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Original paper

Cellular proliferation of Hemangioma endothelial cells (HemECs) under targeted regulation of LncRNA MALAT1 via miR-494-3p/PTEN Axis

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Abstract The current study is aimed to explore the regulation of lncRNA MALAT1 in human HemECs functions. In our study, relative expressions of MALAT1, miR-494-3p and PTEN in HemECs (HemEC) were determined using qRT-PCR methods. MTT assays were used to measure cell viability. The rate of cell apoptosis was assessed using caspase-3 assay. Transfections were performed to mediate lncRNA MALAT1 and miR-494-3p expression in HemEc cells. Also, Bioinformatic analysis and Luciferase reporter were used to predict and validate the bindings between MALAT1 and PTEN, and PTEN and miR-494-3-p. MALAT1 was highly expressed in HemECs. Cell proliferation increased significantly due to MALAT1 overexpression in HemECs while MALAT1 overexpression significantly reduced cell apoptosis in HemECs. On the other hand, contradictory results were observed due to the reduction of MALAT1 in HemEC. We also found that MALAT1 interacts with miR-494-3p/PTEN to mediate cellular functions. Collectively, the results showed that the MALAT1 expression was negatively associated with miR-494-3p and positively matched the PTEN expression. In addition, MALAT1 acted as a ceRNA by binding with miR-494-3p to up-regulate PTEN in HemECs. Our study showed that MALAT1 accelerates the proliferation of HemEC cells by controlling the miR-494-3p/PTEN axis promoting new insight into IH treatment.

Keywords MALAT1, HemECs, miR-494-3p, apoptosis, proliferation.

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Introduction

Infantile hemangioma (IH) is a severe tumor, causing injury to newborns with 4-10% low birth weight [1]. IH could harm normal tissues and organs, due to rapid growth and invasion [2]. To effectively reduce the incidence of complications and control tumor growth, many experts advise remedies and early diagnosis [3]. IH is distinguished by the spontaneous regression of blood vessels [4]. A better understanding of pathogenesis can contribute to evaluating the effective treatment of IH. The tumor is characterized by an increasing number of unique endothelial cells that constitute blood vessels [5]. Therefore, endothelial cells play an important role in the diagnosis and treatment of infantile hemangioma (IH). Previous researchers have shown that lncRNAs expression profile can serve as a tool for predicting various tumors [6]. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a highly preserved lncRNA and also modulates functions in various cancers [7]. Wu et al. found that silencing MALAT1 significantly reduced the expression of vascular endothelial growth factor, infering that MALAT1 promoted angiogenesis in cancers [8]. In IH, MALAT1 knockdown leads to apoptosis and cell regeneration to umbilical vein endothelial cell [9]. In addition, lncRNA MALAT1 modulates tumor angiogenesis in various diseases [10]. However, the fuurther molecular mechanisms of MALAT1 in IH remain exploring.

MicroRNAs are small non-coding RNAs with 18-25 nucleotides in lengt, which can post-transcriptionally regulate mRNA [11]. According to previous studies, miR-494-3p acts a dual role in tumors. In most cancers, miR-494 acts as a gene for suppressing the tumor. Meanwhile, conflicting reports have suggested that elevated miR-494 plays an oncogenic role in the development of several types of cancer including non-small cell lung cancer and colorectal cancer [12, 13]. Previous studies also confirmed that the miR-494-3p had a negative association with PTEN expression in Hemangioma [14]. PTEN is one of the most mutated tumor suppressor genes involved in human cancer. In addition to genetic mutations, suppression of PTEN function by either suppression of the PTEN gene expression or aberrant PTEN subcellular localization is strongly associated with tumorigenesis and disease progression [15]. However, the correlation between miR-494-3p and MALAT1 remains unclear. Therefore, this study aimed to determine the effect of MALAT1 and miR-494-3p on targeting PTEN to HemECs.

Material and Method

Cell Culture

HemECs (HemECs) was purchased from Cell Biologics, Inc. (Cell Biologics, Chicago, USA), and reserved in Endothelial Cell Growth Basal Medium-2 (EBM-2; Lonza Group Ltd, Basel, Switzerland) containing 5% FBS, 20 ng/ml Insulin-like Growth Factor (R3 IGF-1), and 0.5 ng/ml of Vascular Endothelial Growth Factor but do not contain antibiotics or antimycotic and are intended for use in an incubator with a concentration of 5% CO_2 at 37ºC.

Cell Transfection

Specific sh-RNAs, as well as negative controls (NC), were purchased from Biocompare (Francisco, CA, USA) used to knockdown of MALAT1 in HemECs, and vectors pcDNA 3.1 (Addgene, MA, USA) used to add to overexpress MALAT1 or PTEN. Transfections were performed using Lipofectamine® 2000 (Invitrogen; Addgene, MA, USA) at 5×10^6 cells where 50 nM were the final concentration. Subsequent tests were performed within 48 hours of the transfection. To use overexpress MALAT1, HemECs were transferred via pCDNA3-MALAT1 using Lipofectamine® 2000. In addition, mimic miR-494-3p and inhibitors were synthesized by add gene (Invitrogen; Addgene, MA, USA) used for overexpressing or silence miR-494-3p. After the 24 hour of transfection, MALAT1 expression was confirmed by RT-qPCR.

Rt-qPCR

Total RNAs of HemECs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A reverse transcriptase kit (TaKaRa Bio, Kusatsu, Japan) was applied for cDNA synthesis. The mRNA expressions of PTEN were measured by SYBR-Green Premix Ex Taq (Roche, Basel, Switzerland) and qRT-PCR was completed with the Bio-Rad CFX96 system (Biotium, Bio-Rad Laboratories, Inc). Primer sequences of RT-qPCR were as follows: MALAT1,
F: GGAAGGAGCGAGTGCAATTT,
R: ATATTGCCGACCTCACGGAT; miR-494,
F: ATGAGGCTTCAGTACTTTACAG,
R: CATAGCGTAAAAGGAGCAACA, PTEN,
F: TCTGCCATCTCTCTCCTCCT,
R: ACGCCTTCAAGTCTTTCTGC, GAPDH,
F: CAGTCACTACTCAGCTGCCA,
R: GAGGGTGCTCCGGTAG,
U6: F: CTCGCTTCGGCAGCACA,
R: AACGCTTCACGAATTTGCGT. GAPDH was seen as an internal reference to normalize PTEN. The condition of PCRs was as follows: 50ºC for 2 min, 95ºC

for 8.5 min, followed by 95ºC for 15 s, 60ºC for 1 min, for 35 cycles. The extension becomes achieved at 95ºC for 1 min, 55ºC for 1 min, and 55ºC for 10 s. The levels of related expressions were calculated by the $2^{-\Delta\Delta Cq}$ method.

Luciferase reporter assay

The predicting binding site of the MALAT1 binding site was determined using bioinformatics (www.targetscan.org). Wild-type MALAT1 and mutant MALAT1 are individually included in the Luciferase Reporter Assay Kit (Biovision, California, USA). HemECs were cotransfected with miR-494-3p mimics and wt-MALAT1 or mut- MALAT1 by Lipofectamine 2000 (Invitrogen). The related activity of luciferase was measured using a dual-luciferase reporter assay system (BPS Bioscience, Inc. San Diego, CA, United States). Details are presented as a ratio of Renilla luciferase activity and firefly luciferase activity. The Luciferase reporter assay ensures that the direct binding of miR-494-3p to PTEN 3'-UTRwere performed as described.

Apoptosis Assay by Caspase-3

The Caspase-3 Activity Assay Kit (Biovision, California, USA) was used to measure caspase-3 activity in HemECs cells using a cell lysis buffer. After centrifugation, lysed cells were frozen for 10 minutes. The DEVD-pNA substrate (Biocompare, CA, USA) and the reaction buffer were added to the cell medium and placed at 37ºC for 2 hours. Absorbance was measured at 400nm wavelength by using a spectrophotometer or automated microplate reader (BMG LabTech, Ortenberg, Germany).

MTT assay

Boster's MTT Cell Proliferation Assay Kit (Boster Biological Technology, Pleasanton CA, USA) was used to check the cell viability. Cells were sown on 96-well plates (2000 cells/well) and cultured at about 4 h. Then, MTT reagent was applied to replace the media, and dimethyl sulfoxide used to dissolve the blue crystal. Finally, cell activity becomes diagnosed with absorbance at 570 nm.

Statistical Analysis

All facts became offered as a mean ± standard deviation (SD) and statistical analysis performed using SPSS version 19.0 (IBM, MA, U.S.A.) and GraphPad Prism 7.0 software (Graphpad, San Diego, CA, USA). Student assessments (two groups) and variance analyzes (multiple groups) were used to compare two or more groups. The difference was considered statistically significant with $p < 0.05$.

Results and Discussion

Effects of MALAT1 Silencing on Proliferation and Apoptosis of HemECs

RT-qPCR was used to analyze HemECs cells, transfected with sh-MALAT1 and sh-NC (control). Further, we determined the effect of MALAT1 on the growth of HemECs. MALAT1 expression was reduced in HemECs after transfection of sh-MALAT1 (Figure 1A). MTT was used to detect the effects of MALAT1 on the proliferation in HemECs, which turned out that MALAT1 downregulation significantly inhibited the cellular activity of HemECs (Figure 1B). Caspase-3 method measured cell apoptosis. The results showed that cell apoptosis was significantly enhanced in HemECs transfected with sh-MALAT1 compared to the control group (Figure 1C).

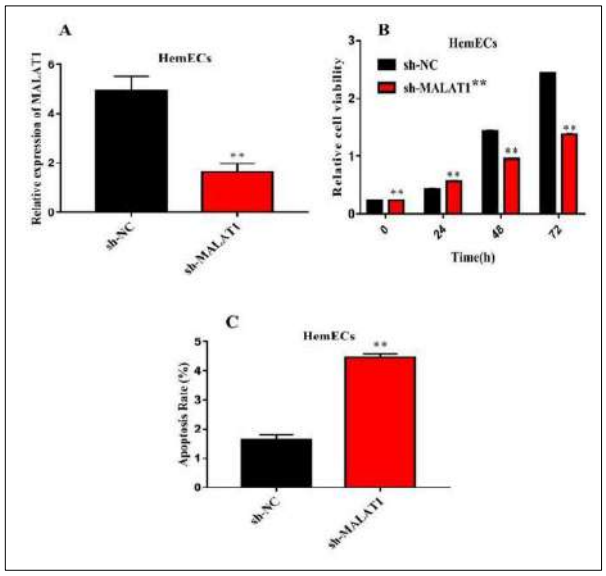

Figure 1. MALAT1 downregulation inhibited cell proliferation and promotedapoptosis of HemECs cells. (**A**) After sh-NC and sh-MALAT1 were transferred to HemECs, MALAT1 was evaluated by qRT-PCR. (**B**) The cell viability of HemECs transfected with sh-NC or sh-MALAT1 was checked by MTT assay. (**C**) Cell apoptosis was detected by the Caspase-3 assay. ** P <0.01.

MALAT1 binds to miR-494-3p in HemECs

Bioinformatics predicted potential binding sites between MALAT1 and miR-494-3p (Figure 2A). Furthermore, we found that mimic of miR-494-3p significantly inhibited the luciferase activity of MALAT1-wt while having no obvious effect on the MALAT1 mutant (Figure 2B). qRT-PCR methods validated that knockdown of MALAT1 resulted in a

notable increase in miR-494-3p expression in cells (Figure 2C). In turn, MALAT1 expression was reduced in mimic group of miR-494-3p compared to the NC group (Figure 2D).

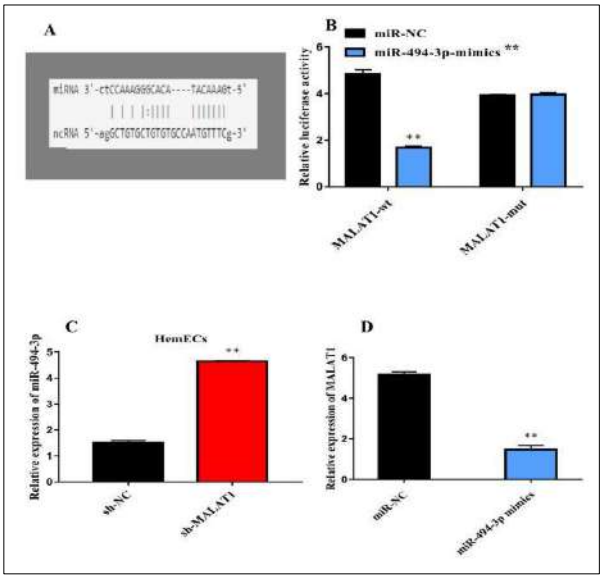

Figure 2. MALAT1 can bind to miR-494-3p in HemECs. (**A**) Targeted sites were predicted between MALAT1 and miR-494-3p. (**B**) dual-luciferase assay. (**C, D**) Expression of MALAT1 and miR-494-3p after MALAT1 knockdown or miR-494-3p overexpression were determined by qRT-PCR. ** P < 0.01.

MALAT1 Regulates PTEN Pathway with Binding to miR-494-3p in Cellular Activity of HemECs

Bioinformatics tools disclosed targeting sites between PTEN and miR-494-3p (Figure 3A). The interaction was also confirmed by Luciferite reporter assay. It has been shown that mimic of miR-494-3p reduced the luciferase activity of wt-PTEN, but did not significantly affect the luciferase activity of mut-PTEN. Also, reduced luciferase activity can be restored by MALAT1 overexpression (Figure 3B). RT-qPCR showed that miR-494-3p transfection was significantly reduced and inhibition was reversed by excessive MALAT1. In contrast, treatment with miR-494-3p inhibitors increased, and stabilization was inhibited following MALAT1 silencing (Figures 3C). The MTT assay was used to confirm the effect of the MALAT1-miR-494-3p-PTEN axis on the cellular activity/cell viability of HemECs. The result showed that the cellular activity of HemECs was reduced after the down-regulation of MALAT1, which was reversed with pcDNA-PTEN or miR-494-3p inhibitors (Figure 3D). Results of Caspase-3 analysis showed that increased apoptosis induced by sh-MALAT1 was reduced with pcDNA-PTEN and miR-494-3p inhibitors (Figure 3E).

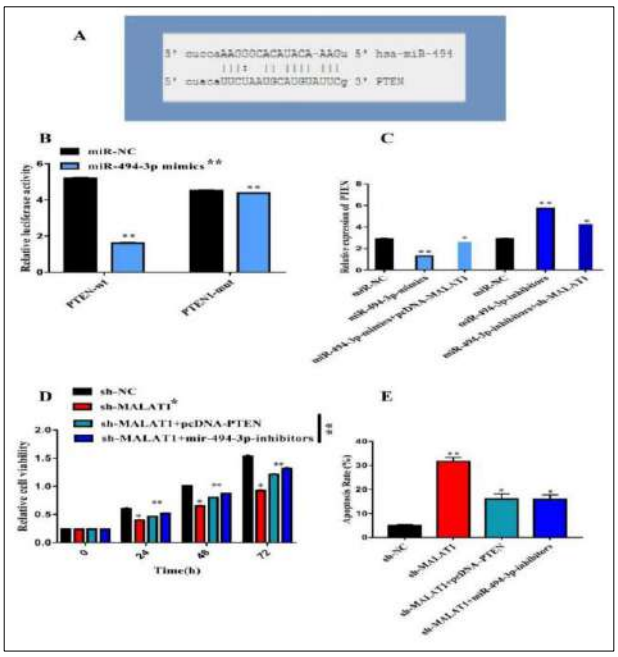

Figure 3. MALAT1 regulates PTEN by binding to miR-494-3p. (**A**) Bioinformatics analysis was used to predict the binding sites between miR-494-3p and PTEN (**B**) The luciferase activity of PTEN may be affected by miR 494-3p mimic. (**C**) PTEN expression after miR-494-3p-mimic and pcDNA-MALAT1 and sh-MALAT1 and miR-494-3p inhibitors was transfected to HemECs. (**D**) The HemECs viability was detected by the MTT assay. (**E**) The apoptotic level was assessed by the Caspase-3 assay. * P < 0.05 no ** P < 0.01 vs. controls.

IH is considered as a vascular lesion (abnormal growth of endothelial cells) in young children and infants [16]. Previous research has determined that MALAT1 plays important role in numerous kinds of cancer [17]. However, the basic function of MALAT1 in infantile hemangioma still needs to be elucidated. Our studies revealed that HemECs were protected by the over-expression of MALAT1. Also, miR-494-3p was negatively regulated by MALAT1. Furthermore, miR-494-3p targeted PTEN and negatively controlled PTEN expressions. Therefore, our findings have shown that MALAT1 plays an important role in the pathogenesis of infantile hemangioma (IH) via miR-494-3p axis.

LncRNA-mediated miRNAs may exert their role in different tumor strategies by the modulation of competing for endogenous RNA (ceRNA). MALAT1 has been reported to play a vital role in the regulation and angiogenesis of endothelial cells. MALAT1 is highly adapted to HCC cancer cells and multicellular cells,

resulting in improved invasion, migration, and proliferation of HCC cell [7]. Also, previous studies determined that MALAT1 expression was expressively enhanced in infantile hemangioma tissues and cells compared to normal skin [18]. However, the exact molecular mechanism by which lncRNA MALAT1 performs its effect on IH remained unclear.

Therefore, a regulatory model of ceRNA (MALAT1-miR-494-3p-PTEN) was developed in our study and we conducted our research. In our work, we investigated the impact of MALAT1 on the cell activity of HemECs cells. First, we silenced the MALAT1 to detect changes in cell function (proliferation, apoptosis) in HemECs. It has been shown that the silencing of MALAT1 specifically inhibit the proliferation of HemECs. We also deliberated the cellular function of MALAT1 in HemECs.

Moreover, miRNA-494 has been described to both promote [19] and inhibit proliferation [20] in the growth of various tumors. In the previous research of IH, miR-494-3p, and the correlation between MALAT1 interactions with miR-494-3p were not clear. Therefore, the bioinformatics analysis performed in this study, suggests that MALAT1 can bind to miR-494-3p, and this study was also performed to detect binding sites of miR-4943p and PTEN. Also, the results further verified that MALAT1 can bind to miR-494-3p and that MALAT1 knockdown enhanced miR-494-3p expression. Binding sites between miR-494-3p and PTEN were verified using Bioinformatics analysis.

PTEN performs a function as a mutated tumor suppressor and previous researches have shown that PTEN also performs an important role in human cancer. PTEN exerted its vital role in the inhibition of migration, cell proliferation, and the promotion of apoptosis. To elucidate the genetic correlation among these genes, double luciferase reporters were performed in this study. Our findings elucidated that MALAT1 regulates PTEN expression efficiently by blocking miR-494-3p expression. Finally, the impacts of MALAT1-miR-494-3p-PTEN on cell viability, as well as apoptosis of HemECs have been confirmed in different experiments.

Conclusion

In conclusion, a recent study was the first to report crosstalk cell functions between MALAT1, miR-494-3p, and PTEN in IH. PTEN is a targeted gene for miR-494-3p, with MALAT1 direct identifying and inhibiting miR-494-3p expression. Silencing MALAT1 suppressed apoptosis and enhanced the proliferation of HemECs by regulating the miR-494-3p/PTEN axis. Therefore, MALAT1 may be used as a therapeutic target in the treatment of IH.

Conflict of Interest

The authors have no conflict of interest to declare.

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