

Original Research Article

Assessment of the therapeutic potential of anti-miR 24 and anti-miR 34 in cardiac diseases

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Abstract

Purpose: To study the therapeutic effects of anti-miR-24 and anti-miR-34 in cardiac diseases

Methods: H9c2 rat cardiomyocyte cell lines were transfected with the synthetic oligonucleotides anti-miR-24 and anti-miR-34 which reduced the expressions of miR-24 and miR-34. Proliferation assay, real time-polymerase chain reaction (RT-PCR) and immunoblotting were carried out to determine the effect of the transfections on cardiomyocyte proliferation, expressions of miR-24 and miR-34, as well as expressions of the target genes, TGF- β 1 and E2F3.

Results: The proliferation ability of the transfected cells was decreased significantly, relative to negative control. In contrast, percentage apoptosis was higher in the negative control group than in transfected cells. The expression profiles of anti-miR-24 and anti-miR-34 were significantly reduced, when compared to negative control. These results were further confirmed using immunoblot which revealed that the expression of the target genes, TGF- β 1 and E2F3, were upregulated in the transfected cells.

Conclusion: These results suggest that the synthetic oligonucleotides (anti-miR-24 and anti-miR-34) might be useful in developing therapeutic drug targets for cardiac diseases by suppressing the expressions of miR-24 and miR-34.

Keywords: H9c2rat cardiomyocyte miRNA, Anti-miR-24, Anti-miR-34, Cardiac diseases

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INTRODUCTION

Myocardial infarction (MI) is one of the most frequently occurring cardiovascular diseases worldwide, and it is associated with high morbidity and mortality [1]. The disease is characterized by myocardial necrosis due to severe hypoxia-ischemia of coronary arteries which lead to heart failure [2]. It has been predicted that between 2020 and 2030, approximately 16-23 million people will be

affected by MI [3]. Therefore, there is need for fast and accurate diagnostic procedures for MI so as to arrest the severity of the disease and save patients' lives.

Currently, there exists a miscellaneous array of biochemical markers for diagnosis of MI. These are creatine kinase, cardiac troponin I, brain natriuretic peptide, creatine kinase MB isoform, and lactate dehydrogenase [4]. However, due to low specificity and sensitivity, these biomarkers

sometimes give rise to false positive diagnosis [5]. MicroRNAs (miRNAs) are the most promising and potential biomarkers with high sensitivity and selectivity for the early diagnosis of cardiovascular diseases. MicroRNAs are small, non-coding RNAs, each with approximately 18-26 nucleotides in length. These miRNAs anneal with the corresponding nucleotides in the target mRNAs, thereby repressing the synthesis of disease associated proteins [6].

Apart from being found in tissues, miRNAs are also present in certain body fluids such as plasma, urine and serum [7]. Abnormally expressed miRNAs were seen in patients suffering from acute myocardial infarction, coronary artery disease, acute coronary syndrome, hypertension, and stroke. Several studies have shown the involvement of miRNA in many cardiovascular diseases. Studies have also revealed that in acute myocardial infarction, miR-208 [8], miR-499 [9] and miR-1 [10] were aberrantly elevated. It has been shown that collagen synthesis was inhibited by miR-29, leading subsequently to myocardial infarction [11]. It has been reported that miR-34a regulated fibrosis after myocardial infarction by targeting the gene Smad4, while elevated expression of miR-24 impaired the function of smooth muscle cells [12]. From these studies, it is clear that miRNAs may be key regulators in cardiovascular diseases. Therefore, it is crucial to seek modern therapeutic interventions for decreasing the expression of miRNAs so as reduce the prevalence of cardiovascular diseases. Recently, antisense therapies using anti-miRNAs were shown to target diseases related to miRNAs *in vivo*. These anti-miRNAs reduced the pathogenicity and abnormal expressions of miRNAs by regulating the translation of mRNAs which are normally targeted by the miRNAs [13].

The present study investigated the therapeutic potential of anti-miR-24 and anti-miR-34 in myocardial infarction, using H9c2 rat cardiomyocyte cell line.

EXPERIMENTAL

Cell culture and transfection

The H9c2 rat cardiomyocyte cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum in a humidified incubator containing 5% CO₂ at 37°C. Transfection was carried out using standard protocols [14]. Cells were seeded into 6-well plate at a density of 1 x 10⁵ cells/well, and transfection was performed after 24 h. Using standard protocols, the cells were transfected

with anti-miR-24, anti-miR-34, miR-24 and miR-34 using Lipofectamine 2000 (Invitrogen, USA). The culture medium was replaced after 4h, and the experiments were carried out in triplicate. The transfected cells were grouped into the following categories: non-transfected cells (control), cells transfected with miRNAs (miR-24 and miR-34 i.e. negative control), and cells transfected with anti-miRNAs (anti-miR-24 and anti-miR-34).

Cell proliferation assay

The proliferation of transfected cells was determined using CCK-8 assay. Cells from each group were seeded in 96-well plates at a density of 5 x 10⁴ cells/ml, and incubated for 24 h. Then, to each well, 10µL of CCK-8 solution was added and the cells were again incubated for 2 h at 37°C. The optical density was read at 450nm in a microplate reader (Bio-Rad, USA).

Determination of cell apoptosis

Transfected cells at logarithmic growth phase were seeded in 6-well plate at a density of 1x10⁵ cells/mL, and incubated for 24 h. The cells were then stained using Annexin V-FITC (Invitrogen, USA) for 15 min, and further subjected to flow cytometric analysis.

Identification of miR-24 and miR-34 targets

The target genes of miR-24 and miR-34 were predicted with Bioinformatics using the databases TargetScan (www.targetscan.org), miRTarBase (www.mirtarbase.mbc.nctu.edu.tw/php) and miRDB (www.mirdb.org).

Isolation of RNA from transfected cells

Total RNA was isolated from transfected H9c2 rat cardiomyocytes using TRIzol reagent as per standard protocol. The purity and concentration of RNA were determined with NanoDrop, while RNA integrity was ascertained with agarose gel electrophoresis. The RNA was then kept frozen at -80°C prior to further analysis.

Quantitative RT-PCR analysis

Quantitative RT-PCR was performed using mirVanaq RT-PCR miRNA Detection Kit (P/N: 1556, Ambion). The RT-PCR for transfected cells and target genes was carried out using standard procedure [15]. Samples of RNA were reverse-transcribed to cDNA by incubating the vials at 37°C for 30 min. The PCR reaction system comprised 4.5 µL 2xSYBR Green Mixture,

Table 1: Primer sequences used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Anti-miR-24	CACACTATATCACATTGCCAG	ATGGTTGTTCTGCTCTCTGTCC
Anti-miR-34	GTAACAGCAACTCCATGTG	CTGTCAACGATACGCTAC
miR-24	TCACACTATATCACATTGCCAGG	TATGGTTGTTCTGCTCTCTGTCTC
miR-34	TGTAACAGCAACTCCATGTGG	GCTGTCAACGATACGCTACG
U6	ATTGGAACGATACAGAGAAGAT	GGAACGCTTCACGAATTTG
β -actin	AACAGTCCGCCTAGAAGCAC	CGTTGACATCCGTAAGA

forward and reverse primers (0.5 μ L each, 5 μ M); 1 μ L of cDNA, and 3.5 μ L of ddH₂O. The reaction was performed on ABI ViiA7 at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The expressions in transfected cells were analyzed using Ct method i.e. $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = [CT(\text{anti-24/anti-34/miR-24/miR-34}) - CT(5S \text{ rRNA})]$ [16]. The experiments were repeated thrice to obtain accurate data, with U6 and β -actin as control. The primers used for qRT-PCR are shown in Table 1.

Immunoblot analysis of TGF- β 1 and E2F3q

Protein samples were isolated using mirVanaPariS isolation kit according to the manufacturer's protocol, and protein concentration was measured using Lowry's assay. Equal amounts of protein samples were resolved using 12 % SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene membrane. The membrane was treated with 5 % BSA (Sigma-Aldrich; Cat. No. 05470) to block non-specific binding of the blot. Thereafter, the membrane was incubated with primary antibodies, i.e., anti-TGF- β 1 (Cat. No. MAB4310) and anti-E2F3 (Cat. No. P0056) overnight, followed by incubation with horse radish peroxidase-conjugated secondary antibody at room temperature. The signals were detected and recorded using enhanced chemiluminescence detection system. β -actin was used as a loading control.

Statistical analysis

Statistical analysis was done using SPSS for Windows 11.0 (SPSS, Inc, Chicago, IL, USA). Measurement data are expressed as mean \pm standard deviation. All the experiments were carried out in triplicate. Values of $p < 0.05$ were taken as indicative of statistical significance.

RESULTS

Effect of transfection on proliferation and apoptosis of H9c2 rat cardiomyocytes

The proliferative ability of H9c2 cardiomyocytes transfected with anti-mir-24 and anti-mir-34 was significantly decreased, when compared with that

of the negative control group (Figure 1A). Moreover, percentage cell apoptosis was markedly decreased in the anti-miR-24 and anti-miR-34 transfected cells. In contrast, percentage apoptosis was high in cells transfected with miR-24 and miR-34 (Figure 1B).

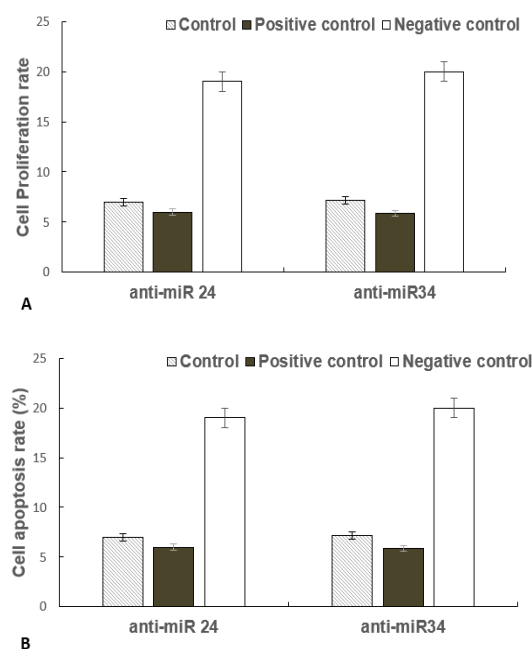


Figure 1: Cell proliferation and apoptosis of H9c2 cardiomyocytes. A. Proliferation of H9c2 cardiomyocytes transfected with anti-miR-24 and anti-miR-34. B. Apoptosis of H9c2 cardiomyocytes transfected with anti-miR-24 and anti-miR-34

Expression patterns of anti-miRNAs in transfected cells

Quantitative qRT-PCR showed that the expression profiles of anti-miR-24 and anti-miR-34 were markedly downregulated, relative to the negative control group. This finding indicates that anti-miRNAs can be used as a therapeutic strategy in myocardial infarction. These results are shown in Figure 2.

Protein expression levels of TGF- β 1 and E2F3 in anti-miRNA transfected cells

In order to confirm the regulation of the target

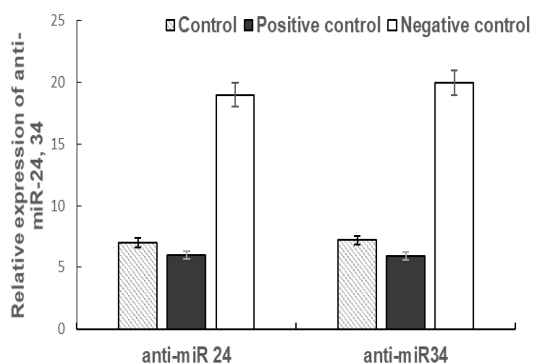


Figure 2: Relative expression patterns of anti-miR-24 and anti-miR-34 in H9c2 rat cardiomyocyte transfected cells

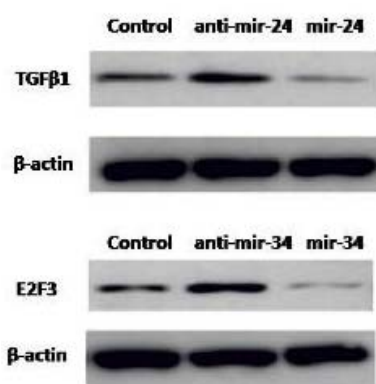


Figure 3: Analysis of protein expression patterns of TGF- β 1 and E2F3 in transfected H9c2 rat cardiomyocyte cells. Beta actin was used as control

genes, immunoblotting was carried out. The expressions of the genes TGF- β 1 and E2F3 were significantly increased in the anti-miR-24 and anti-miR-34 transfected cells, when compared with the negative control (Figure 3). This result indicated clearly that anti-miRNA repressed miRNAs. Thus, the mRNAs of the target genes involved in translation process were not inhibited, resulting in upregulation of their expressions.

DISCUSSION

Myocardial infarction, a cardiovascular disease, causes heart failure in humans [17]. Several studies have shown that aberrant expression of miRNAs is involved in the pathogenesis of many diseases. It has been revealed that, apart from being present in tissues, miRNAs are found in the blood and urine, making them prominent biomarkers for numerous ailments, including cardiovascular diseases [7,18,19]. Therefore, it is necessary to evolve alternate ways for downregulating miRNAs so as to reduce the progression of cardiovascular diseases.

Recently, anti-miRNAs were found to be efficient in the development of remedies for many diseases.

In this study, H9c2 rat cardiomyocyte cell line was used for determination of the therapeutic potential of anti-miR-24 and anti-miR-34 in cardiovascular disease. The results showed that cells transfected with anti-miRNAs showed decreased proliferation rate, and in addition, apoptosis was also reduced, relative to the negative control. The expression profiles of anti-miR-24 and anti-miR-34 were reduced significantly, when compared with the negative control group. The target gene of anti-miR-24 was found to be TGF- β 1, while the target gene for anti-miR-34 was E2F3. The expressions of the target genes were upregulated in cells transfected with anti-miR-24 and anti-miR-34, when compared to the negative control.

Based on these results, it is obvious that the knockdown of anti-miR-24 and anti-miR-34 reduced the expressions of miR-24 and miR-34. As a result, degradation of mRNA was prevented and translation was initiated, which in turn, upregulated the expressions of the target genes TGF- β 1 and E2F3, thereby preventing the progression of the disease. These findings correlate with recent reports which showed that subcutaneous delivery of anti-miR-208a in rats not only silenced the miR-208a in heart tissue, but also prevented pathological changes such as myosin switching and cardiac remodeling, leading to increased survival of the rats [20].

Moreover, administration of exogenous anti-miR-590 and anti-miR-199a promoted cardiac repair in neonatal and adult myocardial-infarcted mice [21]. Administration of anti-miR-210 in myocardial infarcted murine model reduced apoptosis and improved heart function by targeting Efn3 and Ptp1b [22]. Moreover, expression of miR-34a and anti-miR-92a promoted potential therapeutic effects on acute myocardial infarction [23,24].

The present study has revealed the therapeutic potential of anti-miR-24 and anti-miR-34. This might be beneficial for evaluating the therapeutic dose, and for forecasting therapeutic outcome in individuals affected by cardiovascular diseases. Although the precise mechanism underlying the regulation of the target genes under disease situations is not yet clear, this study provides some benefits related to the potential use of anti-miRNAs as new therapeutic tools. The pharmacological properties of anti-miRNAs can adjust based on the mode and outcome of the disease. This might be helpful in assessing the effect and therapeutic dose in affected

individuals. The effectiveness of anti-miRNAs in regulating gene expression will be determined through identification of the mechanism involved. This will be helpful for developing novel therapeutics.

CONCLUSION

Synthetic oligonucleotides, anti-miR-24 and anti-miR-34 are crucial in the development of target drugs for treating cardiac diseases. Based on the results obtained in this study, it may be reasonably concluded that synthetic oligonucleotides (anti-miR-24 and anti-miR-34) might be used for developing therapeutic drug targets for suppressing the expressions of miR-24 and miR-34, thereby reducing the incidence of cardiac diseases.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this research.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chunli Li, Jue Li contributed to this work equally. The research work was designed by the corresponding author Dr. Xiaojun Sun and Jin Huang. In addition, the manuscript was written by Dr. Xiaojun Sun and Jin Huang. The research work was done equally by Dr. Chunli Li and Dr. Jue Li. RT-PCR and Western blotting experiments was carried out by Dr. Jin Huang and Dr. Chunyan Yao, respectively.

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