to be abnormal, which showed that the number of mitochondria decreased markedly, the volume of mitochondria increased, the structures of the mitochondrial ridge were disordered, and sometimes the structure of mitochondrial ridge disappeared along with vacuolar changes (12,13). As a result, it was determined that the effects of 200 and 800 mmol/l on cardiomyocytes for 24 and 48 h were suitable for this experiment.

During the logarithmic growth phase, the cells were incubated in 6-well culture plates. When the cell density reached 60-70%, ethanol was added to the culture plates. The experiment was divided into 6 groups: 0 mmol/l, 24 h group and 48 h group; 200 mmol/l, 24 h group and 48 h group; and 800 mmol/l, 24 h group and 48 h group. The cells were continuously maintained at 37˚C in a 5% CO_2 incubator (Sanyo MCO-18AIC carbon dioxide incubator; Sanyo). Following treatment, the incubated plates were removed from the incubator, and cells extracted using 0.25% trypsin in PBS. Following centrifugation (1,000 x g at room temperature for 5 min) as aforementioned, the supernatant was removed. Following washing, 100 μl of 1X PLB binding buffer (Cell Signaling Technology, Inc.) was added to each tube, and the cells were flicked gently. Subsequently, 3 μl Annexin V-FITC/PI was added to each tube, which where flicked gently again and allowed to stand for 15 min under dark conditions. Approximately 200 μl of 1X binding buffer was added to each tube, and flow cytometry (FlowJo 7.6.2 Software; FlowJo LLC, Ashland, OR, USA) was used to detect and measure the levels of apoptosis.

Fluorescence detection of target gene by dual-luciferase reporter gene. Using bioinformatics software (TargetScan7.1; www.targetscan.org/vert_71/docs/help.html; and miRanda database; www.mirbase.org/), the gene binding sequence of miR-186 was predicted to regulate XIAP, and the gene sequences of the XIAP 3'-UTR and the mutated (mut) XIAP 3'-UTR were designed and synthesized. The two types of synthetic gene fragment were cloned into luciferase reporter carriers of the pmirGLO to construct the wild-type carrier (pmirGLO-wt-XIAP-3'-UTR) and the mutant carrier (pmirGLO-mut-XIAP-3'-UTR). The sequence information were as follows: mut XIAP 3'-UTR,5'-UCAUCUGGAUUUUUUUAAGAAAU-3', pmirGLO-wt-XIAP-3'-UTR,5'-UCAUCUGGAUUUUUUAUUCUUUU-3' and pmirGLO-mut-XIAP-3'-UTR, 5'-UCAUCUGGAUUUUUUUAAGAAAU-3'.

The two recombinant carrier plasmids were co-transfected into AC16 cardiomyocytes with miR-186 mimic (1.2 μg) or miR-186-negative control (mimic control; 1.2 μg) using a transfection reagent Lipofectamine® 3000, respectively. The sequence information for the miR-186-5p mimics was as follows: Forward, 5'-GCGCTAAGGCACGCGGT-3' and reverse, 5'-CAGTGCAGGGTCCGAGGT-3'.

The transfections of the miR and pmirGlo into the cardiomyocyte culture was processed in groups of four as follows: Co-transfection of miR-186-5p mimic control and pmirGLO-wt-XIAP-3'-UTR; co-transfection of miR-186-5p mimic and pmirGLO-wt-XIAP-3'-UTR; co-transfection of miR-186-5p mimic control and pmirGLO-mut-XIAP-3'-UTR; co-transfection of miR-186-5p mimic and pmirGLO-mut-XIAP-3'-UTR. Luciferase activity was measured 48 h post-transfection using a luciferase assay kit (Shanghai Gemma Pharmaceutical Technology Co., Ltd., Shanghai, China) and normalized to *Renilla* luciferase activity.

Cell transfection. When the cells had been digested, they were evenly inoculated into 6-well culture plates and continuously cultivated. When the cultures grew to a unilaminar density of 70%, they were starved for 2 h with serum-free medium. The transfection mixture was composed of ~100 μl serum-free medium, 4.5 μl transfection reagent (Lipofectamine® 3000) and 1.2 μg plasmid, which were incubated for 15 min. This mixture was added to the wells by dripping with a micropipette, and the plates were incubated (37˚C, 5% CO_2) for 5 h. The transfection medium was removed, and the complete medium containing 10% fetal bovine serum (Cell Signaling Technology, Inc.) was added. Cells were maintained in 37˚C constant temperature incubator (5% CO_2 for 24 h) for the follow-up tests.

Western blotting. Protein lysis was used to extract proteins from cells using the radioimmunoprecipitation assay buffer with 1 mmol/l phenylmethylsulfonyl fluoride (Cell Signaling Technology, Inc.) on ice and quantified using the Bradford method. Protein samples were prepared using SDS-PAGE protein loading buffer, and 20 μg protein was added to each gel lane. The gel concentrations used in this experiment were 5% for the concentrated gel, and 10% for the separation gel. The loading volume of the sample fluid in each well was 15 μl. Finally, 5 μl protein standard was added to each of the left- and right-side sample wells. The electrodes were connected, and the voltage was adjusted to 80 V. When the leading edge of the protein marker was transferred from the concentrated gel to the separating gel, the voltage was adjusted to 120 V. Electrophoresis was performed with constant voltage. The segregated condition of the protein marker was examined, and electrophoresis was stopped; the sample was placed on ice and 100 V was applied, and it was transferred a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA) for 90 min. The membrane was blocked with 1.5% bovine serum albumin (BSA; Cell Signaling Technology, Inc.) at room temperature for 2 h. Primary anti-XIAP (1:1,000) and anti-Actin (1:400) were diluted with 1.5% BSA and incubated overnight at 4˚C. The membrane was incubated with the a horseradish peroxidase-conjugated secondary antibody (cat. no. 4410; Cell Signaling Technology, Inc.; 1:10,000) at 37˚C for 2 h and then washed with Tris Buffered Saline-Tween (0.05%; Cell Signaling Technology, Inc.) using a shaking bed 3 times (5 min/wash). The ECL kit was used to develop the membrane, and the results were analyzed using a BioImaging System (ChemiDoc™; Bio-Rad, Laboratories, Inc., Hercules, CA, USA) to display fluorescence imaging of antigen and antibody responses.

Reverse transcription-qPCR (RT-qPCR). PCR was performed using the TRIzol® one-step method to extract total RNA from cardiomyocytes. In brief, 1 μg total RNA was used to synthesize cDNA in 20 μl of RT system; 0.5 μl cDNA and the target gene upstream and downstream primers were added. PCR amplification was performed in a 20 μl reaction volume. The thermocycling conditions used were 45 cycles of 37˚C for 30 min and 85˚C for 5 min. The primer sequences used were as follows: miR-186 forward, 5'-GCG CTAAGGCACGCGGT-3', and reverse, 5'-CAGTGCAGG GTCCGAGGT-3'; XIAP forward, 5'-GGCACGAGCAGG GTTTCTT-3' and reverse, 5'-TCCAACTGCTGAGTCTCC ATATTG-3'; β-actin forward, 5'-ATAGCACAGCCTGGA TAGCAACGTAC-3' and reverse, 5'-CACCTTCTACAATGA GCTGCGTGTG-3'. The amplification and dissolution curve were confirmed. Relative expression of genes was measured using the $2^{-\Delta\Delta cq}$ method (14).

Statistical analysis. The software used for statistical analysis of the data was SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data were presented as the mean ± standard deviation of 3 repeated experiments and analyzed using one-way analysis of variance method, which was used for the comparison of multiple groups. Dunnett's test was used for the post hoc comparisons between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Ethanol-induced apoptosis in AC16 cardiomyocytes. In the present study, the mitochondrial structures of ethanol-treated cardiomyocytes under electron microscopy revealed that the number of mitochondria decreased markedly, the volume of mitochondria increased, the structures of the mitochondrial ridge were disordered, and sometimes the structure of mitochondrial ridge disappeared along with vacuolar changes (data not shown); these results were consistent with those of previous studies (12,13). AC16 cardiomyocytes were exposed to different ethanol concentrations (0, 200 and 800 mmol/l) for different durations (24 and 48 h), and analyzed the changes in the levels of apoptosis for each ethanol concentration and duration of time using flow cytometry. As presented in Fig. 1, following ethanol treatment, the levels of apoptosis in AC16 cardiomyocytes increased compared with the control group, which was dependent on ethanol concentration and duration. The levels of apoptosis increased with the increase in ethanol concentration and duration of action.

XIAP is a direct target gene of miR-186-5p. The bioinformatics software Target Scan7.1 database predicted that the 332-338 base position of the 3'-UTR of the XIAP gene mRNA is a possible binding site for miR-186-5p. Moreover, the complementary binding sequence between miR-186-5p and the 3'-UTR of the XIAP gene mRNA was also identified in the miRanda database. Compared with the miR-186 complementary binding sequence, the binding stability between miR-186-5p and XIAP is better, thus miR-186-5p may downregulate the expression of XIAP.

To further confirm whether XIAP is a direct target gene for miR-186-5p, the dual-luciferase reporter gene assay

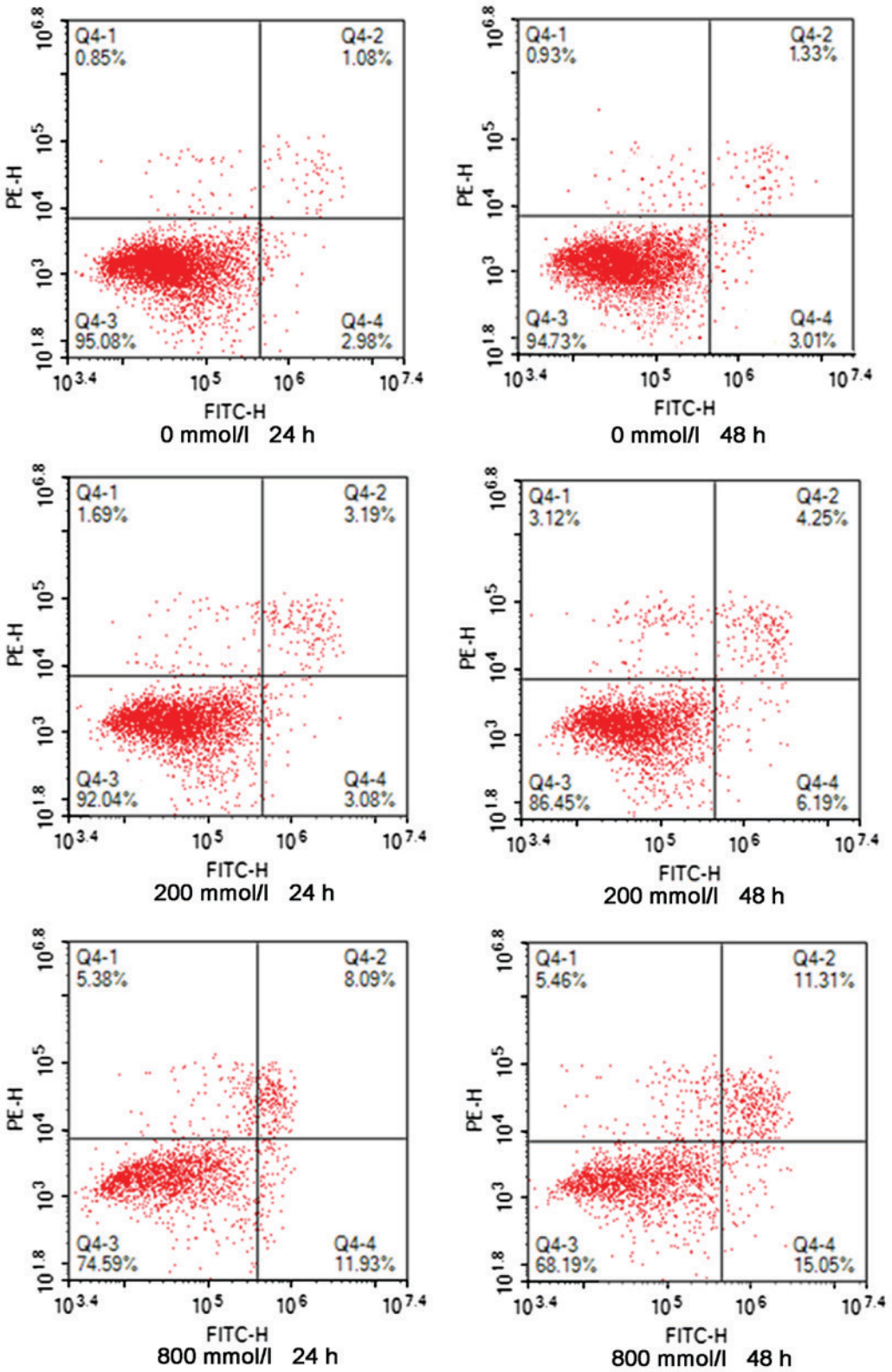

Figure 1. Flow cytometry analyses of the apoptosis levels following ethanol treatment. Q4-1, necrotic cells and mechanically injured cells; Q4-2, late-stage apoptotic cells; Q4-3, living cells; Q4-4, early-stage apoptotic cells. The 0 mmol/l (24 or 48 h) groups were the control group, while 200 mmol/l (24 or 48 h) and 800 mmol/l (24 or 48 h) groups were the experimental groups. The results demonstrated that the level of apoptosis in AC16 cardiomyocytes increased following ethanol treatment compared with the control groups, and this was dependent on ethanol concentration and length of ethanol exposure. PE, phycoerythrin.

and RT-qPCR for target gene verification were performed. Firstly, a wild-type pmirGLO-wt-XIAP-3'-UTR and a mutant pmirGLO-wt-XIAP-3'-UTR were constructed and transfected into AC16 cardiomyocytes along with either a miR-186-5p mimic control or miR-186-5p mimic (Fig. 2). The results indicated that fluorescence intensity in the group with the wild-type pmirGLO-wt-XIAP-3'-UTR combined with miR-186-5p mimic significantly decreased ($P<0.05$) compared with the other

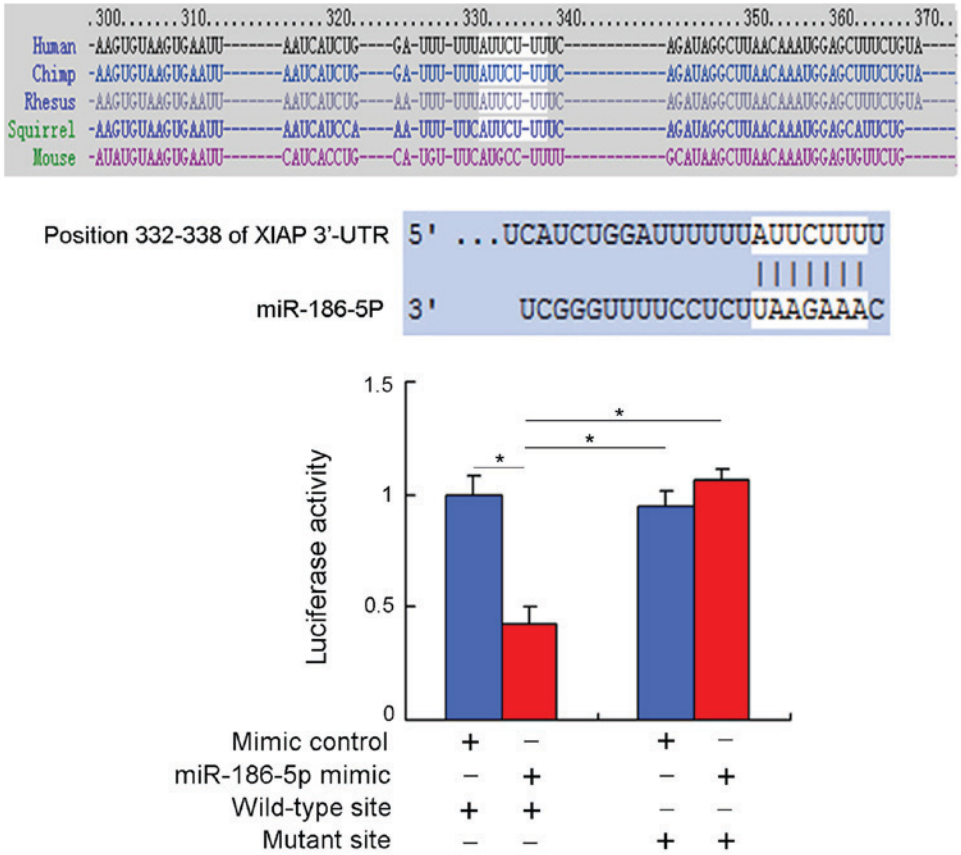

Figure 2. Luciferase reporter gene experiment verifying that XIAP is a direct target of miR-186-5p. The results indicated that fluorescence intensity in the wild-type pmirGLO-wt-XIAP-3'-UTR combined with miR-186-5p mimic group significantly decreased compared with the other three groups and no significant difference were observed in fluorescence intensity between the other three groups. *P<0.05, as indicated. XIAP, X-linked inhibitor of apoptosis protein; UTR, untranslated region; miR, microRNA.

three groups, and no significant differences were observed in fluorescence intensity between the other three groups (P>0.05). Therefore there may be a direct binding site of miR-186 on the 3'-UTR of XIAP and miR-186 may inhibit the expression of XIAP. Therefore, XIAP is a direct target of miR-186.

Ethanol intake upregulates the expression of miR-186-5p in AC16 cardiomyocytes. AC16 cardiomyocytes were treated with ethanol, and the expression levels of miR-186-5p in the cells were detected by RT-qPCR. Expression levels of miR-186-5p differed between the ethanol treatment and control groups (P<0.05), increasing with the higher ethanol concentrations (Fig. 3A) and with longer treatment durations (Fig. 3B). Therefore, increases in ethanol concentration and length of exposure led to the upregulation of miR-186 expression in AC16 cardiomyocytes.

Ethanol intake downregulates the expression levels of the apoptosis-associated protein XIAP in AC16 cardiomyocytes. In order to ascertain the specific mechanism of ethanol-induced apoptosis in cardiomyocytes, the levels of apoptosis-associated markers were detected in AC16 cardiomyocytes treated with ethanol by western blotting. The results (Fig. 4A and B) demonstrated that ethanol treatment reduced the protein levels of XIAP in AC16 cardiomyocytes, and the decrease was dependent on ethanol concentration and duration of exposure.

Similarly, the mRNA level of XIAP in ethanol-treated AC16 cardiomyocytes was detected by RT-qPCR. The results (Fig. 4C and D) confirmed that the expression levels of XIAP significantly decreased between ethanol treatment groups and control group (P<0.05), and that XIAP levels further reduced with the increase in ethanol concentration and duration of treatment. It is suggested that ethanol may downregulate the mRNA expression levels of XIAP in AC16 cardiomyocytes, and this downregulation is dependent on ethanol concentration and length of ethanol exposure.

Transfection of miR-186 mimic/inhibitor and XIAP upregulates the expression levels of miR-186 mimic and XIAP, and downregulates the expression levels of miR-186 inhibitor in AC16 cardiomyocytes. miR-186-5p mimic/inhibitor and XIAP vectors were transfected into AC16 cardiomyocytes through transient transfection. RT-qPCR and western blotting were used to detect the expression levels of miR-186-5p and XIAP in the cells. The results (Fig. 5) were as follows: The mRNA expression levels of miR-186-5p increased in cells following transfection with miR-186-5p mimic, and decreased in cells following transfection with miR-186-5p inhibitor (Fig. 5A). The differences were significant compared with the control group (where the miR mimic control served as the control group; P<0.05); the mRNA expression levels (Fig. 5B) and protein levels (Fig. 5C) of XIAP increased in the cells after transfection with the XIAP vector. The differences in mRNA expression

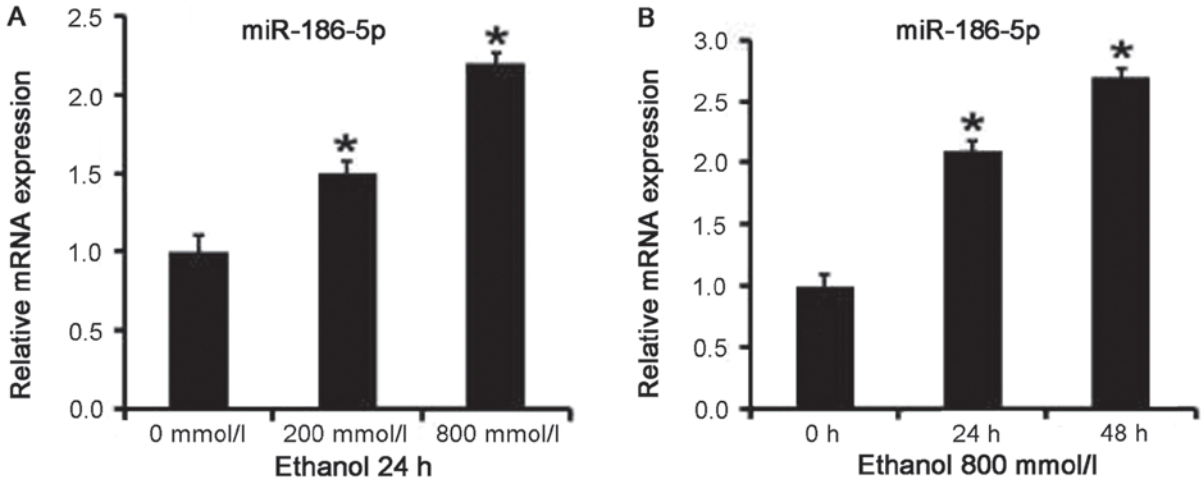

Figure 3. Reverse-transcription quantitative polymerase chain reaction detects the mRNA expression levels of miR-186-5p in ethanol-treated AC16 cardiomyocytes. (A) The mRNA expression levels of miR-186-5p in ethanol-treated AC16 cardiomyocytes after 24 h intervention with different concentrations of ethanol. The 0 mmol/l treatment was the control group, and 200 and 800 mmol/l were the experimental groups. The mRNA expression levels of miR-186-5p differed between the experimental groups and the control group, increasing with higher concentrations of ethanol. (B) The mRNA expression levels of miR-186-5p in 800 mmol/l ethanol-treated AC16 cardiomyocytes following different time interventions. The 0 h was the control group, and 24 and 48 h were the experimental groups. The mRNA expression levels of miR-186-5p also differed between each experimental group and the control, and increasing with longer exposures to ethanol. *P<0.05 vs. the respective control group. miR, microRNA.

were significant compared with the control group (P<0.05). Thus, transfections with either the miR-186 mimic/inhibitor or XIAP in AC16 cardiomyocytes were successful.

miR-186-5p may be involved in the ethanol-induced apoptosis of AC16 cardiomyocytes. In previous sections, it was demonstrated that ethanol treatment increased the apoptosis levels of AC16 cardiomyocytes and upregulated the expression levels of miR-186-5p. In order to investigate the role of miR-186-5p in the process of ethanol-induced apoptosis of cardiomyocytes, AC16 cardiomyocytes were treated with ethanol (800 mmol/l), and alterations in apoptosis levels were examined using flow cytometry. While miR-186-5p mimic was transfected into AC16 cardiomyocytes prior to ethanol treatment (800 mmol/l for 24 h), and the levels of apoptosis further increased. The results demonstrate that ethanol induced cardiomyocyte apoptosis and the levels of apoptosis further increased following transfection of miR-186-5p mimic (Fig. 6). Therefore, ethanol may induce cardiomyocyte apoptosis and miR-186-5p may further promote ethanol-induced cardiomyocyte apoptosis.

Upregulation of XIAP inhibits the ethanol-induced apoptosis of AC16 cardiomyocytes. In order to further examine the role of XIAP in ethanol-induced apoptosis in the heart tissue, AC16 cardiomyocytes were treated with ethanol (800 mmol/l), and the levels of apoptosis were detected via flow cytometry. Meanwhile, the XIAP plasmid was transfected into AC16 cardiomyocytes prior to ethanol treatment (800 mmol/l), and the alterations in apoptosis levels were detected via flow cytometry. The results were as follows: The apoptosis levels of ethanol-treated AC16 cardiomyocytes increased, but the apoptosis levels decreased following transfection with the XIAP plasmid (Fig. 7). Thus, ethanol may induce cardiomyocyte apoptosis and XIAP may partially reverse the ethanol-induced apoptosis of cardiomyocytes.

miR-186-5p regulates the expression of XIAP in AC16 cardiomyocytes. According to the aforementioned experiments, miR-186-5p and XIAP may serve a role in the apoptosis of ethanol-treated AC16 cardiomyocytes. The dual-luciferase reporter gene experiment detected that miR-186-5p may be directly combined with the 3'-UTR (332-338) of the XIAP mRNA and further inhibit the expression of XIAP directly. Therefore, it was hypothesized that XIAP may be a target gene for miR-186-5p and thus regulate apoptosis in AC16 cardiomyocytes. To confirm this hypothesis, miR-186-5p mimic was transfected into AC16 cardiomyocytes to upregulated its expression, and the protein and mRNA expression levels of XIAP in these cells was detected using western blotting and RT-qPCR, respectively.

The western blotting data demonstrated that AC16 cardiomyocyte transfection with miR186-5p mimic followed by treatment with ethanol (800 mmol/l, 24 h) upregulated the expression levels of miR-186-5p, causing a decrease in the protein levels of XIAP (Fig. 8A). In addition, AC16 cardiomyocytes were transfected with miR-186-5p inhibitor and treated with ethanol (800 mmol/l, 24 h), thereby downregulating the expression levels of miR-186-5p, and causing the protein expression levels of XIAP to increase (Fig. 8B).

Similarly, RT-qPCR was used to detect the mRNA expression levels of XIAP in the cells. The results were as follows: AC16 cardiomyocytes transfected with miR186-5p mimic were treated with ethanol (800 mmol/l, 24 h), which upregulated the expression levels of miR-186-5p, causing a significant decrease in the mRNA expression levels of XIAP (P<0.05; Fig. 8C); AC16 cardiomyocytes transfected with miR-186-5p inhibitor were treated with ethanol (800 mmol/l, 24 h), downregulating the expression levels of miR-186-5p, but causing a significant increase in the mRNA expression levels of XIAP (P<0.05; Fig. 8D). Therefore, miR-186-5p may decrease the expression levels of XIAP in ethanol-treated AC16 cardiomyocytes, and the introduction of the miR-186-5p inhibitor partially reversed

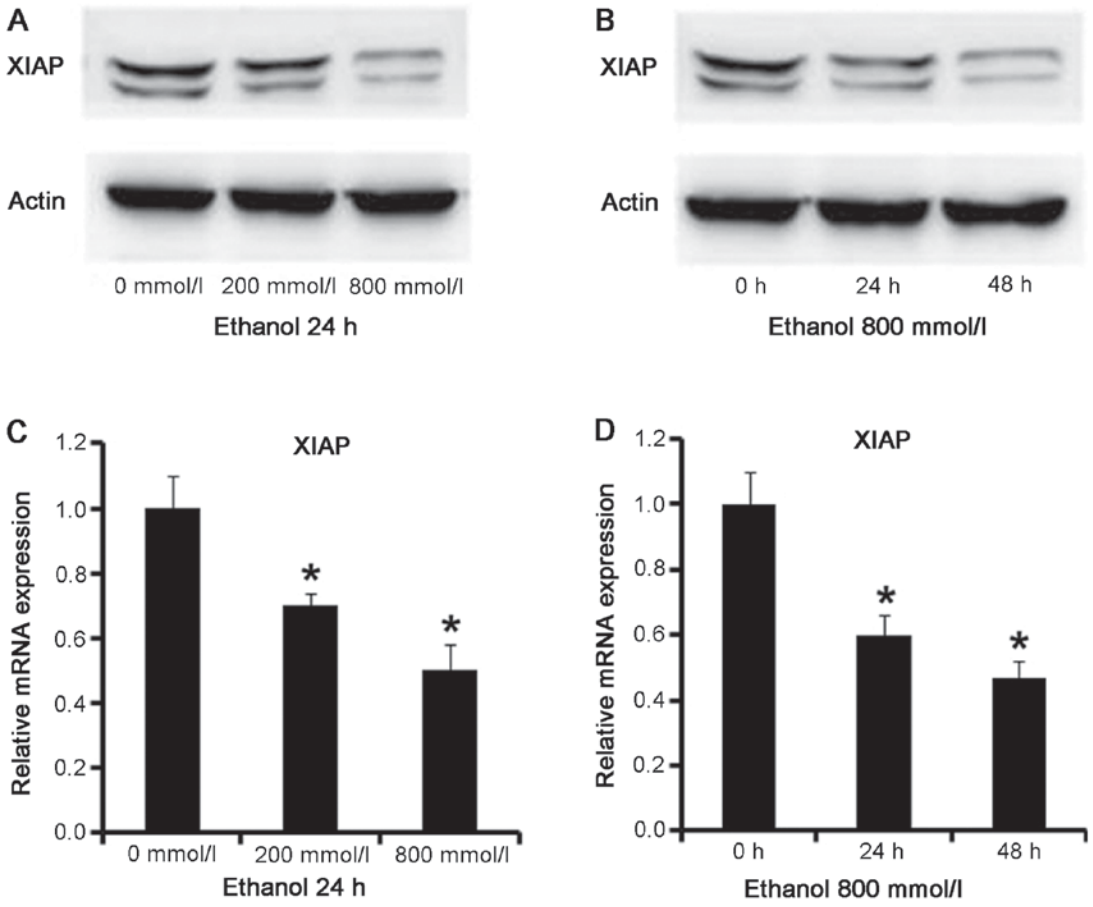

Figure 4. Western blotting and reverse-transcription quantitative polymerase chain reaction to detect the expression levels of XIAP in ethanol-treated AC16 cardiomyocytes. (A) Protein expression levels of XIAP in ethanol-treated AC16 cardiomyocytes after 24 h intervention with different concentrations of ethanol. The 0 mmol/l treatment was the control group, and the 200 and 800 mmol/l were the experimental groups. The protein levels of XIAP differed between the experimental groups and the control group, decreasing in the experimental groups. (B) Protein expression levels of XIAP in 800 mmol/l ethanol-treated AC16 cardiomyocytes following different time interventions. The 0 h treatment was the control group, and the 24 and 48 h groups were the experimental ones. The protein expression levels of XIAP differed between the experimental groups and the control group, decreasing following ethanol treatment. (C) mRNA expression levels of XIAP in ethanol-treated AC16 cardiomyocytes after 24 h intervention with different concentrations of ethanol, and (D) mRNA expression levels of XIAP in 800 mmol/l ethanol-treated cardiomyocytes after different time interventions. The 0 mmol/l group was the control group, and 200 and 800 mmol/l were the experimental groups. The expression levels of XIAP differed between the experimental groups and the control group, decreasing in the experimental groups with increasing ethanol concentrations and longer treatment times. *P<0.05 vs. the respective control group. XIAP, X-linked inhibitor of apoptosis protein; miR, microRNA.

this phenotype, resulting in higher expression levels of XIAP in ethanol-treated cardiomyocytes.

Discussion

ACM refers to myocardial lesions characterized by hypertrophy of the heart, arrhythmia, and congestive heart failure caused by long-term ingestion of large amounts of ethanol. A study demonstrated that drinking >90-120 g per day for 5-15 years may increase the risk of cardiac structural and functional modifications (15). Data have demonstrated that the number of people who succumb to excessive drinking is rising year by year (16). Therefore, more research is required to elucidate the pathogenesis of alcoholic cardiomyopathy to allow for better early diagnosis and treatment. However, the pathogenesis of ACM remained to be clarified.

Various studies have analyzed the pathogenesis of ACM, which includes cardiac toxicity induced by the principal metabolites of ethanol (17,18), apoptosis (19), mitochondrial dysfunction (20) and abnormal energy metabolism of cardiomyocytes, nutrient imbalance, ventricular remodeling (21,22), disorders of the neurohumoral system (23,24), abnormal cell signal transduction and immunological and inflammatory reactions (25). Studies have reported that ethanol-induced apoptosis serves a key role in the pathogenesis of ACM (26-29). A study on the mechanism of ethanol-induced cardiomyocyte apoptosis demonstrated that the levels of tumor necrosis factor-α, the apoptotic protein Bcl-2-associated X and caspase-3 are significantly increased in ACM (30). In the present study, AC16 cardiomyocytes were treated with ethanol and its effects on apoptosis analyzed. The results demonstrated that the levels of apoptosis increased following treatment with ethanol. With the prolongation of ethanol treatment time, the cardiomyocyte apoptosis increased. These results are consistent with previous studies (31-34).

In recent years, studies on miRs have focused on disease processes, including pathogenesis, development and prognosis. By the end of 2006, scientists were realizing that miRs serve important roles in the pathological development of various heart diseases, and important progress on myocardial

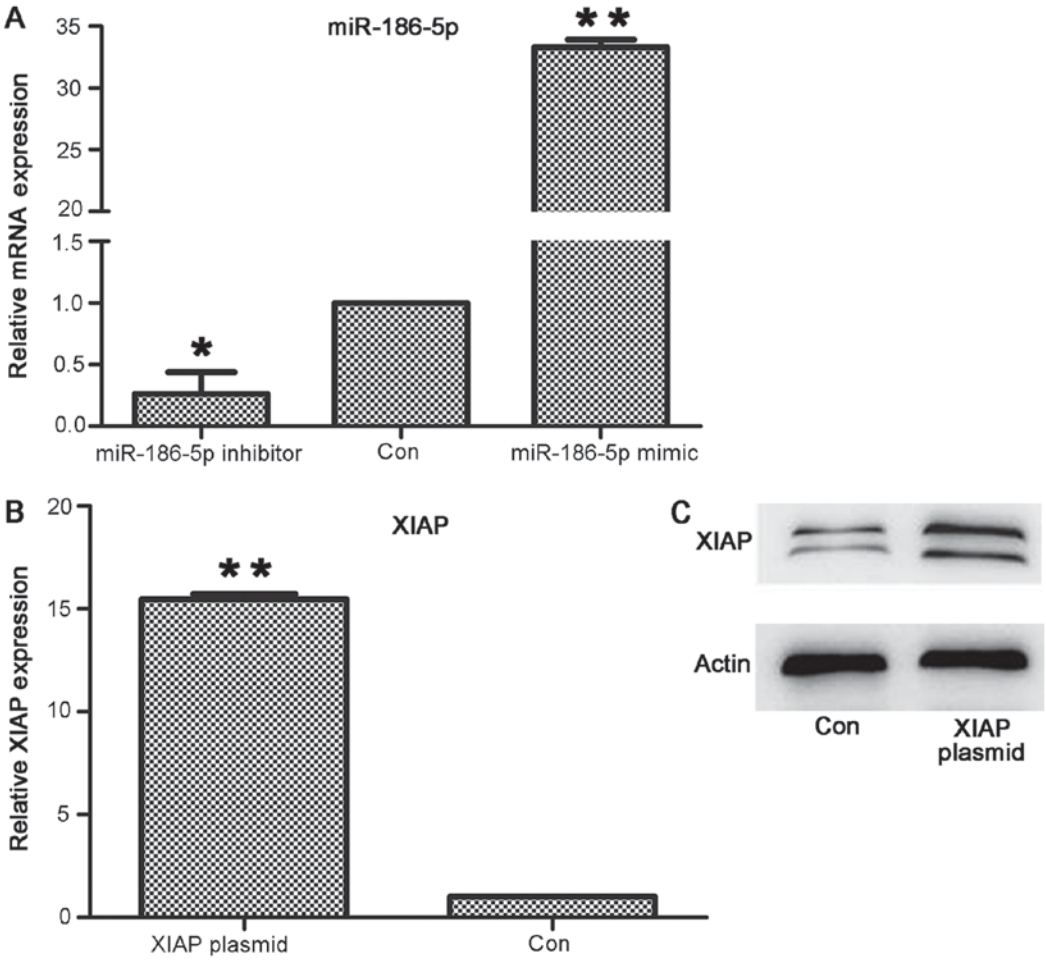

Figure 5. RT-qPCR and western blotting detect the expression levels of miR-186-5p or XIAP in cardiomyocytes following transfection with miR-186 mimic/inhibitor or XIAP plasmid. (A) RT-qPCR determined that the mRNA expression levels of miR-186-5p in AC16 cardiomyocytes increased following transfection with miR-186-5p mimic, while decreasing following transfection with miR-186-5p inhibitor. *P<0.05 and **P<0.01 vs. Con. (B) RT-qPCR was used to analyze the mRNA expression levels of XIAP in AC16 cardiomyocytes, demonstrating an increase following transfection with XIAP plasmid. **P<0.01 vs. Con. (C) Western blotting was used to assess the levels of XIAP protein in AC16 cardiomyocytes, once again illustrating an increase following transfection with the XIAP plasmid. RT-qPCR, reverse-transcription quantitative polymerase chain reaction; XIAP, X-linked inhibitor of apoptosis protein; miR, microRNA; Con, control.

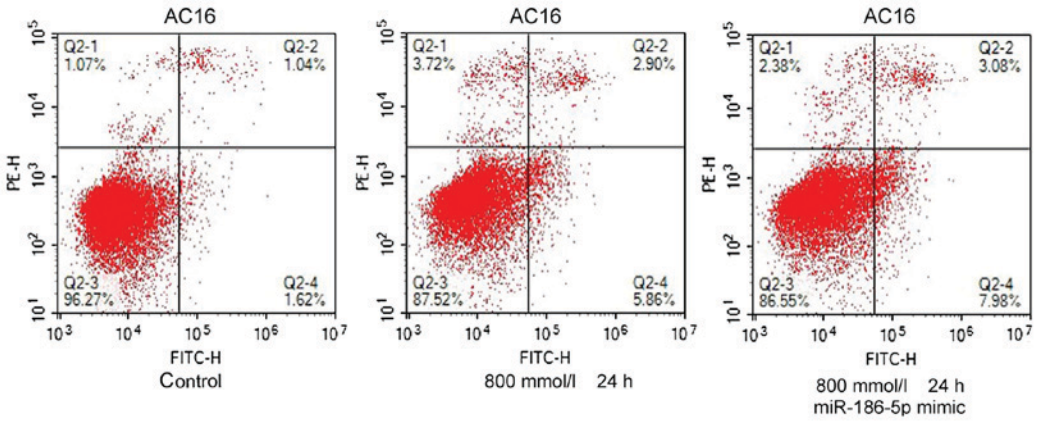

Figure 6. Transfecting miR-186-5p mimic affects the levels of ethanol-induced apoptosis of cardiomyocytes. Q2-1, necrotic cells and mechanically injured cells; Q2-2, late-stage apoptotic cells; Q2-3, living cells; Q2-4, early-stage apoptotic cells. The apoptosis levels of ethanol (800 mmol/l, 24 h)-treated AC16 cardiomyocytes increased, while the apoptosis levels further increased following transfection with the miR-186-5p mimic plasmid. miR, microRNA; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

hypertrophy, arrhythmia, heart failure and other diseases was achieved (35). This caused miRs to become an important research focus in the field of cardiovascular disease, at home and abroad. Notably, Zhang *et al* (36) investigated the effects of

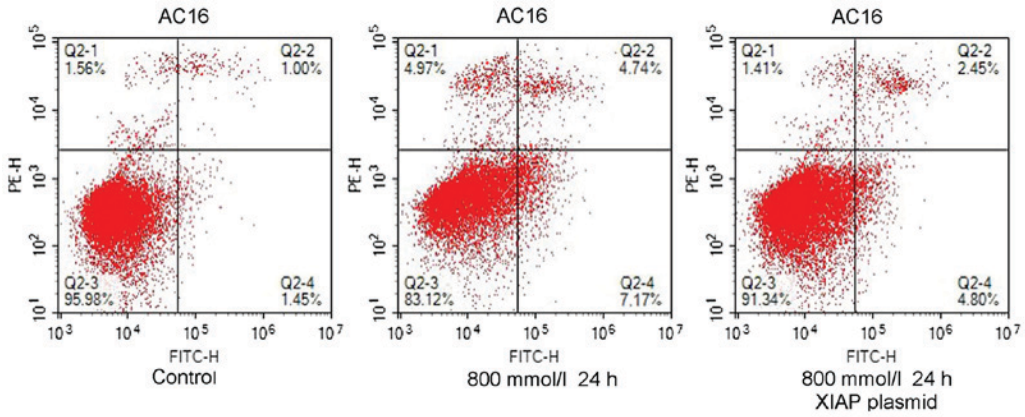

Figure 7. Transfecting the XIAP plasmid reduces the levels of ethanol-induced apoptosis of cardiomyocytes. Q2-1, necrotic cells and mechanically injured cells; Q2-2, late-stage apoptotic cells. Q2-3, living cells; Q2-4, early-stage apoptotic cells. The apoptosis levels of ethanol (800 mmol/l, 24 h)-treated AC16 cardiomyocytes increased, but the apoptosis levels decreased following transfection with the XIAP plasmid. XIAP, X-linked inhibitor of apoptosis protein; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

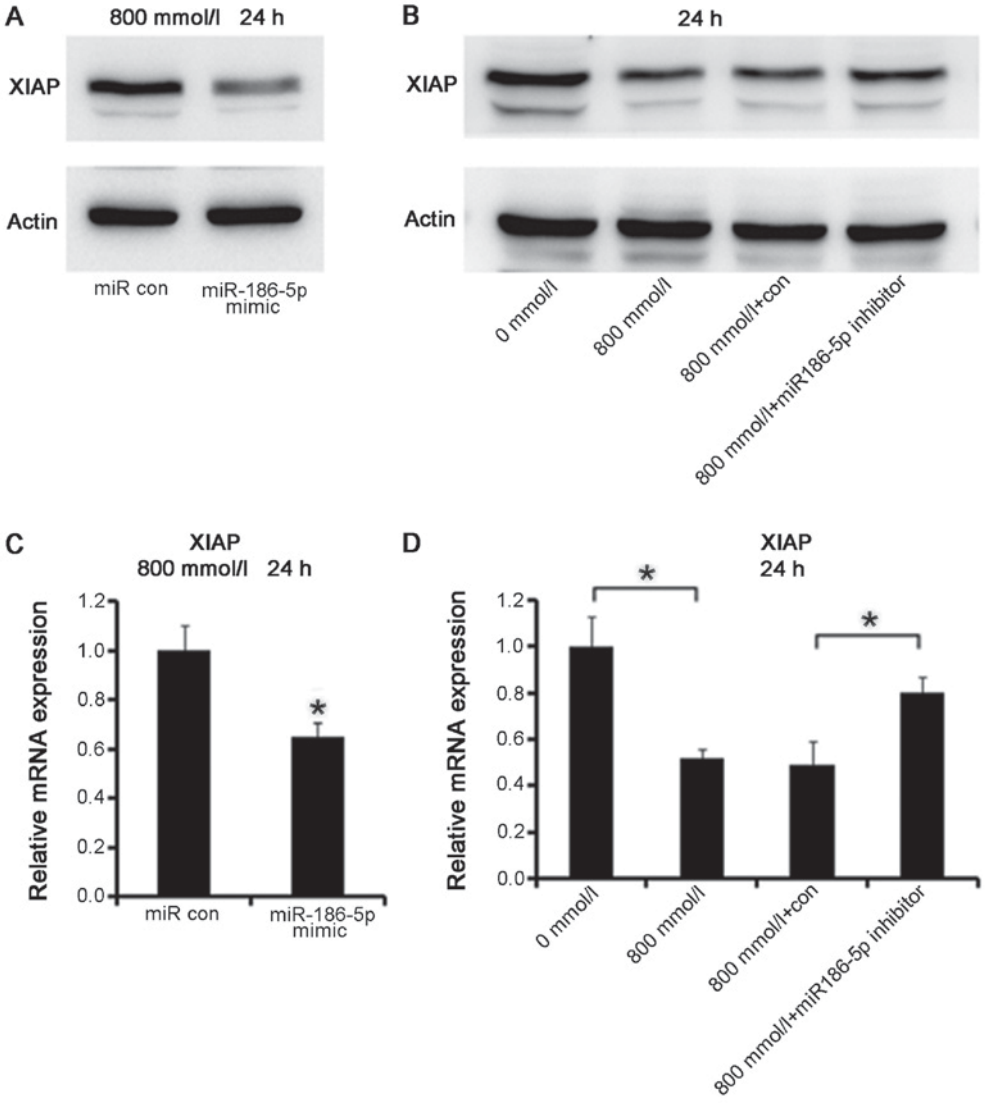

Figure 8. Western blotting and reverse-transcription quantitative polymerase chain reaction to detect the expression levels of XIAP in cardiomyocytes transfected with miR-186 mimic/inhibitor. (A) The protein expression levels of XIAP in ethanol (800 mmol/l, 24 h)-treated AC16 cardiomyocytes decreased following transfection with miR-186-5p mimic into cells. (B) The protein expression levels of XIAP in ethanol (800 mmol/l, 24 h)-treated AC16 cardiomyocytes increased following transfection with miR-186-5p inhibitor into cells. (C) The mRNA expression levels of XIAP in ethanol (800 mmol/l, 24 h)-treated AC16 cardiomyocytes decreased following transfection miR-186-5p mimic into cells. *P<0.05 vs. miR Con. (D) The mRNA expression levels of XIAP in ethanol (800 mmol/l, 24 h)-treated AC16 cardiomyocytes increased following transfection with miR-186-5p inhibitor into cells. *P<0.05, as indicated. XIAP, X-linked inhibitor of apoptosis protein; miR, microRNA; con, control.

miR-186 overexpression or inhibition on the apoptosis of A549 cells, and demonstrated that the significant downregulation of miR-186 expression was associated with curcumin-induced apoptosis. Based on these research findings, it may be determined that miR-186 has a complex association with apoptosis, and its varying roles in apoptosis may be associated with the regulation of different downstream signaling pathways or with the existence of different subtypes of miR-186.

The goal of this study was to investigate whether the expression of miR-186-5p is associated with alcohol-induced apoptosis of AC16 cardiomyocytes. In our previous experiment (Liu *et al*, unpublished data), the expression levels of partial miRs, such as miR-133a, miR-125, miR-195, miR-186-5p and miR-488-3p, in ethanol-treated cardiomyocytes were screened, and it was observed that the expression levels of miR-186-5p increased significantly. In the present study, transfecting miR-186-5p mimic into the ethanol-treated cardiomyocytes led to the upregulation of miR-186-5p, causing the level of apoptosis to further increase. Thus, it may be that miR-186-5p serves an important role in promoting the ethanol-induced apoptosis of cardiomyocytes.

XIAP is a member of the IAP family, and the most potent known IAP protein in human tissues (3-6). XIAP is able to inhibit apoptosis induced by viral infection (37) or by the overexpression of caspase family proteins (38). In the present study, the expression levels of XIAP in ethanol-treated AC16 cardiomyocytes were observed to be decreased. In order to further confirm whether XIAP serves a role in the process of ethanol-induced cardiomyocyte apoptosis, the expression levels of XIAP were upregulated via plasmid transfection. As a result, cardiomyocyte apoptosis was reduced following ethanol treatment and compared with untransfected ethanol-treated cardiomyocytes. Therefore, XIAP may inhibit alcohol-induced cardiomyocyte apoptosis.

Abnormal expression levels of miR-186-5p and XIAP were simultaneously detected in ethanol-treated cardiomyocytes. Moreover, through the dual-luciferase reporter gene experiment, the fluorescence intensity of the group of wild-type pmirGLO-wt-XIAP-3 'UTR in combination with miR-186-5p mimic was observed to be decreased compared with the other three groups, a finding which was substantiated by RT-qPCR. Thus, there may be a direct site on the 3'-UTR region of XIAP, which may allow interactions with miR-186-5p, and miR-186-5p may target XIAP to inhibit its expression. To verify this hypothesis, AC16 cardiomyocytes transfected with miR186-5p mimic were treated with ethanol (800 mmol/l, 24 h), which upregulated the expression levels of miR-186-5p, and observed that XIAP was downregulated at the protein and mRNA levels. However, XIAP was upregulated at the protein and gene levels in cardiomyocytes following transfection of miR-186-5p inhibitor and then treatment with ethanol (800 mmol/l, 24 h). Therefore, XIAP is a direct target gene of miR-186-5p, and miR-186-5p along with XIAP may control the process of ethanol-induced apoptosis in cardiomyocytes. The present study further elucidated the pathogenesis of ACM. A limitation of the present study is that a control experiment in non-myocardial cells is lacking. As this research work focused on the study of ACM, no non-myocardial cell lines were available. This will be improved upon in future experiments.

In conclusion, ethanol intake may have caused the apoptosis of AC16 cardiomyocytes, and the apoptosis levels were dependent on ethanol dose and duration of action. In terms of the specific mechanism of action, it was indicated that the expression levels of miR-186-5p were upregulated, and the expression levels of XIAP were downregulated in cardiomyocytes following ethanol treatment. XIAP may be a target gene of miR-186, and together may regulate the process of ethanol-induced apoptosis in cardiomyocytes. This study provides a novel therapeutic target for the prevention and treatment of ACM.

Acknowledgements

Not applicable.

Funding

This research was supported by The Natural Science Fund Guidance Plan of Liaoning Province (grant no. 20180550419).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YL performed the experiments, organized the data and wrote the article. BY conceived and designed the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Naimi TS, Nelson DE and Brewer RD: The intensity of binge alcohol consumption Among U.S. adults. Am J Prev Med 38: 201-207, 2010.
2. Steiner JL and Lang CH: Etiology of alcoholic cardiomyopathy: Mitochondria, oxidative stress and apoptosis. Int J Biochem Cell Biol 89: 125-135, 2017.
3. Edison N, Curtz Y, Paland N, Mamriev D, Chorubczyk N, Haviv-Reingewertz T, Kfir N, Morgenstern D, Kupervaser M, Kagan J, *et al*: Degradation of Bcl-2 by XIAP and ARTS promotes apoptosis. Cell Rep 21: 442-454, 2017.
4. Cesa LC, Shao H, Srinivasan SR, Tse E, Jain C, Zuidereg ERP, Southworth DR, Mapp AK and Gestwicki JE: X-linked inhibitor of apoptosis protein (XIAP) is a client of heat shock protein 70 (Hsp70) and a biomarker of its inhibition. J Biol Chem 293: 2370-2380, 2018.
5. Yang WZ, Zhou H and Yan Y: XIAP underlies apoptosis resistance of renal cell carcinoma cells. Mol Med Rep 17: 125-130, 2018.

6. Wang Z, Song J, Zhang L, Huang S, Bao L, Chen F and Zhao X: Increased expression of microRNA-378a-5p in acute ethanol exposure of rat cardiomyocytes. Cell Stress Chaperones 22: 245-252, 2017.
7. Jing L, Lin C, Lu Y, Huo P, Zhou L, Wang Y and Tian Y: Investigation of microRNA expression profiles associated with human alcoholic cardiomyopathy. Cardiology 30: 223-233, 2015.
8. Zheng J, Li XD, Wang P, Liu XB, Xue YX, Hu Y, Li Z, Li ZQ, Wang ZH and Liu YH: CRNDE affects the malignant biological characteristics of human glioma stem cells by negatively regulating miR-186. Oncotarget 6: 25339-25355, 2015.
9. Jiang J, Mo H, Liu C, Wu B, Wu Z, Li X, Li T, He S, Li S, You Q, *et al*: Inhibition of miR-186-5p contributes to high glucose-induced injury in AC16 cardiomyocytes. Exp Ther Med 15: 627-632, 2018.
10. Karnewar S, Vasamsetti SB, Gopoju R, Kanugula AK, Ganji SK, Prabhakar S, Rangaraj N, Tupperwar N, Kumar JM and Kotamraju S: Mitochondria-targeted esculetin alleviates mitochondrial dysfunction by AMPK-mediated nitric oxide and SIRT3 regulation in endothelial cells: Potential implications in atherosclerosis. Sci Rep 6: 24108, 2016.
11. Perez Pinzon MA, Stetler RA and Fiskum G: Novel mitochondrial targets for neuroprotection. J Cereb Blood Flow Metab 32: 1362-1376, 2012.
12. Karnewar S, Vasamsetti SB, Gopoju R, Kanugula AK, Ganji SK, Prabhakar S, Rangaraj N, Tupperwar N, Kumar JM and Kotamraju S: Mitochondria-targeted esculetin alleviates mitochondrial dysfunction by AMPK-mediated nitric oxide and SIRT3 regulation in endothelial cells: Potential implications in atherosclerosis. Sci Rep 6: 24108, 2016.
13. Perez-Pinzon MA, Stetler RA and Fiskum G: Novel mitochondrial targets for neuroprotection. J Cereb Blood Flow Metab 32: 1362-1376, 2012.
14. 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.
15. McNair P, Jones E, Truong Q and Singh H: Incidental finding of single coronary artery in a patient with alcoholic cardiomyopathy presenting as acute heart failure. Clin Imaging 42: 224-227, 2017.
16. George A and Figueredo VM: Alcoholic cardiomyopathy: A review. J Card Fail 17: 844-849, 2011.
17. Guo R and Ren J: Alcohol dehydrogenase accentuates ethanol-induced myocardial dysfunction and mitochondrial damage in mice: Role of mitochondrial death pathway. PLoS One 5: e8757, 2010.
18. Ge W and Ren J: mTOR-STAT3-notch signalling contributes to ALDH2-induced protection against cardiac contractile dysfunction and autophagy under alcoholism. J Cell Mol Med 16: 616-626, 2012.
19. Guo R, Hu N, Kandadi MR and Ren J: Facilitated ethanol metabolism promotes cardiomyocyte contractile dysfunction through autophagy in murine hearts. Autophagy 8: 593-608, 2012.
20. Zhang B, Turdi S, Li Q, Lopez FL, Eason AR, Anversa P and Ren J: Cardiac overexpression of insulin-like growth factor 1 attenuates chronic alcohol intake-induced myocardial contractile dysfunction but not hypertrophy: Roles of Akt, mTOR, GSK3beta, and PTEN. Free Radic Biol Med 49: 1238-1253, 2010.
21. Hu C, Ge F, Hyodo E, Arai K, Iwata S, Lobdell H IV, Walewski JL, Zhou S, Clugston RD, Jiang H, *et al*: Chronic ethanol consumption increases cardiomyocyte fatty acid uptake and decreases ventricular contractile function in C57BL/6J mice. J Mol Cell Cardiol 59: 30-40, 2013.
22. Piano MR and Phillips SA: Alcoholic cardiomyopathy: Pathophysiologic insights. Cardiovasc Toxicol 14: 291-308, 2014.
23. Piano M: Alcoholic cardiomyopathy: Incidence, clinical characteristics, and pathophysiology. Chest 121: 1638-1650, 2002.
24. Cheng CP, Cheng HJ, Cunningham C, Shihabi ZK, Sane DC, Wannenburg T and Little WC: Angiotensin II type 1 receptor blockade prevents alcoholic cardiomyopathy. Circulation 114: 226-236, 2006.
25. Jing L, Jin CM, Li SS, Zhang FM, Yuan L, Li WM, Sang Y, Li S and Zhou LJ: Chronic alcohol intake-induced oxidative stress and apoptosis: Role of CYP2E1 and calpain-1 in alcoholic cardiomyopathy. Mol Cell Biochem 359: 283-292, 2012.
26. Lai Y, Guo H, Li J, Dai J, Ren C and Wang Y: Comparison of surgical results in patients with hypertrophic obstructive cardiomyopathy after classic or modified morrow septal myectomy. Medicine (Baltimore) 96: e9371, 2017.
27. Ji F, Liu Q, Feng Z, Han X and Li Z: Genetic association between 1425G/A SNP in PRKCH and hypertrophic cardiomyopathy in a Chinese population. Oncotarget 8: 114839-114844, 2017.
28. Mu J, Zhang G, Xue D, Xi M, Qi J and Dong H: Sudden cardiac death owing to arrhythmogenic right ventricular cardiamyopathy: Two case reports and systematic literature review. Medicine (Baltimore) 96: 8808, 2017.
29. Dahraoui S, Uwingabiye J, Belarj B, Biaz A, Rachid A, Dami A, Bouhsain S, Ouzzif Z and Elmachatni Idrissi S: Unexpected discovery of multiple myeloma following cardiomyopathy. Clin Case Rep 6: 86-90, 2018.
30. Fu KY, Zamudio R, Henderson-Frost J, Almuedo A, Steinberg H, Clipman SJ, Duran G, Marcus R, Crawford T, Alyesh D, *et al*: Association of caspase-1 polymorphisms with Chagas cardiomyopathy among individuals in Santa Cruz, Bolivia. Rev Soc Bras Med Trop 50: 516-523, 2017.
31. Adachi K, Hashiguchi S, Saito M, Kashiwagi S, Miyazaki T, Kawai H, Yamada H, Iwase T, Akaike M, Takao S, *et al*: Detection and management of cardiomyopathy in female dystrophinopathy carriers. J Neurol Sci 386: 74-80, 2018.
32. Baltrūnienė V, Bironaitė D, Kažukauskienė I, Bogomolovas J, Vitkus D, Ručinskas K, Žurauskas E, Augulis R and Grabauskienė V: The role of serum adiponectin for outcome prediction in patients with dilated cardiomyopathy and advanced heart failure. Biomed Res Int 2017: 3818292, 2017.
33. Bollen IAE, van der Meulen M, de Goede K, Kuster DWD, Dalinghaus M and van der Velden J: cardiomyocyte hypocontractility and reduced myofibril density in end-stage pediatric cardiomyopathy. Front Physiol 8: 1103, 2017.
34. Buccheri D and Zambelli G: The link between spontaneous coronary artery dissection and takotsubo cardiomyopathy: Analysis of the published cases. J Thorac Dis 9: 5489-5492, 2017.
35. Van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA and Olson EN: A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Naatl Acad Sci USA 103: 18255-18260, 2006.
36. Zhang J, Du Y, Wu C, Ren X, Ti X, Shi J, Zhao F and Yin H: Curcumin promotes apoptosis in human lung adenocarcinoma cells through miR-186* signaling pathway. Oncol Rep 24: 1217-1223, 2010.
37. Crook NE, Clem RJ and Miller LK: An apoptosis-inhibiting baculovirus gene with a zinc finge-like motif. J viorl 67: 2168-2174, 1993.
38. LaCasse EG: Pulling the plug on a cancer cell by eliminating XIAP with AEG35156. Cancer Lett 332: 215-224, 2013.