Original Article

MiR-197-3p affects angiogenesis and inflammation of endothelial cells by targeting CXCR2/COX2 axis

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Abstract: Background: Decreased circulating miR-197-3p was found in patients with recurrent deep vein thrombosis (DVT), but the specific role of miR-197-3p needs further exploration. Materials and Methods: Venous blood samples were collected from DVT patients and healthy controls, and peripheral blood mononuclear cells (PBMCs) were isolated to examine the expression patterns of miR-197-3p, CXCR2 and COX2 by qRT-PCR. Human umbilical vein endothelial cells (HUVECs) were further used as a cellular model to investigate the role of the miR-197-3p/CXCR2/COX2 axis in regulating cell viability, angiogenesis, and inflammation, which were determined by MTT assay, Matrigel-based tube formation assay, and enzyme-linked immunosorbent assay, respectively. Dual-luciferase reporter assay was used to examine the interactions between miR-197-3p and CXCR2. Expression of NF-κB p65 was examined by western blot to investigate whether the NF-κB pathway was involved in the regulatory effect of miR-197-3p on DVT. Results: miR-197-3p was decreased in PBMCs of patients with DVT, while CXCR2 and COX2 were increased compared to the healthy controls. In HUVECs, overexpression of miR-197-3p reduced CXCR2 levels and inhibited cell viability, angiogenesis, and release of inflammatory cytokines including TNF-α, IL-1β, and IL-6, which were reversed by miR-197-3p inhibition. Dual-luciferase reporter assay indicated miR-197-3p directly bound to CXCR2. CXCR2 further upregulated the expression of COX2 and activated the NF-κB pathway, promoting cell viability, angiogenesis and release of inflammatory cytokines in HUVECs. The effect of miR-197-3p inhibition on cell viability, angiogenesis and inflammation of HUVECs could be reversed by CXCR2 silencing. Conclusion: MiR-197-3p affected viability, angiogenesis and inflammation of endothelial cells by targeting CXCR2/COX2 axis in vitro. Our findings provided a novel theoretical basis to investigate more effective therapies for DVT.

Keywords: miR-197-3p, CXCR2, COX2, deep vein thrombosis, NF-κB pathway, HUVECs

Introduction

Venous thromboembolism is defined as deep vein thrombosis (DVT) or pulmonary embolism (PE), which refers to the formation of a blood clot in a lower extremity. It is one of the major cardiovascular diseases worldwide and affects about 10 million people every year [1, 2]. As the population grows and life expectancy extends, we may see a further increase in the prevalence of DVT. Endothelial cells are essential for regulating thrombosis and angiogenesis. A previous study suggested that the most considered cause of venous thrombosis is damage and dysregulation of vascular endothelial cells [3]. Nowadays, anticoagulants such as heparin or warfarin and thrombolytics have played a role in the treatment of DVT [4]; however, how to solve the bleeding risk caused by drugs remains tricky. Additionally, anticoagulants and thrombolytics are insufficient to reduce the sequelae of DVT [5, 6]. Therefore, in future studies, it is imperative to better understand the pathophysiology of DVT and to explore key molecules that might serve as targets for prevention or treatment.

Micro RNAs (miRNAs) are endogenous non-coding RNAs composed of approximately 22 nucleotides that regulate the expression of downstream genes through translation inhibition or messenger RNA (mRNA) degradation, they play key roles in many pathological processes, and function as biomarkers for many diseases, including cardiovascular diseases [7-9]. Studies have shown that miRNAs have the potential to
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regulate platelet activation and aggregation, and multiple miRNAs can target genes encoding key hemostatic factors, demonstrating that miRNAs may be involved in the hemostasis process [10, 11]. Furthermore, accumulating studies have reported the dysregulated expression of miRNAs in DVT, suggesting that miRNAs might be involved in the progression of DVT and should be explored more comprehensively as new biomarkers. miR-197-3p, which has attracted increasing attention in recent years, was downregulated in blood samples of DVT patients and is closely related to the recurrence of DVT [12], but its functional mechanisms in DVT are poorly reported.

The CXC chemokine receptor type 2 (CXCR2) is a G protein-coupled receptor and belongs to the CXCR family, which acts as a major receptor of ELR-CXC chemokines that mediate angiogenesis [13]. CXCR2 and its ligands are important for the activation and trafficking of inflammatory mediators, suggesting that CXCR2 participates in the development of different inflammatory diseases. Inflammation is considered to be closely correlated with the initiation of DVT [5, 14]. A previous study demonstrated that the activation of CXCR2 signaling might increase the frequency of thrombosis in mice [15]. However, the specific regulatory mechanism of CXCR2 in DVT has not been reported.

Cyclooxygenase 2 (COX2) has been demonstrated to be a biomarker for a variety of malignancies. For example, the COX2 inhibitor, celecoxib, can prevent angiogenesis and induce apoptosis in breast cancer cells in a mice model [16]. Xu et al. suggested that through activating COX2, CXCR2 could promote the metastasis and chemical resistance of breast cancer [17]. Furthermore, the highly selective inhibition of COX2 could help Danshensu exert anti-thrombotic and anti-platelet aggregation effects [18]. NF-κB regulates many biological processes including inflammation. NF-κB up-regulated the expression of inflammatory cytokines, such as factor-α (TNF-α) and interleukin 1-β (IL-1β), and leads to an extensive inflammatory response, thereby inducing apoptosis [19]. The NF-κB signaling pathway can be activated by many molecules. A previous study indicated that COX2 might aggravate the formation of venous thrombosis through the NF-κB pathway [20]. Taking these studies together, we hypothesize that the CXCR2/COX2 axis might be involved in the pathogenesis of DVT, but no relevant studies have been reported.

Here, we determined the interaction between miR-197-3p and CXCR2 in DVT for the first time, and demonstrated that miR-197-3p regulates the NF-κB pathway through the CXCR2/COX2 axis, thereby affecting cell proliferation, angiogenesis and inflammation. These findings provide a novel theoretical basis to investigate more effective therapies of DVT.

Materials and Methods

Patients and Specimen collection

Venous blood samples (n=25) were collected from patients with DVT upon DVT diagnosis and before receiving any medication, who were confirmed to have no history of hypertension, diabetes mellitus, and other chronic diseases, between October 2019 and January 2020 at Beijing Jishuitan Hospital. Normal healthy samples (n=25) were obtained from health volunteers. This study was approved by the Ethics Committee of Beijing Jishuitan Hospital, and informed consent was obtained from all participants. Samples were collected from both patients and volunteers after overnight fasting. Peripheral blood mononuclear cells (PBMCs) were isolated within 4 hours after collection and cultured in RPMI Medium 1640. qRT-PCR analysis (described below) was then performed to examine the expression patterns of miR-197-3p, CXCR2 and COX2.

Cell lines and transfection

Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and then cultured in D-MEM/F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), and 100 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified incubator with 5% CO₂. To explore the regulatory role, chemosynthetic miR-197-3p mimic, inhibitor and their negative controls (NCs), as well as specific plasmids used for overexpressing CXCR2 and COX2 were transfected into HUVECs at a final concentration of 2 µg/µL using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA). All plasmids were synte-
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Cells were collected 48 h after transfection and then subjected for further study. The sequence of the miR-197-3p inhibitor was 5'-GCUGGGUGGAGAAGGUGGUGA-3', and the sequence of the inhibitor NC was 5'-CAGU-ACUUUUGUGUAGUACAA-3'.

RNA extraction and quantitative real-time PCR (RT-PCR) assay

Total RNA from PBMCs and HUVECs was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), then RNA quality was detected using NanoDro2000c (Thermo Scientific, Waltham, USA) according to the manufacturer’s protocols. Next, the RNA was converted into cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo), and qRT-PCR process was performed on an ABI 7900 system using SYBR Green Real-Time PCR master mixes (Thermo). The reaction parameters were as follows: 94°C for 6 min; 35 cycles of 35 s at 92°C, 35 s at 58°C and 35 s at 70°C, and 10 min at 70°C. U6 and GAPDH served as an internal control. The transcriptional level of targeted genes was analyzed by the 2^ΔΔCt method.

Western blot

The standard Matrigel method was performed as an in vitro angiogenesis assay to assess the tube-forming capacity of HUVECs. Briefly, HUVECs were seeded onto Matrigel-coated plates (BD Bioscience, Franklin Lakes, NJ, USA) in EBM medium and incubated at 37°C for 24 h. Then, photographs were taken at low magnification with a DFC-290 camera (Leica Microsystems, Wetzlar, Germany). Unclosed tubes between two branching points were counted.

Enzyme-linked immunosorbent assay (ELISA)

Cell supernatant samples from treated HUVECs were used to measure the levels of TNF-α, IL-1β and IL-6 using an ELISA kit (#SEKM-0034, #SEKM-0002, and #SEKM-0007, Solarbio, Inc., China) following the manufacturer’s protocol.

Dual-luciferase reporter assay

The wild type (WT) and mutant (MUT) sequences of CXCR2 which contained miR-197-3p binding sites were amplified and inserted into pGLO vector to establish recombinant plasmids WT-CXCR2 and MUT-CXCR2. Then HUVECs were co-transfected with the luciferase report-
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er plasmid and miR-197-3p mimic or NC. After growing 48 h, luciferase activity of treated cells was tested by Dual-Luciferase Reporter Assay System (Promega, WI, USA).

Statistical analysis
All experiments were performed in at least three biological replicates. All experimental data were expressed as mean ± standard deviation (SD). Student’s t-test was utilized to analyze the difference between two groups and one-way analysis of variance with Turkey post hoc test was performed to evaluate the differences among at least 3 groups through GraphPad (Ver. Prism 7, GraphPad Prism Software, La Jolla, CA, USA). P value less than 0.05 was considered as statistically significant.

Results
MiR-197-3p decreased while CXCR2 and COX2 increased in patients with DVT
MiR-197-3p, CXCR2 and COX2 expressions in the serum of patients with DVT were measured using qRT-PCR. The experimental data were showed as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 1. MiR-197-3p decreased while CXCR2 and COX2 increased in patients with deep venous thrombosis (DVT). A-C. MiR-197-3p, CXCR2 and COX2 levels in patients with DVT were measured using qRT-PCR. The experimental data were showed as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

MiR-197-3p overexpression clearly repressed the cell viability, angiogenesis and inflammation in HUVECs.

MiR-197-3p influenced cell viability, angiogenesis and inflammation by targeting CXCR2 in vitro
Considering the high expression of CXCR2 in the serum of patients with DVT, we reasonably suspected that there might be a targeted regulatory relationship between miR-197-3p and CXCR2. To test the hypothesis, we first measured CXCR2 expression in HUVECs after overexpression or inhibition of miR-197-3p. We observed a lower level of CXCR2 after transfecting with miR-197-3p mimic, while the opposite results were obtained in miR-197-3p inhibitor group (Figure 2B). In conclusion, miR-197-3p overexpression clearly repressed the cell viability, angiogenesis and inflammation in HUVECs.

MiR-197-3p affected cell viability, angiogenesis and inflammation of HUVECs
To investigate whether miR-197-3p participated in the process of DVT, we chose HUVECs to conduct the subsequent experiments. MiR-197-3p mimic or miR-197-3p inhibitor was transfected into the cells, and then we measured the transfection efficiencies using qRT-PCR. We found that miR-197-3p was up-regulated in the mimic group while it was down-regulated in the inhibitor group (Figure 2A). Subsequently, we observed that the viability of HUVECs decreased after enhancing miR-197-3p expression, while miR-197-3p inhibition increased cell viability (Figure 2B). Next, we analyzed angiogenesis of HUVECs, as shown in Figure 2C, overexpression of miR-197-3p reduced the tube-forming capacity, while silencing of miR-197-3p enhanced the capacity which was characterized by the fine vascular network with fewer unclosed loops. Moreover, ELISA was performed to analyze the levels of proinflammatory cytokines, TNF-α, IL-1β and IL-6 in the HUVECs supernatant. The results indicated that TNF-α, IL-1β and IL-6 levels decreased after transfecting with miR-197-3p mimic, while the opposite results were obtained in miR-197-3p inhibitor group (Figure 2D).
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we concluded that CXCR2 directly bound to miR-197-3p, and decreased miR-197-3p may attribute to CXCR2 overexpression. Next, to further investigate the role of CXCR2, we enhanced CXCR2 expression in HUVECs, and detected the effective overexpression efficiency of CXCR2 (Figure 3D). Moreover, the result indicated that overexpression of EZH2 could promote cell viability (Figure 3E), enhance the tube-forming capacity (Figure 3F) and increase TNF-α, IL-1β and IL-6 levels (Figure 3G), suggesting that CXCR2 overexpression contributed to the proliferation, angiogenesis and inflammation in vitro. Taken together, miR-197-3p

Figure 2. MiR-197-3p affected the proliferation, angiogenesis and inflammation in HUVECs. A. miR-197-3p mimic or miR-197-3p inhibitor was transfected into HUVECs and the transfection efficiencies were detected by qRT-PCR. B. Cell viability was measured by MTT. C. The tube-forming capacity was detected using Matrigel-based tube formation assay. D. ELISA confirmation of TNF-α, IL-1β, IL-6 expression in the supernatant of HUVECs. The experimental data were showed as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.
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Figure 3. MiR-197-3p influenced the proliferation, angiogenesis and inflammation by regulating CXCR2 in vitro. A and B. qRT-PCR and western blot were used to measure CXCR2 expression in HUVECs after overexpressing or silencing miR-197-3p. C. Dual-luciferase reporter assay was used to analyze the interaction between miR-197-3p and CXCR2. D. The overexpression efficiency of CXCR2 was analyzed. E. Cell viability was analyzed after CXCR2 overexpression. F. The effect of CXCR2 overexpression on angiogenesis. G. TNF-α, IL-1β, IL-6 levels were measured after CXCR2 overexpression. The experimental data were shown as mean ± SD. *P < 0.05, **P < 0.01 and *** P < 0.001.
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CXCR2 upregulated COX2 to regulate the proliferation, angiogenesis and inflammation in vitro

To further explore how CXCR2 played a promoting role in DVT formation, we then examined COX2 levels in cells transfected with CXCR2. We found that the level of COX2 increased after enhancing CXCR2 expression, indicating that CXCR2 might upregulate COX2 in HUVECs (Figure 4A, 4B). Then, we overexpressed COX2 levels in vitro, and observed that COX2 overexpression significantly increased the cell viability (Figure 4C), enhanced the tube-forming capacity (Figure 4D) and up-regulated TNF-α, IL-1β and IL-6 expression (Figure 4E). Overall, the CXCR2/COX2 axis was involved in the process of DVT in vitro.

MiR-197-3p affected venous thrombosis by targeting the CXCR2/COX2 axis in which the NF-κB pathway may be involved

Based on our data that miR-197-3p affected the inflammation of HUVECs, we then began to reveal whether NF-κB was involved in the mechanism by which miR-197-3p regulated inflammation. As shown in Figure 5A, inhibition of miR-197-3p increased CXCR2 and COX2 levels, as well as the phosphorylation of NF-κB, which suggests that miR-197-3p silencing activated the NF-κB pathway. More importantly, the promotional effect on related genes expression...
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caused by miR-197-3p inhibition was reversed through silencing CXCR2 in vitro. Next, the functional effect of the miR-197-3p/CXCR2/COX2 pathway was determined by rescue assay in vitro. The results showed that the increased cell viability induced by miR-197-3p inhibition was reduced by CXCR2 silencing (Figure 5B).

We further observed that CXCR2 silencing further reversed the enhancement in angiogenesis induced by miR-197-3p inhibitor (Figure 5C). CXCR2 knockdown reversed the promotional effect of miR-197-3p silencing on TNF-α, IL-1β and IL-6 levels (Figure 5D). Our data indicated that miR-197-3p may affects DVT formation by

Figure 5. MiR-197-3p affected venous thrombosis through regulating NF-κB pathway by targeting CXCR2/COX2 axis. A. CXCR2 and COX2 expressions, as well as the phosphorylation of NF-κB in HUVECs transfected with miR-197-3p inhibitor+sh-CXCR2 were determined. GAPDH used as an endogenous control. B-D. Cell viability, angiogenesis and inflammation were measured after transfecting with miR-197-3p inhibitor+sh-CXCR2. The experimental data were showed as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.
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**Discussion**

Since the traditional DVT treatment regimen has few benefits to the injured endothelium and blood vessels, it is important to explore more effective targeted therapy strategies. Here, we demonstrated that miR-197-3p was decreased in the serum of patients with DVT while CXCR2 and COX2 were up-regulated. Furthermore, miR-197-3p enhancement contributed to reducing viability, angiogenesis and inflammation of HUVECs. More importantly, we have first suggested that miR-197-3p directly targeted the CXCR2/COX2 axis in HUVECs and might regulate the NF-κB signaling pathway, thereby further affecting the formation of venous thrombosis. These observations suggested that miR-197-3p may be a target for the treatment of DVT.

Growing evidence has shown that miRNAs are dysregulated and function as potential biomarkers in human vascular diseases [21]. miRNAs play vital roles in the regulation of cell proliferation, migration, apoptosis, angiogenesis and inflammation, which are also essential for the progression of DVT [22-24]. For example, Chen et al. indicated that miR-126 showed lower expression in the DVT mice model, and the enhancement of miR-126 suppressed apoptosis of HUVECs and DVT by targeting the phosphatidylinositol 3 kinase/AKT serine/threonine kinase 1 (PI3K/Akt) pathway [25]. miR-197-3p was initially found to be down-regulated in primary biliary cirrhosis [26], and subsequent reports indicated that miR-197-3p was overexpressed, not expressed or at a low level in a variety of cancers [27-29], while Li et al. demonstrated that miR-197-3p deletion promoted endothelial cell growth in Kawasaki disease [30], suggesting that miR-197-3p performs different roles in multiple human diseases. However, for venous thrombosis, only one publication demonstrated that miR-197-3p was down-regulated and associated with recurrent DVT [12]. In this study, down-regulation of miR-197-3p was also observed in DVT tissues. Furthermore, miR-197-3p enhancement reduced cell viability, angiogenesis and inflammation of HUVECs, while miR-197-3p inhibition led to opposite results. The data demonstrated that miR-197-3p may participate in the progression of DVT. However, the specific functional mechanism still needs to be further explored.

Besides the decreased levels of miR-197-3p, we also observed that CXCR2 and COX2 were increasingly expressed in DVT. Therefore, we hypothesized that the regulation of the balanced levels between miR-197-3p and CXCR2/COX2 might be crucial for the targeted therapy of DVT. A study had shown that the miR-940/CXCR2 pathway is involved in the regulation of invasion and migration of hepatocellular carcinoma cells [31]. Furthermore, CXCR2 may severe as a target of certain miRNAs to mediate angiogenesis in vitro through multiple signal pathways [32]. Gollomp et al. suggested that the retrograde migration of neutrophils into venous thrombi can be abrogated with the blockade of CXCR2 [33]. Most importantly, Yago et al. demonstrated that the activation of CXCR2 increased the frequency and size of thrombi [15]. Based on these reports and our experiments, we found that miR-197-3p bound to CXCR2 and subsequently influenced the proliferation, angiogenesis and inflammatory response of HUVECs.

Our data suggested that the activation of COX2 caused by CXCR2 might induce the enhancement of angiogenesis and inflammation, consistent with the results of a previous study [34]. The expression of COX2 was mostly induced by proinflammatory stimuli, growth factors or cytokines [35, 36]. Overexpression of COX2 had been reported in a different type of tumor epithelial cells and may be involved in cancer initiation and metastasis [37]. For the investigation of DVT, a study suggested that the highly selective inhibition of COX2 could help Danshensu exert anti-thrombotic and anti-platelet aggregation effects [18]. This coincides with our findings that overexpression of COX2, or CXCR2 could promote proliferation, angiogenesis and inflammation. Additionally, our data suggested that miR-197-3p inhibition promoted the phosphorylation level of NF-κB p65, indicating activation of the NF-κB pathway. Cheng et al. demonstrated that cotinine aggravated thrombus and inflammation in rats with DVT through the activation of the TLR-4/NF-κB inflammatory signaling pathway [38]. A previous study indicated that COX2 might regulate the formation of venous thrombosis.
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through the NF-κB pathway [20]. Taken together, these data support our conclusion by which miR-197-3p promoted cell viability, angiogenesis and inflammation through the NF-κB pathway by targeting the CXCR2/COX2 axis, suggesting that miR-197-3p might function as a biomarker for DVT treatment. However, the conclusion needs to be supported by more in vivo data, which will be our future work.

Our study has some limitations. First, in the study we mainly used HUVECs as a cellular model without investigating the role of miR-197-3p in vivo; while HUVECs obviously cannot well represent the process of DVT development, therefore studies with better models (especially animal models) are needed to validate our findings. Second, we did not further explore how CXCR2 regulated COX2, while there is a study that reported CXCR2 might activate COX2 [17], but this should be further examined as well. Third, in our study, the investigation into the role of NF-κB pathway was an initial investigation, which also warrants future studies to explore the underlying mechanisms.

In conclusion, miR-197-3p was decreases in blood sample of DVT patients, and miR-197-3p binds to CXCR2/COX2 and regulates cell proliferation, angiogenesis and inflammation of HUVECs in which the NF-κB pathway might be involved. These observations provide a feasible theoretical support for better treatment of DVT.

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Disclosure of conflict of interest

None.

Abbreviations

miRNAs, micro RNAs; VTE, venous thromboembolism; DVT, deep vein thrombosis; PE, pulmonary embolism; HUVECs, human umbilical vein endothelial cells; CXCR2, CXC chemokine receptor type 2; COX2, cyclooxygenase 2.

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