

RESEARCH ON THE ROLE OF MIR-212-5P IN REGULATING VASCULAR ENDOTHELIAL CELL PROLIFERATION IN DEEP VEIN THROMBOSIS

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ABSTRACT

Objective: To investigate the effect of MiR-212-5p on the proliferation function of vascular endothelial cells (Vascular Endothelial Cells (VECs) in DVT. **Methods:** The expression levels of MiR-212-5p in the peripheral blood of DVT patients and healthy subjects were detected using RT-qPCR. In EA.hy926 endothelial cells, MiR-212-5p was silenced and overexpressed, and the cell proliferation ability was assessed using the CCK8 assay. **Results:** The expression level of MiR-212-5p in the peripheral blood of patients with DVT was significantly higher than that of healthy subjects ($p < 0.001$). Overexpression of MiR-212-5p in EA.hy926 endothelial cells resulted in decreased cell proliferation ($p < 0.05$), while silencing MiR-212-5p enhanced cell proliferation ($p < 0.05$). **Conclusions:** MiR-212-5p is highly expressed in DVT and promotes the formation of DVT by inhibiting the proliferation of endothelial cells. MiR-212-5p may serve as a potential target for the treatment of DVT.

Key words: deep vein thrombosis, endothelial cells, MiR-212-5p, proliferation

INTRODUCTION

Deep venous thrombosis (DVT) is a common vascular disease whose pathogenesis involves abnormal coagulation of the blood in the deep venous system. According to statistics, the annual incidence of DVT is between 0.1% and 0.27%, and about 40% of patients will have venous claudication, which seriously affects the quality of life of patients [1]. Men had a slightly higher risk of DVT than women, and this risk showed an increasing trend with age [2]. If a deep vein thrombosis in the lower limbs dislodges, it can travel through the inferior vena cava to the pulmonary artery system, resulting in acute pulmonary embolism (PE). This condition poses a significant threat to patient life, with an annual incidence rate of pulmonary embolism ranging from approximately 28 to 79 per 100,000 individuals, and a mortality rate as high as 10% [3,4].

Despite extensive studies of DVT worldwide, its pathogenesis is still not completely revealed. At present, the most widely accepted theory of pathogenesis was proposed by the German medical scientist Rudolf Weierxiao in the 19th century, who believed that thrombosis is related to three factors: slow blood flow, blood hypercoagulability and vascular intimal injury, and the interaction of these factors promotes the development of thrombosis [5]. With further medical research, researchers began to focus on the complex interactions between VECs, platelets, and inflammatory responses. They generally agree that DVT formation is associated with an imbalance of coagulation and anticoagulant mechanisms in

vivo, and that VECs play a central role in maintaining this balance[6]. Furthermore, the VECs produce nitric oxide[7–9], prostacyclin[7,10,11] And the exonucleosidase CD394[12] And other three main antithrombotic factors, they work together to effectively prevent thrombosis[13]. However, previous studies and a large number of literature reports have proved that vascular endothelial cells are closely related to the formation of DVT, and the proliferation of vascular endothelial cells can repair the damaged and missing vascular endothelium, promote angiogenesis, and then contribute to the vascular remodeling of DVT[14–17]

The role of microRNA as a biomarker in the pathogenesis and diagnosis of DVT has been widely concerned. It can be involved in the regulation of the proliferation rate, invasion ability, migration direction, as well as the apoptotic process of these cells by regulating the expression of specific genes within VECs. for instance. Qin et al Using microRNA microarray to analyze the differential expression of microRNA in the serum of DVT patients and healthy people, the serum levels of miR582, miR-195 and miR-532 were significantly increased in DVT patients, which has important significance for the diagnosis of DVT[18]. We found that the plasma levels of miR-136-5p were reduced in DVT patients, while miR-424-5p was significantly increased, and the plasma levels of D-dimer and activated protein C-protein C inhibitor complex (APC-PCI) were also closely related[19]. With the complex role of miR-212 in tumor promotion and suppressive functions, studies suggest that downregulation of miR-212 is associated with resistance to and adverse effects of multiple anticancer therapies[20–22]. For example, a decrease in miR-212 expression was observed in patients who responded well to treatment with ifosfamide (an alkylating agent, commonly used for cancer therapy). However, there are still some problems in current research on microRNA as a diagnostic marker of DVT, such as inconsistent diagnostic value of microRNA found in different studies, insufficient sample size included in experiments, and large deviations in studies used techniques such as microRNA microarrays. Therefore, the research goal of this topic is to study and explore the potential mechanism of MiR-212-5p in DVT by combining clinical samples and cell experiments, to explore the differential genes related to disease development and possible early diagnosis markers, to provide new clues for the risk factors and key molecular mechanisms of deep vein thrombosis, so as to provide new ideas for the clinical prevention and treatment of the disease in the future.

OBJECTIVE

To investigate the effect of MiR-212-5p on the proliferation function of vascular endothelial cells (Vascular Endothelial Cells (VECs) in DVT

METHODOLOGY

1. Clinical blood sample collection

The study was approved by the Ethics Committee of Liuzhou Workers Hospital with the ethics approval number KY2024091. Patients and volunteers using the samples in this study signed a written informed consent form. 10ml of peripheral blood from 15 DVT patients attending Liuzhou Workers Hospital from August 2024 to December 2024 were collected. Inclusion criteria: 1) Meet the diagnostic criteria in the 3rd edition of the "Guidelines for the Diagnosis and Treatment of Deep Vein Thrombosis". 2) belonging to the acute phase mixed simple lower limb DVT; 3) unilateral limb onset by ultrasound or imaging; 4) aged 18-60 years; 5) not treated with thrombolytic drugs; 6) plasma D-dimer >0.5mg/L. Fifteen patients with general data of age and gender matching the case group were collected as the normal control group. Exclusion criteria: 1) the presence of active ulcers in the lower limbs; 2) the combination of cardiac, hepatic and renal function abnormalities; 3) blood-related diseases; 4) pregnant women; 5) a history of long-term medication, especially oral anticoagulant and antiplatelet agent drugs; 6) involuntary participation in this study.

2. Total RNA extraction and RT-qPCR

Total RNA was extracted from the serum of 15 cases of DVT and 15 healthy control cases using Trizol LS. The total RNA was then reverse transcribed and quantified using the miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN, KR211-02) and the miRcute Plus miRNA qPCR Kit (SYBR Green) (TIANGEN, FP411-02). RT-qPCR was performed on an ABI7500 real-time fluorescence quantitative

PCR instrument, and the relative expression levels of miRNA were calculated using the 2- $\Delta\Delta C_t$ method. Each sample was analyzed in triplicate. The primer for MiR-212-5p was purchased from BGI, with the following sequence: 5'- GACCTTGGCTCTAGACTGCTTACT-3'.

3. Endothelial culture and passage

The purchased EA.hy926 endothelial cells were seeded in T25 culture flasks and cultured in DMEM medium supplemented with 10% FBS (Gibco, Auckland, NZ) and 1% penicillin-streptomycin solution. The cells were maintained in a 5% CO₂ incubator at 37°C. When the cells reached 90% confluence, they were washed twice with 5 ml of PBS, followed by the addition of 1 ml of trypsin for 3 minutes to detach the cells. The digestion was stopped by adding complete medium, and the cells were subcultured at a 1:3 ratio.

4. siRNA transfection

The day before transfection, the cells were plated at 2105 Individual / wells were spread in 6-well plates for transfected when cell growth density reaches about 70-80% in 6-well plates. 7.5 μ L Lipofectamine™ 3000 reagent was diluted in 125 μ L Opti-MEM™ medium and then 150 pmol of siRNA solution was added to 125 μ L Opti-MEM™ medium. The transfection reagent mixture was prepared by adding siRNA premix to the diluted Lipofectamine™ 3000 reagent and incubating for 15 min at room temperature. The transfection reagent complex was dropped into six-well plates of cultured cells for transfection. Cell morphology was observed 4 – 6 hours, the medium was replaced and RNA was extracted 24 hours later for transfection.

5. for the CCK 8 experiments

Successfully transfected EA.hy926 cells were seeded at 3000 cells / well in 96-well cell culture plates with Cell Counting Kit-8 solution (Merck KGaA, Darmstadt, Germany), 37°C, 5% CO₂. The cells were incubated for 3h. The absorbance at 450 nm at 0,24,48 and 72 h was measured using a microplate reader (Thermo Fisher Scientific 2).

6. Statistical analysis

Statistical analysis was performed using SPSS 26.0 software, GraphPad Prism 8, and Image J software. Data are presented as the mean $\pm$ SD. Comparisons between two groups was performed using a two-tailed t-test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

1. Demographic data

We collected peripheral blood from 15 DVT patients and 15 healthy control volunteers, and the demographic characteristics of all participants are shown in Table 1. There was no statistically significant difference in the baseline data between the two groups ($p > 0.05$).

Table 1:
Demographic characteristics of the two case groups

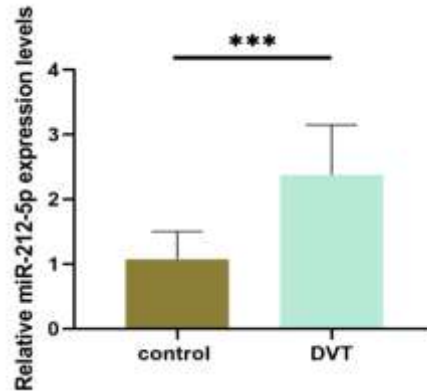
feature	DVT (n=15)	group	Non- DVT (n=15)	group	p price
Age (year)	51.9 $\pm$ 7.7		48.0 $\pm$ 9.9		0.264
sex					0.602
-man (%)	9(60.0)		8(53.3)		
-woman (%)	6(40.0)		7(46.7)		
Body mass index (BMI)	24.4 $\pm$ 3.4		24.5 $\pm$ 2.3		0.929
History of smoking					0.121
-smoke (%)	7(46.7)		3(20.0)		
-Smoking (%)	8(53.3)		12(80.0)		
history of past illness					0.267
-Physical health (%)	8(53.3)		13(100.0)		
-hypertension (%)	2(13.3)		1(0)		
-diabetes mellitus (%)	3(20.0)		1(0)		
-coronary disease (%)	1(6.7)		0(0)		
-Other (trauma, COPD, etc.,%)	1(6.7)		0(0)		

2. MiR-212-5 is highly expressed in the peripheral blood of patients with DVT

To verify the expression of hsa-mir-212-5p in DVT, we collected serum from 15 DVT and 15 control cases, and RT-qPCR showed that MiR-212-5p was significantly higher in DVT than controls ($p < 0.001$) (Figure 1).

Figure 1

RT-qPCR test for miR-204-5p expression in the peripheral blood of both groups

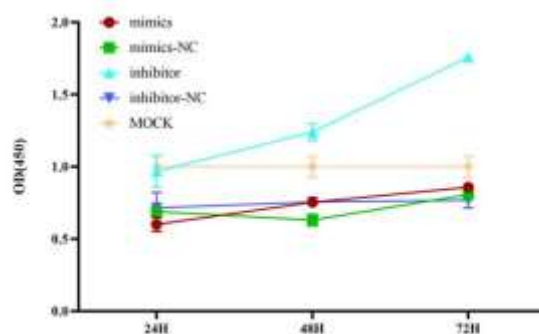


3. The proliferative capacity of endothelial cells was significantly enhanced after silencing MiR-212-5p

To explore the role of MiR-212-5p in DVT, we performed the siRNA-mediated knockdown experiment of MiR-212-5p in the EA.hy926 endothelial cell line. At 48 hours after transfection, the CCK-8 experiment showed that the proliferation capacity of endothelial cells was increased after inhibitor transfection compared with inhibitor-NC transfection ($p < 0.05$). (graph 2)

Figure 2

The OD value of EA.hy926 after transfection was determined by CCK-8.



4. The proliferative capacity of endothelial cells decreased significantly after overexpression of MiR-212-5p

We performed the MiR-212-5p overexpression experiments in the EA.hy926 endothelial cell line. At 48 hours after transfection, mimics expression was increased compared to mimics-NC ($p < 0.05$). The results of CCK-8 assay showed that the proliferation capacity of endothelial cells decreased after mimics transfection compared with the transfection of mimics-NC. (graph 2)

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Discussion

DVT is a common peripheral vascular disease caused by the abnormal clotting of blood in the venous lumen, resulting in the obstruction of venous return[23]. Because its pathogenesis is usually more

insidious, clinical diagnosis faces many challenges. The German pathologist Virchow proposed in 1845, ——— vascular wall injury, hemodynamic changes and abnormal blood composition, are still at the core of studying the mechanism of thrombosis[5]. VECs, a layer of barrier to the vascular intima, play a key role in maintaining vascular health and function. They maintain the mobility of blood by fine modulating their anti-thrombotic and procoagulant properties, a balance crucial for the prevention of thrombosis. These not only provide a smooth inner surface for blood flow, but also perform a variety of physiological functions, such as secretion of a variety of bioactive molecules, regulating metabolic processes and organ functions, and participating in the growth, development and regulation of pathological changes[24]. It was found that vascular endothelial cells further influence the process of thrombus formation through a complex series of functions, such as proliferation, invasion, migration, and apoptosis[25]. During deep vein thrombosis, the longer the venous thrombosis contacts with the venous wall, the greater the damage to the venous wall[26]. In this process, vascular endothelial cells migrate to the space surface and gradually proliferate to fill the vascular lumen occupied by the thrombus, thus promoting vascular repair and remodeling, and with the migration and proliferation of vascular endothelial cells, the blood flow inside or around the thrombus can be reperused[27]. Therefore, exploring how the proliferation function of endothelial cells mediate the occurrence of DVT will further clarify the pathogenesis of DVT and provide new ideas and new targets for clinical treatment.

The miRNA is involved in almost all the physiological and pathological processes of the human body. The miRNA produced by various tissues, organs and cells can enter the blood and become the circulating mi RNA. Circulating mi RNA are encapsulated in membranous microbubbles or form complexes with proteins[28,29]. However, miR-212, as an important subtype of microRNA, its expression plays an important role in many diseases, for example, the dysfunction of miR-212 may lead to some neurodegenerative diseases such as Alzheimers disease, epilepsy, tau disease, and schizophrenia[30];Some investigators elevated miR-212 expression in pancreatic cancer patients, which may have opposite effects on pancreatic cancer cell proliferation[31]. Nevertheless, the role of miR-212 in DVT disease is still poorly studied. In this study, hsa-mir-212-5p was an abnormal expressed miRNA of DVT and highly expressed in DVT. Therefore, we speculate that hsa-mir-212-5p also plays a key role in the pathogenesis of DVT, and it is necessary to further explore its function and mechanism.

The role of miR-212 is not limited to the regulation of a single gene, but rather by affecting the expression of a range of genes, with subsequent profound effects of endothelial cell function and behavior. For example, mutations or loss of function of the VHL (von Hippel-Lindau) gene can lead to the upregulation of miR-212 expression, which in turn promotes angiogenesis and progression[32]. The miR-212 can also target negative regulators in the PI3K-AKT pathway, such as PTEN (phosphatase and tensin homologs), thus reducing the inhibition of the PI3K-AKT pathway and leading to the continuous activation of AKT signaling pathway. This change not only promotes the proliferation and migration of vascular endothelial cells, but also enhances the angiogenic capacity, which then plays an important role in tumor development and revascularization of ischemic tissue[33]. In this study, we performed silencing and overexpression of miR-212-5p in EA.hy926 endothelial cell lines and found that the proliferation capacity of EA.hy926 endothelial cells was increased after silencing of miR-212-5p and decreased after overexpression, similar to previous findings. This finding provides further insight into the role of miR-212-5p in the pathogenesis of DVT.

However, other unknown regulatory pathways in DVT may still exist and need further investigation. For example, the specific mechanism by which miR-212-5p regulates endothelial cell proliferation is not fully understood, and its effects on other key endothelial cell functions (e.g., apoptosis, migration, and invasion) were not examined. Future work should include in vivo experiments (e.g., miR-212-5p knockout or overexpression in animal models) to observe DVT formation and progression. Additionally, exploring the interactions between miR-212-5p and other molecules could reveal its role in the DVT molecular network and uncover more mechanisms underlying DVT development.

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