Original Article
Effects of long non-coding RNA MALAT1-mediated PI3K/Akt pathways on the biological function of VSMCs in venous segments of AVFs in ESRD hemodialysis patients

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Abstract: Objective: The aim of the current study was to investigate expression levels of long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in vascular smooth muscle cells (VSMCs) of arteriovenous fistula (AVFs) in end-stage renal disease (ESRD) hemodialysis patients, examining its related regulatory mechanisms. Methods: VSMCs in venous segments of AVFs and venous segment tissues of AVFs were collected for primary culturing and in vitro culturing, respectively. Of these, VSMCs stenosis in venous segments of AVFs were considered as the reconstruction group (study subjects), while newly built VSMCs in venous segments of AVFs were considered as the new group. A VSMCs model with 20% FBS induced phenotypic transformation in vitro was utilized. Effects of downregulated MALAT1 on proliferation, migration, invasion, and apoptosis of VSMCs were analyzed by transfecting siRNA. MALAT1, α-SMA, SM-MHC, SM22α, OPN, PI3K, p-PI3K, Akt, p-Akt, TNF-α, IL-1β, IL-10, MDA, SOD, and CAT in VSMCs were detected. Results: Expression levels and protein levels of SM22α, SM-MHC, and α-SMA in the reconstruction group were remarkably downregulated, while expression levels of OPN were remarkably upregulated. Expression of MALAT1 in the reconstruction group was significantly higher than that in the new group. After transfection with siRNA, expression of MALAT1 was remarkably decreased. Moreover, cell proliferation, migration, and invasion were inhibited, while apoptosis rates were increased. Expression of PI3K, p-PI3K, Akt, p-Akt, TNF-α, IL-1β, and MDA, downregulated by MALAT1, was remarkably decreased, compared with the phenotypic transformed VSMCs model. Levels of IL-10, SOD, and CAT were significantly upregulated. Conclusion: MALAT1 can mediate PI3K/Akt pathways, affecting the phenotypic transformation and cytological function of VSMCs. It can act as a regulator of inflammation and oxidative stress in VSMCs.

Keywords: MALAT1, PI3K/Akt, hemodialysis, AVFs, VSMCs

Introduction
A malignant outcome of the worldwide epidemic of chronic kidney disease (CKD), end-stage kidney disease (ESRD) can be caused by multiple factors. These may include diet, systemic inflammation, endocrine disorders, and accumulation of toxins [1]. Epidemiological statistical analysis has shown that CKD affects more than one tenth of the U.S. population, to varying degrees. ESRD patients are expected to reach 971,000-1,259,000 by 2030 [2, 3]. Currently, the main treatment for ESRD patients is hemodialysis. The utilization rate of renal replacement therapy may only reach 0.1% of the total population due to the high costs. Renal transplantation is not the first-line treatment for ESRD patients due to resource scarcity and the requirement of a high matching degree [4]. Arteriovenous fistula (AVFs) is the most commonly used permanent dialysis vascular access in clinical practice. However, venous stenosis may lead to higher primary dysfunction, which may bring higher hospitalization rates and mortality rates to hemodialysis patients [5, 6]. Intimal hyperplasia is the histological feature of AVFs stenosis. The dysregulation of this process will give rise to a series of cascading
MALAT1 can regulate the phenotypic modulation of VSMCs

events, including inflammatory response, caused by vascular smooth muscle injury and oxidative stress, leading to occurrence of phenotypic transformation of vascular smooth muscle cells (VSMCs) [7, 8]. Therefore, examining the molecular mechanisms of phenotypic transformation of VSMCs in AVFs stenosis is of great significance for the prevention and treatment of AVFs stenosis.

Long non-coding RNAs (lncRNAs) can regulate various cellular functions and pathophysiological processes in the body. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), a member of the IncRNA family, plays an important role in regulating the cellular biological process of vascular remodeling [9-11]. The team of Xue put forward [12] that silencing expression of MALAT1 can alleviate vascular lesions and improve vascular remodeling in hypertensive rats. They suggested that the mechanisms may involve the inhibition of Notch signaling pathways. Other studies have reported the regulatory effects of MALAT1 on angiogenesis, growth, and endothelial cell function, suggesting that its mechanisms are related to the regulation of MALAT1 on the phenotypic transformation and cell cycle arrest of endothelial cells from phenotypic proliferation to migration [13, 14]. Puthanveetil and his team [15] reported that MALAT1 can maintain the imbalance of inflammatory mediators of endothelial cells IL-6 and TNF-α induced by hyperglycemia through stimulating the secretion levels of serum amyloid protein antigen 3. It was also shown to regulate the production of reactive oxygen, suggesting that MALAT1 may be a regulator of inflammatory response and oxidative stress.

At present, there are few studies concerning the molecular regulation mechanisms of MALAT1 in AVFs stenosis. The present study collected VSMCs in venous segments of AVFs in ESRD hemodialysis patients for exploration, aiming to provide a new target for treatment of AVFs stenosis.

Methods

Phenotypic transformation of VSMCs in venous segments of AVFs in hemodialysis patients

VSMCs in venous segments of AVFs stenosis from 67 cases of ESRD hemodialysis patients, admitted to Taizhou Hospital of Zhejiang Province (reconstruction group), and sixty-five cases of newly established VSMCs in venous segments of AVFs (new group), from January 2012 to January 2019, were collected. Inclusion criteria: Patients with a hemodialysis time of 2-5 years; Patients with AVFs for at least 3 months; Patients with blood flow of AVF less than 200 mL/min; Patients with at least 50% stenosis. All patients and families agreed to participate in the experiment and provided informed consent. The present experiment was approved by the hospital Ethics Committee. Exclusion criteria: Patients with obvious thrombosis; Patients complicated with other malignant tumors; Patients with incomplete clinical data. Inclusion criteria were applicable to the reconstruction group. The new group included patients with hemodialysis for less than two months, excluding patients with significant vascular stenosis and vascular disease. VSMCs of 15 cases in each group were cultured using the modified tissue explants adherent method [16], placed in DMEM (Xinfan Biological Technology Co., Ltd., Shanghai, China, 1273) containing 10% FBS, and cultured in an incubator (Fuze Trading Co., Ltd., Shanghai, China, 3111) at 5% CO₂ and 37°C. Moreover, 25% trypsin (Beinuo Biotechnology Co., Ltd., Shanghai, China, R20109) was used for digestion and passage, while 3-8 generations of cells were used for experimentation. VSMCs cell morphology was observed with an inverted phase contrast microscope (Yuyan Instruments Co., Ltd., Shanghai, China). Specific α-SMA antibodies were identified, aiming to verify the success rate of primary culture of VSMCs. When the expression rate of α-SMA antibodies in primary cells of VSMCs was above 95%, the primary culture model of VSMCs was deemed successful.

Establishment, transfection, and cell grouping of phenotypic transformation model of VSMCs in vitro

VSMCs of the remaining 52 patients in the reconstruction group and the remaining 50 patients in the new group were treated with VSMCs cell model of phenotypic transformation induced by 20% FBS in vitro. It was transfected with siRNA-MALAT1 (inhibitory sequence), NC (blank control), and si-NC (negative control). A Lipofectamine™ 2000 kit (BioMag Scientific Inc., Wuxi, China, 11668019) was used
MALAT1 can regulate the phenotypic modulation of VSMCs

to transfect the cells. The procedure was conducted in strict accordance with manufacturer instructions. Other groupings: Primary cultured VSMCs (Control), phenotype-transformed VSMCs cell models (Model), and their cell grouping after transfection of siRNA-MALAT1, NC, and si-NC (siRNA-MALAT1/Model+siRNA-MALAT1, NC/Model+NC, si-NC/Model+si-NC).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cultured VSMCs using the TRizol kit (Kangliang Biological Technology Co., Ltd., Shanghai, China, KL058). Purity, concentration, and integrity levels of total RNA were determined using a UV-1500 spectrophotometer (Hengfei Biotechnology Co., Ltd., Shanghai, China, R1230). RNA was reverse transcribed into cDNA, according to the instructions of the reverse transcription kit (Qiming Biotechnology Co., Ltd., Shanghai, China, OX02700). SYBR Premix Ex Taq TM kit (Shanghai Yihui Biotechnology Co., Ltd., China, OX02700) was adopted to react on a PCR instrument (Microsep Biotechnology Co., Ltd., Wuxi, China, TC9639), with GAPDH or β-Actin used as an internal reference. PCR amplification conditions: Pre-denaturation at 95°C for 10 minutes, then denaturation at 95°C for 15 seconds; Annealing/extension at 60°C for 60 seconds, for a total of 40 cycles. Data was obtained after three repeated experiments. Relative expression levels were calculated using 2^{-\Delta\Delta CT}. All primers were designed and synthesized by JRDUN Biotechnogy (shanghai) Co. Ltd.

Western blot assay

RIPA lysate (Hengfei Biotechnology Co., Ltd., Shanghai, China, PS0012) was added to each group of cells after culturing, extracting the total protein. Protein concentrations were detected by BCA assay (Shanghai Chuan Qiu Biotechnology Co., Ltd., China, C-N202). The protein concentration was adjusted to 4 μg/μL, separated by 12% SDS-PAGE (LMAI Bio Co, Ltd., Shanghai, China, LM0053A) electrophoresis. It was then transferred to a PVDF membrane (Shifeng Biological Technology Co., Ltd., Shanghai, China, A3805). The membrane was sealed with 5% skimmed milk powder (Xinfeng Biological Technology Co., Ltd., Shanghai, China, XF-P438) for 2 hours and SM22α, SM-MHC, α-SMA, OPN, PI3K, p-PI3K, Akt, p-Akt diluted at the ratio of 1:500, β-Actin primary antibodies, at the ratio of 1:1000, were added to block overnight at 4°C. All antibodies were purchased from Hengfei Biotechnology Co., Ltd. The membrane was washed to remove the primary antibody. Horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Yubo Biological Technology Co., Ltd., Shanghai, China, YB0178) at a dilution ratio of 1:100 was added. It was incubated at 37°C for 1 hour and rinsed 3 times with PBS (Zhenyu Biotechnology Co., Ltd., Shanghai, China, 130-070-525) for 5 minutes each. Excess liquid was absorbed from the membrane with filter paper. ECL was used to illuminate and develop in a dark room (Shanghai Yuanye Bio-Technology Co., Ltd). Protein bands were scanned, then the grayscale value was analyzed using Quantity One software. Relative expression levels of the protein = grayscale value of the target protein band/grayscale value of β-actin protein band.

Detection of cell proliferation ability

VSMCs cell suspension was seeded into a 96-well plate using CCK8. After transfection, the cells were incubated at 5% CO₂ and 37°C for 24 hours, 48 hours, 72 hours, and 96 hours, respectively. CCK8 reagent (Yubo Biological Technology Co., Ltd., Shanghai, China, IC-CCK8-Hu) was added to each well, incubating for an additional 1 hour at 37°C in the dark. A microplate reader (Yanhui Biotechnology Co., Ltd., Shanghai, China, HBS-1096A) was applied to detect absorbance at 450 nm.

Detection of cell migration ability

Serum-free DMEM (Yiyan Biotechnology Co., Ltd., Shanghai, China, N676-50ML) was changed 12 hours after transfection, while serum was starved for 24 hours. A 1 mL pipette tip (Fanke Biotechnology Co., Ltd., Shanghai, China, FK-KN253-134) was applied to draw 2 crosses in the orifice plate. Next, 20% FBS + DMEM (Yaji Biotechnology Co., Ltd., Shanghai, China, PM150220C) and 10 ng/mL PDGF-BB + DMEM (Beijing T&L Biotechnology Co., Ltd., China, TL-644) were changed to stimulate smooth muscle cell migration. At 0 hours, 15 hours, and 30 hours, the entire cross was taken with a microscope. Image-Pro Plus 6.0 soft-
ware was applied to analyze the cross picture, calculate the area of scratch-free cell area, and statistically analyze the ability of cell proximal migration.

**Detection of cell invasion ability**

VSMCs after 48 hours of transfection were collected and adjusted to $3 \times 10^4$, then inoculated to a 24-well plate. It was digested with 0.25% Trypsin-EDTA (Qiming Biotechnology Co., Ltd., Shanghai, China, RF(m)11426) and transferred to the upper compartment. The upper compartment was added with 200 µL RPMI1640 nutrient solution, while the lower compartment was added with 500 mL RPMI1640 (containing 10% FBS). They were cultured at 37°C for 48 hours. Cells that failed to penetrate the upper compartment were removed, rinsed 3 times with PBS, fixed with paraformaldehyde for 10 minutes, and rinsed with double distilled water 3 times. After drying, they were stained with 0.5% crystal violet. Invasion levels were observed with a microscope.

**Detection of cell apoptosis ability**

After transfection, VSMCs grown to the logarithmic phase were stimulated with 0.3% H$_2$O$_2$, washed with PBS, and tested with an Annexin V-FITC Apoptosis detection kit (Xiyuan Biotechnology Co., Ltd., Shanghai, China, XY-ICT-9122) for BD Biosciences FACS Calibur flow cytometry (Shiwei Experimental Instrument Technology Co., Ltd., Shanghai, China). The experiment was repeated 3 times and the average value was taken.

**Detection of oxidative stress index**

Determination of malondialdehyde (MDA) was performed by thiobarbituric acid. Determination of superoxide dismutase (SOD) was conducted using the pyrogallol autoxidation method. Determination of catalase (CAT) was performed by improved colorimetry. All detection kits were purchased from Shanghai Qiaoyu Biotechnology Co., Ltd.

**Statistical analysis**

GraphPad 6 was applied for data analysis and image rendering. All data are expressed as mean ± standard deviation (Mean ± SD). Independent sample t-tests were used for comparisons between two groups. One-way ANOVA was used for intergroup comparisons, represented by F. LSD-t tests were used for pair-wise comparisons afterwards. Repeated measurement ANOVA was utilized for expression of multiple time points. Bonferroni’s method was used for back testing. P<0.05 indicates statistical significance.

**Results**

**Expression of proliferation and migration indicators in venous segment VSMCs**

Expression levels of SM22α, SM-MHC, and α-SMA in the venous segment VSMCs of patients in the reconstruction group were significantly lower than those in the new group. Expression levels of OPN were significantly higher than those in the new group. Differences were statistically significant (P<0.05) (Figure 1).

**Expression and transfection of MALAT1 in AVFs stenosis reconstruction group and new group**

Expression of MALAT1 in the AVFs stenosis reconstruction group was significantly higher than that in the new group. Expression of MALAT1 in the VSMCs phenotypic transformation model in vitro transfected with siRNA-MALAT1 was remarkably inhibited, with statistically significant differences (P<0.05) (Figure 2).

**Effects of MALAT1 on cell biological function of VSMCs phenotypic transformation model in vitro**

Proliferation, migration, and invasion levels of the VSMCs phenotypic transformation model in vitro transfected with siRNA-MALAT1 were notably inhibited, compared with NC and si-NC. Apoptosis rates were notably upregulated, with statistically significant differences (P<0.05). Differences between NC and si-NC were not statistically significant (P>0.05) (Figure 3).

**Expression of PI3K/Akt signaling pathway related proteins**

Expression of PI3K, p-PI3K, Akt, and p-Akt proteins in the model group transfected with siRNA-MALAT1 was significantly lower than that in the model group (P<0.05), but not significantly different from the control group (P>0.05) (Figure 4).
MALAT1 can regulate the phenotypic modulation of VSMCs

Figure 1. Expression of proliferation and migration indicators in venous segment VSMCs of the two groups. A. mRNA expression of VSMCs proliferation and migration indicators in the venous segments of the two groups. B. Protein expression of VSMCs proliferation and migration indicators in the venous segments of the two groups. C. Corresponding protein figure.

Figure 2. Expression and transfection of MALAT1 in the AVFs stenosis reconstruction group was significantly higher than that in the new group. A. Expression of MALAT1 in AVFs stenosis reconstruction group was significantly higher than that in the new group. B. Conditions of transfection.

Effects of MALAT1 on TNF-α, IL-1β, IL-10, and other inflammation-related factors

TNF-α and IL-1 levels in the model group transfected with siRNA-MALAT1 were notably lower than those in the model group. Levels were notably higher than those in the control group. Results concerning IL-10 were reversed, with statistically significant differences (P<0.05) (Figure 5).

Effects of MALAT1 on oxidative stress indexes of MDA, SOD, AND CAT

SOD and CAT levels in the model group transfected with siRNA-MALAT1 were significantly higher than those in the model group and significantly lower than those in the control group. Results concerning IL-10 were reversed, with statistically significant differences (P<0.05) (Figure 6).

Discussion

VSMCs have both contractile and synthetic phenotypes. Normal and mature VSMCs are often contractile and have low proliferation and migration functions. They mainly mediate the self-regulation of blood vessel diameter, blood flow, and blood pressure. The synthetic type generally shows strong proliferation and migration ability, mainly regulating synthesis of the extracellular matrix. Research on the regulatory mechanisms of phenotypic transformation of VSMCs will help to find a solution to the stenosis and dysfunction of AVFs [17, 18]. More and more researchers have focused on phenotypic transformation of VSMCs and the results are endless. For example, in the study of Liu [19], C1q/TNF related protein 9 was shown to inhibit cholesterol-induced phenotypic transformation of VSMCs by regulating the activation of AMP-dependent kinases. The team of Gong [20] reported that upregulation of lncRNA CASC2 could inhibit hypoxia-induced vascular remodeling by inhibiting expression of phenotypic transformation related indicator α-SMA, as well
MALAT1 can regulate the phenotypic modulation of VSMCs

as proliferation and migration of VSMCs. Zhu and other researchers [21] have reported that silencing MALAT1 could promote the transformation of VSMCs from contractile type to synthetic type by inhibiting autophagy, suggesting that MALAT1 has the potential to regulate the phenotypic transformation of VSMCs. Other studies have shown that SM22α, SM-MHC, and α-SMA are contractile protein in phenotypic transformation of VSMCs, while OPN is a synthetic protein in phenotypic transformation of VSMCs. Detection of these four proteins is conducive to the quantitative observation of phenotypic transformation of VSMCs [22, 23]. In the present study, expression of SM22α, SM-MHC, α-SMA, and OPN was detected at the transcriptional level and protein level. SM22α, SM-MHC, and α-SMA of the reconstruction group showed lower levels than the new group, while OPN showed higher levels. Results suggest that phenotypic transformation of VSMCs in the reconstruction group had a clear synthetic trend. In addition, expression of MALAT1 in the reconstruction group was significantly higher than that in the new group. Moreover, expression of MALAT1 was significantly downregulat-
MALAT1 can regulate the phenotypic modulation of VSMCs

ed after transfection of siRNA, suggesting that MALAT1 may be involved in the regulation of phenotypic transformation of VSMCs.

The present study analyzed the effects of MALAT1 on the biological function of VSMCs through further cytological studies. Results revealed that VSMCs transfected with siRNA-MALAT1 showed lower ability of proliferation, migration, and invasion, as well as higher ability of apoptosis, suggesting that downregulation of MALAT1 expression would affect the cell biological function and phenotypic transformation of VSMCs. VSMCs tend to transform into contractile phenotype. The current study then analyzed the effects of siRNA-MALAT1 on PI3K/Akt pathways of VSMCs. Results exhibited that VSMCs transfected with siRNA-MALAT1 had lower levels of PI3K, p-PI3K, Akt, and p-Akt, indicating that siRNA-MALAT1 might have an activated and inhibitory effect on PI3K/Akt pathways of VSMCs. In the study of Liu and his team [24], it was pointed out that PI3K/Akt signaling pathways are involved in the regulatory mechanisms of phenotypic transformation of human aortic VSMCs, suggesting that the activation and inhibition of this pathway can help reverse the phenotypic transformation process.

Previous studies have shown that vascular inflammatory and oxidative stress-dependent activation stimulation may induce the phenotypic transformation process of VSMCs and promote the proliferation and migration levels of VSMCs [25, 26]. Therefore, the current study also analyzed the effects of siRNA-MALAT1 on inflammation and oxidative stress in VSMCs. Results showed that pro-inflammatory TNF-α and IL-1β, downregulated by MALAT1, were notably reduced, compared with phenotype-transformed VSMCs cell models. Moreover, anti-inflammatory IL-10 was significantly increased, suggesting that knocking down MALAT1 expression could help improve the inflammatory environment of VSMCs. Results of oxi-
MALAT1 can regulate the phenotypic modulation of VSMCs

dative stress revealed that VSMCs transfected with siRNA-MALAT1 had lower levels of MDA and higher levels of SOD and CAT than phenotype-transformed VSMCs models, indicating that downregulation of MALAT1 helped to suppress oxidative stress in the body. In conclusion, MALAT1 has the function of regulating inflammation and oxidative stress after VSMCs injury. Knocking down its expression contributes to the improvement of the above process.

In conclusion, MALAT1 can mediate PI3K/Akt pathways, affecting the phenotypic transformation and cytological function of VSMCs. Downregulation of MALAT1 expression may be a therapeutic target for improvement of AVFs stenosis and dysfunction, inflammation, and oxidative stress in ESRD hemodialysis patients. However, there remains room for improvement in the current study. First, increased research on miRNAs of patient MALAT1 will further complement its potential analytical regulatory mechanisms. Second, expanded research samples would improve research for hemodialysis patients with diseases, such as sepsis. This would greatly increase the potential universality of research results.

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Disclosure of conflict of interest

None.

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MALAT1 can regulate the phenotypic modulation of VSMCs


