Research paper

MicroRNA-98 regulates foam cell formation and lipid accumulation through repression of LOX-1

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ABSTRACT

Objective: Several miR/s that regulate gene/s relevant in atherogenesis are being described. We identified a miR (miR-98) that targets LOX-1, a receptor for ox-LDL, and speculated that it might be relevant in atherogenesis.

Approach and results: MicroRNA-98 was predicted by bioinformatics tools. The effects of miR-98 (by use of mimics and inhibitors) on LOX-1 expression and foam cell formation in mouse peritoneal macrophages were assessed. ApoE−/− mice fed by high fat diet were administered with mmu-agomiR-98 and mmu-antagomiR-98, and expression of LOX-1 and foam cell formation in aorta were quantified. LOX-1 was established to be a direct target of miR-98 by luciferase reporter assay. Enhancement of miR-98 decreased the expression of LOX-1 and inhibited foam cell formation and lipid accumulation. Inhibition of miR-98 had the opposite effects on all parameters.

Conclusions: Reduced expression of miR-98 may relate to LOX-1 expression and foam cell formation and lipid accumulation in aortas of ApoE−/− mice. Plasma level of miR-98 may be a biomarker of atherosclerotic disease process and its modulation may offer a therapeutic strategy for atherosclerosis.

1. Introduction

The precise mechanism of atherogenesis has not been fully elucidated. Ox-LDL stimulates a series of pathological reactions such as foam cell formation [1] and lipid accumulation in arterial wall. Lectin-like ox-LDL scavenger receptor-1 (LOX-1), a type II membrane glycoprotein, binds and internalizes ox-LDL in macrophages, and is responsible in large part in the induction of pathological steps leading to atherosclerosis [2–4].

Recently, microRNAs (miRs), a group of endogenous, small, non-coding, single stranded RNAs, have been shown to participate in atherogenesis [5–11] suggesting varied functions of different miRs on markers of atherogenesis. We predicted novel microRNA, miR-98, using bioinformatics tools as it targets LOX-1, an important receptor for ox-LDL. We found that macrophages treated with ox-LDL had lower expression of miR-98, and higher expression of LOX-1. Then we subsequently studied its role in foam cell formation and lipid accumulation in vitro and in vivo settings using loss-gain function method.

2. Materials and methods

2.1. Bioinformatics prediction

We used three different Bioinformatics databases (Targetscan, miRanda and miRFinder) to predict that the 3’-UTR of LOX-1 is a direct target for miR-98.

2.2. Culture of mouse peritoneal macrophages

Macrophages were collected from C57BL/6 mice (Shanghai Laboratory Animal Center, Shanghai, China). Briefly, mice were injected intraperitoneally with 1 mL 4% Brewer modified thioglycollate medium (BD Biosciences, San Jose, CA) to stimulate accumulation of macrophages. Three days later, 5 mL cold 1× PBS was injected intraperitoneally, and macrophages were harvested. Isolated cells were cultured in the 1640 medium (ATCC, Beijing, China) along with 10% fetal calf serum, 4 mM L-glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin (ATCC, Beijing, China) for 2 h. After incubation at 37 °C in a humidified atmosphere with 5% CO2, cells were seeded onto six-well plate at a density of 5 × 105 cells/well before being used in
further experiments. Mice were evaluated by cervical dislocation. The study was approved by the Research Ethics Committees and the Chinese Academy of Sciences (Anhui Medical University).

2.3. MTT assay for ox-LDL and miR-98 modulator toxicity measurement

Cell cytotoxicity was evaluated by using the CellTitre 96 AQueous One Solution Reagent (Promega, Madison, WI) containing a tetrazolium compound 3-(4,5-dimethyl-2-yi)-5- (3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTT) and an electron coupling reagent (phenazine ethosulfate; PES). Confluent macrophages plated in 96 well tissue culture plates (BD Biosciences, Spikes, MD) were washed three times in PBS and incubated in serum free medium with different concentration of ox-LDL (0–40 µg/mL) for 24 h or transfected with different concentration of miR-98 mimic or inhibitor (0–200 nM). MTT assay was performed by adding 10 µL/well of reagent and absorbance at 490 nm was recorded using an ELISA plate reader.

2.4. Dil-ox-LDL uptake

Dil-ox-LDL was used to determine the ability of mice macrophages to take up ox-LDL as described previously [12]. In brief, cells were incubated with 10 µg/mL Dil-ox-LDL for 2 h. Upon completion of incubation, cells were gently washed with 1× PBS three times to remove free Dil-ox-LDL and analyzed using fluorescent microscope and quantified by red intensity per cell.

2.5. miR-98 and LOX-1 siRNA transfection in macrophages

To study the effects of miR-98 on the expression of LOX-1 and foam cell formation, mouse peritoneal macrophages were transfected with the MISSION® Lenti hsa-miR-98 mimic (HM0982) and inhibitor (HILTUD0460), and MISSION® Lenti mmu-miR-98 mimic (MLMIR0250) and inhibitor (MILTUD0250) (Sigma Aldrich, St. Louis, MO) using Lipofectamine 2000 (Invitrogen Corp, Shanghai, China) as the transfection agent (transfection efficiency ≈75%). The most appropriate concentration of miR-98 transfection was determined by miR-98 expression (RT-qPCR) and cell proliferation (MTT assay) in dose-finding experiment (0, 12.5, 25, 50, 100, 200 nM). Scrambled miRNA Mimics Negative Control #1 (lot # HMC0002) and scrambled MISSION® Synthetic microRNA Inhibitor ath-miR416 (lot # NCTUD001) served as negative controls (Sigma Aldrich, St. Louis, MO). Cells treated with only Lipofectamine 2000 were used as mock control to normalize the background of transfection reagent. After 12 h transfection, medium was replaced with fresh medium and cells were incubated with different concentrations of ox-LDL (0–10 µg/mL) (Alfa Aesar, Beijing, China) at 37 °C for another 24 h. For LOX-1 siRNA (ThermoFisher, Waltham, MA) transfection, macrophages were incubated with ox-LDL (10 µg/mL) along with appropriate concentration of miR-98 inhibitors; Silencer® Negative Control #1 siRNA (ThermoFisher, Waltham, MA) was used as negative control.

2.6. MiR-98 and foam cell formation

Primary macrophages were transfected with the mmu-miR-98 mimic (50 nM) or mmu-miR-98 inhibitor (50 nM) (Sigma Aldrich, St. Louis, MO) or siRNA of LOX-1, and appropriate controls, for 12 h followed by treatment with ox-LDL (10 µg/mL) for 24 h. Oil red O staining was used to study foam cell formation and intensity of red color was calculated according to standard protocol [13].

2.7. Studies in ApoE−/− mice

Male ApoE−/− mice were purchased and given a high-fat diet (0.15% cholesterol and 21% cocoa butter) mixed with regular chow ad lib for 12 weeks from the age of 6 weeks. The mice were administered agomiR-98 (5′-TGGGAACACTCCAGCGAGCTGATTGTTAGC-3′) (n = 7) or antagomiR-98 (5′-AGTCTCAACTGTTCTGGGCGAAGTTCATTGACCATCAGT-3′) (n = 7) (Ribobio. Co, Guangzhou, China) via tail vein (0.8 mg/kg in 0.2 mL saline every day) from the beginning of high-fat diet feeding according to a previous study [14]. A sterile cannula was placed in the tail vein to collect blood to avoid frequent puncture of the vein. The cannula was replaced every week. Scrambled agomirRNA (n = 7) and scrambled antagomirRNA (n = 7) were used as negative controls. The body weight and serum lipid profiles (total cholesterol, triglyceride, non HDL-c, HDL-c) were measured every two days during delivery of miR-98 modulators. At 18 weeks of age (12 weeks of feeding and injection of miR-98 modulators), mice were sacrificed and aortic tissues and plasma were rapidly isolated. MicroRNA-98 expression was measured in plasma after mice were sacrificed. Systemic delivery efficiency, calculated as described previously [7], was estimated to be about 60%. Side effects of miR-98 modulators were determined by measurements of body weight, caspase-3 activity in different tissues (brain, heart, lung, liver, kidney and spleen), and liver function (H&E and liver enzyme measurement) [15,16]. To test which cell types take up agomiR-98 and antagomiR-98, we isolated endothelial cells, smooth muscle cells as described previously [17]. ApoE−/− mice were sacrificed by exsanguination under anesthesia ketamine-HCl 100 mg/kg and xylazine 20 mg/kg i.p. injection.

All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee, and conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study was approved by Anhui Medical University ethics review board.

2.8. Immunohistochemistry

Multiple aortic sections (5 sections from pre-defined regions in each mouse) were stained with LOX-1 antibodies using mouse/rabbit specific HRP/DBT detection IHC kit (Abcam, Hongkong, China) as described previously [18]. Image J. v 1.46 (NIH, MD) software was used to quantify the results of immunohistochemical staining.

2.9. Oil red O staining of aortas from ApoE−/− mice

Whole aortas and aortic roots from different groups of mice were isolated and cut into multiple slices, and used to detect the presence of lipids using oil red O staining as described previously [13,19]. Area of aorta stained with oil red O area was quantified with KS400 Software.

2.10. Enzyme-linked immunosorbent assay

Circulating soluble LOX-1 (sLOX-1) was measured in mice (treated with agomiR-98 and antagomiR-98 and appropriate controls) by ELISA using mice sLOX-1 ELISA kits (Ray Biotech, Shanghai, China).

2.11. Luciferase reporter assay

Luciferase reporter assay was used to confirm that LOX-1 is the direct target of miR-98. Mouse LOX-1 mRNA 3′-UTR reporter and its mutant were obtained as described previously [20,21].

2.12. Quantitative real time polymerase chain reaction

Total RNA was isolated from cultured mouse peritoneal macrophages and mice aortic tissues using miRNAeasy Micro Kit (QIAGEN, Shanghai, China). For detection of miRs, cDNA was synthesized by TaqMan MicroRNA assays (Invitrogen Corp, Shanghai, China) on the Applied Biosystems 7900 real time PCR system according to the manufacturer’s instructions. The detection of other RNAs was performed using SYBR Green PCR Master Mix (Applied Biosystems, Beijing, China). To determine miR expression levels, we spiked C. elegans miR-
39 (Invitrogen Corp, Shanghai, China) into the sample before RNA isolation and used it as a reference gene for normalization, while for others GAPDH was used as reference. Real-time qPCR primers were designed and purchased from B&M Biotech Co (Beijing, China) using compensated nucleotides following stem-loop structures shown in Table 2.

### 2.13. Western blotting

Expression of LOX-1 was measured in the lysates of cultured mice peritoneal macrophages and mice aortic tissues. Antibodies were obtained from Santa Cruz Biotechnology, Shanghai, China. Protein extraction and western blotting were performed as common protocol. 30 µg of total protein was separated by SDS-PAGE (12%). β-actin was used to normalize the relative expression of proteins. Image-J software was used to quantify the intensities of the protein bands.

### 2.14. Statistical Analysis

All experiments were performed in more than 5 sets of mouse peritoneal macrophages or mice aortas. Data were analyzed using 2-tailed t-test or single-way ANOVA (multiple means). All data are presented as mean ± SD. S-N-K was used as a post-hoc test for multiple comparisons. A q or P value < 0.05 was considered significant.

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### Table 1

| Lipid parameters       | Saline     | Ctrl 1 | AgomiR-98 | Ctrl 2 | Antago-miR-98 |
|------------------------|------------|--------|-----------|--------|---------------|
| Body weight (g)        | 38.4 ± 1.1 | 36.2 ± 1.8 | 34.8 ± 1.6 | 36.7 ± 0.7 | 41.4 ± 0.9   |
| Total cholesterol (mg/dl) | 1476.5 ± 35.6 | 1352.5 ± 84.3 | 662.1 ± 74.6 | 1333.6 ± 73.2 | 1892.1 ± 95.8 |
| Non-HDL (mg/dl)        | 886.3 ± 61.2 | 956.5 ± 66.8 | 426.3 ± 32.5 | 849.8 ± 3.6  | 1266.7 ± 2.7  |
| HDL-c (mg/dl)          | 44.5 ± 5.8  | 47.7 ± 4.9   | 85.2 ± 3.3  | 45.2 ± 1.7   | 36.8 ± 2.5    |

* P < 0.05, Mean ± SD (n = 5), vs. Ctrl 1.

† P < 0.05, Mean ± SD, vs. Ctrl 2; Ctrl 1: scramble miRNA for agomiR-98; scramble miRNA for antagomiR-98; HDL-c, high density lipoprotein cholesterol.

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### Table 2

| Primer sequences for real-time PCR. |
|-------------------------------------|
| Forward Primers | Reverse Primers |
|-----------------|-----------------|
| mmu-miR-98      | 5′-ACACTCCAGCTGGGTTGGATATGGCAAT-3′   |
| LOX-1           | 5′-CTGGATTGGATTGCATCGGAA-3′          |
| GAPDH           | 5′-AGGTGGGTGAGCGGATTG-3′             |

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Fig. 1. Toxicity of ox-LDL and miR modulators in macrophages. (A) MTT assay to evaluate ox-LDL toxicity on macrophage and choose most appropriate concentration for in vitro study. *P < 0.05 vs. 0 µg/mL ox-LDL, † P < 0.05 vs. 10 µg/mL ox-LDL, n = 7. (B) Evaluation of Dil-ox-LDL uptake for macrophage. *P < 0.05 vs. 0 µg/mL ox-LDL, n = 7. (C and D) Expression of miR-98 and LOX-1 mRNA in ox-LDL treated macrophages. *P < 0.05 vs. 0 µg/mL ox-LDL, n = 7; (E) MTT assay to evaluate miR-98 mimic and inhibitor toxicity on macrophage and choose most appropriate concentration for in vitro study. *P < 0.05 vs. 0 nM miR-98 mimic or inhibitor, n = 7. Scale bar = 100 µm; Abbrev: CTRL, control group; ox-LDL, oxidized low density lipoprotein; LOX-1, Lectin-like ox-LDL scavenger receptor-1.
3. Results

3.1. Ox-LDL and macrophage toxicity

We used MTT assay as an index of cellular toxicity after treatment with different concentrations of ox-LDL (0–40 µg/mL). We observed that 10 µg/mL of ox-LDL induced proliferation of macrophages, whereas ≥20 µg/mL of ox-LDL were toxic; 10 µg/mL concentration of ox-LDL also had the most pronounced effect on Dil-ox-LDL uptake (Fig. 1A and B). We used 10 µg/mL ox-LDL as the concentration in all subsequent experiments.

3.2. miR-98 and LOX-1 expression in ox-LDL treated macrophages

As shown in Fig. 1C and D, the expression of miR-98 was decreased and the mRNA expression of LOX-1 was increased in 10 µg/mL ox-LDL treated macrophages.

3.3. Selection of concentrations of miR-98 modulators

The concentrations of miR-98 mimic and its inhibitor for use in subsequent experiments were determined by MTT assay. As shown in Fig. 1E, ≤50 nM of miR-98 mimic or inhibitor showed no toxicity in mouse macrophages, while ≥100 nM concentrations of miR-98 modulators were toxic (P < 0.05 vs. 50 nM miR-98 modulator). Based on these observations, we used 50 nM concentration of miR-98 mimic and the inhibitor in all subsequent experiments.

3.4. Influence of miR-98 on LOX-1 expression and foam cell formation in macrophages

Major features of atherosclerotic process are the formation of foam cells from macrophages and their accumulation in the lipid laden regions; these processes are regulated in a large part by LOX-1 [22]. In the present study, mouse peritoneal macrophages following treatment with ox-LDL showed intense expression of LOX-1 and transformed into foam cells. The expression of LOX-1 (mRNA and protein) was blocked by miR-98 mimic, and enhanced by transfection with the inhibitor (Fig. 2A and B). Importantly, treatment with the miR-98 mimic inhibited foam cell formation and miR-98 inhibitor promoted it (P < 0.05 vs. control) (Fig. 2C). Finally, LOX-1 deletion reversed the effect of miR-98 inhibitor on foam cell formation (Fig. 2D).

3.5. Delivery of miR-98 modulators into the mice and side effects

We established a colony of ApoE−/− mice fed high fat diet to evaluate the effects of miR-98 on lipid deposition on the aorta as a marker of development of atherosclerosis. Delivery efficiency of miR-98 modulators with the administration of agomiR and antagoniR was determined to be ≈60% (Fig. 3A and B). Since systemic administration of microRNA modulators has been known to exert some off-target effects [23–25], we checked for any major side-effects in the mice by measurements of body weight, caspase-3 activity in different tissues such as brain, heart, lung, kidney and spleen, as well as liver function (H&E and liver enzyme measurement). There was no significant effect on any of these parameters with the administration of miR-98 modulators.
3.6. Influence of miR-98 on LOX-1 expression in mice

Next, we examined LOX-1 expression in the mice, and found extensive LOX-1 expression along the intima of the aorta of ApoE-/- mice fed high fat diet; this LOX-1 expression was reduced by treatment of mice with agomiR-98 and enhanced by antagomiR-98 (both P < 0.05 vs. ApoE-/- mice given scrambled miR) (Fig. 4).

3.7. Influence of miR-98 on lipid accumulation in mice aortas and plasma lipids

As shown in Fig. 5, high fat diet fed ApoE-/- mice developed extensive lipid accumulation in the aortic root and luminal surface of aortas (approximately 40% of aorta covered with fat deposits). The agomiR-98 administration to the mice significantly reduced lipid accumulation (= 10% aorta covered with fat deposits, P < 0.05 vs. ApoE-/- mice given scrambled miR), and antagoniR-98 administration enhanced it (=75% of aorta covered with fat deposits, P < 0.05 vs. ApoE-/- mice given scrambled miR). Of note, plasma total cholesterol and non-HDL-c levels fell and HDL-c level increased by treatment of mice with agomiR-98, while treatment of mice with antagoniR-98 showed the opposite effects (Table 1) (P < 0.05 vs. scrambled miR-98 modulators).

3.8. Luciferase reporter assay for LOX-1 activity

To confirm that LOX-1 is a direct target of miR-98, we used luciferase reporter assay to detect LOX-1 activity in macrophages incubated with ox-LDL. As shown in Fig. 6, LOX-1 luciferase level decreased in cells treated with miR-98 mimic, while it increased in cells treated with the miR-98 inhibitor throughout the increased concentration of miR-98 modulators (P < 0.05 vs. control). Importantly, the mutation of 3′UTR of LOX-1 showed similar luciferase level with or without miR-98 mimic transfection (P < 0.05 vs. pmiR-LOX-1 3′UTR with miR-98 mimic group).

4. Discussion

Atherogenesis is a complex process that includes dysfunction/activation of endothelial cells resulting in adhesion of monocytes to the endothelial cell surface and subsequent migration of monocytes to the sub-endothelial space. The monocytes then differentiate into macrophages which internalize ox-LDL via a variety of scavenger receptors including LOX-1, and transform into "foam cells" resulting in so-called “fatty streak” [26]. Studies from our laboratory showed that deletion of LOX-1 significantly reduced the development of atherosclerosis in the...
In this study, based on bioinformatics, we predicted that LOX-1 is a target of miR-98. To determine the relationships between miR-98 and LOX-1, we treated macrophages with ox-LDL, and measured miR-98 and LOX-1 expression. We observed that ox-LDL treatment enhanced LOX-1 and simultaneously reduced miR-98 in a mirror image fashion. These observations led us to study the link between miR-98 with LOX-1 on one hand and between miR-98 and the determinants of atherogenesis on the other hand. We examined the possible link between miR-98 and LOX-1 by transfecting macrophages with miR-98 mimic or its
inhibitor prior to incubation with ox-LDL. Indeed, treatment of cells with miR-98 mimic inhibited LOX-1 expression, whereas treatment with the inhibitor enhanced LOX-1 expression. These observations indicated that LOX-1 may be a target of miR-98. Few previous studies focused on miR-98 function on atherosclerosis except one study stating that miR-98 rescued proliferation and alleviated ox-LDL-induced apoptosis in endothelial cells by targeting LOX-1 [27]. This study is the first time to elucidate the effect of miR-98 on foam cell formation, the most important process in atherogenesis.

Formation of foam cells is the most important determinant of the development of fatty streak [26]. We studied foam cell formation from macrophages in vitro, and observed that macrophage transformation into foam cells was significantly inhibited by transfection of macrophages with miR-98 mimic and enhanced by its inhibitor. To further confirm that miR-98 regulates atherosclerosis through LOX-1, we transfected mouse peritoneal macrophages with LOX-1 siRNA along with miR-98 inhibitor, and found that the effects of miR-98 on foam cell formation were repressed by LOX-1 deletion. This observation provides a strong evidence that LOX-1 is the mediator in miR-98 regulated atherogenesis.

The high fat diet fed ApoE−/− mouse is a common model to study the development of atherosclerosis and the role of different therapeutic strategies. We used this model to study the effect of miR-98 on atherogenesis. We observed that miR-98 regulated the development of atherosclerosis in the ApoE−/− mice fed high fat diet except one study stating that miR-98 rescued proliferation and alleviated ox-LDL-induced apoptosis in endothelial cells by targeting LOX-1 [27]. This study is the first time to elucidate the effect of miR-98 on foam cell formation, the most important process in atherogenesis.

To verify if LOX-1 is a direct target of miR-98, we transfected pmir-LOX-1 3′UTR or pmir-LOX-1 mutant 3′UTR into macrophages with or without miR-98 mimic to see if the LOX-1 activity is inhibited by up-regulation of miR-98, and whether the mutant of functional part of LOX-1 reverses this effect. As expected, luciferase activity was reduced in miR-98 mimic-transfected group and this effect was completely reversed after mutation of LOX-1 3′UTR, the target region for miR-98. Moreover, there was a sustained decrease or an increase in LOX-1 activity when the macrophages were transfected with miR-98 mimic or inhibitor, respectively. These observations taken together suggest that LOX-1 is indeed a direct target of miR-98. LOX-1 was found to be targeted by some other miRNAs such as let-7, miR-590–5p and miR-21 [12,28,29]. However, none of them disclosed that it is regulated by miR-98 in macrophages. As an important procedure in atherogenesis, prohibition of macrophages transforming into foam cells is considered as a significant method to inhibit its formation. Our study demonstrated that increasing the expression of miR-98 may block this procedure, and subsequently repress the atherogenesis. However, MicroRNAs are tuners of gene expression, and their biological effects are often synergic. One of the major drawbacks with therapeutic use of microRNA in atherosclerosis and other disease states is the fact that many molecules of this class have additional unwanted impact, including tumorigenesis. In this regard, whether miR-98 has other effects remains to be determined. In our preliminary studies, we did not observe any major untoward effect of miR-98 modulators in the brain, heart, liver, lungs, kidney and spleen of mice (Fig. 3).

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Author contributions

Y.D. and X.Q.W performed all experiments and analyzed the data. Y.D. and J.L.M prepared the original manuscript. J.L. revised the final manuscript. D.S.D helped to histological analysis. All authors approved the nal manuscript.

Competing financial interests

The authors declare no competing financial interests.

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