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Effect of miR-26a on diabetic rats with myocardial injury by targeting PTEN

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Abstract. – OBJECTIVE: To investigate the effect of micro ribonucleic acid (miR)-26a on diabetes-induced myocardial injury in rats by targeting the gene of phosphate and tension homology detected on chromosome ten (PTEN).

MATERIALS AND MATERIALS: Male Wistar rats aged 8-9 weeks old were divided into the control group (n=10), GK group (n=10), and miR-26a agomir group (n=10) according to the body weight. MiRanda and TargetScan target gene prediction software were used to predict and analyze the target gene of miR-26a-5p. The expressi[illegible] of miR-26a and PTEN in the myocardial tis[illegible] the diabetic rats were detected by quan[illegible]ve Reverse Transcription-Polymerase Chain [illegible]- tion (qRT-PCR). Hematoxylin-eosin (HE) stai[illegible] was adopted to observe the pathological cha[illegible] es in the myocardial tissues. In[illegible]on, the te[illegible] minal deoxynucleotidyl tran[illegible]TP nick end labeling (TUNEL) assa[illegible]s co[illegible]cted to detect myocardial apopto[illegible] while t[illegible]expression of PTEN protein wa[illegible]ected histochemistry, and the p[illegible]ssions of PTEN, b-cell lympho[illegible] (Bc[illegible]-2-associated X protein (Bax) [illegible] cysteiny[illegible]rtate specific proteinase[illegible]aspase-3) we[illegible]ted by Western blottin[illegible]

RESULTS: [illegible]getS[illegible]tabase analysis results show[illegible]d that miR-2[illegible] and PTEN 3'UTR had 6 pa[illegible]f complemen[illegible]ases with the same s[illegible]nce. Compared [illegible] those in the contr[illegible]oup, t[illegible] messenger RNA (mRNA) expres[illegible] of PT[illegible]n the GK group was notably inc[illegible] (p[illegible]5), wh[illegible]that of miR-26a was subst[illegible]educed[illegible]0.05). In comparison with thos[illegible]he GK[illegible]oup, the mRNA expres-[illegible]f PTE[illegible]nificantly decreased, but tha[illegible]miR-26[illegible] significantly raised in miR-26a [illegible]omir grou[illegible] (p<0.05). Through observation[illegible]ptical microscope, it was mani-fe[illegible]e control group, the myocardial [illegible]s were intact with clear texture but no frac-[illegible] and the solid necrosis did not appear in [illegible]dial cells. In the GK group, the myocardia[illegible]rs were disorderedly arranged and incomplete[illegible]lear edge and burrs. The myocardi[illegible] fibers [illegible] miR-26a agomir group were more regular, w[illegible]s breakage and solid ne[illegible]According to [illegible]EL staining results, t[illegible]UNEL-stained brown granules in rats in the [illegible]group were remarkably increased, relative to [illegible] control gro[illegible]p<0.05). Compared with the [illegible]group, miR-[illegible] agomir group had marked-[illegible]reased th[illegible]UNEL-stained brown particles (p[illegible]. It w[illegible]und in immunohistochemical resu[illegible]N protein was in lighter color after staining in the control group, with a clear myocardial cell stripe structure. Compared with that [illegible]ol group, PTEN protein in the GK group [illegible]eper color after staining, and in comparison with that in the GK group, the color of PTEN protein in miR-26a agomir group became significantly lighter. Moreover, the Western blotting results demonstrated that, compared with those in the control group, the Caspase-3 and Bax protein expressions in the GK group were significantly raised, while Bcl-2 protein expression was notably reduced (p<0.05). Besides, in comparison with the GK group, miR-26a agomir group evidently elevated Caspase-3 and Bax protein expressions and a notably increased Bcl-2 protein expression (p<0.05).

CONCLUSIONS: We showed that miR-26a can protect against myocardial injury in diabetic rats by regulating PTEN.

Key Words:
MiR-26a, PTEN, Myocardial injury, Diabetes.

Introduction

Diabetes is a kind of chronic and metabolic disorder disease, typically characterized by significantly increased blood lipid and glucose, as well as abnormal glucose metabolism in the body[1,2]. The disease is mainly caused by the destruction of islet cells and abnormal insulin secretion[3,4].

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Statistics[5-7] have revealed that the incidence rate of diabetes shows a year-by-year increasing trend, and that of the accompanying cardiovascular and cerebrovascular diseases is also on the rise year by year. Korkmaz-Icoz et al[8] have shown that the incidence rate of myocardial infarction in diabetic patients is higher than those in patients with other diseases, and the clinical manifestations of myocardial infarction include severe heart failure, a larger myocardial infarction area, rapid development of the disease, refractoriness, and deadlines. According to some reports, the occurrence and development of diabetes are related to numerous signal pathways.

The gene of phosphate and tension homology deleted on chromosome ten (PTEN) is an anti-cancer-related gene closely associated with various cancers. It has been found in many investigations that the expression of PTEN in tissues from patients with cervical cancer, liver cancer, ovarian cancer, and colon cancer is significantly different from that in normal tissues. Moreover, some works[9-11] have indicated that PTEN is closely correlated with diabetes. PTEN may be a gene target for the treatment of diabetes. It can participate and function in the insulin-signaling way when it is damaged[12]. In addition, PTEN can activate the PI3K/Akt pathway by suppressing the expression of TGF-β1, thus causing renal fibrosis in diabetic rats[13].

In spite of the association of PTEN gene with many diseases in the body, the myocardial injury in diabetic rats has been rarely reported. Currently, there is no literature on the interaction mechanism between PTEN and myocardial injury in diabetic rats. In this study, the effect of micro ribonucleic acid (miR-26a on myocardial injury in diabetic rats by PTEN was preliminarily investigated, thus providing a theoretical basis for the pathogenesis of diabetes and a certain experimental basis for the treatment of heart failure accompanied by diabetes.

Materials and Methods

Experimental Animals As Well As Main Reagents and Instruments

A total of 30 SPF-grade male Wistar rats aged 8-10 weeks and weighing (200±20) g were selected. They were randomly divided into the control group (n=10) and model group (n=20) according to the body weight. The rats in the control group were fed with common feed, while those in the model group were fed with high-glucose and high-fat feed. 30 d later, the blood was collected from the caudal vein. Fasting blood glucose ≥ 11.1 mmol/L[12] indicated the successful modeling of diabetes. According to the body weight, the model group was randomly subdivided into the GK group (n=10) and miR-26a agomir group (n=10). MiR-26a agomir was injected into the tail vein in miR-26a agomir group, and the same volume of normal saline was injected into the myocardium in the control group and the GK group. 24 h later, the rats were sacrificed and the heart was taken out and divided into two parts. One part was stored in liquid nitrogen and the other part was fixed in paraformaldehyde solution.

The main reagents and instruments used were: miR-16a agomir (Guangzhou Ribio Co., Ltd., Guangzhou, China), PI (Sigma, St. Louis, MO, USA), eosin staining solution (Shenyang No.5 Reagent Factory, Shenyang, China), fluorescence quantitative Polymerase Chain Reaction (qPCR) reagents and consumables (TaKaRa, Otsu, Shiga, Japan), Western blotting primary antibody goat anti-rabbit immunoglobulin G (IgG; Abcam, Cambridge, MA, USA), secondary antibody rabbit anti-mouse horseradish peroxidase (HRP) and miRNeasy Mini Kit (Tiangen Biotechnology, Beijing, China), TD-5A bench top low speed centrifuge (Jintan Changzhou Instrument Factory, Jintan, China), TGL-16G centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China), PT-3502B microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), electrothermal constant-temperature dry box (Shanghai Yuejin Medical Devices Factory, Shanghai, China), electric boiling sterilizer (Shanghai Chemical Import and Export Co., Ltd., Shanghai, China), and HH-4 digital thermostatic water bath (Jintan Walter Experimental Instrument Co., Ltd., Jintan, China).

Prediction of Downstream Target Genes of MiR-26a

MiR-26a and PTEN were analyzed with TargetScan database. Based on the results, PTEN involved in the regulation of myocardial cell function was selected, as the research object through literature report and gene function analysis.

Fluorescence qPCR

The rat heart samples in liquid nitrogen were taken out, and the total RNA in the heart was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA), followed by Reverse Transcription (RT) according to the RT kit instructions. With

RT product complementary deoxyribose nucleic acid (cDNA) as a template, the target gene was amplified based on the instructions of the qRT-PCR kit. The reaction system for fluorescence qPCR: 10 μL of qPCR Master Mix (2×), 1 μL of PCR reverse primer, 2 μL of cDNAs, 11 μL of ddH_2O. The final result was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences for qRT-PCR are listed in Table I.

Hematoxylin-Eosin (HE) Staining

The heart samples fixed for 24 h were taken out, washed with clear water, dehydrated with ethanol, embedded in paraffin, and sliced into sections. Subsequently, the sections were stained with hematoxylin at room temperature for 10 min, washed with clear water for 30 min, soaked in 1% hydrochloric acid for 30 s, and washed with clear water for 30 s. Then, they were stained with eosin at room temperature for 5 min, dehydrated with ethanol, and sealed with neutral glue in a ventilated environment. Finally, the changes in the myocardial tissues were observed under a microscope.

Detection of Apoptosis Via Terminal Deoxynucleotidyl Transferase dUT End Labeling (TUNEL) Assay

The sections prepared in 1.2.3 were dehy ed with ethanol at gradient concentrations (7 80%, 90%, and 95%), treated with proteina K for 15-30 min and rinsed h te-Buff- ered Saline (PBS) twice. Th ter, TUNEL staining solution was used 0 min aining, followed by washing wi for 3 the sections were add dr n 50 μL of converter-POD for min o on at 37°C and washed by P or 3 times. that, diaminobenzidin was added fo min of reaction at 25 follo y washing with PBS for 3 times and sealing eutral glue in the ventilated ironment. Ulti the apoptosis of myo ial cells was observed under the microsc The p ammed necrosis of myocardial cells was examined by TUNEL assay, and the brown nucleus after staining represent were the TUNEL-positive cells.

Table I. P quenc qRT-PCR.

P	Sequence
m	5'CGTCCTTCAAGTAATCCAGGA3'
	GCAGGGTCCGAGGTATTC3'
EN	5'TGGAAAGGGACGAACTGGTG3'
	5'CATAGCGCCTCTGACTGGGA3'
DH	5'AGGTCGGTGTGAACGGATTTG3'
	5'TGTAGACCATGTAGTTGAGGTCA3'

Immunohistochemistry

The sections made in 1.2.3 w hydrated and washed with PBS for 3 es with each Ultra V Block was adde drops for s room temperature for n, foll ed by ri with PBS for 3 times 5 mi n. Thereafter, the sections were a op with t orresponding PTEN tibo ted at 00) for incubation at 2 for 1.5 ed PBS for 3 times wit in each, and d with the correspon labeled anti y (diluted at 1:5000) om t ture for 20 min, followed by rinsing with PBS imes with 5 min each. Sub ly, the sectio e added with drop- DAB for incubation at room temperature 5 min and se d with running water. Lastly, expression o EN protein was observed under the microsco

Wes ng

The heart tissues of the rats were removed from iquid nitrogen, added with radioimmunoprecipi- y (RIPA) lysate containing phenylmeth- sul l fluoride (PMSF) (Beyotime, Shanghai, China), and homogenized using a glass homogenizer for 4 min. Then, the homogenized liquid was taken out and centrifuged at 12000 rpm for 10 min. The supernatant was sucked out, mixed well with 5×protein loading buffer and boiled for 3 min. After SDS-PAGE, the sample proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), which was then put into 5% skim milk powder solution and sealed at room temperature for 1 h. After that, the PVDF membrane was added with the corresponding antibody (diluted at 1:1000), incubated overnight at 4°C, and washed with TBST for 3 times with 5 min each. Thereafter, the corresponding antibody containing HRP (diluted at 1:5000) was added for blocking at 37°C for 1 h, and the color was developed using the color-developing solution. At last, the Quantity One software was employed for the quantitative analysis.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA) was adopted to analyze the experimental data. The intra-group comparison was expressed as mean ± standard deviation ($\bar{x}\pm s$), while the in-

Position 41-47 of PTEN 3' UTR	5' ...ACACCAUGAAAAUAAACUUGAAU...
hsa-miR-26a-5p	3' UCGGAUAGGACCUAAUGAACUU
Position 1261-1268 of PTEN 3' UTR	5' ...ACUGUUAGGGAAUUUUACUUGAA...
hsa-miR-26a-5p	3' UCGGAUAGGACCUAAUGAACUU
Position 2619-2626 of PTEN 3' UTR	5' ...UUACAUGUCUGAAGUUACUUGAA...
hsa-miR-26a-5p	3' UCGGAUAGGACCUAAUGAACUU
Position 3800-3807 of PTEN 3' UTR	5' ...CUAAAGGACUUUUUGUACUUGAA...
hsa-miR-26a-5p	3' UCGGAUAGGACCUAAUGAACUU

Figure 1. Correlation between miR-26a and PTE

ter-group comparison was conducted by One-way analysis of variance. $p<0.05$ suggested that the difference was statistically significant.

Results

Correlation Between MiR-26a and PTEN

The TargetScan database was utilized to seek the binding sites between miR-26a-5p and PTEN 3'UTR (Figure 1). There were 6 pairs of co- mentary bases with the same sequence i 3'UTR and miR-26a-5p, indicating that m 6a has a correlation with PTEN.

Messenger RNA (mRNA) Expressions of MiR-26a and PTEN in t ardium of Diabetic Rats

Compared with the cont roup, th group had notably increased t NA PTEN ($p<0.05$) and e the mRNA expressio 6a ($p<0.05$). comparison with tho the C up, the mRNA expression of PTEN was signific ecreased ($p<0.05$), but that -26a was sig ntly raised in miR-2 omir group ($p<0.05$) (Figure 2).

ct of MiR- Agomir on the M rdial C Morphology in D s

HE-stained sections were observed under an tical microscope, and it was found that in the up, the myocardial fibers were intact th r texture but no fracture, and the solid necrosis did not appear in the myocardial cells (Figure 3A). In the GK group, the myocardial fibers were disorderedly arranged and incomplete with an unclear edge and burrs (Figure 3B). The myocardial fibers in the miR-26a agomir group were in order, with less breakage and solid necrosis (Figure 3C).

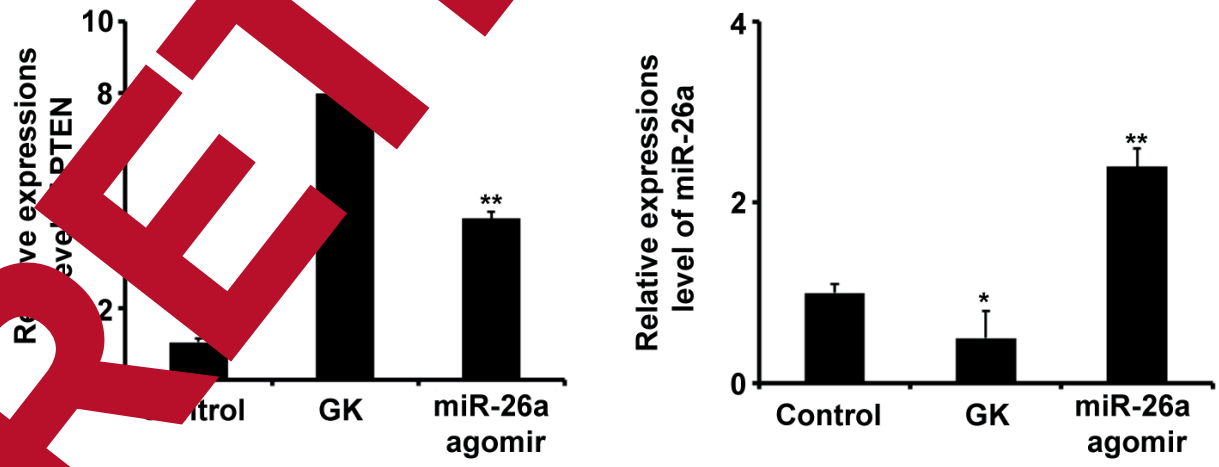


Figure 2. MiRNA expressions of PTEN and miR-26a in the myocardium of diabetic rats. Note: $^{*}p<0.05$ and $^{**}p<0.01$ *vs.* control group.

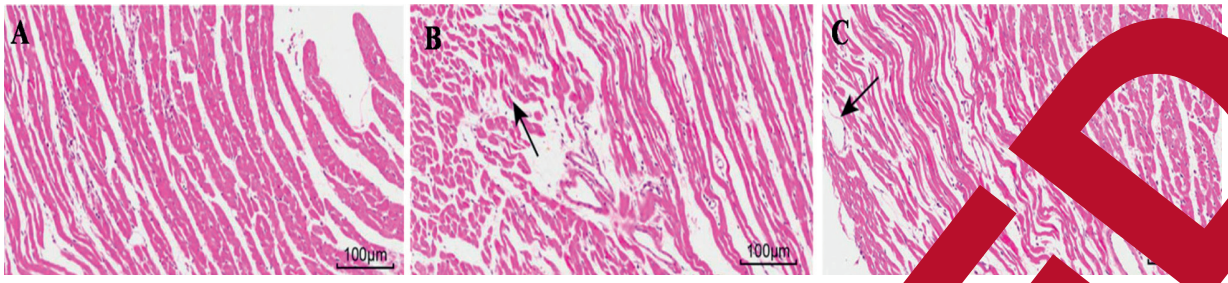


Figure 3. Morphological changes in the myocardial tissues of diabetic rats in each group (×). Note: *(A)* control group, *(B)* GK group, and *(C)* MiR-26a agomir group.

Apoptosis of Myocardial Cells in Diabetic Rats Detected via TUNEL

Through the observation of the TUNEL-stained sections, it was found that compared with those in control group (Figure 4A), TUNEL-stained brown granules in the rats in the GK group were remarkably increased ($p<0.05$) (Figure 4B). Compared with the GK group, miR-26a agomir group markedly decreased the TUNEL-stained brown particles ($p<0.05$) (Figure 4C).

Effect of MiR-26a Agomir on PTEN in the Myocardial Cells of Diabetic Rats

The optical microscope was used to observe the immunohistochemical sections. It was discovered that PTEN protein was in lighter color after staining in the control group, with a clear myocardial cell stripe structure (Figure 5A). Compared with that in the control group, PTEN protein in GK group was in deeper color after staining (Figure 5B), and the color of PTEN protein in agomir group became lighter relative to the GK group (Figure 5C).

Influence of MiR-26a Agomir on the Myocardial Cell Apoptosis in Diabetic Rats

As shown in Figure 6, compared with those in control group, B-cell lymphoma 2 (Bcl-2) protein was notably reduced, but the cysteinyl aspartate specific proteinase-3 (Caspase-3) and Bcl-2-associated protein (Bax) in GK group were significantly increased ($p<0.05$), suggesting markedly reduced myocardial apoptosis. Besides, in comparison with GK group, miR-26a agomir group had evidently reduced the Caspase-3 and Bax proteins and notably increased Bcl-2 protein ($p<0.05$), indicating that the myocardial cell apoptosis is significantly relieved (Table II).

Discussion

Diabetes is a common chronic and metabolic disorder disease, and it is divided into four types. Type 1 diabetes is manifested as the complete loss of the islet function, whose clinical treatment method is long-term insulin injection. Type 2 diabetes refers to the loss of part of the islet function. Type 3 diabetes is characterized by a temporary increase in blood glucose in women during the gestation period and self-healing without medication. Type 4 diabetes is generally caused by hormone drugs. Type 3 and type 4 diabetes are uncommon with relatively low incidence rates. Most patients in China suffer from type 2 diabetes, and type 1 diabetes mainly attacks teenagers. According to the data from the China Report on Diabetes[14] in 2010-2018, the estimated number of diabetic patients nationwide is 280 million. It is thus

Table II. Optical density values of Bcl-2, Bax, and Caspase-3 protein bands in each group.

Protein	Optical density value	Control group	GK group	MiR-26a agomir group
Bcl-2	Target protein/β-actin	0.90±0.15	1.97±0.27[a]	1.38±0.21[ab]
Bax	Target protein/β-actin	1.38±0.39	0.76±0.50[a]	1.12±0.46[b]
Caspase-3	Target protein/β-actin	0.42±0.17	0.97±0.37[a]	0.76±0.22[ab]
	Target protein/β-actin	0.37±0.24	0.72±0.41[a]	0.56±0.22[ab]

Note: [a]$p<0.05$ *vs.* control group and [b]$p<0.01$ *vs.* GK group.

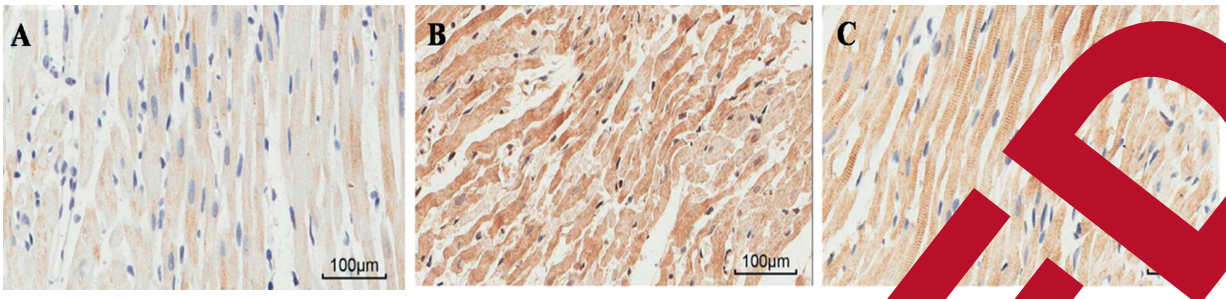
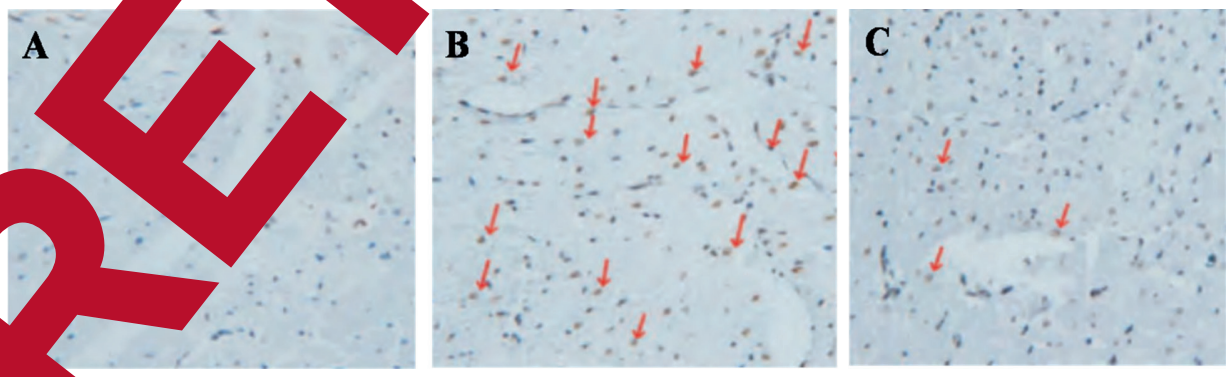


Figure 4. Myocardial tissue apoptosis of diabetic rats in each group observed *via* TUNE ing (4 Note: ***(A)*** Control group, ***(B)*** GK group, and ***(C)*** MiR-26a agomir group.

clear that there are a relatively large proportion of diabetic patients in China, and the prevention and treatment for diabetes are especially pivotal. As a metabolic disease, diabetes is characterized by an increased blood glucose content[15,16].

PTEN is an anti-cancer gene closely associated with many cancers. It has been considered as a tumor suppressor gene since its discovery in 1997[16]. PTEN can also regulate insulin signals in fat, bone, liver, and other tissues and participate in the occurrence and development of insu a- lated diseases[17]. Recently, miRNAs have a increasingly more attention. MiRNAs ca m- plement and pair with target mRNAs, cleav degrade target RNAs, and suppress the funct of mRNAs after transcription[18]. A ording to W et al[19], the inhibition of miR-2 ulate the expression level of CTGF a educe generation of myocardial fiber agens. NA-26 is able to activate the R K/E pathway by targeting ryv1, ng to interstitial fibrosis of the ased h

In this work, w e use of the can database, miR-26a TEN 3'UTR e found to have m ding sites wi the same sequence, lying iR-26a has a significant correlation with PTE miR-26a can function by g PTEN. Q R was implemented examine the expressions of miR-26a and N in the my ardial tissues in each group. as discovere at miR-26a expression had a tive associa with PTEN mRNA expression the myo ium of diabetic rats. In other wor NA expression was decreased with the increase of miR-26a expression, and vice rsa. With type 2 diabetes as an example, after g the PTEN expression by injecting R- agomir into the heart, PTEN protein expression in the rat myocardium was remarkably reduced ($p<0.05$), and the injury degree was decreased. The expressions of the apoptosis-related proteins, caspase-3, and Bax, were evidently lowered, while the expression of the anti-apoptosis protein Bcl-2 was significantly raised ($p<0.05$). The above findings indicate that miR-26a can target PTEN, reduce the diabetes-induced apoptosis of myocardial cells by inhibiting the PTEN expression, and alleviate myocardial cell injury. In



Fi Expression of PTEN protein in the myocardial tissues of diabetic rats in each group (400×). Note: ***(A)*** Control group, ***(B)*** G oup and ***(C)*** MiR-26a agomir group.

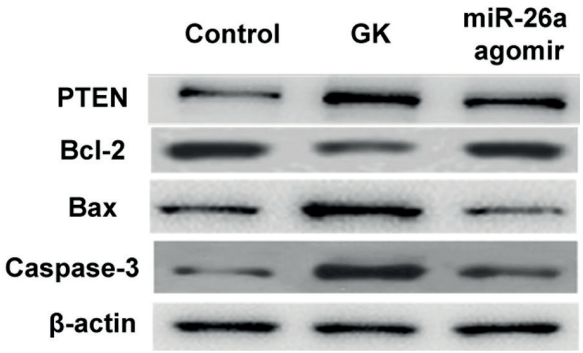


Figure 6. Protein expressions of Bcl-2, Bax, and Caspase-3 in the myocardial tissues detected by Western blotting.

this work, only the correlat ion between miR-26a and PTEN and apoptosis-related indicators were detected, which revealed that miR-26a could target PTEN and protect against myocardial injury in diabetic rats. However, there are a large number of determinants of diabetes and many signaling pathways affecting the occurrence and development of diabetes, for these reasons, the signaling pathway related to PTEN protection was no[t] ified in this report. This study laid a cer[tain] oretical and experimental foundation for [further] research on the mechanism of miR-26a in pr[otect]ing against myocardial injury in diabetic rats [by] targeting PTEN.

Conclu[sion]s

We demonstrated [that] [can] protect against myocardial i[njur]y in di[abetic] rats by regulating PTEN.

Conflict of [I]nterests

The Autho[rs] [de]clare that they have [no] conflict of interests.

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