microRNA-301a-3p is a potential biomarker in venous ulcers vein and gets involved in endothelial cell dysfunction

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ABSTRACT

Venous ulcer is a common contributor to chronic venous insufficiency (CVI) of lower limbs, which seriously affects the life quality of patients. In this study, we researched the expression characteristics of microRNA-301a-3p (miR-301a-3p) in patients with CVI and investigated the impact of miR-301a-3p on the dysfunction of human umbilical vein endothelial cells (HUVECs). The plasma level of miR-301a-3p in normal controls, patients with varicose great saphenous vein, and patients with the venous ulcer of lower limbs were measured. We adopted Interleukin-1β (IL-1β), H₂O₂, and oxygen and glucose deprivation (OGD) to induce endothelial cell injury in vitro. In this way, we evaluated the influence of miR-301a-3p on HUVEC viability, apoptosis, inflammatory response, and oxidative stress. Our data showed that miR-301a-3p was substantially overexpressed in patients with lower limb venous ulcers. The viability of HUVECs decreased, and miR-301a-3p was up-regulated after IL-1β, H₂O₂, and OGD treatment. miR-301a-3p inhibition greatly ameliorated the dysfunction and cell damage of HUVECs, promoted IGF1/PI3K/Akt/PPARγ, and down-regulated NF-κB/MMPs. The phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) or the peroxisome proliferator-activated receptor-γ (PPARγ) inhibitor (GW9661) reversed the anti-inflammatory, antioxidant, and anti-apoptotic effects mediated by miR-301a-3p down-regulation. The nuclear factor-κB (NF-κB) inhibitor lessened cell injury mediated by miR-301a-3p overexpression. In terms of the mechanism, miR-301a-3p targeted the 3′UTR of Insulin-like growth factor-1 (IGF1) and repressed the profile of IGF1. Thus, miR-301a-3p mediates venous endothelial cell damage by targeting IGF1 and regulating the IGF1/PI3K/Akt/PPARγ/NF-κB/MMPs pathway.

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Highlights

- miR-301a-3p expression is uplifted in the plasma of great saphenous vein varicosity and lower extremity vein ulcer patients.
- miR-301a-3p overexpression enhances HUVEC apoptosis, inflammation, and oxidative stress.
- miR-301a-3p targets IGF1, initiates PI3κ/ AKT/PPARγ, and hampers NF-κB/MMPs.
- PI3κ/AKT/PPARγ inhibition weakens the protective function of miR-301a-3p down-regulation against HUVEC damage.

1. Introduction

The chronic venous insufficiency (CVI) of lower limbs, a prevailing clinical disease with a high incidence rate, is a leading contributor to skin eczema, pigmentation, ulcers, and other nutritional disorders [1]. Currently, the pathogenesis of CVI remains poorly understood, possibly pertaining to multiple factors like age, gender, genetics, hypoxia, and matrix metalloproteinases (MMPs) [2]. Lower limb venous ulcers are the severest manifestation of CVI, and seriously influence patients’ living quality [3–5]. During CVI progression, increased venous pressure of varicose veins and alterations in venous valve functions lead to weakness of vascular walls and a lot of stasis in the veins, which augment the damage to endothelial cells and promote vascular wall remodeling [6,7]. Therefore, a probe into the molecular mechanism of venous endothelial cell dysfunction and injury during venous ulcer progression is critical.

As evidenced by increasing studies, microRNAs (miRNA/miR), non-coding RNA molecules 20–25 nt in length, can modulate endothelial cell apoptosis, inflammation, oxidative stress, and dysfunction [8–12]. Notably, several miRNAs present an aberrant expression in patients suffering from CVI [13]. microRNA-301a-3p (miR-301a-3p), a member of the miRNA family, has been discovered to boost tumor cell proliferation, migration, invasion and other malignant phenotypes to exert its pro-cancer functions [14,15]. Interestingly, it also partakes in regulating endothelial cell functions. For instance, inhibiting miR-301a-3p improves the functions of HUVECs by promoting insulin-like growth factor-1 (IGF-1) expression [16]. Nevertheless, we need to conduct more studies on the profile of miR-301a-3p in CVI and lower limb vein ulcer patients and its influence on endothelial cell damage elicited by inflammation/hypoxia.

IGF-1, a peptide hormone, is instrumental in normal pre- and postnatal growth and progression [17]. After combining with its receptor, IGF-1 mediates cell proliferation, differentiation, and survival and enhances migration and tube formation, exerting a prominent function in endothelial cell physiology by inducing PI3K/AKT pathway activation [18,19]. Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor inextricably associated with lipid metabolism, glucose metabolism, endothelial cell functions, and other risk factors for cardiovascular diseases. Formononetin initiates the PPARγ signaling pathway to mitigate inflammation, oxidative stress, and apoptosis in HUVECs treated with oxidized low-density lipoprotein (ox-LDL) [20]. PPAR-γ upregulation suppresses NF-κB activation, and relieves murine vascular endothelial dysfunction induced by lipopolysaccharide (LPS) [21]. Moreover, IGF1 is modulated by miRNAs, such as miR-186 [22], as well as miR-301a-3p [16].

Reportedly, matrix metalloproteinases (MMPs) are a critical link in skin target organ injury. MMPs, an enzyme family dependent on zinc and calcium ions, have outstanding functions in vascular remodeling. At least 26 members of the family have been discovered. As per their substrates, they can be grouped into collagenases (matrix metallopeptidase 1 (MMP-1), MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), intermesenchymal lysins (MMP-3), MMP-10, MMP-11), membrane metalloproteinases (MMP-14, MMP-15, MMP-16, MMP-17), and matrix lysozymes (MMP-7, MMP-26), etc [23,24]. As is reported, the inflammatory status of varicosity may be associated with MMPs expression up-regulation and cytokine
imbalance [7]. The levels of MMP-1, -2, -3, -8, -9, -12, -13 are substantially heightened in lower limb venous ulcer patients as opposed to normal controls and can be distinctly attenuated by pressure treatment [25]. This finding reveals that MMPs inhibition can ameliorate malignant varicosity progression. Prior works have shown that IGF1 activates the IGF-1 R/AKT pathway to repress MMP3 expression, thereby impeding lumbar disc degeneration (LDD) development. Therefore, we try to understand whether the miR-301a-3p/IGF-1/PI3K/AKT/PPARY/NF-κB axis can regulate MMPs to function in CVI.

Here, an ex-vivo endothelial cell damage model was established employing HUVECs. We discovered that miR-301a-3p was dramatically up-regulated, whereas IGF1 was considerably down-regulated in the damage models. miR-301a-3p overexpression exacerbated HUVEC damage mediated by IL-1β/H2O2/OGD. Bioinformatics analysis signified that IGF1 was a downstream target of miR-301a-3p, which also suppressed PI3K/AKT/PPARY expression. Therefore, we supposed that miR-301a-3p gets involved in endothelial cell injury by affecting HUVECs’ viability, apoptosis, oxidative stress, and inflammation. We hope the study will offer novel diagnostic and therapeutic targets for CVI-elicited venous ulcers.

2. Materials and methods

2.1. Sample collection

The study had received the approval from the Ethics committee Institutional Review Board of the Heilongjiang Provincial Hospital (Approval No. 2019–153). Forty-five patients with recurrent venous insufficiency in their lower limbs were recruited from May 2018 to December 2020. The clinical rating of CVI refers to category of CEAP (Clinical-Etiology-Anatomy-Pathophysiology) classification [26]. C0 group: symptoms but no signs of phlebocholosis; C1 group: angioflectasis and reticular veins; C2 group: superficial varicos veins; C3 group: venous edemas; C4: skin alterations (pigmentation, eczema, lipoderma, white atrophy); C5 group: skin alterations coupled with healed ulcers; C6 group: skin changes and active ulcers. There were three experimental groups: the normal group (n = 15), the lower limb great saphenous vein varicosity (C1-C3) group (n = 15), and the lower limb venous ulcer (C4-C6) group (n = 15). Prior to the surgery, the blood specimens were harvested from the two groups as well as healthy controls. Inclusion criteria: 18–80 years old; body mass index ≤ 35; CVI patients or healthy controls. Exclusion criteria: history of cancer; other chronic diseases; history of drug or alcohol abuse; documented infection within 30 days of admission, recent hospitalization or surgery (within 30 days of admission); infection upon presentation; patients with other acute medical problems at the time of admission (e.g. trauma, myocardial infarction, etc.); When we were selecting participants, all the patients experienced a detailed ultrasound Doppler (Esaote MyLab25, Genoa Italy) scanning of the deep, superficial, and perforating vein system and received a vascular specialist visit. The use of compression stockings during the examination was excluded in that it carried an underlying bias.

2.2. Cell culture and treatment

HUVECs, supplied by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were kept in a Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 u/ml penicillin, and 100 μg/ml streptomycin (Gibco, USA). The cells were cultivated under normoxic and humidified conditions at 37°C with 5% CO2 for 24 hours. Cells in the logarithmic growth stage were treated with 0.25% trypsin (Thermo Fisher HyClone, Utah, USA) for digestion and passage. IL-1β (10 ng/ml, Cat. No. C600002-0002), ordered from Sangon Biotech (Shanghai), was taken to treat HUVECs for 48 hours, followed by one-hour treatment with hydrogen peroxide solution (60 μM, Sigma, Cat. No. 3587191). Then, the original medium was substituted by a fresh one for 24 hours’ culture. The PI3K inhibitor LY294002 (1 μM, Cat. No. HY-
10108), the PPARy inhibitor GW9662 (3.3 nM, Cat. No. HY-16578), and the NF-κB inhibitor BAY-11-7082 (1 μM, Cat. No. HY-13453) were all supplied by MedChemExpress (SH, USA). HUVECs were treated with those inhibitors for 24 hours [11].

2.3. Cell transfection

HUVECs in the logarithmic growth phase were digested, passed, and inoculated into 6-well plates with a density of 5 × 10⁵/well. The cells were transfected when they achieved steady growth. Ribobio Biotechnology Co., Ltd (Guangzhou) took on the design and synthesis of miR-301a-3p mimics, inhibitors, and corresponding negative controls (miR-NC, in-NC). IGF1 overexpression plasmids (IGF1) and the negative control (NC) were purchased from GenePharma (Shanghai, China). IGF1-overexpression plasmids or NC were transfected into HUVECs using Lipofectamine® 3000 (Invitrogen; ThermoFisherScientific, Inc.) as instructed by the supplier. The cells were cultured in an incubator with 5% CO₂ at 37°C. Following 48 hours’ transfection, the original medium was replaced by a fresh and complete one for another 24 hours’ culture. With total RNA extracted from the cells, quantitative real-time polymerase chain reaction (qRT-PCR) was implemented to check alterations in the profile of each molecule in the transfected cells [27].

2.4. The OGD model in vitro

To simulate ischemia conditions in vitro, an OGD model was constructed, as mentioned before [28]. For OGD activation, a glucose-free medium (RPMI-1640 without FBS) was taken to substitute the old culture medium, and HUVECs were then moved into an airtight experimental hypoxia compartment rinsed in a gas mixture of 95% N₂ and 5% CO₂ for 8 hours at 37°C. For OGD termination, the cells were treated with a glucose-contained medium containing EL-102 or vehicle of the identical concentration, subjected to re-oxygenation, and put back in the incubator with a normal growth atmosphere. HUVECs that were not exposed to OGD were taken as the normal control.

2.5. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromid (MTT)

A complete medium was adopted to dilute HUVECs into a cell suspension (density: 4 × 10⁴/ml), which was then inoculated into 96-well plates with a density of 200 μl/well. As the majority of the cells adhered to the wall 24 hours later, 0.5% FBS culture solution was employed for culture to synchronize the cells. After 24 hours, the culture solution was removed, with 200 μL of a culture solution with serum that contained corresponding drugs administered to each well (8 wells in each group). Later, 10 μl of 5 mg/ml MTT solution was given to each well for 4 hours’ incubation in an incubator with 5% CO₂ and saturated humidity at 37°C. As the culture solution was discarded, 150 μl of dimethyl sulfoxide (DMSO) was administered to each well for 10 minutes’ oscillation and incubation. The optical density (OD) at 450 nm was not gauged with a microplate reader until the precipitation was thoroughly dissolved [29].

2.6. Flow cytometry for apoptosis examination

After being treated with IL-1β, H₂O₂, and OGD, HUVECs were digested using trypsin, centrifuged at 1500 r/min for 3 minutes, and harvested. The Annexin V-FITC Apoptosis Detection Kit (Beyotime, Cat. No. C1062S) was utilized as instructed: after twice phosphate-buffered saline (PBS) washing, 400 μl of pre-cooled PBS was administered, followed by the addition of 10 μl Annexin V-fluorescein isothiocyanate (AnnexinV-FITC) and 5 μl propidium iodide (PI), for 30 minutes’ incubation in darkness at 4°C; then, a flow cytometry instrument was taken for examination, and the apoptotic rate was calculated through computer software processing. The apoptotic rate = the number of apoptotic cells/(the number of apoptotic cells + the number of normal cells) × 100%. All steps were completed as guided by the kit [30].
2.7. RT-qPCR

TRIZol reagent was utilized to extract total RNA from the cells. The PrimeScript™ RT Reagent kit (Invitrogen, Shanghai, China) was operated to reverse-transcribe the RNA into cDNA as stipulated by the manufacturer. qRT-PCR was implemented through the Bio-Rad CFX96 quantitative PCR system and SYBR instructions. qRT-PCR was done with the following conditions: 5 minutes’ pre-denaturation at 95°C, 15 seconds’ denaturation at 95°C, and 30 seconds’ annealing at 60°C [31]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken as the internal parameter of IGF1, MMP1, MMP3, MMP9, IL-1β, and tumor necrosis factor α (TNFα). The 2^(-ΔΔCt) approach was utilized for statistics. All RT-qPCR reactions were duplicated three times. Ribobio Biotechnology Co., Ltd took on the design and synthesis of primers (Table 1).

2.8. Western blot

HUVECs, inoculated into 6-well plates with a density of 5 × 10^5/well, were treated with IL-1β, H₂O₂, and OGD, digested with trypsin, harvested, and flushed in cold PBS three times. Next, 100–200 μL of Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) was administered to lyse the cells with ultrasound in ice-cold water, with the Bradford method applied to determine the protein concentration. Proteins of the identical amount were taken from each group for 10% dodecyl sulfate, sodium salt (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE) and then moved onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After being sealed at 4°C for an hour, the membranes were incubated with primary antibodies (concentration: 1:1000) Anti-IGF1 antibody (ab133542), Anti-Insulin-like growth factor-1 receptor (IGF1R) antibody (ab182408), Anti-p-IGF1R antibody (ab39398), Anti-Bax antibody (ab32503), Anti-Bcl2 antibody (ab32124), Anti-Caspase3 antibody (ab32351), Anti-inducible nitric oxide synthase (iNOS) antibody (ab178945), Anti-cyclooxygenase-2 (COX2) antibody (ab179800), Anti-Pi3K antibody (ab191606), Anti-p-Pi3K antibody (ab182651), Anti-AKT antibody (ab8805), Anti-p-AKT antibody (ab38449), Anti-PPARγ antibody (ab272718), Anti-NF-κB antibody (ab32536), Anti-p-NF-κB antibody (ab76302), Anti-MMP1 antibody (ab52631), Anti-MMP3 antibody (ab52915), Anti-MMP9 antibody (ab76003), and Anti-GAPDH antibody (ab181602) overnight at 4°C. Tris-buffered saline with Tween-20 (TBST) was utilized to flush the membranes twice, which were then incubated with the fluorescein-labeled goat anti-rabbit secondary antibody (ab205718, 1:2500) at room temperature (RT) for an hour. All these antibodies were supplied by Abcam (Cambridge, UK). The membranes were flushed three times. The enhanced chemiluminescence (ECL) color developing agent (Millipore, Bedford, MA, USA) was deployed for exposure, and a membrane scanner was adopted for imaging [32].

Table 1. Primer sequences of each gene.

| Gene Name Primer Name | Primer Sequence (5’→3’) |
|------------------------|-------------------------|
| mirR-301a-3p | forward: 5’-ACACTCCAGCTTGCGCAATAGTATTGTGCT-3’
| | reverse: 5’-CTCAACTGTTGCTGGGGA-3’ |
| IGF1 | forward: 5’-TTGTAATCCTTGTGCGCTGTA-3’
| | reverse: 5’-TGCTGGTCTGACGACTGTC-3’ |
| MMP1 | forward: 5’-GATGAGACCGCCAGATGTCG-3’
| | reverse: 5’-GCTTGGACCTCGAGACCTC-3’ |
| MMP3 | forward: 5’-CCCGTGAATGTTGGCGGCA-3’
| | reverse: 5’-TCATGTGAGACGCGGAG-3’ |
| MMP9 | forward: 5’-GAGTCCCGAGAGATGTTGA-3’
| | reverse: 5’-AAGGTGACGAAAGGGCCG-3’ |
| IL-1β | forward: 5’-AGGCGACCTAAGGGGGA-3’
| | reverse: 5’-CCCGGATCATGTCCTCGTG-3’ |
| TNFα | forward: 5’-AGGACCGACTTAAGAGGGGA-3’
| | reverse: 5’-CCCGGATCATGTCCTCGTG-3’ |
| GAPDH | forward: 5’-TGATTCATGTCGACGCGT-3’
| | reverse: 5’-CCCGGATCATGTCCTCGTG-3’ |
| U6 | forward: 5’-CTCCGGTGGCAGACGA-3’
| | reverse: 5’-AAGCTTCAGAATTTGTCG-3’ |

2.9. Reactive oxygen species (ROS) level detection

ROS levels in the cells were examined employing theDCFDA/H2DCFDA-Cellular ROS Assay Kit (ab113851, Abcam), as instructed. HUVECs, inoculated into 96-well plates with a density of 5 × 10^5/well, were treated with IL-1β, H₂O₂, and OGD. After 1X Buffer was administered, the cells were flushed twice, and the diluted DCFDA solution was given for 45 minutes’ incubation at 37°C in darkness. The cells were later flushed with 1X
Buffer once or twice. A fluorescence microscope (Olympus, Japan) was taken to monitor DCFDA fluorescence signal [33].

2.10. The detection of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), lactic dehydrogenase (LDH), and minimum descent altitude (MDA) levels

HUVECs, inoculated into 24-well plates with a density of $5 \times 10^5$/well, were treated with IL-1β, H$_2$O$_2$, and OGD, digested employing trypsin, harvested, and rinsed in cold PBS three times. Predicated on prior literature, we measured the levels of SOD, GSH-PX, LDH, and MDA in the cells with the use of the SOD (Cat. NO. A001-3-1), GSH-PX (Cat. NO. A005-1-2), LDH (Cat. NO. A020-1-2), and MDA (Cat. NO. A003-1-2) kits, respectively, which were all supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protein concentration was gauged through the Bovine Serum Albumin (BSA) method (Beyotime, Shanghai, China) [28].

2.11. Dual luciferase activity assay

The sequence of IGF1 was amplified and then slotted into the pmirGLO dual-luciferase miRNA target expression vector to produce pMIR-IGF1-wt. TargetScan forecast the binding site of IGF1 and miR-301a-3p, according to which we designed the IGF1 fragment (pMIR-IGF1-MT) containing the mutant target region. Then, HUVECs were transfected with pMIR-IGF1-wt, pMIR-IGF1-MT, miR-301a-3p mimics, and miR-NC with the use of lipofectamine* 3000 for 48 hours. The Promega dual luciferase reporter system was introduced to analyze the relevant luciferase activity [32].

2.12. Statistical analysis

The SPSS20.0 statistical software (SPSS Inc., Chicago, IL, USA) was adopted for analysis, with the measurement statistics presented as mean ± standard deviation (X ± S). One-way Analysis of Variance (ANOVA) was taken for comparison among multiple groups, while an independent t-test was implemented to compare two groups. Pearson analysis was utilized to evaluate correlation. $P < 0.05$ was regarded as statistically meaningful [30].

3. Results

miR-301a-3p expression in CVI patients and the endothelial cell injury model was measured. The in-vitro miR-301a-3p overexpression and low expression cell models were established. We examined viability, apoptosis, inflammation, oxidative stress, and the profile of the IGF1/IGF-1 R/PI3K/AKT/PPARγ/NF-κB pathway in endothelial cells treated with IL-1β/H$_2$O$_2$/OGD stimulation. Furthermore, bioinformatics analysis predicted the targeted correlation between miR-301a-3p and IGF1, which was further corroborated through dual luciferase activity assay.

3.1. miR-301a-3p expression was up-regulated in the plasma of great saphenous vein varicosis and lower limb venous ulcer patients

To probe the expression features of miR-301a-3p in CVI patients, we obtained venous blood samples (5 ml) from normal controls, great saphenous vein varicosis patients (C1-C3), and lower limb venous ulcer patients (C4-C6) and measured miR-301a-3p and IGF1 expressions in the samples. RT-qPCR reflected that in contrast with the normal control group, C1-C3 and C4-C6 patients experienced a notable increase in miR-301a-3p expression and a remarkable decline in IGF1 expression in their serum ($P < 0.05$, Figure 1(a, b)). RT-qPCR confirmed the profiles of matrix metalloproteinases MMP1, MMP3, and MMP9 as well as inflammatory factors IL-1β and TNFα in the serum of C1-C3 and C4-C6 patients. By contrast to the normal group, the profiles of MMP1, MMP3, MMP9, IL-1β, and TNFα were dramatically elevated ($P < 0.05$, Figure 1(c–e)). Linear regression analysis evaluated the correlation between miR-301a-3p and IGF1, MMP1, MMP3, MMP9, IL-1β, and TNFα. As a result, miR-301a-3p expression negatively correlated with IGF1 expression but positively correlated with the profiles of MMP1, MMP3, MMP9, IL-1β, and TNFα ($P < 0.05$, Figure 1(h–m)). All these findings suggested that miR-301a-3p/IGF1 might modulate the
development of great saphenous vein varicosity and lower limb venous ulcers, bearing a significant relation to inflammation.

### 3.2. miR-301a-3p overexpression exacerbated HUVEC viability decrease and apoptosis increase mediated by IL-1β, H₂O₂, and OGD

To investigate the expression features of miR-301a-3p in the in-vitro endothelial cell damage model, we treated HUVECs with IL-1β, H₂O₂, and OGD and constructed an in-vitro endothelial cell damage model. MTT examined cell viability, denoting that IL-1β, H₂O₂, and OGD caused a substantial drop in HUVEC viability (Figure 2 (a–c)). RT-PCR determined miR-301a-3p expression and revealed that IL-1β and H₂O₂ contributed to a marked increase in miR-301a-3p’s expression (Figure 2(d,e)). OGD also elevated its expression (Figure 2(f)). We reckoned that miR-301a-3p might participate in HUVEC dysfunction.

### 3.3. miR-301a-3p overexpression exacerbated HUVEC viability decline and apoptosis increase mediated by IL-1β, H₂O₂, and OGD

To confirm the impact of miR-301a-3p in HUVEC damage, we transfected vascular endothelial cells with miR-301a-3p mimics for upregulating its expression (Figure 3(a)). IL-1β, H₂O₂, and OGD were administered to treat the transfected HUVECs for 48 hours. MTT assay, flow cytometry, and western blot showed that IL-1β, H₂O₂, and OGD brought about a stark decrease in vascular endothelial cell viability, but promoted
apoptosis, restrained Bcl2 expression, but enhanced Caspase3 and Bax expressions (Figure 3(b–j)), which demonstrated that all of IL-1β, H2O2, and OGD boosted vascular endothelial cell apoptosis. Following the transfection of miR-301a-3p mimics, HUVEC viability was increased, whereas the apoptosis was greatly augmented (by contrast to the miR-NC+IL-1β/H2O2/OGD group, Figure 3(e–j)). Our observation displayed that miR-301a-3p aggravated vascular endothelial cell damage mediated by IL-1β, H2O2, or OGD.

3.4. miR-301a-3p down-regulation mitigated HUVEC viability decline and apoptosis increase mediated by IL-1β, H2O2, and OGD

To further confirm the function of miR-301a-3p in HUVEC damage mediated by IL-1β, H2O2, and OGD, we transfected vascular endothelial cells with the miR-301a-3p inhibitor (Figure 4(a)). IL-1β, H2O2, and OGD were taken to treat HUVECs for 48 hours, and MTT gauged their viability. Transflecting the miR-301a-3p inhibitor culminated in an increase in HUVEC viability (versus the in-NC+IL-1β/H2O2/OGD group) (Figure 4(b–d)). Flow cytometry and western blot examined cell apoptosis, indicating that by contrast to the in-NC+IL-1β/H2O2/OGD group, the transfection of the inhibitor caused a reduction in HUVEC apoptosis (Figure 4(e–g)), a notable increase in Bcl2’s level, and a decline in Bax and Caspase3 levels (Figure 4(h–j)). These findings exhibited that miR-301a-3p inhibition alleviated vascular endothelial cell damage mediated by IL-1β, H2O2, or OGD.

3.5. The influence of miR-301a-3p on HUVEC inflammation and oxidative stress mediated by IL-1β, H2O2, and OGD

We detected inflammation and oxidative stress mediators in HUVEC. RT-PCR confirmed the enhanced expression of IL-1β, TNFa, and MMP1, 3, 9 in HUVECs (in contrast with the miR-NC+IL-1β/H2O2/OGD group), whereas miR-
301a-3p inhibition vigorously reduced their levels in the cells (versus the in-NC+IL-1β/H2O2/OGD group) (Figure 5(a–c)). Western blot showed that IL-1β, H2O2, and OGD dramatically elevated COX2 and iNOS. As compared with the miR-NC+IL-1β/H2O2/OGD group, miR-301a-3p overexpression boosted, IL-1β, TNFα, MMP1, 3, 9, COX2 and iNOS expressions in HUVECs treated with IL-1β/H2O2/OGD. Transfection of the miR-301a-3p inhibitor showed protective effects against HUVEC inflammation and oxidative stress (Figure 5(d–f)). Additionally, IL-1β, H2O2, and OGD increased ROS levels in HUVECs. miR-301a-3p overexpression facilitated ROS generation in HUVECs treated with IL-1β/H2O2/OGD (versus the miR-NC+IL-1β/H2O2/OGD group), whereas miR-301a-3p inhibition lessened ROS production in the cells (versus the in-NC+IL-1β/H2O2/OGD group) (Figure 5(g–i)). These discoveries reflected that miR-301a-3p overexpression facilitated HUVEC inflammation and oxidative stress mediated by IL-1β, H2O2, and OGD, which were alleviated by miR-301a-3p inhibition.

3.6. miR-301a-3p dampened the IGF1/IGF-1 R/PI3K/AKT/PPARγ pathway and up-regulated the NF-κB pathway

To confirm the mechanism of miR-301a-3p modulating vascular endothelial cell damage, we implemented western blot to measure the profile of the
IGF1/IGF-1 R/PI3K/AKT/PPARγ/NF-κB pathway in HUVECs. By contrast to the control group, IL-1β, H2O2, and OGD suppressed IGF1 and PPARγ expressions, reduced IGF-1 R, PI3K, and AKT phosphorylation, and strengthened NF-κB phosphorylation in HUVECs. miR-301a-3p overexpression dramatically augmented NF-κB phosphorylation, weakened IGF-1 R, PI3K, and AKT phosphorylation, and lowered IGF1 and PPARγ expressions (versus the miR-NC+IL-1β/H2O2/OGD group), whereas miR-301a-3p inhibition culminated in the opposite outcomes (against the in-NC+IL-1β/H2O2/OGD group) (Figure 6(a–f)). All these findings suggested that miR-301a-3p overexpression restrained the IGF1/IGF-1 R/PI3K/AKT/PPARγ pathway and up-regulated the NF-κB pathway, but miR-301a-3p inhibition initiated the IGF1/IGF-1 R/PI3K/AKT/PPARγ pathway and suppressed NF-κB pathway activation.

3.7. The PI3K or PPARγ inhibitor weakened PI3K/AKT/PPARγ pathway activation mediated by the miR-301a-3p inhibitor

To probe the function of the PI3K/AKT/PPARγ pathway in vascular endothelial cell damage, we treated HUVECs, the PI3K inhibitor LY294002 (1 μM) or the PPARγ inhibitor GW9661 (3.3 nM), for 24 hours. The viability and apoptosis of HUVECs were determined. LY294002 or GW9661 mostly repressed miR-301a-3p inhibition-mediated protective effects in HUVECs (Figure 7(a–c)). RT-PCR and western blot showed...
Figure 5. The influence of miR-301a-3p on HUVEC inflammation and oxidative stress mediated by IL-1β, H2O2, and OGD. Vascular endothelial cells were transfected with miR-NC, miR-301a-3p mimics, in-NC, and the miR-301a-3p inhibitor. Then, transfected HUVECs were treated with IL-1β, H2O2, and OGD for 48 hours, respectively. (a–c): RT-PCR confirmed the profiles of inflammatory factors IL-1β, TNFα, and MMP1, 3, 9 in HUVECs. (d–f): Western blot determined the profiles of inflammatory proteins COX2 and iNOS in HUVECs. (g–i): The DCFDA/H2DCFDA-Cellular ROS Assay Kit verified ROS levels in HUVECs. *P < 0.05, **P < 0.01, ***P < 0.001. N = 3.
the profiles of IL-1β, TNFα, MMP1, 3, 9, and COX2 and iNOS in HUVECs were promoted following Y294002 or GW9661 treatment (versus the miR-301a-3p-in group) (Figure 7(d–e)). We used SOD, GSH-PX, LDH, and MDA detection kits to examine the levels of ROS, SOD, GSH-PX, LDH, and MDA in HUVECs. LY294002 or GW9661 lowered SOD and GSH-PX levels and enhanced ROS, LDH, and MDA levels (against the miR-301a-3p-in group) (Figure 7(f–j)). Western blot showed that miR-301a-3p inhibition heightened IGF1 and PPARγ expressions, augmented IGF-1 R, PI3K, and AKT phosphorylation, and attenuated NF-κB phosphorylation in HUVECs. LY29400 exerted no influence on IGF1 expression and IGF-1 R phosphorylation, impaired PI3K and AKT phosphorylation, reduced PPARγ expression, and strengthened NF-κB phosphorylation. GW9661 restrained PPARγ expression, and augmented NF-κB phosphorylation (Figure 7(k)). All these discoveries signified that the PI3K or PPARγ inhibitor weakened the anti-inflammatory, anti-oxidative, and anti-apoptotic functions mediated by the miR-301a-3p inhibitor.

Figure 6. miR-301a-3p suppressed the IGF1/IGF-1 R/PI3K/AKT/PPARγ pathway and up-regulated the NF-κB pathway. Vascular endothelial cells were transfected with miR-NC, miR-301a-3p mimics, in-NC, and the miR-301a-3p inhibitor. Then, transfected HUVECs were treated with IL-1β, H2O2, and OGD for 48 hours, respectively. (a–f): Western blot confirmed the profile of the IGF1/IGF-1 R/PI3K/AKT/PPARγ/NF-κB pathway in the miR-301a-3p overexpression and inhibition cell models. *P < 0.05, **P < 0.01, ***P < 0.001. N = 3.
Figure 7. The PI3K or PPARγ inhibitor weakened PI3K/AKT/PPARγ pathway activation mediated by the miR-301a-3p inhibitor. The PI3K inhibitor LY294002 (1 μM) or the PPARγ inhibitor GW9661 (3.3 nM) was taken to treat HUVECs, transfected with the miR-301a-3p inhibitor, for 24 hours. (a): MTT measured HUVEC viability. (b): Flow cytometry tracked apoptosis. (c): Western blot determined the profiles of Bax, Bcl2, and Caspase3 in HUVECs. (d): RT-PCR checked the profiles of IL-1β, TNFα, and MMP1, 3, 9 in HUVECs. (e): Western blot verified COX2 and iNOS expressions in HUVECs. (f): The DCFDA/H2DCFDA-Cellular ROS Assay Kit ascertained ROS levels in HUVECs. (g–j): The levels of SOD, GSH-PX, LDH, and MDA were examined through the SOD, GSH-PX, LDH, and MDA detection kits. (k): Western blot gauged the profile of the IGF1/IGF-1R/PI3K/AKT/PPARγ/NF-κB pathway in HUVECs. *P < 0.05, **P < 0.01, ***P < 0.001. N = 3.
Figure 8. NF-κB inhibition attenuated damage mediated by miR-301a-3p. HUVECs transfected with miR-301a-3p mimics were treated with the NF-κB inhibitor BAY-11-7082 (1 μM) for 24 hours. (a): MTT examined cell viability. (b,c): Flow cytometry and western blot monitored HUVEC apoptosis. (d): RT-PCR confirmed the profiles of IL-1β, TNFα, and MMP1, 3, 9 in HUVECs. (e–f): Western blot determined COX2 and iNOS expressions in HUVECs. (g): The DCFDA/H2DCFDA-Cellular ROS Assay Kit ascertained ROS levels in HUVECs. (h–k): The levels of SOD, GSH-PX, LDH, and MDA were determined through the SOD, GSH-PX, LDH, and MDA detection kits. K: Western blot confirmed the profile of the NF-κB pathway in HUVECs. *P < 0.05, **P < 0.01, ***P < 0.001. N = 3.
3.8. NF-κB inhibition weakened miR-301a-3p-mediated injury

To confirm the function of NF-κB in vascular endothelial cell damage, we treated HUVECs with the NF-κB inhibitor BAY-11-7082 (1 μM) for 24 hours. Cell viability BAY-11-7082 strengthened their viability, reduced apoptosis, and oxidative reactions (versus the miR-301a-3p group) (Figure 8(a–c)). RT-PCR showed that in contrast with the miR-NC group, BAY-11-7082 lowered their expressions (versus the miR-301a-3p group) (Figure 8(d)). Western blot confirmed COX2 and iNOS expressions in HUVECs, reflecting that by contrast to the miR-NC group, there was a notable increase in COX2 and iNOS expressions in HUVECs transfected with miR-301a-3p mimics. By contrast to the miR-301a-3p group, BAY-11-7082 brought down IL-1β, TNFα, and MMP1, 3, 9 (Figure 8(e–f)). Furthermore, BAY-11-7082 reduced ROS, LDH, and MDA levels, whereas enhanced SOD and GSP-PX levels in HUVECs (compared to the miR-301a-3p group, p < 0.05, Figure 8(g–j)), weakened NF-κB phosphorylation (Figure 8(l)). These findings revealed that the NF-κB inhibitor ameliorated cell damage mediated by miR-301a-3p overexpression.

3.9. miR-301a-3p targeted IGF1

The website (http://starbase.sysu.edu.cn/) indicated that IGF1 might be an underlying target of miR-301a-3p. With the wt and mt sequences of IGF1 mRNA 3’-UTR established, luciferase reporter assay signified that miR-301a-3p mimics substantially lessened the luciferase activity of IGF1-wt but exerted little influence on that of IGF1-mt (Figure 9(a,b)). RT-PCR confirmed IGF1 expression in the miR-301a-3p overexpression and inhibition cell models. By contrast to the corresponding control group, miR-301a-3p overexpression suppressed IGF1’s expression, whereas its expression was enhanced by miR-301a-3p inhibition (Figure 9(c)). These findings denoted that miR-301a-3p targeted the 3’UTR terminal of IGF1 and restrained its expression.
4. Discussion

Chronic venous ulcers are the severest complication of the chronic venous insufficiency of lower limbs, a multitude of factors like inflammation, apoptosis, oxidative stress, and hemodynamic alterations are found during pathological disorder [34]. Existing therapies for CVI encompass compression garments, compression pumps, and wound dressings with and without biologic components, which, however, can merely ameliorate some symptoms for the time being. Their efficacy is underwhelming because of the low compliance of the disease and the high recurrence rate of ulcers [35,36]. Other surgical methods like valve generation and repair, external valvuloplasty, and venous valve transplantation have not been extensively accepted on the back of poor long-term effects and technical issues [37]. Thus, a probe into the occurrence and progression of CVI and lower limb venous ulcers is critical for overcoming relevant symptoms fundamentally.

Vascular endothelial cells are simple squamous epithelial cells lining the inner surfaces of the heart, blood vessels, and lymphatics. They constitute the innermost layer of all vessels and function importantly in vascular health maintenance and the occurrence and development of cardiovascular diseases [38,39]. Pathological alterations, including oxidative stress, calcium imbalance, inflammation, ischemia-hypoxia, and mitochondrial dysfunction can destroy the homeostasis of endothelial cells, contributing to endothelial cell damage and death [40,41]. Endothelial cell dysfunction manifests itself in umpteen vascular diseases like pulmonary hypertension, post-myocardial infarction angiogenesis, chronic venous disease of lower limbs, myocardial ischemia-reperfusion injury, and diabetes-induced microvascular complications [42,43]. In this research, we treated HUVECs with IL-1β, H2O2, and OGD, respectively, to establish an *in-vitro* endothelial cell damage model. In contrast with the control group, IL-1β/H2O2/OGD boosted HUVEC apoptosis, inflammation, and oxidative stress and attenuated HUVEC viability.

Over the past years, the functions of microRNAs in endothelial cells have drawn more and more attention. Many microRNAs can modulate gene expression at the post-transcriptional level, influencing the functions of endothelial cells [44]. miRNA-34a-5p (miR-34a-5p) is vigorously up-regulated in vascular endothelial cells treated with LPS. As for the mechanism, miR-34a-5p inhibition up-regulates Forkhead box M1 (FOXM1) to alleviate endothelial cell apoptosis and inflammation induced by LPS and strengthen the cells’ viability and angiogenic activity [45]. miR-101-3p overexpression down-regulates tet methylcytosine dioxygenase 2 (TET2) to boost ROS generation and NF-κB activation, hence contributing to vascular endothelial cell dysfunction [46]. miR-301a-3p also exerts anti-inflammatory functions in the development of umpteen malignancies like gastric cancer [47] and pancreatic cancer [48]. For instance, miR-301a-3p overexpression down-regulates connexin 43 (Cx43) to repress astrocytes’ apoptosis and elicit their proliferation, thereby playing a significant part in bacterial meningitis occurrence and progression [49]. miR-301a-3p is considerably up-regulated in HUVECs elicited by oxidized low-density lipoprotein (ox-LDL). In terms of the mechanism, miR-301a-3p targets Krueppel-like factor 7 (KLF7) to boost oxidative stress, inflammation, and apoptosis in HUVECs treated with ox-LDL [50].

Predicated on the above works, we probed into the function of miR-301a-3p in great saphenous vein varicosity and lower limb venous ulcers. Our outcomes indicated that miR-301a-3p was substantially augmented in the plasma of great saphenous vein varicosity patients and lower limb venous ulcer patients, and its expression was increased in the *in-vitro* HUVEC damage model treated with IL-1β, H2O2, and OGD. miR-301a-3p overexpression stepped up apoptosis, inflammation, and oxidative stress mediated by IL-1β, H2O2, and OGD in vascular endothelial cells and repressed the cells’ viability, whereas miR-301a-3p inhibition contributed to the opposite situation. All these findings denoted that miR-301a-3p exerted a prominent regulatory function in HUVEC dysfunction and injury.

IGF-1, an insulin-producing factor, widely partakes in the angiogenesis of endothelial cells [51]. IGF1 receptor (IGF1R), which mediates IGF-1’s bioactivity, is a tyrosine kinase-containing heterotetramer on the cell surface, pertaining to multiple cytoplasmic signaling cascades [52,53].
Ginsenoside F1 (GF1) initiates the IGF-1/IGF1R pathway to facilitate angiogenesis in human umbilical vein endothelial cells and human microvascular endothelial cells, thereby ameliorating cerebral ischemia [54]. The PI3K/AKT signaling pathway is of great significance in modulating endothelial cell homeostasis and angiogenesis [55–57]. PPARγ, a downstream molecule in the PI3K/AKT pathway, is a nutrient sensor playing a pivotal part in modulating the body's metabolic homeostasis. Its destruction will result in vascular lesions, fatty acid/lipid metabolism disorders, and insulin resistance [58,59]. As evidenced by many studies, boosting PPARγ will impede the activation of NF-κB to relieve HUVEC inflammation [60,61]. Here, we discovered that miR-301a-3p overexpression dampened the profile of the IGF1/IGF-1 R/PI3K/AKT/PPARγ pathway and initiated the NF-κB pathway. PI3K or PPARγ inhibition attenuated miR-301a-3p inhibition-mediated anti-inflammatory, anti-oxidative, and anti-apoptotic functions. NF-κB inhibition alleviated HUVEC injury mediated by miR-301a-3p overexpression. Through bioinformatics analysis, we uncovered that IGF1 was an underlying target of miR-301a-3p, and the latter targeted IGF1 and suppressed its expression. Therefore, we speculated that miR-301a-3p targeted IGF1 to repress IGF-1 R/PI3K/AKT/PPARγ pathway activation and up-regulate the NF-κB pathway, hence boosting vascular endothelial cell damage mediated by IL-1β, H2O2, or OGD.

MMPs, a class of cell-secreted peptide endonuclease enzymes containing zinc metal ion catalytic centers, show an outstanding value in not only extracellular matrix and basement membrane degradation but also the modulation of cell growth factor development, evolution, morphogenesis, tissue remodeling, and angiogenesis [62,63]. For instance, targeting and suppressing MMPs in cells can efficaciously deter tumor metastasis and angiogenesis, thereby favoring the appropriate design of nanodrugs utilized for cancer treatment [64]. More to the point, MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL) may be relevant to the clinical course of venous ulcers. In contrast with patients without ulcers, the contents of MMP-9 and NGAL are obviously heightened in the plasma and wound fluids of those with venous ulcers [65]. By contrast to subjects without venous ulcers, those with varicose ulcers go through a notable increase in the ratio of gelatinases (MMP-2 and MMP-9) and their inhibitors (TIMP metalloproteinase inhibitor 1 (TIMP-1) and TIMP metalloproteinase inhibitor 2 (TIMP-2)), but the ratio of MMP-2 and TIMP-2 is lowered in subjects whose ulcers have been repressed [66]. Aligned with the above works, our findings signified that the profiles of MMP1, 3, 9 were substantially heightened in the plasma of great saphenous vein varicosis and lower limb venous ulcer patients but reduced by degrees following great saphenous vein high ligation and exfoliation. This reflected that MMPs extensively participated in venous ulcer progression, which was also confirmed by Raffetto JD et al. [67]. In-vitro studies displayed that IL-1β, H2O2, and OGD elicited an increase in MMP1, 3, 9 expressions, weakening HUVECs' viability and strengthening their apoptosis, but such an effect was inverted by miR-301a-3p inhibition. miR-301a-3p inhibition greatly ameliorated HUVEC damage mediated by IL-1β, H2O2, and OGD and lowered NF-κB/MMPs expression [68].

5. Conclusion

To summarize, we have revealed that miR-301a-3p overexpression facilitates HUVEC dysfunction and injury, whereas miR-301a-3p inhibition can mitigate HUVEC apoptosis, inflammation, and oxidative stress. As for the mechanism, miR-301a-3p targets and suppresses IGF1 to lower the profile of the IGF1R/PI3K/AKT/PPARγ pathway and elevate that of NF-κB/MMPs, thereby bolstering vascular endothelial cell damage. Thus, miR-301a-3p and IGF-1 will presumably become novel targets for hampering venous endothelial cell dysfunction and alleviating venous ulcers.

Abbreviation list

AnnexinV-FITC: Annexin V-fluorescein isothiocyanate; ANOVA: Analysis of Variance; BSA: Bovine Serum Albumin; COX2: cyclooxygenase-2; CVI: chronic venous insufficiency; Cx43: connexin 43; DCFDA: 2',7'-Dichlorodihydrofluorescein diacetate; DMSO: dimethyl sulfoxide; ECL: enhanced chemiluminescence; FBS: fetal bovine serum; FOXM1: Forkhead box M1; GAPDH: glyceraldehyde-
3-phosphate dehydrogenase; GF1: Ginsenoside F1; GSH-PX: glutathione peroxidase; H2DCFDA: 2',7’-dichlorodihydrofluorescein diacetate; HUVECs: human umbilical vein endothelial cells; IGF-1: Insulin-like growth factor-1; IGFIR: Insulin-like growth factor-1 receptor; IL-1β: Interleukin-1β; iNOS: inducible nitric oxide synthase; KLF7: Kruempel-like factor 7; LDD: lumbar disc degeneration; LDH: Lactic dehydrogenase; LPS: Lipopolysaccharide; LRP6: LDL receptor related protein 6; MDA: Minimum Descent Altitude; miR-101-3p: microRNA-101-3p; miR-126: microRNA-126; miR-186: microRNA-186; miR-21: microRNA-21; miR-301a-3p: microRNA-301a-3p; miR-34a-5p: microRNA-34a-5p; miRNA/miR: microRNAs; MMPs: matrix metalloproteinases; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NF-kB: Nuclear factor-kappaB; NGAL: neutrophil gelatinase-associated lipocalin; OD: optical density; OGD: oxygen and glucose deprivation; ox-LDL: oxidized low-density lipoprotein; PBS: phosphate-buffered saline; PI: propidium iodide; PI3K: phosphatidylinositol 3-kinase; PPARγ: Peroxisome proliferator-activated receptor-γ; PVDF: polyvinylidene fluoride; qRT-PCR: quantitative real-time polymerase chain reaction; RIPA: Radio-Immunoprecipitation Assay; ROS: Reactive oxygen species; RT: room temperature; SDS-PAGE: dodecyl sulfate, sodium salt (SDS)-Polyacrylamide gel electrophoresis; SOD: Superoxide Dismutase; TBST: Tris-buffered saline with Tween-20; TET2: tet methylcytosine dioxygenase 2; TIMP-1: TIMP metalloproteinase inhibitor 1; TIMP-2: TIMP metalloproteinase inhibitor 2; TNFa: Tumor necrosis factor α.

Authors contribution
Conceived and designed the experiments: Qingshan Wang; Performed the experiments: Ying Wang, Jingchen Du; Statistical analysis: Yu Liu, Shuhui Yang; Wrote the paper: Ying Wang, Jingchen Du. All authors read and approved the final manuscript.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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Ethics statement
Our study was approved by the Institutional Review Board of Heilongjiang Provincial Hospital (Approval No. 2019-153).

Data availability statement
The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

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