RETRACTED ARTICLE: Long non-coding RNA H19 down-regulates miR-181a to facilitate endothelial angiogenic function

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ABSTRACT

H19 is the first identified long non-coding RNA (lncRNA) whose function in diverse cancers and non-cancerous disease states has been widely studied. The objective of this study was to study the functional role of H19 in vascular endothelial cells. We found that H19 overexpression significantly increased HMEC-1 cells viability, migration and tube-formation capacity. Meanwhile, H19 overexpression up-regulated the protein levels of MMP-2, MMP-9, VEGF and eNOS, and down-regulated RNA level of miR-181a. These alterations, induced by H19 overexpression were abolished by miR-181a overexpression, while they were enhanced when miR-181a was silenced. And also, overexpression of H19 activated JNK and AMPK signalling, which could be eliminated by miR-181a overexpression and accelerated by miR-181a suppression. In conclusion, overexpression of H19 improved HMEC-1 cells viability, migration and tube-formation capacity. H19 exerted pro-angiogenic effects possibly by down-regulating miR-181a, and thus activating JNK and AMPK signalling pathways.

Introduction

Peripheral artery disease (PAD) is a narrowing of the arteries other than those that supply the heart or the brain. PAD usually occurs in the arteries of lower limbs, feet, kidneys and intestines, and significantly impairs the ability to walk, and leads to amputation, ulceration, severe hypertension, renal failure and even stroke [1]. It is estimated that PAD affected ~5.3% of 45 to 50-year-olds in the developed counties, and ~4.6% of 45 to 50-year-olds in the developing countries [2]. Depending on the disease stage of PAD, lifestyle changes, taking cilostazol, pentoxifylline or vitamins and thrombectomy are recommended to improve symptoms of PAD.

Long non-coding RNAs (lncRNA) are a class of non-coding RNAs that widely exist in eukaryotic cells with length longer than 200 nt. Although many lncRNAs have been discovered recently, little is known about their functions [3]. A widely accepted function of lncRNAs is that they can work as competing endogenous RNAs (ceRNAs) to sequester mRNAs from degradation by miRNAs. And because of this, lncRNAs have been found to be involved in multiple processes of cellular biology, which has been overlooked for several decades. H19 is the first identified lncRNA, which is located in human chromosome 11p15.5 [4]. H19 has been considered as an oncogenic or a tumor suppressive lncRNA in diverse cancers. For example, H19 promoted the progression of lung cancer [5], colorectal cancer [6], non-small-cell lung cancer [7] and acute myeloid leukemia [8]. On the contrary, H19 inhibited cell viability, migration, and invasion of thyroid cancer cells [9]. Apart from tumor-related properties, H19 is also involved in other non-cancerous disease states, such as temporal lobe epilepsy [10], acute lung injury [11], coronary artery disease [12], liver cirrhosis [13], etc. In 1996, Han and his colleagues have pointed out that although not normally expressed by adult smooth muscle cells (SMCs), H19 was reexpressed by modified cells of the atherosclerotic plaque [14]. Later, Gao et al., reported that common polymorphisms of H19 were associated with the risk and severity of coronary artery disease in a Chinese population [15]. Furthermore, in vitro studies have indicated that H19 overexpression increased human umbilical vein endothelial cells (HUVECs) growth [16], and silence of H19 inhibited the adipogenesis and inflammation response in ox-LDL-treated Raw264.7 cells [17]. Collectively, these previous studies suggested H19 as a key regulator for atherosclerosis.

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In this paper, the involvement of H19 in the migration and tube formation of human microvascular endothelial HMEC-1 cells was studied to evaluate the potential role of H19 in PAD. Furthermore, the downstream genes and signaling pathways were revealed to further understand H19’s function. A growing number of literature have suggested H19 conferred its functional impacts through a miRNA-dependent regulation [6,18,19]. It has been demonstrated that miR-181a contributed to the migration-promoting effect of H19 on LPS-stimulated lung fibroblast cells [11]. Besides that, miR-181a has been found to be highly expressed in atherosclerotic plaques [20] and its expression was related to the attenuation of the atherosclerotic lesion [21]. Therefore, the regulatory network between H19, miR-181a and two signalling pathways (JNK and AMPK pathways) involved in the pathogenesis of cardiovascular diseases [22–24] were studied.

Materials and methods

HMEC-1 cell culture

HMEC-1 cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in MCDB131 medium (no glutamine, Gibco, Grand Island, NY, USA) supplemented with 10 ng/mL epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA), 1 μg/mL hydrocortisone (Solarbio, Beijing, China), 10 mM glutamine (Sigma-Aldrich), and 10% fetal bovine serum (FBS, Gibco) as recommended by ATCC. HMEC-1 cells were seeded in 75 cm² flasks at 37°C in a humidified incubator with 5% CO₂.

Transfection

Full-length H19 sequence was lightened into pcDNA™3.1(+) plasmid (Invitrogen, Carlsbad, CA, USA) for construction of H19 expression vector. The empty pcDNA™3.1(+) plasmid acted as its negative control (NC). miR-181a mimic (sense: 5'-AAC AUU CAA CGC UGU CGG UGA GU-3') and miR-181a inhibitor (5'-ACU CAC CGA CAG CGU UGA AUG UU-3') were obtained from GenePharma (Shanghai, China). Mimic NC and inhibitor NC with scrambled sequences were used as corresponding controls. The vectors and oligonucleotides were transfected into HMEC-1 cells with a final concentration of 2 μg/mL and 200 nM, respectively. Transfection was performed by using Lipofectamine 3000 (Invitrogen) in 6-well plates under antibiotic-free condition. After 48 h, the culture medium was replaced by the complete culture medium to stop transfection.

RT-qPCR

Following 48 h of transfection, total RNAs in HMEC-1 cells were extracted by TRIzol reagent (Invitrogen). For the test of VEGF, eNOS, and H19, 2 μL extracted RNA was reverse transcribed into cDNA by using PrimeScript™ 1st Strand cDNA Synthesis kit (TaKaRa, Dalian, China). qPCR was performed by SYBR® Advantage® qPCR Premix (TaKaRa) and was carried out using the following conditions: 95°C for 30 s; 40 cycles at 95°C for 10 s; 60°C for 30 s. For the test of miR-181a, QIAGEN reverse transcription kit and miScript SYBR Green PCR Kit (both from QIAGEN, Hilden, Germany) were used. qPCR was carried out as follows: 95°C for 15 min; followed by 40 cycles at 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s; and finally 70°C for 30 s. β-actin and U6 were used as internal controls. Data were calculated according to the 2−ΔΔCT method.

CCK-8 assay

The transfected cells (1 × 10⁴) were seeded in 96-well plates, and were cultured at 37°C for 48h. Thereafter, 10 μL CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD) was added to detect cell viability. The cultures were maintained at 37°C for another 4 h, and then the absorbance of each well was measured by a Microplate Reader (Bio-Rad, Hercules, CA) at 450 nm.

Migration assay

The transfected cells were starved in serum-free medium for 24 h and then were suspended in serum-free medium. The cell suspension was placed in the upper chamber of HTS Transwell™-24-well Permeable Inserts (0.4μm Pore Polyester Membrane, Corning, New York, USA). The lower chamber was filled with a complete culture medium. After 48 h of incubation at 37°C, the cells in the upper side were removed carefully by a cotton swab. The transferred cells in the lower side were stained by 0.5% crystal violet for 5 min at room temperature and were counted microscopically.

In vitro angiogenesis (tubulogenesis) assay

HMEC-1 cells were seeded in a Matrigel (BD Biosciences, San Jose, CA, USA) extracellular matrix-coated 6 well plates at a density of 4 × 10⁴ cells/well. After culturing for 72 h, cells were subjected to phase-contrast microscopy. Tubulogenesis was determined by the rate of tube-like cells to the nucleus.

Western blot

Following 48 h of transfection, the proteins in HMEC-1 cells were extracted by RIPA lysis buffer (Beyotime, Shanghai, China) and the purity of the protein was verified by BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). An equal amount of the protein sample was subjected to SDS-PAGE and the separated proteins were transferred onto PVDF membranes (Millipore, MA, USA). The membranes were blocked in 5% non-fat milk for 1 h at room temperature, after which primary antibodies were used to probe VEGF (sc-80442, Santa Cruz Biotechnology, Santa Cruz, CA, USA), eNOS (ab199956), MMP-2 (ab2462), MMP-9 (ab73734), JNK (ab199380), p-JNK (ab47337), c-Jun (ab32137), p-c-Jun (ab32385), AMPK (ab110036), p-AMPK (ab131357), and β-actin (ab8227, Abcam, Cambridge, MA, USA), overnight at 4°C. Then, the membranes were incubated with the secondary antibodies for 1 h at room temperature. Protein bands were developed by using BeyoECL Plus kit (Beyotime). The
Intensity of the bands was quantified by Image Lab™ software (Bio-Rad Laboratories, Hercules, CA).

**Statistical analysis**

Data represented as mean ± SD from three independent experiments. Statistical analysis was performed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The p-values were calculated by ANOVA following Duncan’s post-hoc test. p-Values of < .05 were considered statistically significant.

**Results**

**Tube formation of HMEC-1 cells in Matrigel**

HMEC-1 cells were cultured in Matrigel-coated plates for 24 h. With time, tubes to nucleus ratio were significantly increased (p < .05, Figure 1(A,B)). By performing RT-qPCR and Western blot, we found that both the mRNA and the protein levels of VEGF and eNOS were significantly increased as time increased (p < .05, Figure 1(C–E)). These data suggested the tube formation of HMEC-1 cells in Matrigel.

**Overexpression of H19 promoted cell migration and tube formation of HMEC-1 cells**

To analyze whether H19 was involved in the angiogenesis of HMEC-1 cells, the expression of H19 in HMEC-1 cells was overexpressed by pc-H19 transfection (p < .05, Figure 2(A)). Overexpression of H19 significantly increased HMEC-1 cells viability and migration (p < .05, Figure 2(B,C)) and significantly up-regulated the protein levels of MMP-2 (p < .05) and MMP-9 (p < .05, Figure 2(D,E)). Meanwhile, H19 overexpression significantly up-regulated VEGF (p < .05) and eNOS (p < .05, Figure 2(F–H)) and promoted tubes to nucleus ratio (p < .05, Figure 2(G)). These data indicated that H19 might contribute to the angiogenesis of HMEC-1 cells.

**Overexpression of H19 promoted cell migration and tube formation of HMEC-1 cells by down-regulation of miR-181a**

miR-181a has been previously identified as a target of H19 [11]. Herein, we want to know whether miR-181a also participated in the pro-angiogenic effect of H19 on HMEC-1 cells.

![Figure 1](image)

Figure 1. Tube formation of HMEC-1 cells in Matrigel. HMEC-1 cells were cultured in Matrigel-coated plates for 24 h. (A, B) tube formation, as well as the (C) mRNA and (D, E) protein levels of VEGF and eNOS were respectively detected by tubulogenesis assay, RT-qPCR and Western blot. (100× magnification, scale bar = 100 μm). Data represented as mean ± SD. Different letters above columns indicated significant difference between groups.
RT-qPCR data in Figure 3 showed that miR-181a level was significantly down-regulated in HMEC-1 cells transfected with pc-H19, as compared to the cells transfected with pcDNA3.1 ($p < .05$).

Next, the expression of miR-181a in HMEC-1 cells was altered by oligonucleotides transfection. As compared to the corresponding controls, miR-181a expression was significantly increased by mimic transfection ($p < .05$), while it was significantly decreased by inhibitor transfection ($p < .05$, Figure 4(A)). Figure 4(B–E) showed that the increased cell viability and migration, as well as the up-regulation of MMP-2 and MMP-9 induced by H19 overexpression, were all eliminated by transfection with miR-181a mimic (all $p < .05$). However, these alterations induced by H19 overexpression
were all accelerated by miR-181a inhibitor transfection \( (p < .05) \). Also, the up-regulation of VEGF and eNOS, and the increased tubes/nucleus ratio by H19 overexpression were attenuated by miR-181a overexpression \( (p < .05) \) while they were accelerated by miR-181a suppression \( (p < .05) \), Figure 4(F–H). These data suggested that H19 promoted the migration and tube formation of HMEC-1 cells by down-regulation of miR-181a.

Figure 4. Overexpression of H19 promoted cell migration and tube formation of HMEC-1 cells by down-regulation of miR-181a. (A) HMEC-1 cells were transfected with mimic, inhibitor or the corresponding controls specific for miR-181a. The expression of miR-181a was detected by RT-qPCR. HMEC-1 cells were co-transfected with pc-H19 and miR-181a mimic/inhibitor, or transfected with the corresponding controls. (B) cell viability, (C) relative migration, (D, E) protein levels of MMPs, (F, G) protein levels of VEGF and eNOS, and (H) tube formation were respectively assessed by CCK-8 assay, transwell assay, Western blot, and tubulogenesis assay. Data are represented as mean ± SD. *\( p < .05 \) when compared to the indicated group.
Overexpression of H19 activated JNK and AMPK pathways by down-regulation of miR-181a

We finally studied the possible underlying pathways of H19 which impacted HMEC-1 cells migration and tube formation. By performing Western blot analysis, we observed that phosphorylation levels of JNK, c-Jun, and AMPK were all significantly increased by H19 overexpression (\(p < .05\), Figure 5(A–D)). And also, the increased phosphorylation levels were all attenuated by miR-181a overexpression (\(p < .05\)), while they were enhanced by miR-181a suppression (\(p < .05\)). Collectively, these results implied that H19 activated JNK and AMPK pathways in a miR-181a-dependent fashion.

Discussion

The pathogenesis of atherosclerosis has not been fully understood, but multiple pieces of evidence have suggested that SMC, monocyte, foam cell, lymphocyte, macrophage and vascular endothelial cell are important cell types that contribute to the formation of the atherosclerotic lesion [25,26]. A previous study has demonstrated that H19 was reexpressed in SMCs which were modified by atherosclerotic plaque [14]. Besides, the importance of H19 in the proliferation, adipogenesis and inflammation of HUVECs [16] and macrophages [17] have been reported. However, the role of H19 in vascular endothelial cells has not been studied yet. This study demonstrated that H19 overexpression significantly increased HMEC-1 cells viability, migration and tube formation capacity.

Meanwhile, H19 overexpression up-regulated the protein levels of MMP-2, MMP-9, VEGF and eNOS. By performing RT-qPCR, miR-181a was found to be down-regulated by H19 overexpression, and the functions of H19 in HMEC-1 cells were abolished by miR-181a overexpression, while they were enhanced when miR-181a was silenced. And also, overexpression of H19 activated JNK and AMPK signalling through a miR-181a-dependent regulation.

Endothelial cell sprouting is a main angiogenesis-driving process that involves the invasion of avascular areas by proliferating and migrating endothelial cells [27]. Herein, we found that H19 promoted the viability and migratory capacity of HMEC-1 cells, which indicated that H19 might be a key regulator in endothelial cell sprouting. Additionally, we found that H19 increased tube formation of HMEC-1 cells, suggesting H19 might contribute to the formation of plaque neovascularization, which is a major event in atherosclerosis. MMPs, a family of zinc-dependent proteases, are key modulators in extracellular matrix turnover, and cells growth, inflammation, migration and angiogenesis [28]. It has been reported that the elevated levels of MMP-2 and MMP-9 increased the proliferation, migration and cell cycle transition of pulmonary arterial endothelial cells which eventually led to angiogenesis [29]. In the current study, we observed that H19 overexpression up-regulated protein levels of these two proteases, further suggesting the pro-angiogenic effect of H19.

Plaque neovascularization is an important contributor to plaque growth and instability. To date, the source of plaque neovascularization is still a mystery. However, it has been
widely-accepted that endothelial cells grow from the existing adventitial vasa vasorum triggered by a gradient of VEGF [30]. eNOS is an upstream promoter of VEGF expression in certain cell types [31,32]. eNOS can synthesize NO and alter endothelial cells dysfunction via controlling of platelet aggregation, monocyte adhesion, and smooth muscle cells proliferation [33]. In the present study, H19 overexpression increased the protein levels of VEGF and eNOS in HMEC-1 cells, suggesting H19 promoted tube formation, possibly by regulating VEGF and eNOS.

To further understand the impacts of H19 on the migration and tube formation of human microvascular endothelial HMEC-1 cells, downstream effector of H19 was explored. In the present work, we focused on miR-181a, as it has been previously identified as a target of H19 [11] and its expression is closely associated with cardiovascular diseases. miR-181a has been found to be highly expressed in atherosclerotic plaques [20]. Another study showed that miR-181a expression was related to the attenuation of the atherosclerotic lesion [21]. Further investigations suggested that miR-181a might play a role in the pathological progress of atherosclerosis by regulation of endothelial dysfunction [20]. In the current study, we found that miR-181a was down-regulated in response to H19 overexpression. Besides, the impacts of H19 towards HMEC-1 cells migration and tube formation were all abolished when miR-181a was overexpressed while they were enhanced by miR-181a’s silence, indicating H19 exerted pro-angiogenic effects at least in part by down-regulating miR-181a.

JNKs are traditionally considered as stress-activated protein kinases, and they are implicated in the regulation of cell proliferation, apoptosis, migration, inflammation, and even cancer development [34]. AMPK is a phylogenetically conserved serine/threonine kinase, which has been recognized as a key regulator of lipid and energy metabolism [35]. Both JNK and AMPK signalling pathways have emerged as promising molecular targets for the treatment of cardiovascular diseases [22–24]. In the current study, the activation of these two signals was observed in H19-overexpressing cells, and the activation was abolished by miR-181a overexpression, which indicated that H19 activated JNK and AMPK signalling in a miR-181a-dependent manner. Actually, the regulatory effects of miR-181a on the activation of JNK signalling pathway have been previously revealed. miR-181a overexpression was capable of suppressing JNK signalling pathway in various experimental models, like human trabecular meshwork cells [36] and human neuroblastoma SK-N-Sh cells [37]. However, this study, for the first time, revealed the inhibitory effects of miR-181a on AMPK signalling. Further study is required to confirm whether JNK and AMPK contribute to the pro-angiogenic effects of H19.

To sum up, this study demonstrated that overexpression of H19 improved HMEC-1 cells viability, migration and tube formation capacity, showing pro-angiogenic effects. Besides, we suggested that H19 overexpression down-regulated miR-181a expression which, in turn, activated JNK and AMPK signalling and ultimately promoted neovascularization. These findings provided in vitro evidence that H19 might be one of the potential targets for the treatment of atherosclerosis-related diseases, such as PAD.

Disclosure statement
No potential conflict of interest was reported by the authors.

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