MiR-let-7i inhibits CD4$^+$ T cell apoptosis in patients with acute coronary syndrome

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Abstract

Background. Abnormal CD4$^+$ T cells appear in the peripheral blood of patients with acute coronary syndrome (ACS). Studies have confirmed that CD4$^+$ T cells are resistant to apoptosis, but the specific mechanism has not been elucidated yet.

Objectives. The microRNA (miR)-let-7i plays an important regulatory role in the cardiovascular system and is widely involved in cell proliferation and apoptosis. In this study, we aimed to investigate its functional and regulatory roles in CD4$^+$ T cell apoptosis.

Materials and methods. Apoptosis of CD4$^+$ T cells was detected using TUNEL assay. Western blot analyses were used to detect the expression of Bcl-2 and Bax. Real-time polymerase chain reaction and western blot analyses were used to detect the expression of miR-let-7i, Fas and FasL. A miR-let-7i mimic or inhibitor was transfected into CD4$^+$ T cells, and miR-let-7i activity was investigated using Cell Counting Kit-8 (CCK-8) and TUNEL assays.

Results. Apoptosis of CD4$^+$ T cells in ACS patients was significantly decreased. Overexpression of miR-let-7i inhibited CD4$^+$ T cell apoptosis and improved cell survival rates, while inhibition of miR-let-7i facilitated cell apoptosis. We also found that miR-let-7i negatively regulated Fas and FasL gene expression in CD4$^+$ T cells.

Conclusions. The present study identified that miR-let-7i significantly reduces Fas and FasL expression in ACS CD4$^+$ T cells and inhibits apoptosis in these cells. Therefore, miR-let-7i may serve as a possible therapeutic target for the treatment of ACS.

Key words: Fas, FasL, CD4$^+$ T cell, apoptosis, miR-let-7i

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Background

Acute coronary syndrome (ACS) refers to clinical conditions characterized by the rupture of atherosclerotic plaques and the secondary formation of complete or incomplete occlusive thrombosis. Atherosclerotic plaque rupture is the most important physiological and pathological mechanism in ACS, accounting for more than 70% of acute coronary events. The ACS-vulnerable plaques are characterized by thin, fibrous caps covering large lipid nuclei, inflammatory cell infiltration (including T cells, macrophages and other immune cells) and endothelial smooth muscle cell apoptosis. Studies have shown that T lymphocytes play an important role in the development of atherosclerosis, and inflammatory cytokines secreted by $CD4^+$ T cells can directly affect the stability of atherosclerotic plaques by activating macrophages, thereby leading to the occurrence of ACS.[1] In addition to macrophage activation and cytokine secretion, $CD4^+$ T cells have been reported to accumulate in early and advanced human atherosclerotic plaques and contribute to the progression and destabilization of these atherosclerotic lesions.[2] Studies have shown that $CD4^+$ T cells in the peripheral blood of patients with ACS lack the co-stimulatory receptor CD28.[3] Furthermore, $CD4^+$ T cells have a long life span and secrete large amounts of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), which leads to a chronic inflammatory response that causes plaque rupture.[4] It is currently believed that the longevity of $CD4^+$ T cells is related to apoptotic resistance;[5,6] however, this specific mechanism needs further study.

MicroRNAs (miRNAs) are a class of non-coding small RNAs of about 18–25 nucleotides in length that can regulate gene expression after transcription through degradation or inhibition of translation of the target mRNA via base pairing specific to that mRNA.[7] MiR-let-7 was first discovered in *Caenorhabditis elegans* and is highly conserved in a variety of species, including flies and mammals.[8] The human miR-let-7 family includes 13 members – let-7a, let-7b, let-7c, let-7d, and miR-98 and other members. Recent studies have shown that let-7i plays an important regulatory role in tissue differentiation and tumorigenesis as well as cell proliferation and apoptosis.[9] MiR-let-7i can act on different apoptosis-related genes, such as insulin-like growth factor-1 receptor (*IGF1R*),[10] B-cell lymphoma-extra large (*Bcl-xl*)[11] and YAP1,[12] supporting its role in regulating apoptosis.

Cell apoptosis, as a part of the self-balancing mechanism of growth and development, maintains a constant number of cells in the body by eliminating damaged cells in tissues that are difficult to control for a prolonged time.[13,14] Apoptotic signal transduction includes 3 pathways: the Fas/FasL pathway, the mitochondrial pathway and the granulase B pathway,[15,16] among which is Fas/FasL the main pathway of apoptosis.

Objectives

Fas is the most representative death receptor in the TNF superfamily. After binding to its ligand FasL on the surface of the cell membrane, the Fas molecules aggregate and form a trimer that initiates apoptosis signal transmission leading to cell death with positive Fas expression.[17] Kovalcsik et al. found that $CD4^+$ T cells resist apoptosis through the death receptor pathway (Fas-mediated) in ACS patients.[18] Furthermore, studies have shown that there are binding sites between miR-let-7i and Fas/FasL.[19,20] Nevertheless, how miR-let-7i and Fas/FasL mediate the anti-apoptotic effects of $CD4^+$ T cells in ACS remains unclear. Therefore, the aim of this study was to explore whether miR-let-7i regulates the expression of the Fas/FasL genes to promote the anti-apoptotic effects of $CD4^+$ T cells in the peripheral blood of ACS patients.

Materials and methods

Clinical study population

Based on patients' angina symptoms and the diagnostic criteria of the American Heart Association, we enrolled 36 patients (41–78 years old) diagnosed with ACS, 23 men and 13 women. The enrollment criteria were ACS confirmed with angiography at the time of enrollment and obvious clinical symptoms within 24 h before the consultation. Patients with infectious diseases, tumors, connective tissue diseases, and autoimmune diseases were excluded. A total of 30 healthy study participants (20 men and 10 women) were recruited from the physical examination center of Second Affiliated Hospital of Harbin Medical University, China. Stable angina pectoris (SAP) patients enrolled in this study experienced chest discomfort or pain that could be relieved with rest or administration of nitroglycerin; SAP patients (diagnosed via coronary angiography) were enrolled from our hospital. There was no statistically significant difference in gender or age of patients enrolled in this study ($p > 0.05$). In order to exclude the interference of other risk factors on experimental data, statistical analysis was conducted on triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) (Table 1), and the results showed no statistical difference among each group ($p > 0.05$).

The present study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Harbin Medical University and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from all patients for the use of their clinical tissues.

Table 1. Clinical and biochemical characteristics of the study subjects. Values are expressed as mean ±SD or median (Q1–Q3). TC and LDL were analyzed with ANOVA. Age, TG and HDL were analyzed using Kruskal–Wallis test. The χ^2 test was used for gender statistics

Variable	Total	Normal	SAP	ACS	p-value
Patients, n	97	30	31	36	–
Gender	62 male/35 female	20 male/10 female	19 male/12 female	23 male/13 female	0.91
Age [years]	58 (48–65)	54.5 (45.75–61)	60 (52–65)	57.5 (47–65.75)	0.24
TC [mmol/L]	4.38 ±0.80	4. 50 ±0. 72	4.31 ±1.06	4.34 ±0.58	0.61
TG [mmol/L]	1.65 (1.22–2.07)	1.65 (1.27–1. 97)	1.48 (0.98–1.97)	1.76 (1.34–2.14)	0.12
HDL [mmol/L]	1.13 (1.06–1.27)	1.13 (1.08–1.50)	1.13 (1.05–1.24)	1.13 (1.05–1.19)	0.19
LDL [mmol/L]	2.66 ±0.62	2.81 ±0.46	2.51 ±0.81	2.67 ±0.52	0.18

TC – total cholesterol; TG – triglyceride; HDL – high-density lipoprotein; LDL – low-density lipoprotein; SD – standard deviation; ANOVA – analysis of variance; ACS – acute coronary syndrome; SAP – stable angina pectoris.

CD4⁺ T cell isolation and culture

Peripheral blood mononuclear cells were isolated by density centrifugation using Human Peripheral Blood Lymphocyte Dissociation Solution (TBD Science, Tianjin, China). CD4⁺ T cells were purified from peripheral blood mononuclear cells using BD IMag™ anti-human CD4 Particles-DM (Becton Dickinson Biosciences, San Jose, USA) and a Cell Separation Magnet (Becton Dickinson Biosciences) according to the manufacturer's instructions. Cells were cultured at 37°C in a humidified incubator at 5% CO_2 in RPMI-1640 supplemented with 100 U IL-2, 2 µg/mL phytohemagglutinin (PHA), and 10% fetal bovine serum (FBS). Next, the cells were washed and subjected to flow cytometric analysis and RNA extraction.

Flow cytometry

CD4⁺ T cell purity determination

Isolated cells were stained with PE Mouse Anti-Human CD4 antibody (Elabscience Biotechnology, Wuhan, China) according to the manufacturer's instructions. Cells were subsequently analyzed using multicolor flow cytometry, and their purity was assessed. The purity of sorted CD4⁺ T cells was 90–95% (Fig. 1).

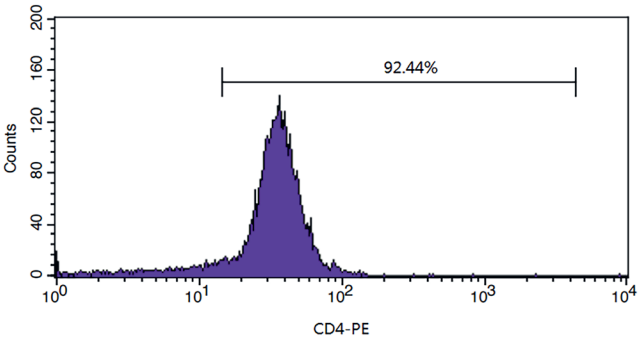

Fig. 1. The purity of sorted CD4⁺ T cells

Perforin detection

Briefly, 100 µL of fresh EDTA anticoagulant whole blood was added to the test and control tubes, respectively. Then, 20 µL of FITC-CD4 and PEcy5-CD28 (BD Biosciences) were added to the test tube and 20 µL of isotype control antibody was added to the control tube; the tubes were incubated. The erythrocytes were lysed and perforated with FACS (Becton Dickinson Biosciences), 20 µL PE-Perforin mAb (Legend Biotech, USA) was added and the cells were incubated. Finally, paraformaldehyde was used to fix the cells, which were analyzed using flow cytometry.[21]

qRT-PCR

Total RNA was extracted from CD4⁺ T cells using Trizol reagent (Sigma–Aldrich, St. Louis, USA) and quantified using a NanoDrop spectrophotometer (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions. The total RNA was then reverse-transcribed into complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using FastStart Universal SYBR Green Master (Roche), with an initial denaturation step at 95°C for

Table 2. Primer sequence of the target gene

Gene name	Forward primer	Reverse primer
Fas	TCTGGTTCTTACGTCTGTTGC	CTGTGCAGTCCCTAGCTTTCC
FasL	ATCCTACCAAGGCAACCA	CCCTGTCCAACCTCTGTG
β-actin	GGACCTTCTAAGCCCTTTTGG	GCCTGCTCCTCTTACTCCTCAC
miR-let-7i	CGGGCTGAGGTAGTAGTTTG	CAGCCACAAAAGAGCACAAT
U6	GAGATACCGACTTGTTCCTTACG	CTCGCCTTTCTCAACCTCTTCTT

10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. The sequences of the used primers (Shanghai Generay Biotech, Shanghai, China) are shown in Table 2. U6 small nuclear RNA was used as an internal control to normalize miR-let-7i levels. β-actin was used as an internal control to normalize Fas and FasL.

Western blot

The protein expressions of Fas, FasL, Bax, Bcl-2, and β-actin in cells were assayed with western blot analysis. Total protein of $CD4^+$ T cells was extracted in RIPA lysis buffer (Beyotime, Shanghai, China) and then quantified using BCA assay (Beyotime). A total of 50 μg of protein was separated using 8% or 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with 5% skimmed milk at room temperature (20–25°C) for 1 h and then incubated with the corresponding primary antibody overnight at 4°C. Membranes were washed 3 times with Tris-buffered saline with Tween 20 (TBST), and then incubated with the appropriate secondary antibodies. Protein bands were visualized using the electrochemiluminescence (ECL) system. Beta-actin was used as an internal control.

Cell transfection

$CD4^+$ T cells were transfected with miR-let-7i mimic or mimic negative control (NC) or with inhibitor or inhibitor NC, using Entranster™-R4000 (Engreen Biosystem Ltd, Beijing, China) according to the manufacturer's instructions. Cells were then collected and washed with ice-cold phosphate-buffered saline (PBS) for subsequent analysis.

CCK-8 assay

To test the $CD4^+$ T cell survival ratio, we used the Cell Counting Kit-8 (CCK-8; Dalian Meilun Biotechnology, Dalian, China) according to the manufacturer's instructions. $CD4^+$ T cells were digested and seeded into 96-well plates at a density of 1×10^5 per well in 100 μL of the medium. Cells were then transfected with mimic, mimic NC, inhibitor or inhibitor NC for 12 h 24 h or 36 h. The optical density (OD) value was read at 450 nm. Each experiment was repeated 3 times. The cell survival ratio was calculated as follows:

$$\text{cell survival ratio} = [(As - Ab)/(Ac - Ab)] \times 100\%.$$

As – the OD value of the treatment group; Ac – the OD value of control group; Ab – the OD value of the blank group.

TUNEL staining for DNA fragmentation

Poly-L-lysine was used to coat the cell culture plate (24-well plate) for 30 min, and the liquid was discarded. The transfected cells were inoculated and plated on the 24-well plate and cultured for 3 h to fix the cells on the plate. The air-dried cell samples were fixed with freshly prepared 4% paraformaldehyde in PBS for 1 h. The cells were incubated with 3% H_2O_2 in methanol for 10 min, incubated with 0.1% Triton X-100 for 2 min on ice and washed twice with PBS. A total of 50 μL of TUNEL reaction solution (Roche) was added to each well and incubated at 37°C for 1 h in the dark. Cell samples were analyzed using fluorescence microscopy.

Statistical analyses

Statistical analyses were performed using IBM SPSS v. 24.0 (IBM Corp., Armonk, USA) and GraphPad Prism v. 5.0 (GraphPad Software, San Diego, USA), and results were considered significant when $p < 0.05$. The results are presented as mean ± standard error of the mean (SEM) from 3 or more independent experiments. Student's t-test was used to analyze the data between 2 groups. Clinical and biochemical characteristics were analyzed using the Kruskal–Wallis test, analysis of variance (ANOVA) or χ^2 test. Parametric tests mentioned in this paper satisfy the assumptions.

Results

Percentage of $CD4^+$ T cells between ACS patients and healthy subjects

We found that $CD4^+$ T cells accounted for 63.7% in healthy subjects, while the percentage of $CD4^+$ T cells was 58.4% in ACS patients.[22] These results indicate that there was no significant difference in the percentage of $CD4^+$ T cells between normal subjects and ACS patients. It is possible that the absolute lymphocytosis was not significantly different between the studied groups.

The apoptosis level of $CD4^+$ T cells was markedly decreased in ACS patients

The apoptosis level of $CD4^+$ T cells was assessed using TUNEL staining (Fig. 2). TUNEL-stained images showed a greater number of TUNEL-positive cells (green) in cells from the healthy controls than in cells from ACS patients. Statistical analysis showed that the number of apoptotic $CD4^+$ T cells in ACS patients was significantly decreased when compared with $CD4^+$ T cells from healthy control individuals. Next, we detected the expression levels of apoptotic proteins, Bcl-2 and Bax with western blot. As shown in Fig. 3, the $CD4^+$ T cells from ACS patients showed higher expression of the anti-apoptotic protein Bcl-2 ($t = 6.585$, degrees of freedom (df) = 4, $p = 0.0028$) and markedly lower expression of the pro-apoptotic protein Bax than those in healthy controls ($t = 7.703$, $df = 4$, $p = 0.0015$). This suggests that $CD4^+$ T cells in peripheral blood of ACS patients are resistant to apoptosis.

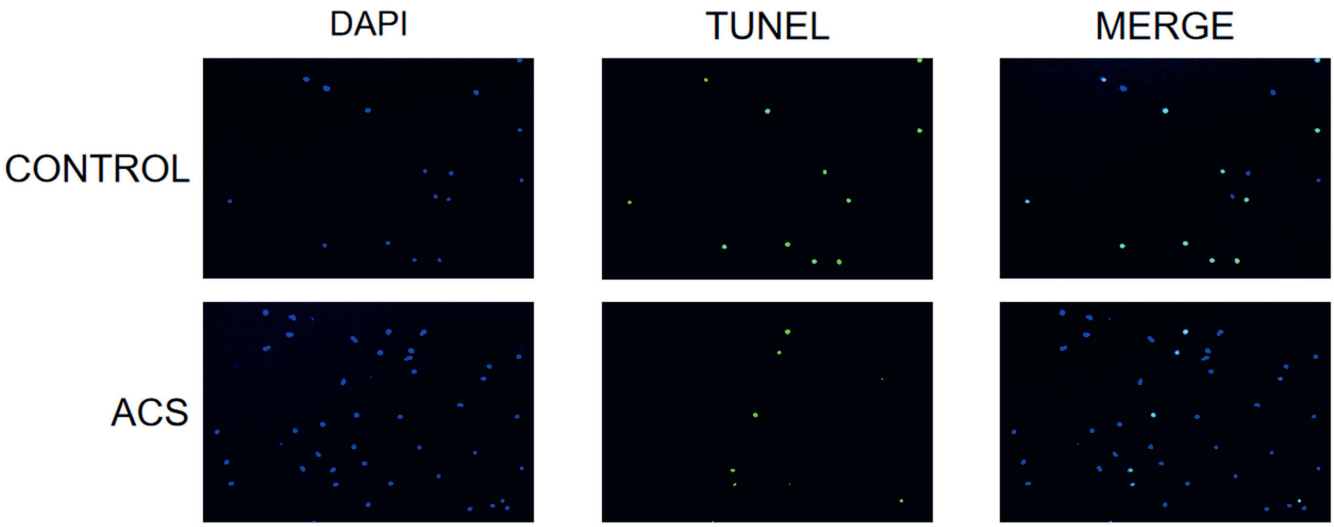

Fig. 2. TUNEL assay was used to detect the apoptosis in cells. Representative images of immunofluorescence staining showing expression of apoptotic cells (stained in green). Nuclei that labeled with DAPI (blue)

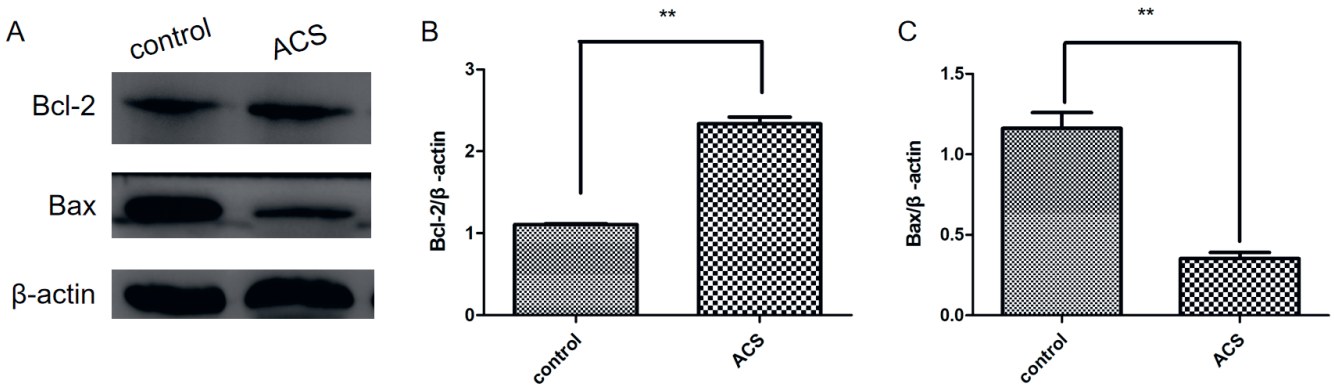

Fig. 3. A. Western blot analysis showing the expression levels of Bcl-2 and Bax proteins; B and C. Relative integrated density values were calculated using ImageJ (National Institutes of Health, Bethesda, USA) (n = 3). ** $p < 0.01$ compared to control. Data are expressed as mean ±standard error of the mean (SEM). Statistical analysis was done using Student's t-test.

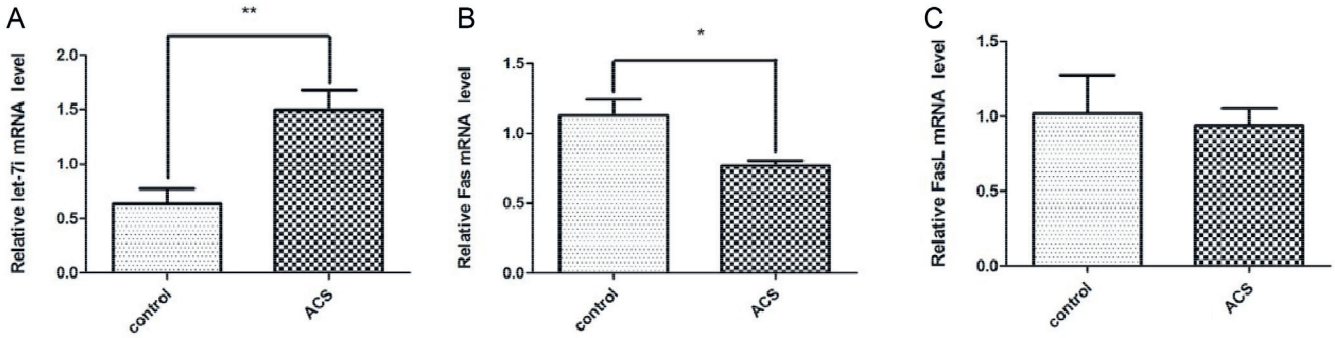

Fig. 4. The expression levels of miR-let-7i, Fas and FasL. Relative miR-let-7i (A), Fas (B) and FasL (C) expression levels in ACS $CD4^+$ T cells compared with normal $CD4^+$ T cells as determined using quantitative real-time polymerase chain reaction (qRT-PCR). *$p < 0.05$ and **$p < 0.01$ compared to control. Data are expressed as mean ±standard error of the mean (SEM). Statistical analysis was done using Student's t-test

MiR-let-7i was upregulated and Fas was downregulated in $CD4^+$ T cells of patients with ACS

As shown in Fig. 4, miR-let-7i expression was markedly higher ($t = 3.701$, $df = 8$, $p = 0.006$) and Fas mRNA expression levels ($t = 2.579$, $df = 11$, $p = 0.0257$) decreased in ACS $CD4^+$ T cells when compared with those of healthy controls. However, the expression of FasL ($t = 0.9227$, $df = 12$, $p = 0.3743$) was not significantly different from that of the healthy controls.

Fas and FasL were negatively regulated by miR-let-7i in ACS CD4+ T cells

The qRT-PCR results revealed that overexpression of ##miR-let-7i significantly reduced the mRNA expression levels of Fas ($t = 3.509$, $df = 4$, $p = 0.0247$) and FasL ($t = 3.801$, $df = 7$, $p = 0.0067$) when compared with that of mimic NC. In contrast, miR-let-7i inhibitors had the opposite effect and increased expression levels of Fas ($t = 2.428$, $df = 12$, $p = 0.0319$) and FasL ($t = 5.550$, $df = 4$, $p = 0.0052$) (Fig. 5). Similarly, the protein expression of Fas (mm: $t = 3.392$, $df = 6$, $p = 0.0146$; in: $t = 2.417$, $df = 10$, $p = 0.0363$) and FasL (mm: $t = 4.927$, $df = 4$, $p = 0.0388$; in: $t = 3.773$, $df = 4$, $p = 0.0196$) as determined with western blot analysis supported this finding (Fig. 6). Based on the obtained results, we conclude that miR-let-7i might negatively regulate the mRNA and protein expression of Fas and FasL.

MiR-let-7i enhanced the survival rate of ACS CD4+ T cells

Based on the miR-let-7i expression levels in CD4+ T cells determined with qRT-PCR, the cells were transfected with miR-let-7i mimic or mimic NC or with inhibitor or inhibitor NC to investigate the biological functions of miR-let-7i in CD4+ T cells. Next, the influence of miR-let-7i on CD4+ T cells was investigated using CCK-8 assays. The survival rate of CD4+ T cells transfected with miR-let-7i mimic was significantly higher than that of the miR-let-7i mimic NC group (12 h: $t = 4.677$, $df = 6$, $p = 0.0034$; 24 h: $t = 10.17$, $df = 6$, $p < 0.001$; 36 h: $t = 10.74$, $df = 6$, $p < 0.001$). In contrast, CD4+ T cells transfected with the miR-let-7i inhibitor (12 h: $t = 6.295$, $df = 7$, $p = 0.0004$; 24 h: $t = 8.496$, $df = 8$, $p < 0.0001$; 36 h: $t = 3.725$, $df = 4$, $p = 0.0204$) exhibited a notable decrease compared with those in the inhibitor

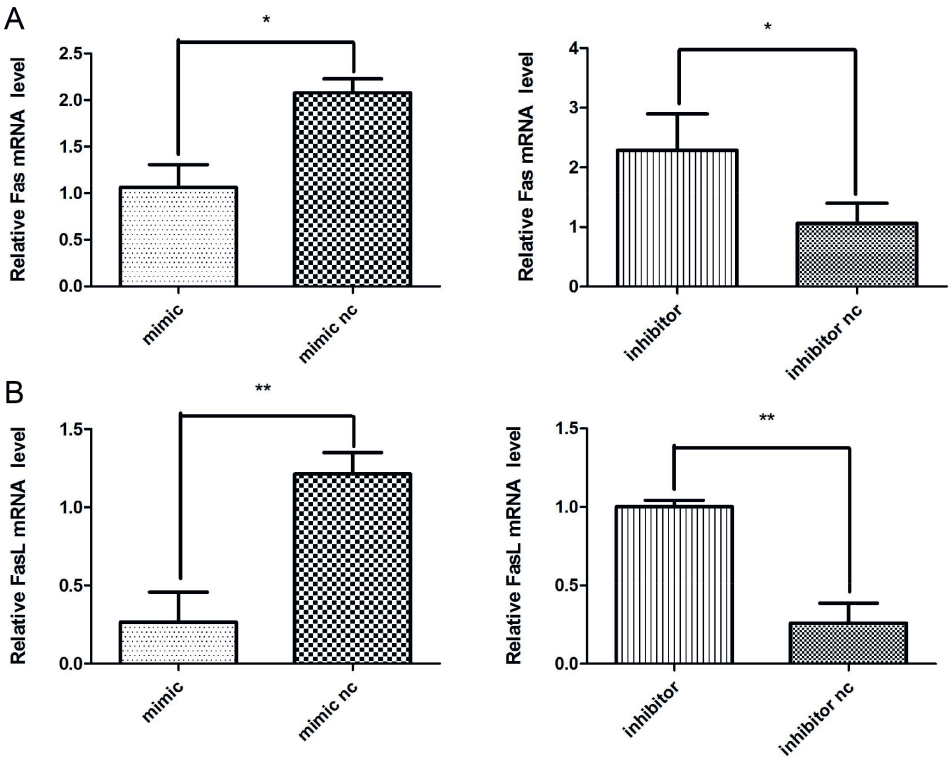

Fig. 5. Effect of miRNA-let-7i on the expression levels of Fas, FasL mRNA after transfection. ACS CD4+ T cells were transfected with miR-let-7i mimic, mimic NC, inhibitor, or inhibitor NC. The mRNA expression levels of Fas and FasL were decreased in cells transfected with miRNA-let-7i mimic compared with the mimic NC, but there was a higher expression in cells transfected with the miRNA-let-7i inhibitor compared with the inhibitor NC. *p < 0.05 and **p < 0.01 compared to control. Data are expressed as mean ±SEM. Statistical analysis was done using Student's t-test

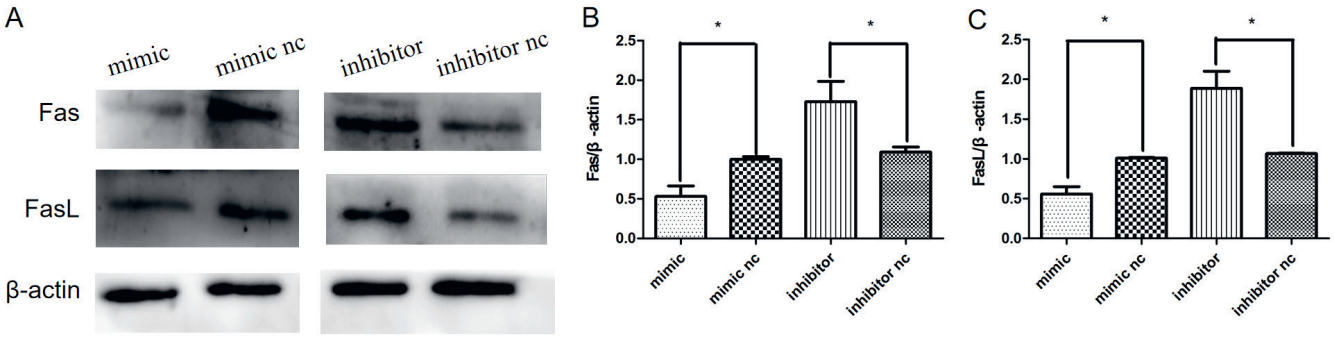

Fig. 6. Transfected ACS CD4+ T cells with miR-let-7i mimic, mimic NC, inhibitor, or inhibitor NC. A. Western blot analysis showed the expression levels of Fas and FasL proteins; B and C. The miRNA-let-7i mimic group showed a significantly decreased expression of Fas and FasL, and the miR-let-7i inhibitor group showed a significantly increased expression of these proteins compared with those in control cells (n = 3); *p < 0.05 compared to control. Data are expressed as mean ±standard error of the mean (SEM). Statistical analysis was done using Student's t-test

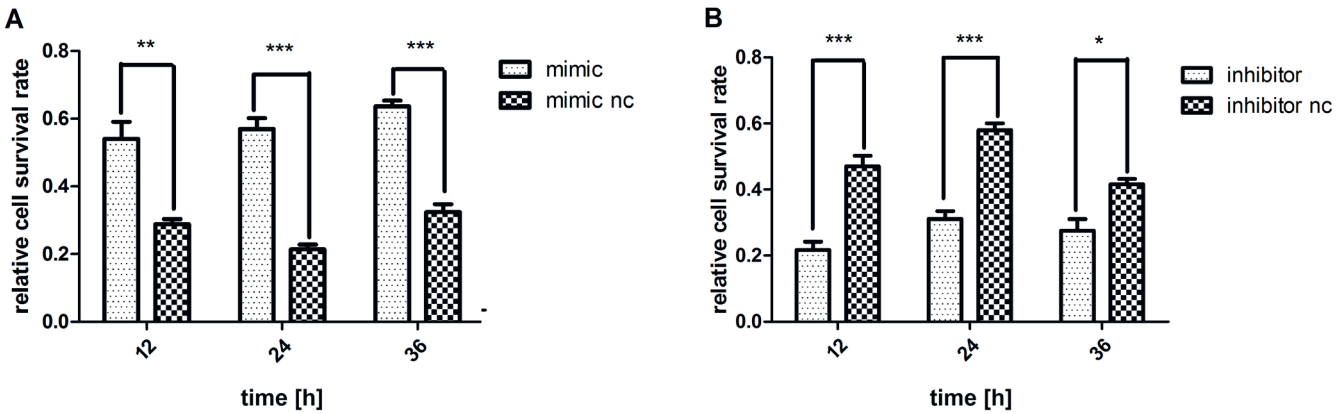

Fig. 7. Effect of miRNA-let-7i on the ACS CD4+ T cell survival ratio. CD4+ T cells were transfected with miR-let-7i mimic, mimic NC, inhibitor, or inhibitor NC for 12 h, 24 h or 36 h. CCK-8 assay was conducted to detect cell survival ratio (n > 3).*p < 0.05, **p < 0.01 and ***p < 0.001 compared to control. Data are expressed as mean ±standard error of the mean (SEM). Statistical analysis was done using Student's t-test

nc group (Fig. 7). These findings indicated that miR-let-7i might play a protective role in ACS CD4+ T cells.

MiR-let-7i inhibited apoptosis of ACS CD4+ T cells

To determine the effect of miR-let-7i on the apoptosis of CD4+ T cells, we transfected miR-let-7i mimic, mimic NC or inhibitor, inhibitor NC into CD4+ T cells. The apoptosis of CD4+ T cells was determined using TUNEL staining. As demonstrated in Fig. 8, TUNEL staining images showed a greater number of TUNEL-positive cells (green color) in the miR-let-7i-inhibitor-transfected group than in the inhibitor NC group. Interestingly, overexpression of miR-let-7i dramatically decreased the number of TUNEL-positive cells.

MiR-let-7i regulates the expression of the apoptosis proteins Bcl-2 and Bax in ACS CD4+ T cells

To further confirm that miR-let-7i can regulate CD4+ T cell apoptosis, the expression of apoptotic proteins was analyzed with western blot. As shown in Fig. 9, the expression of the anti-apoptotic protein Bcl-2 ($t = 3.659$, $df = 4$, $p = 0.0216$) was higher and the pro-apoptotic protein Bax ($t = 5.466$, $df = 6$, $p = 0.0016$) was lower in the mimic group compared with the mimic NC group. In contrast, miR-let-7i inhibitor reduced the expression of Bcl-2 ($t = 7.008$, $df = 4$, $p = 0.0022$) and increased the expression of Bax ($t = 16.59$, $df = 4$, $p < 0.0001$) when compared with the inhibitor NC group. In recent years, Bax has been found to interact with Bcl-2 to form a heterodimer or with itself to form a homodimer, and increased levels of the Bax homodimers often lead to apoptosis.[23] Accordingly, we examined the ratio of Bax/Bcl-2 (Fig. 9D). The results showed that miR-let-7i downregulated the Bax/Bcl-2 ratio. These results further confirmed that miR-let-7i plays a crucial role in regulating CD4+ T cell apoptosis.

Discussion

In this study, we demonstrate that compared with healthy control, the apoptosis rate of CD4+ T cells was reduced in ACS patients. We found high levels of miR-let-7i in CD4+ T cells in the peripheral blood of ACS patients. Upregulation of miR-let-7i inhibited apoptosis of CD4+ T cells in ACS, which may be one of the reasons for the resistance to apoptosis of these cells. The present study showed that Fas expression levels were reduced in ACS patients, while the expression of FasL was not significantly different from that in normal subjects, a finding which is consistent with previous results.[18] Our research confirms that miR-let-7i contributes to the partial protection of CD4+ T cells from apoptosis by targeting Fas/FasL.

Studies on T cells have confirmed the critical roles of miR-let-7i in the regulation of apoptosis. Zhang et al. found that miR-let-7i mediates CD4+ T cells to resist apoptosis through the IL-2 signaling pathway during HIV-1 infection.[24] Recent reports have indicated that miR-let-7i might protect T cells from apoptosis in ankylosing spondylitis by targeting IGF1R.[10] However, the regulatory mechanisms of miR-let-7i in CD4+ T cells in ACS are still unclear. In this study, we determined the expression of miR-let-7i. The expression level of miR-let-7i in CD4+ T cells in ACS was significantly upregulated when compared with the healthy control group. The abnormal expression of miR-let-7i may be associated with anti-apoptotic effects in CD4+ T cells. Therefore, the biological activity of miR-let-7i in CD4+ T cells was investigated by transfection and other tests. Our results showed that miR-let-7i overexpression facilitated cell survival and anti-apoptotic effects, while the opposite effects were observed in the miR-let-7i inhibitor group.

Apoptosis is a programmed and orderly cell death regulated by several genes. Cells undergo apoptosis via 'exogenous' (death receptor) or 'endogenous' (mitochondrial) pathways. The Fas/FasL signaling pathway plays an important role in apoptosis.[16] Fas, also known as Apo1 or CD95,

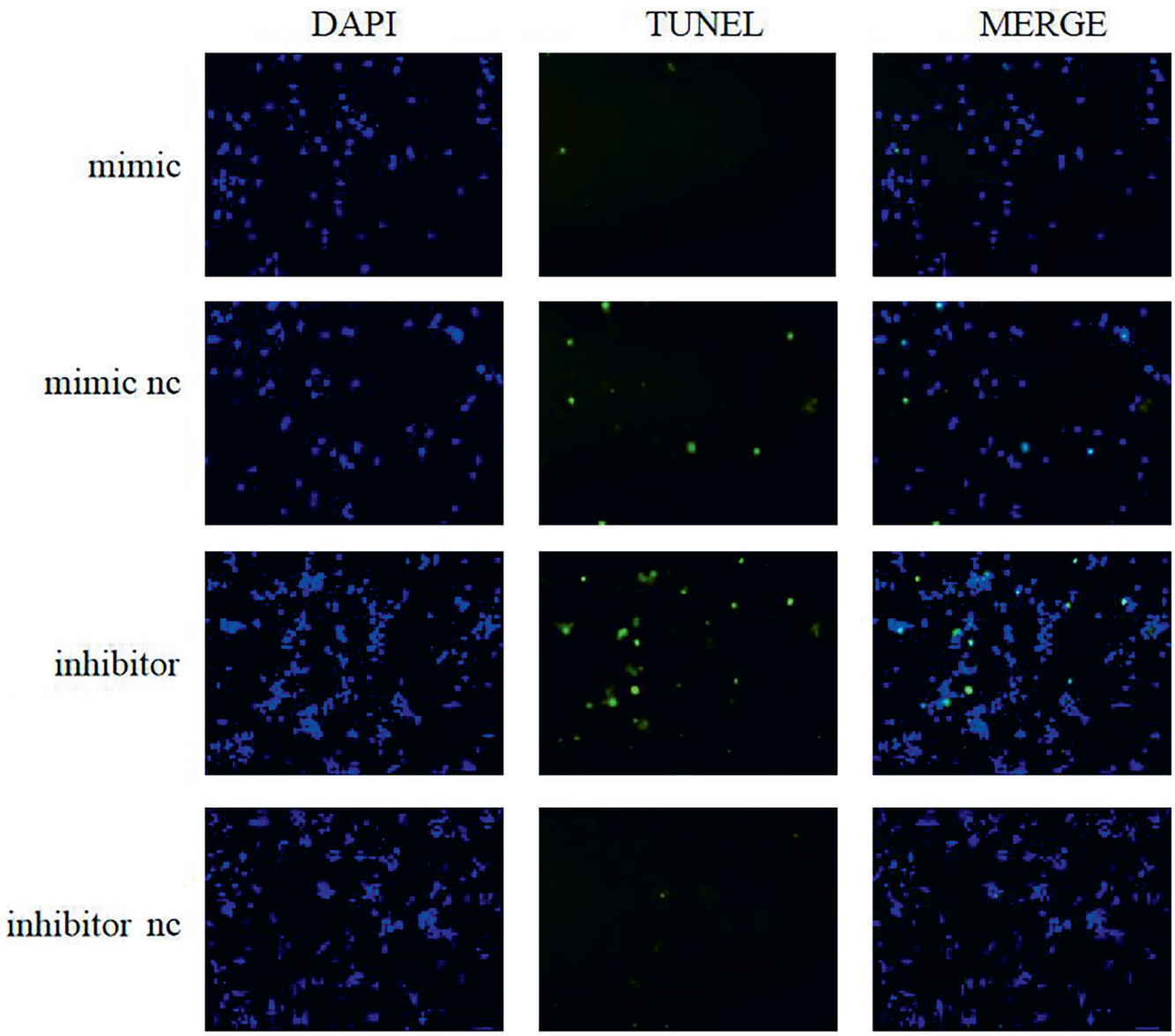

Fig. 8. Effect of miRNA-let-7i on ACS CD4+ T cell apoptosis. The number of TUNEL-positive cells transfected with miRNA-let-7i mimic was decreased compared with those with mimic NC. However, the number of TUNEL-positive cells transfected with miR-let-7i inhibitor was significantly increased. Representative images of immunofluorescence staining showing expression of apoptotic cells (stained in green). Nuclei were labeled with DAPI (blue)

belongs to the TNF receptor family and is a transmembrane glycoprotein widely distributed on the surface of different cells.[25] Fas initiates the extrinsic apoptotic pathway to induce apoptosis by interacting with its natural ligand, FasL.[26] Liu et al. found that Fas/FasL induced myocardial cell apoptosis in the process of myocardial cell ischemia-reperfusion (I/R) in a rat model.[25] Zhang et al. indicated that the upregulation of miR-25 inhibits cerebral I/R injury-induced apoptosis by downregulating Fas/FasL.[27] In this study, we identified the decreased expression of Fas and a slight difference in FasL in ACS patients when compared with healthy subjects.

MiR-let-7 is one of the largest and most highly expressed families of miRNAs.[28] All family members are considered to have similar functions because of the identical seed region (nucleotides 2 to 7) required for miR-target mRNA interaction.[9] It has been shown that miR-let-7i belongs to the let-7 family. Wang et al. first demonstrated that let-7 regulates Fas expression and the sensitivity of Fas-mediated apoptosis.[19] Furthermore, Zhang et al. indicated that miR-98, a member of the let-7 family, regulates Fas/FasL gene expression in myocardial cells and modulates cell apoptosis.[29] Zhang et al. also proved that Faslg, encoding the Fas ligand, is the target of let-7i-5p and plays an anti-apoptotic role.[20] Fas/FasL signaling is a major regulator of apoptosis.[30] We confirmed that miR-let-7i negatively regulates Fas/FasL at the RNA and protein levels and plays a significant role in the apoptosis of CD4+ T cells in ACS. However, the association between cell apoptosis and Fas/FasL expression and whether interference with

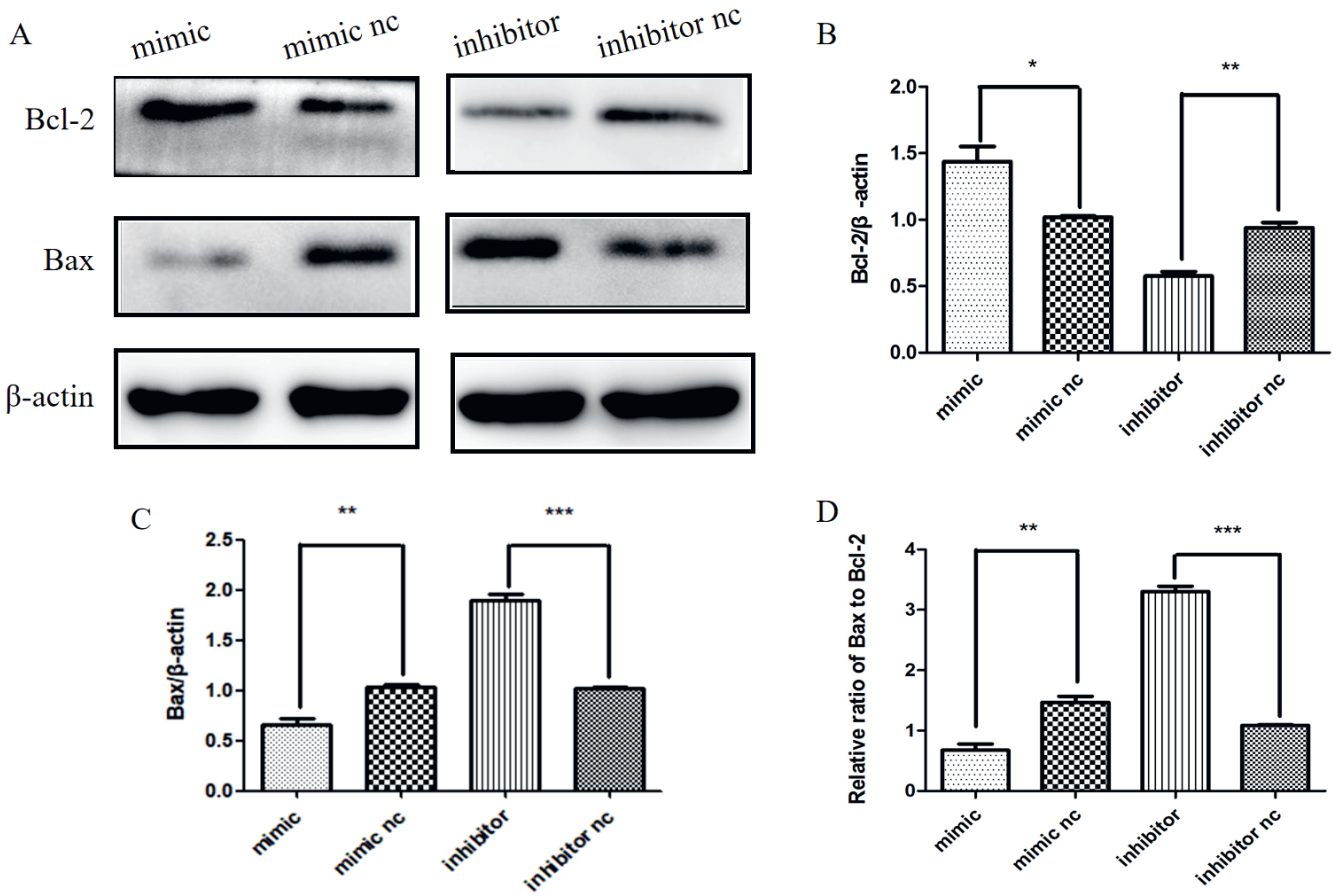

Fig. 9. Effect of miRNA-let-7i on ACS CD4⁺ T cell apoptosis proteins Bcl-2 and Bax and the ratio of Bax/Bcl-2. A. Western blot analysis shows the expression levels of Bcl-2 and Bax proteins; B and C. Relative integrated density values were calculated using ImageJ. The ratio of Bax/Bcl-2 (D) (n = 3); *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control. Data are expressed as mean standard error of the mean (SEM). Statistical analysis was done using Student's t-test

the Fas/FasL signaling pathways affects the role of miR-let-7i in apoptosis remain to be established. Future studies are needed to clarify the molecular mechanisms involved.

Previous studies have found that CD4⁺ T cells lacking the costimulatory receptor CD28 are present in the peripheral blood of patients with ACS. It has also been shown that these CD28-deficient CD4⁺ T cells can accumulate preferentially in unstable ruptured atherosclerotic plaques. This particular subset of CD4⁺ T cells can selectively invade unstable plaques, indicating that they may have a direct role in plaque destabilization following the local activation by antigens.[31] These cells can also express killer cell immunoglobulin-like receptors (KIRs) that release IFN-γ.[32] Our study has shown that the majority of CD4⁺ T cells in ACS patients express perforin but lack CD28, while CD4⁺ T cells in both healthy controls and SAP patients express CD28 but lack perforin expression.[21] Thus, the decreased expression of the CD28 costimulatory molecule has an important effect on the function of CD4⁺ T cells. Because of the decreased expression of CD28 protein, the CD4⁺ T cells in ACS patients are resistant to apoptotic signals, and live longer in vivo. We will focus on the understanding of the relationship between the decreased protein expression of CD28 and the mechanisms of survival and apoptosis of CD4⁺ T cells in future research studies.

Limitations

In addition to CD4+CD28–T cells, there are many other subsets of CD4+T cells. In this study, we only study the CD4+T cells. Therefore, we need to further investigate the important role of CD4+T cells and their subsets in acute coronary syndromes.

Conclusions

In conclusion, our findings demonstrate that miR-let-7i significantly reduces Fas and FasL expression in ACS CD4⁺ T cells and inhibits apoptosis in these cells. Our work indicates that suppressing miR-let-7i may be a potential approach to treat atherosclerosis. Since a large number of genes are involved in apoptosis, we cannot exclude the possibility that miR-let-7i regulates cell apoptosis by targeting other apoptosis genes. Therefore, further studies are needed to confirm our findings.

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