Original Article

Study on the effect of LncRNA AK094457 on OX-LDL induced vascular smooth muscle cells

Mei Liu1*, Yiqun Song2*, Zhongyuan Han3

Departments of 1Emergency, 2North Emergency Hospital, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin 300000, China; 3Department of Cardiology, The Second People’s Hospital of Nantong, Nantong 226002, Jiangsu, China. *Equal contributors and co-first authors.

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Abstract: Atherosclerosis as the common disease has aroused many attentions worldwide. Gene target therapy has become the promising filed for atherosclerosis treatment. Herein, LncRNA AK094457 as a new promising therapy target is investigated in OX-LDL induced vascular smooth muscle cells. The Results showed that LncRNA AK094457 downregulated by shRNA-AK094457-1 have inhibiting effects on proliferation, migration, ROS level and inflammation level in OX-LDL induced vascular smooth muscle cells (VSMCs). In addition, the down regulation of lncRNA suppressed expressions of relevant proteins that are involved in TLR4/MyD88 signal pathway and enhanced expressions of relevant proteins in Nrf2/HO-1 pathway. Taken together, Down regulation of lncRNA AK094457 against effects induced by OXL-LDL in atherosclerosis via Nrf2/HO-1 and TLR4/MyD88 signal pathway is a promising avenue for atherosclerosis treatment.

Keywords: LncRNA AK094457, OX-LDL, atherosclerosis, shRNA-AK094457-1

Introduction

Atherosclerosis as chronic disease severely threatening human health has aroused widely attention, especially for coronary atherosclerosis. While the mechanism of atherosclerosis remains unclear. Inflammatory, metabolism disorder and some other factors including infection, age, and gender results in the formation of atherosclerosis [1]. Hypertension, diabetes mellitus, smoking and hypercholesterolemia are identified as the risk factors of atherosclerosis [2]. The dysfunction of endothelial and vascular smooth muscle cells (VSMCs) contributes to atherosclerosis formation. For instance, the high blood pressure causes endovascular shear stress and the tension of vascular wall is increased by perivascular tissue. The mechanical stimulation causes endothelial dysfunction resulting in the formation of atherosclerosis. Diabetes mellitus is reported to involve in endothelial dysfunction that contributes to diabetic atherosclerosis finally [3]. VSMCs is associated with plaque stability and contributes to atherosclerosis by thickening intimal and involving in the formation of atherosclerosis plaques, which was reported as the promising therapeutic target for atherosclerosis [4, 5].

Plaque is the primary lesion of atherosclerosis. As it has been suggested that a sequence of grossly visible different atherosclerotic plaques is identified as the pretention of the development in atherosclerosis [2]. Rupture of vulnerable atherosclerotic lesions is the main cause of cardiovascular mortality and morbidity. Study on plaque stability is valuable for atherosclerosis treatments. Plaques are composed of extra cellular matrix, lipid, vascular smooth muscle cells, endothelial cells and several other cell types [4]. VSMCs as one of the components of plaques play pivotal roles in promoting the progress of atherosclerotic lesion and generate many types of cells found within the plaque core [4, 6-8]. Oxidized LDL (ox-LDL) is reported to play pivotal role in atherosclerotic lesion by causing dysfunction of VSMC and endothelial as well as contributing to plaque instability [9].
Therefore, OX-LDL induced vascular smooth muscle cells was investigated herein as the cell model for atherosclerosis.

Atherosclerosis is an inevitably progressive disease and most of the therapeutic drugs delay the process of atherosclerosis without radical treatment. The therapy and diagnosis at gene level are promising. Non-coding RNA (ncRNA) as the research hot topics is potential effective biomolecule for drug target treatment and genetic therapy. The function of vascular smooth muscle cells is closely related to non-coding RNA [10].

Non-coding RNA including long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) and microRNAs (miRNAs) is classified into small ncRNAs and long ncRNA (lncRNA) according to their size. More than 90% of the mammalian genome is transcribed as non-coding RNA. LncRNA with numerous gene regulatory functions exists in the cytoplasm as well as nucleus and regulates gene expressions at transcriptional and posttranscriptional levels. Accumulating evidences demonstrated that lncRNA as promising biomarker possesses regulatory function in vascular smooth muscle cell and plays an important role in atherosclerosis [11]. As reported, proliferation and migration of vascular smooth muscle cell was regulated by lncRNA BANCR which can be utilized as a novel target [12]. LncRNA AK094457 as a new RNA was found to be overexpressed in the endothelial cells [13]. Whilst the effect of lncRNA AK094457 on OX-LDL induced VSMCs is still unknown. Herein, the effect of lncRNA AK094457 in OX-LDL induced VSMCs was investigated for the first time.

**Method and material**

**Cell culture and treatment**

Human aortic vascular smooth muscle cells (hVSMCs) obtained from ATCC with an initial density of $1 \times 10^5$ cells/well were cultured in DMEM medium containing 10% fetal bovine serum (Sigma-Aldrich), 100 μg/mL penicillin (Sigma-Aldrich), 100 μg/mL streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO₂. The cells were divided into control group and experimental groups treated with ox-LDL for 12 h, 24 h and 48 h, respectively.

**Small interfering RNA (siRNA) transfections and OX-LDL treatment**

The cells were lysed and seeded in the medium without antibiotics. 10 nM siLncRNA-AK094457-1 and 10 nM siLncRNA-AK094457-2 were transfected into hVSMCs. The interference vectors were used as the control. Then the PCR was performed to evaluate the transfection effect. After transfection for 72 h, the cells as described above were induced by OX-LDL for 12 h, 24 h and 48 h, respectively. The cells with OX-LDL treatment without transfection were shown as the OX-LDL induced control group. The cells without OX-LDL treatment or transfection were used as the control group.

**CCK8 assay**

Each well of the cells in the group of control, OX-LDL, transfection and vector control group after transfection and OX-LDL treatment as described above were incubated with 20 μL CCK8 agent for 4 h. The Absorbance at 450 nm were obtained using a microplate reader (Multiskan MK3, Thermo Fisher Scientific).

**Western blot**

The cells in all groups were collected and centrifuged for 10 min at 12000 rpm to collect the cell lysates. The total proteins were submitted to polyacrylamide gel (10% SDS-PAGE). After separation, the proteins were transferred to nitrocellulose membranes. Then tris-buffered saline-Tween-20 solution containing 5% non-fat dry milk was added to block the membranes. The membranes blocked well were washed for three times and incubated with the primary antibodies. After washed, the membranes were incubated with the secondary antibody. The bands were obtained by enhanced chemiluminescence and Image J software (National Institutes of Health, Bethesda) was used to quantify.

**Real-time PCR analysis**

The cells in all groups were collected and centrifuged at 12000 rpm to obtain the total RNA. The RNA miniprep kit (Sigma-Aldrich) was used to separate the target RNA according to the manufacturer’s instructions. The conditions of PCR were set up as described before [13].
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Immunofluorescence assay

The medium was removed, and the cells were fixed by 4% Polychlorinated formaldehyde at 4°C, overnight. Then the cells were blocked for 0.5 h. The cells were incubated with anti-α-SMA antibody (Sigma-Aldrich) (1:100) at 4°C overnight. After the cells were washed with PBS for three times, the DAPI staining solution (Abcam) was added. The fluorescence microscope (Olympus BX61) was used to take images.

Transwell assay

Transwell assay was applied to evaluate the cell migration. The cells of all groups collected were plated in the upper chamber with serum-free culture medium. The regular culture medium was added into the lower chamber. The cells in the chamber were incubated for 24 h. The cells in the bottom of the transwell plates were fixed with methanol. 0.1% crystal violet was used to stain the cells. The inverted microscope (IX71, Olympus, Japan) was applied to observe and take images.

Evaluation on the level of ROS, TNFα, IL-1 and IL-8

The ROS level was detected by the ROS Activity Assay Kit (Abnova, China) according to the instruction of manufacture. The level of TNFα, IL-1 and IL-8 in the cell supernatants were detected by the corresponding kits according to the instructions of manufacture.

Statistical analysis

GraphPad prism and SPSS were used for data processing. All data in this study were mean ± standard deviation. One-way or two-way analysis of variance were applied for significant difference analysis of data. P<0.05 was set as significant difference.

Results

Enhanced expression of AK094457 in OX-LDL induced cells

The results showed that the expression of AK094457 was highest at 24 h than that at 12 h or 48 h. So, VSMCs induced by OX-LDL for 24 h were chosen as the cell model to process the following experiment. Whilst, compared with control, AK094457 expression was higher in OX-LDL induced VSMCs, indicating that Lnc-RNA AK094457 may be a critical LncRNA in atherosclerosis (Figure 1A).

The effect of LncRNA AK094457 on the proliferation in OX-LDL induced cells

In order to access the effect of LncRNA AK094457 on cell proliferation, shRNA-AK094457-1 and shRNA-AK094457-2 as two RNA interference plasmids were selected to inhibit LncRNA AK094457 expression. The results showed that shRNA-AK094457-1 had better effect on inhibiting LncRNA AK094457 expression (Figure 1B). The results for CCK8 assay
demonstrated that the proliferation of OX-LDL induced VSMCs was decreased by shRNA-AK094457-1 in contrast to VSMCs induced by OX-LDL only, indicating that after inhibition the expression of LncRNA AK094457, the proliferation level in OX-LDL induced VSMCs was decreased (Figure 1C). Whilst, cyclin-dependent kinase 2 (CDK-2) as the cell proliferation controlling protein was increased in OX-LDL induced cells (Figure 2). Cyclin E1 and P27 as cell proliferation relevant proteins were evaluated as well (Figure 2). The Cyclin E1 was increased in OX-LDL induced VSMCs and decreased in OX-LDL induced VSMCs treated by shRNA-AK094457-1. P27 as negative regulatory proteins of cell cycle was increased in OX-LDL induced VSMCs treated by shRNA-AK094457-1, compared with OX-LDL induced.
group treated with the interference vector. As shown by the immunofluorescence assay (Figure 3), the α-SMA level was higher in OX-LDL induced VSMCs group. The α-SMA level was decreased in OX-LDL induced VSMCs group treated with shRNA-AK094457-2. All the results demensted that the LncRNA AK094457 have promoting effect on proliferation in OX-LDL induced VSMCs.

The effect of LncRNA AK094457 on the migration in OX-LDL induced VSMCs

Transwell assay was applied to evaluate the migration of VSMCs induced by OX-LDL (Figure 4A). The migration ability was enhanced in OX-LDL induced VSMCs in comparation to the control group. Compared with the group induced by OX-LDL only, the migration was decreased in the OX-LDL induced group that was treated with shRNA-AK094457-1, confirming that LncRNA AK094457 promoted the migration of VSMCs. The expressions of MMP7/MM9 as migration associated proteins were enhanced in the OX-LDL induced group. Compared with the group induced by OX-LDL, after the expression of LncRNA AK094457 was inhibited by shRNA-AK094457-1 in OX-LDL induced VSMCs, MMP7/MM9 was decreased, further confirming that the migration ability was decreased when the LncRNA AK094457 expression was down-regulated (Figure 4B).

The effect of LncRNA AK094457 on the expression of inflammatory factors and oxidative stress in OX-LDL induced VSMCs

TNFα, IL-1 and IL-8 as indicators of inflammation were assessed (Figure 6A-C). The level of TNFα, IL-1 and IL-8 were higher in OX-LDL induced VSMCs. The inflammatory factors levels were declined in OX-LDL induced VSMCs transfected with shRNA-AK094457-1, indicating that LncRNA AK094457 down-regulated by transfection with shRNA-AK094457-1 have at-
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Figure 4. The effect of LncRNA AK094457 on the migration (A) and expressions of MMP7/MM9 (B) (*P<0.05, **P<0.01, ***P<0.001 vs control group; #P<0.05, ##P<0.01, ###P<0.001 vs shRNA+ox-LDL group).

Figure 5. The effect of LncRNA AK094457 on the expressions of angiogenic proteins in OX-LDL induced VSMCs (*P<0.05, **P<0.01, ***P<0.001 vs control group; *P<0.05, **P<0.01 vs shRNA+ox-LDL group).
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Figure 6. The effect of LncRNA AK094457 on the expressions of inflammatory factors (A-C) and ROS in OX-LDL induced VSMCs (D) (**P<0.001 vs control group; *P<0.05, **P<0.01, ***P<0.001 vs shRNA+ox-LDL group).

tenuation effect on inflammatory factors generation. ROS was detected to evaluate the oxidative stress (Figure 6D). As shown by the results that ROS generation was higher in OX-LDL induced group or OX-LDL induced group transfected with empty vector than any other group. The enhanced generation of ROS was attenuated in the OX-LDL induced group transfected with shRNA-AK094457-1. As all data presented, downregulation of the LncRNA AK094457 expression by transfection with shRNA-AK094457-1 have inhibiting effect on the expression of inflammatory factors or ROS generation in OX-LDL induced VSMCs.

The effect of LncRNA AK094457 on the Nrf2/ HO-1 and TLR4/MyD88 signal pathway

Nrf2/HO-1 played a pivotal role in oxidative stress and diseases [14, 15]. The expression of
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Nrf2/HO-1 in OX-LDL induced VSMCs was also assessed (Figure 7). The results showed that the expressions of Nrf2 and HO-1 were highest in control group. The expressions of Nrf2 and HO-1 were higher in OX-LDL induced VSMCs group transfected with shRNA-AK094457-1 than in OX-LDL induced group with or without transfection with empty vector, indicating that down-regulation of LncRNA AK094457 expression can enhance the expression of Nrf2 and HO-1, resulting in enhanced antioxidative stress. TLR4/MyD88 was reported to regulate vascular smooth muscle cell function [16]. The results showed that the expression of TLR4 and MyD88 was enhanced in OX-LDL induced group with or without transfection with empty vector compared with control group (Figure 7). Compared with any other group, the expressions of TLR4 and MyD88 were declined in OX-LDL induced group transfected with shRNA-AK094457-1, demonstrating that LncRNA AK094457 down regulated by shRNA-AK094457-1 inhibited the TLR4/MyD88 signal pathway.

Discussion

Atherosclerosis as leading cause of death remains unsolved. Atherosclerosis is reported to be associated with VSMCs dysregulation [17-19]. VSMCs is the vital component of arteries, keeping the integrity of the arterial wall. In this study, VSMCs dysregulation induced by OX-LDL was as the cell model of atherosclerosis. As reported that it was via cytokine and inflammatory mechanisms that VSMCs contributed to atherosclerosis immunity [7, 20]. Evidently, cytokine and inflammatory played an important role in atherosclerosis progress as well [21]. As reported that vascular functions which were related to stability of the atherosclerotic plaque were regulated by cytokines [22]. Consistent with previous studies, the inflammation level in OX-LDL induced cells were higher than that in control group. Migration induced by inflammatory exudate and proliferation of VSMCs also contributed to atherosclerosis progress [23,
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24]. Consist with the previous report, the migration and proliferation were enhanced in OX-LDL induced VSMCs. Taken together, the OX-LDL induced VSMCs as cell model of atherosclerosis were constructed successfully.

As genetic factors were the major cause of atherosclerosis formation, the studies on gene level could illuminate the mechanism of atherosclerosis and conducive to find avenues for preventing and treating atherosclerosis. Currently, long non-coding RNA is found to play a key role in atherosclerosis. LncRNA H19 was reported to promote atherosclerosis via regulation of MAPK and NF-kB signaling pathway [25]. LincRNA-p21 was found to play a pivotal role in proliferation and apoptosis of vascular smooth muscle cells as well as atherosclerosis via enhancing p53 activity [26]. So far, there is no investigation on the role of LncRNA AK094457 in OX-LDL induced VSMCs.

In this study, we first investigated the effect of LncRNA AK094457 in OX-LDL induced VSMCs. We found that LncRNA AK094457 was overexpressed in OX-LDL induced VSMCs. Compared with group induced by OX-LDL with/without empty vector transfection, down-regulation of LncRNA AK094457 expression inhibited the proliferation and migration of VSMCs, indicating that LncRNA AK094457 is the potential therapy target in atherosclerosis. In order to investigate the effect of LncRNA AK094457 on proliferation of VSMCs for further evidences, Cyclin E1, CDK-2 and P27 as cell proliferation relevant proteins were detected as well. The results presented that Cyclin E1 and CDK-2 induced by OX-LDL were reduced by down-regulation of LncRNA AK094457 expression, the results for negative regulatory proteins P27 were on the contrary, further confirming the promotion effect of LncRNA AK094457 on proliferation in OX-LDL induced cells. Whilst the angiogenic proteins and α-SMA induced by OX-LDL were attenuated by down-regulation of LncRNA AK094457.

As known that VSMCs could generate the inflammatory factors that contributes to progression of atherosclerosis. In this study, the level of TNFα, IL-1 and IL-8 and ROS generation that induced by OX-LDL in VSMCs were all reduced by down-regulation of LncRNA AK094457, suggesting that the inflammation level was able to be reduced through inhibiting the expression of LncRNA AK094457.

It is known that VSMCs migration contributes to atherosclerosis progression. The migration ability was decreased by downregulation of LncRNA AK094457 (Figure 4) further suggesting that LncRNA AK094457 may be a good target for treatment of atherosclerosis.

The mechanism of the effect of LncRNA AK094457 on OX-LDL induced VSMCs were also investigated herein. As Nrf2/HO-1 and TLR4/MyD88 signal pathway were involved in atherosclerosis. Herein the relevant proteins of Nrf2/HO-1 and TLR4/MyD88 signal pathway were also evaluated herein. Nrf2 and HO-1 proteins induced by OX-LDL were enhanced by downregulation of LncRNA AK094457 expressions. It was reported that Nrf2/HO-1 pathway was activated, resulting in up-regulation of antioxidant enzymes, anti-apoptotic proteins and protective effect of atherosclerosis [27]. In this study, down-regulation of LncRNA AK094457 increased the expression of Nrf2 and HO-1, indicating that Nrf2 and HO-1 up-regulated by LncRNA AK094457 down-regulation may be the potential mechanism of antioxidant effect. The TLR4/MyD88 signal pathway was reported to be involved in inflammation response induced by OX-LDL [28]. Consist with the literature, the expression of TLR4 and MyD88 were up-regulated in cells induced by OX-LDL, compared with control group. Whilst, the expression of TLR4 and MyD88 induced by OX-LDL were reduced by down-regulation of LncRNA AK094457. Taken together, all these results confirmed that down regulation of LncRNA AK094457 which is high expressed in OX-LDL induced VSMCs against atherosclerosis via Nrf2/HO-1 and TLR4/MyD88 signal pathway is the potential avenue for atherosclerosis therapy.

Conclusion

In this study all the results confirmed that down regulation of LncRNA AK094457 inhibited the effects induced by OXL-LDL in VSMCs via Nrf2/HO-1 and TLR4/MyD88 signal pathway, providing a promising avenue for treatment and diagnosis of atherosclerosis.

Disclosure of conflict of interest

None.
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Address correspondence to: Zhongyuan Han, Department of Cardiology, The Second People’s Hospital of Nantong, No. 43 Xinglong Street, Tangzha, Nantong 226002, Jiangsu, China. Tel: 05138555-4333; E-mail: zhongyuanhanjsnt@163.com

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