Friend or Foe: A Cancer Suppressor MicroRNA-34 Potentially Plays an Adverse Role in Vascular Diseases by Regulating Cell Apoptosis and Extracellular Matrix Degradation

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Background: MicroRNAs (miRNAs) have emerged as central regulators of many processes. MiRNA-34 (miR-34) functions as a well-known tumor suppressor. The aim of this study was to investigate the mechanisms underlying how miR-34 participates in vascular disorders.

Material/Methods: Three miR-34 family members (miR-34a, miR-34b, and miR-34c) were overexpressed or silenced in human vascular smooth muscle cells (VSMCs) and umbilical vein endothelial cells (UVECs), respectively, before the proliferation and apoptosis of cells were detected by using Cell Counting Kit-8 assay and Annexin V- fluorescein isothiocyanate/propidium iodide flow cytometry. The protein expression of apoptosis biomarkers was detected by western blot. Dual-luciferase reporter assay was performed to determine the candidate target of miR-34, and enzyme-linked immune sorbent assay was used to evaluate the effect of miR-34 on the expression of the target gene.

Results: Overexpression of miR-34 family members repressed proliferation and promoted apoptosis of VSMCs and UVECs, whereas miR-34 knockdown led to the opposite results. In addition, miR-34a inhibited the expression of alpha-1 antitrypsin (AAT), a serine protease inhibitor that suppresses the degradation of extracellular matrix, through a miR-34-binding site within the 3'-UTR of AAT.

Conclusions: MiR-34 promoted apoptosis of VSMC and UVEC cells by inhibiting AAT expression. This finding provides an update on the understanding of the clinical value of miR-34, which might assist to uncover novel and effective therapeutic strategies for the treatment of vascular diseases.

MeSH Keywords: Apoptosis • MicroRNAs • Vascular Diseases
Background

Recent findings of diverse atherosclerosis-related miRNAs provided novel insights into the diagnosis and treatment of cardiovascular diseases [1]. MicroRNAs (miRNAs) are small, typically 22 nucleotides in length, endogenous non-coding RNAs that can inhibit gene expression at post-transcriptional levels by interacting with 3'-UTR of the target mRNA, resulting in either degradation of mRNA or translation suppression [2]. It was previously well-known that miRNAs play a crucial role in a wide array of physiological and pathological processes including cell proliferation, differentiation, stem cell maintenance, and tumorigenesis [3,4]. An increasing number of studies have revealed that miRNAs are indispensably involved in angiogenesis by regulating the biological functions of angiogenesis-related cells as well as angiogenic factors, which are responsible for a number of vascular diseases such as aneurysm, aortic dissection, atherosclerosis, phlebothrombosis, stroke, cardiovascular disease, and other angiogenic disorders [5–7].

In mammals, 3 miR-34 family members are generated from 2 transcriptional units: miR-34a is transcribed from its own transcript, while miR-34b and miR-34c share a common primary transcript. Being identified as direct transcriptional targets of the onco-suppressor p53, miR-34 family members are implicated in the induction of G1 cell cycle arrest, senescence and apoptosis, as well as the regulation of cancer stem cells self-renewal functions in response to DNA damage and oncogenic stress [8–10]. In addition, enhanced expression of miR-34 has been reported to suppress tumor growth and induce chemo-sensitization and apoptosis in cells harboring a loss-of-function mutant of p53 [11].

MiR-34 is also well-known for its anti-angiogenic functions in the development of tumors [12]. It substantially mitigates tumor sphere proliferation, migration, and particularly angiogenesis by regulating the expression of downstream target genes in various tumors such as head and neck squamous cell carcinoma (HNSCC) and colon cancer [13,14]. Recent studies have demonstrated that miR-34a expression is increased in atherosclerotic portions of vessels and in plaques [15,16]. However, research addressing the role of miR-34 in vascular diseases are still unavailable. Accordingly, our study investigated how miR-34 acts on general cell functions and searched for its downstream targets in cells that are related to the pathogenesis of vascular diseases, typified by vascular smooth muscle cells (VSMCs) and umbilical vein endothelial cells (UVECs).

Material and Methods

Cell culture

Human VSMCs, UVECs, and HEK293T cells were maintained in Dulbecco’s modified eagle medium (DMEM) (Gibico, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibico, Grand Island, NY, USA). Cells were harvested for RNA or protein analysis at approximately 90% confluence. UVEC cells were isolated from donated umbilical cords and cultivated in extracellular matrix (ECM) medium (Sciencell, San Diego, CA, USA) supplemented with endothelial cell growth supplement ECGS (Sciencell, Cat No. 1052, San Diego, CA, USA) plus 5% FBS, until used at passage 7.

Infection of cultured cells

The DNA fragment that encode human alpha-1 antitrypsin (AAT) cloned into the pIREs vector was synthesized by GenePharma Co. (Shanghai, China). VSMC and UVEC cells were seeded in 6-well culture plates and sequentially infected with lentivirus encompassing different miR-34 members or sponge, produced by Weiguang Bio. (Shenzhen, Guangdong, China), at 80% confluency (approximately 500 000 cells per well).

MiRNA extraction and quantification

MiRNAs were extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA). Real-time polymerase chain reaction (RT-PCR) was carried out using the Stem-loop method [17] with 1 μL RT product. The data were normalized to RNU6B small nuclear RNA to calculate fold changes using the method of ΔΔCt.

Dual-luciferase reporter assay

Online database RegRNA was used to predict the potential binding sites for miR-34. For dual-luciferase reporter assays, the full-length 3'-untranslated (3'-UTR) of AAT containing miR-34 binding sites was cloned into the downstream of a psiCheck2 (Promega, Madison, WI, USA) to generate psiCheck2-AAT 3'-UTR, which was co-transfected with miRNA expression vectors into VSMC cells. The vector carrying the firefly luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase activities were determined by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturers’ protocol. All the reactions were performed in triplicate.

Cell Counting Kit-8 (CCK-8) assay

Cell proliferation assay was performed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocols. We seeded 1×10^4 transfected...
cells into 96-well plates and analyzed at the indicated time points. The absorbance of samples was measured at 450 nm using a multilabel plate reader.

Flow cytometry assay

Cells were washed twice in phosphate buffered saline (PBS) and centrifuged at 670 g for 5 minutes. Each pellet was collected and resuspended in PBS. For detecting apoptosis, 400 μL of cells were mixed with 100 μL incubation buffer with 2 μL of Annexin V and 2 μL propidium iodide (IP, BD Pharamingen, San Jose, CA, USA). Recombinant human AAT was used to prepare a standard curve. Data of AAT levels obtained from duplicate wells are presented as means ± standard deviation (SD).

Statistical analysis

Data are presented as means ± SD. All in vitro experiments included at least 3 replicates per group. Groups were compared using the 2-tailed Student’s t-test for parametric data. When comparing multiple groups, data were analyzed by analysis of variance (ANOVA) with Bonferroni’s post-hoc test. A value of P<0.05 was considered statistically significant.

Results

MiR-34 suppressed the cell proliferation of VSMCs and UVECs

MiR-34 is a classical regulator of tumor cell apoptosis, and for this study wondered if it could also induce apoptosis in cells that were involved in the pathogenesis of vascular diseases, including VSMCs and UVECs. Thus, we first overexpressed the 3 miR-34 family members (miR-34a, miR-34b, and miR-34c) in VSMCs and UVECs. As shown in Figure 1A, qRT-PCR results showed that there was a dramatic increase in the expression of miR-34a, miR-34b, and miR-34c (P<0.01). The impact of miR-34 on cellular proliferation of VSMCs was determined by performing a CCK-8 assay. Overexpression of miR-34a, miR-34b, or miR-34c caused an obvious decrease in cell proliferation (P<0.01, Figure 1B).

MiRNA sponges were widely used to inhibit the expression of miRNA. Consequently, we suppressed miR-34 expression in VSMCs and UVECs by using lentivirus expressing miR-34 sponge. Knockdown efficiency was assessed by detecting the expression of miR-34 using qRT-PCR. As shown in Figure 1C, the expression level of miR-34a, miR-34b, and miR-34c was compromised due to the introduction of miR-34 sponge. In contrast to miR-34 overexpression, the number of viable VSMC and UVEC cells was remarkably augmented due to miR-34 knockdown, (P<0.05, Figure 1D). This result demonstrated that all the miR-34 family members exhibited an inhibitory effect on the proliferation of VSMC and UVEC cells.

MiR-34 promoted cell apoptosis of VSMCs and UVECs

To test the effect of miR-34 on the apoptosis of VSMC and UVEC cells, AnnexinV-FITC/PI flow cytometry was carried out. Our results revealed that the percentage of apoptotic VSMC cells was increased by enhanced expression of miR-34a, miR-34b, or miR-34c (P<0.01, Figure 2A). Of note, there was an approximately 15- to 25-fold increase in the percentage of apoptotic

Western blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) before total protein was extracted. Then protein samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After the membrane was blocked for 1 hour using 5% skim milk, it was incubated with primary antibodies against caspase-3 and cleaved caspase-3, and b-actin (Cell Signaling Technology) at 4°C overnight. Finally, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for another 1 hour. Band signals were visualized using electrochemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA).

Enzyme-linked immune sorbent assay (ELISA)

The harvested cells were pelleted and resuspended in RIPA lysis buffer, followed by incubating on ice for further lysing, and centrifuged at 15 000 rpm for 5 minutes at 4°C. After centrifugation, the content of released AAT in the supernatant was measured using a human AAT quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). After the membrane was blocked for 1 hour with PBS and blocked for at least 30 minutes with 200 μL of PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), followed by the addition of standard dilutions of recombinant AAT and test samples, and incubated 2 hours at 37°C. After washing 3 times with PBS, each well was incubated with 100 μL of biotinylated anti-human AAT polyclonal antibody (1: 200) for 60 minutes and then washed 3 times. After staining with streptavidin-horseradish peroxidase (1: 200) for 30 minutes, 100 μL of substrate working solution was added to each well and incubated for 15 to 20 minutes in the dark. The reaction was then stopped by the addition of 100 μL of 2 M H₂SO₄ and the optical density (OD) at 450 nm was measured with a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA). Recombinant human AAT was used to prepare a standard curve. Data of AAT levels obtained from duplicate wells are presented as means ± standard deviation (SD).
Figure 1. MiR-34 suppresses cell proliferation of VSMCs and UVECs. (A) The expression levels of miR-34a, miR-34b, and miR-34c were evaluated by qRT-PCR. (B) The impact of miR-34 overexpression on cellular proliferation of VSMCs and UVECs was determined by performing a CCK-8 assay. (C) MiR-34 sponge was used to suppress miR-34 expression in VSMCs and UVECs. Knockdown efficiency was assessed using qRT-PCR. (D) The number of viable VSMC and UVEC cells after miR-34 knockdown was measured by CCK-8 assay. Data were presented as mean ± standard deviation. * P<0.05, ** P<0.01. VSMCs – vascular smooth muscle cells; UVECs – umbilical vein endothelial cells; qRT-PCR – quantitative real-time polymerase chain reaction; CCK-8 – Cell Counting Kit-8.
cells in the VSMC cells overexpressing miR-34b. Furthermore, overexpression of these miR-34 family members also significantly promoted the apoptotic status of UVEC cells (Figure 2B).

As the activated form of caspase-3, cleaved caspase-3 acting as an apoptosis biomarker was detected by western blot. As shown in Figure 2C and 2D, the proportion of cleaved caspase-3 was significantly increased with overexpression of miR-34. Data were presented as mean ± standard deviation. ** P<0.01. VSMCs – vascular smooth muscle cells; UVECs – umbilical vein endothelial cells; Annexin V-FITC/PI – Annexin V-fluorescein isothiocyanate/propidium iodide.

**Figure 2.** MiR-34 promotes cell apoptosis of VSMCs and UVECs. Annexin V-FITC/PI flow cytometry was carried out to test the percentage of cell apoptosis of (A) VSMC cells and (B) UVECs cells. The proportion of cleaved caspase-3 in (C) VSMC cells and (D) UVEC cells was detected by western blot after overexpression of miR-34. Data were presented as mean ± standard deviation. ** P<0.01. VSMCs – vascular smooth muscle cells; UVECs – umbilical vein endothelial cells; Annexin V-FITC/PI – Annexin V-fluorescein isothiocyanate/propidium iodide.

To further explore the relationship between miR-34 and AAT expression in vitro, we measured AAT protein levels in both VSMC and UVEC cells undergoing transfection with different compounds by performing ELISA assay. Results showed that overexpression of miR-34 significantly suppressed AAT level compared with untreated cells, whereas restriction of miR-34 exhibited opposite effects (Figure 3C, 3D, P<0.01). Taken together, these

**MiR-34 directly targeted the 3’-UTR of AAT gene**

Extracellular matrix (ECM) is critical in the regulation of various vascular diseases as it can affect vessel wall thickness and elasticity. It is well known that miR-34 inhibits cell proliferation and promotes apoptosis. Although many target genes of miR-34 have been identified in the tumorigenic processes, whether it can directly modulate ECM components in vascular diseases remains unknown. Putative target genes of miR-34 were predicted by performing the tool RegRNA and TargetScan. Among numerous familiar ECM components, AAT with a relatively long 3’-UTR was predicted to be a candidate target of miR-34 (Figure 3A), which was then verify via dual-luciferase activity assay. A reporter vector containing a luciferase cDNA followed by the 3’-UTR of AAT was constructed and then co-transfected with miR-34 response element into HEK293 cells. As a result, miR-34a, miR-34b, or miR-34c obviously impaired luciferase activity of the reporter vector carrying AAT (Figure 3B).

To further explore the relationship between miR-34 and AAT expression in vitro, we measured AAT protein levels in both VSMC and UVEC cells undergoing transfection with different compounds by performing ELISA assay. Results showed that overexpression of miR-34 significantly suppressed AAT level compared with untreated cells, whereas restriction of miR-34 exhibited opposite effects (Figure 3C, 3D, P<0.01). Taken together, these
data suggested that miR-34 decreases AAT expression by directly binding to the AAT 3’-UTR.

Discussion

Conventionally, miR-34 is known as a cancer suppressor since it promotes cell apoptosis and thus impairs tumor regeneration [18]. Epigenetic inactivation of miR-34 is related to the initiation and progression of diverse human cancers types such as prostate cancer, lung cancer, breast cancer, colon cancer, and glioblastoma [18–20]. In lung cancer, enforced expression of miR-34 suppressed cancer cell growth [21], whereas miR-34 depletion resulted in a more aggressive behavior of cancer stem cells [22]. Thus, miR-34 has been deemed as an alternative therapy candidate to conquer tumor progression [23].

The anti-angiogenesis effect of miR-34 on endothelial cells has been confirmed for a long time, and is often used to explain its involvement in diverse diseases. For example, miR-34a expression is increased in atherosclerotic portions of vessels and in plaques [15,16], revealing its crucial role in cardiovascular diseases. Researchers proposed that forced expression of miR-34a triggers the senescence of human UVECs and suppresses cellular proliferation through, at least in part, inhibition of SIRT1 protein [24,25]. It was also demonstrated that silencing of the entire miR-34 family could protect the heart against pathological cardiac remodeling and improve function [26]. However, in the present study, we observed some new findings that may be related to the abdominal aortic aneurysm (AAA) pathobiology. In the present study, the expression of 3 members of the miR-34 family was respectively upregulated or downregulated in VSMCs and UVECs, 2 types of vascular diseases-related cell lines, to investigate the role of miR-34 in regulating cell general functions. In this study we found that overexpression of all miR-34 family members impaired cell proliferation and promoted apoptosis, whereas inhibition of miR-34 contributed to an opposite result. This finding is in agreement with previous studies addressing miR-34-mediated suppression of cancer cell growth. Besides, expression in atherosclerotic portions of vessels and in plaques [15,16], revealing its crucial role in cardiovascular diseases. Researchers proposed that forced expression of miR-34a triggers the senescence of human UVECs and suppresses cellular proliferation through, at least in part, inhibition of SIRT1 protein [24,25]. It was also demonstrated that silencing of the entire miR-34 family could protect the heart against pathological cardiac remodeling and improve function [26]. However, in the present study, we observed some new findings that may be related to the abdominal aortic aneurysm (AAA) pathobiology. In the present study, the expression of 3 members of the miR-34 family was respectively upregulated or downregulated in VSMCs and UVECs, 2 types of vascular diseases-related cell lines, to investigate the role of miR-34 in regulating cell general functions. In this study we found that overexpression of all miR-34 family members impaired cell proliferation and promoted apoptosis, whereas inhibition of miR-34 contributed to an opposite result. This finding is in agreement with previous studies addressing miR-34-mediated suppression of cancer cell growth. Besides,
the pathogenesis of AAA results from, to a large extent, the apoptosis of medial smooth muscle cells and endothelial cells. Thus miR-34 is suggested to promote AAA development by accelerating cellular apoptosis.

On the other hand, miR-34 directly targets the 3'-UTR of the AAT gene and suppresses the level of its protein product. AAT is a serine protease inhibitor with an important role in protecting the structures of ECM from degradation by various proteolytic enzymes. Depletion of AAT is responsible for impaired protease-antiprotease homeostasis accompanied by destruction of elastic tissue in several organs such as lungs, liver, as well as vasculature [27]. Alpha-1 antitrypsin deficiency (AATD) is an inherited disorder that causes an extremely reduced level of AAT in the blood and may predispose to a variety of diseases. Research regarding the association of AATD with increased risk of AAA have been conducted since the 1990s. It has also been demonstrated that aberrances in AAT concentration, as well as imbalance between AAT and elastase activity in patients with AAA are responsible for this disease [28]. Moreover, the crucial role of AAT protein in the pathogenic process of AAA has been reviewed recently [29]. Other than AAA, many vascular disorders are significantly associated with the lack of AAT in tissues or plasma. For example, those patients with AATD are more susceptible to developing a cardiovascular disease [30]. Ruptures of atherosclerotic plaque are common events in acute coronary syndromes and strokes. Decreased expression of AAT may promote atherosclerosis formation [31], and recent evidence revealed the relationship between AATD and occurrence of stroke [32].

Conclusions

In aggregate, a major finding of our study was that miR-34 promoted apoptosis of VSMC and UVEC cells. MiR-34 could also inhibit AAT expression by directly targeting its 3'-UTR, implicating its potential role in the pathogenesis of some vascular diseases such as AAA. Our new findings provide an update on the understanding of the clinical value of miR-34, although more evidence is required to verify the involvement of miR-34 in AAA development.

Conflict of interest

None.

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