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MiR-204-5p regulates HUVEC cell inflammation and apoptosis by targeting P4HB

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ABSTRACT

The purpose of this study arose to investigate the mechanism of miR-204-5p targeting P4HB to regulate inflammation and apoptosis in HUVEC cells. Serum specimens were obtained from lower extremity DVT patients and healthy subjects. Targetscan predicted P4HB as a target gene for miR-204-5p. A dual luciferase reporter assay was conducted to determine the modulating effect of miR-204-5p on P4HB. qRT-PCR was used to detect miR-204-5p and P4HB expression. Established CoCl2-induced hypoxia/ischemia model of HUVEC, transfected with miR-204-5p mimics and pcDNA3. 1-P4HB. CCK-8 assay for cell viability. Apoptosis was assayed by flow cytometry, western blot and western blot. Immunofluorescence and ELISA were carried out to detect ROS, MDA, SOD, LDH, GSH-px, TNF-α, IL-1β and IL-6 expression. miR-204-5p was reduced markedly in the sera of DVT patients. miR-204-5p negatively regulated P4HB. P4HB expression was raised in the sera of DVT patients. Exposure to CoCl2 decreased miR-204-5p expression and increased P4HB in HUVEC. Over-expressed miR-204-5p effectively increased cell viability and inhibited apoptosis; its effect was counteracted by continued overexpression of P4HB. In addition, miR- 204-5p mimics clearly reduced CoCl2-induced ROS and inflammation, and pcDNA3. 1-P4HB acted counteractively. miR-204-5p may inhibit HUVEC proliferation, ROS generation and cellular inflammation through negative regulation of P4HB. miR-204-5p promises to become a potential target for DVT therapy.

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Introduction

MicroRNAs (miRNAs) are non-coding single-stranded RNAs discovered in animals, plants and some viruses (1). miRNAs are short (~19-24 nucleotides), endogenous, and evolutionarily conserved RNAs (2). With the development of epigenetic studies, the primary function was proven to be the sequential regulation of gene expression at the post-transcriptional level, through complementary base pairing with messenger RNA II (3). Several miRNAs possess critical roles in various aspects of vascular inflammation and disease progression (4). For example, miR-24 was shown to be a key regulator of vascular inflammation and abdominal aortic aneurysm pathology (5). Furthermore, miRNAs are widely documented to participate in the regulation of apoptosis and polarization of various cells (6). The previous study provides evidence that miR-204-5p suppresses prostate cancer invasion, migration and bone metastasis (7). Also, the protective function of penicillin-3-O-glucoside against injured human umbilical vein endothelial cells (HUVECs) through increased apoptosis and inflammation is impaired by overexpression of miR-204-5p (8). In sepsis, LPS was evidenced to reduce the cell viability of HUVECs by decreasing miR-204-5p expression, inducing apoptosis, inflammatory damage and oxidative stress (9).

Deep vein thrombosis (DVT) is a type of blood clot forming in the deep veins, typically affecting the lower extremities, where the thrombosis begins in the deep veins of the lower legs and spreads proximally (10). However, DVT can also occur in the arms and other parts of the body (11). It is a common venous thromboembolic disease with a rate of approximately 1.6/1000 inhabitants per year (12). In the acute stages of DVT, patients may develop pulmonary embolism, which results in a significant mortality rate (13). Venous thrombosis is rare and is associated with malignancy, compression, and vascular abnormalities (14). At present, the exact mechanism of deep vein thrombosis is poorly understood (15). Endothelial cell injury and dysfunction are the primary factors causing deep vein thrombosis (16). Previous studies have demonstrated that endothelial cell apoptosis decreases the content of intravascular reactive substances, damages multiple intravascular defenses, and reduces the stability of the vascular endothelium (17, 18). Inflammatory agents are also participating in the development of DVT (19, 20). The decreased expression of miR-338-5p was proven to promote DVT formation by increasing interleukin-6 (21).

The role of Prolyl 4-hydroxylase (P4HB) extends beyond its involvement in deep vein thrombosis (DVT) and encompasses various cellular processes. P4HB functions as an essential molecular chaperone protein located within the endoplasmic reticulum (ER), where it plays a crucial role in facilitating proper protein folding in res-

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ponse to ER stress (22). It acts as a catalyst in the post-translational modification of collagen, promoting its stability and assembly by hydroxylating proline residues.

P4HB has garnered significant attention in the context of malignant tumors, as it has been implicated in cancer progression. In several cancer types, including gastric cancer, clear cell renal cell carcinoma, and colon cancer, P4HB has been found to be upregulated and associated with pro-cancer properties (23-25). Its overexpression has been linked to enhanced tumor growth, invasion, and angiogenesis, highlighting its potential as a therapeutic target in cancer treatment.

Furthermore, a specific P4HB isoform, P4HB-021 splice, has been observed to be relatively more expressed in vascular smooth muscle cells (26). This finding suggests a potential involvement of P4HB in vascular-related processes and diseases, such as DVT. Additionally, studies have established a relationship between P4HB and endothelial cells, including human coronary artery endothelial cells and human endothelial cells, smooth muscle cells (27, 28). These findings further underscore the importance of investigating the role of P4HB in the context of vascular pathologies.

Motivated by the previous evidence linking P4HB to cancer and vascular cells, our study aims to explore the expression patterns and functional significance of miR-204-5p in patients with DVT. We hypothesize that miR-204-5p may contribute to the progression of DVT by regulating key processes, namely inflammation and apoptosis, in vascular endothelial cells. Understanding the mechanisms by which miR-204-5p modulates these cellular events could provide valuable insights into the pathogenesis of DVT and potentially uncover novel therapeutic strategies.

Therefore, this study aims to unravel the intricate interplay between miR-204-5p, P4HB, and DVT. By elucidating the involvement of miR-204-5p in regulating the inflammation and apoptosis of vascular endothelial cells, we seek to shed light on the underlying molecular mechanisms driving DVT progression. The findings from this study hold the potential to uncover new therapeutic targets and pave the way for the development of innovative interventions to mitigate the burden of DVT and improve patient outcomes.

Materials and Methods

Clinical samples

Permission for the study was obtained from the Ethics Committee of Suqian Hospital, Xuzhou Medical University, and informed consent from all participants was obtained. The peripheral blood samples were collected from a total of 30 patients with DVT and 30 healthy volunteers.

Cell culture and treatment

HUVECs were obtained from the Shanghai Institute of Life Sciences Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 mg/mL penicillin/streptomycin (Life Technologies, Gaithersburg, MD, USA), and cultured at 37°C in a humidified atmosphere containing 5% CO2. HUVECs were incubated with 250 μM CoCl2 for 12 hours to construct a hypoxic injury model (29).

Cell transfection

miR-204-5p overexpression vector (miR-204-5p mimic), miRNA negative control (miR-NC), the P4HB overexpression vector (pcDNA3.1-P4HB) and empty control vector (pcDNA 3.1) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). HUVECs were seeded into 24-well plates for 24 h at 5×10^4 cells per good density. Then, 50 ng/μL plasmid, 50 nM miR-204-5p mimic and negative control were transfected into the cells following the instructions with Lipofectamine® 3000. After 8 h of transfection, replaced the medium with DMEM (10% FBS) (Gibco, Rockville, MD, USA). Cells were collected after 48 hours for RT-qPCR.

Dual-luciferase reporter gene assay

StarBase v3.0 (http://starbase.sysu.edu.cn/) was employed to forecast the target genes of miR-204-5p. Sequences of wild-type and mutant 3'UTRs of human P4HB in which the miR-204-5p binding site was contained were amplified by PCR and cloned into the pmiRGLO vector. HUVEC was transfected with 100 ng of wild-type pmiRGLO-P4HB-3'-UTR or mutant pmiRGLO-P4HB-3'-UTR and 100 nM miR-204- 5p mimic or mimic control (Genepharma, Shanghai, China). Transfection of 50 ng of pRL-TK vector was used as an internal control. Luciferase activity was measured 48 h after transfection using a dual luciferase assay kit.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA isolation and synthesis were conducted according to commercial standard methods. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). TaqMan miRNA Reverse Transcription Kit (cat. no. 4366597; Applied Biosystems, Foster City, CA, USA) was used for the preparation of cDNA that reverses transcribed from miRNA. Then obtained cDNA was subjected to qRT-PCR using SYBR Premix Ex Taq (cat.no. DRR420A; TaKaRa, Tokyo, Japan) to measure miR-204-5p and P4HB mRNA. Analysis of relative gene expression following the $2^{-\Delta\Delta Ct}$ method (30). U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was treated as a normalization control. The primer sequences used are as follows: miR-204-5p forward 5'-ACACTCCAGCTGGGTTCCCTTTGTCATCCTAT-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; P4HB forward, 5'-GGCTATCCCACC- ATCAAGTTC-3' and reverse, 5'-TCACGATGTCATCAGCCTCTC-3'; caspase-3 forward, 5'-TGCATACTCCACAGCACCTGGTTA-3' 5'-CATGGCACAAAGCGACTGGATGAA-3'; Bcl-2 forward, 5'-ATGACCAGACACTGACCATCCACT-3' and reverse, 5'-ATGTAGTGGTTCTCCTGGTGGCAA-3'; GAPDH forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Western blot analysis

Total proteins were extracted from cell lines by lysis with RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China), and quantified using BCA protein assay reagent (Beyotime Institute of Biotechnology, Shanghai, China). And loaded onto 10% SDS gels with 30

μg/lane. Then transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA) by electrophoresis. The membranes was blocked for 2 h with 5% skimmed milk, then incubated overnight at 4°C with antibody against P4HB (cat. no. ab137110; 1:1,000; Abcam, Cambridge, MA, USA), anti-cleaved caspase-3 (cat. no. 9661; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-caspase-3 (cat. no. 9662; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Bcl-2 (cat. no. 4223; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), followed by horseradish peroxidase-labeled anti-rabbit secondary antibody (Cat. no. sc-2004/sc-2005; 1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). GAPDH (cat. no. sc-47724; 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were treated as internal references.

Cell Count Kit-8 (CCK-8) assay

The CCK-8 assay was used to assess the viability of the cells. HUVECs in 96-well plates were seeded with 5 × 103 cells per well. 24 hr later, each well was incubated for 2 hr with the addition of 10 uL CCK-8. The absorbance was measured at 450 nm using a microplate reader.

Cell apoptosis assay

Cell apoptosis was analyzed by annexin V-FITC/PI apoptosis detection kit (cat. no. 556547; BD Biosciences, Franklin Lakes, NJ, USA). Transfected HUVECs were cultured under $CoCl_2$ or not for 24 h. Staining was performed with FITC-Annexin V and PI. Then analyzed on a BD FACS Verse flow cytometer.

Intracellular reactive oxygen species (ROS) measurement

Intracellular ROS generation was detected by a fluorescence probe DCFH-DA as described (31). Briefly, HUVECs were incubated with 10 μM DCFH-DA (30 min; 37°C; in the dark). Then, the photographs were captured by a fluorescence microscope (80i, Nikon, Japan).

Enzyme-linked immunosorbent (ELISA) experiment

The ELISA assay kits (for IL-6, IL- 1β and TNF-α) were got from Miltenyi Biotec, Inc (cat. no. 130- 101-688, 130-094-065, 130-094-053).

The levels of malondialdehyde (MDA), lactate dehydrogenase (LDH) and glutathione peroxidase (GSH-px), superoxide dismutase (SOD) levels in the serum, were detected by the lipid peroxidation MDA kit (cat. no. S0131S; Beyotime Institute of Biotechnology, Shanghai, China), LDH cytotoxicity assay kit (cat. no. C0016; Beyotime Institute of Biotechnology, Shanghai, China), GSH assay kit (cat. no. S0052; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and total SOD assay kit with WST-8 (cat. no. S0101S; Beyotime Institute of Biotechnology, Shanghai, China), respectively.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and anaylsised by Statistic Package for Social Science (SPSS) v. 19.0 (IBM, Armonk, NY, USA) and GraphPad Prism 6 (La Jolla, CA, USA). A Student's t-test was used to assess comparisons between groups. One-way ANOVA and Tukey's post hoc test were used to analyze differences in multiple comparisons. $P < 0.05$ represents statistically significant differences.

Results

Abnormally low expression of miR-204-5p in the serum of DVT patients

To investigate the expression of miR-204-5p in patients with DVT, the mRNA expression of miR-204-5p was analyzed in the serum of healthy volunteers and patients with DVT. Notably, miR-204-5p expression was lower in DVT patients than in healthy volunteers (Figure 1A). The online bioinformatics StarBase v3.0 forecasts that P4HB is the target of miR-204-5p. To determine the regulatory relationship between P4HB and miR-204-5p, the 3'-UTR of P4HB contained wild-type (WT) or mutant miR-204-5p target sequences was cloned into the pmiRGLO vector (Figure 1B). The luciferase activity of the WT 3'-UTR reporter gene was obviously decreased after transfection with the miR-204-5p mimic, whereas the luciferase activity of the mutant reporter gene was unaffected (Figure 1C). As shown in Figure 1D, the mRNA expression of P4HB was raised in DVT patients, compared with healthy volunteers. These results suggested that miR- 204-5p negatively regulated P4HB and was quite likely involved in DVT.

miR-204-5p was down-regulated in $CoCl_2$-injured HUVECs

To uncover the role of miR-204-5p in DVT, the HUVECs hypoxic injury model was constructed. The miR-204-5p and P4HB expression were tested and the results told that miR-204-5p was downregulated in HUVECs after exposure to $CoCl_2$, while P4HB was upregulated (Figure 2A). These results are consistent with those obtained from the patient sample. As presented in Figure 2B, transfection of HUVECs with miR-204-5p mimics markedly elevated the expression of miR-204-5p efficiently compared with the control group. In addition, mRNA and protein expression of P4HB was upregulated in HUVECs with pcDNA3. 1-P4HB significantly (Figure 2C).

miR-204-5p affects the proliferation and apoptosis of $CoCl_2$-injured HUVECs via P4HB

The CCK-8 assay revealed that high miR-204-5p expression markedly increased the proliferation rate of HUVEC cells exposed to $CoCl_2$. Meanwhile, enhanced

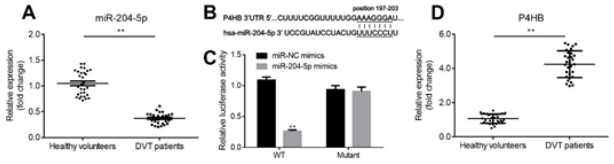

Figure 1. miR-204-5p negatively regulates P4HB. (A) Detection of miR-204-5p expression in serum of VT patients by qRT-PCR. (B) Targetscan predicted the binding site of miR-204-5p to the target gene P4HB. (C) Construction of wild-type and mutant luciferase reporter gene plasmids in the 3'UTR region of P4HB and validation of the relationship between miR-204-5p and P4HB targets. (D) Expression of P4HB in the serum of DVT patients and healthy people by qRT-PCR.

P4HB expression reduced the cell proliferation rate (Figure 3A). Also, we found that apoptosis in HUVECs was induced by CoCl2 and apoptosis was attenuated by miR-204-5p mimics and the co-culture fraction of pcDNA3. 1-P4HB exacerbated the apoptosis rate (Figure 3B). Next, the protein and RNA expression of apoptosis-related genes were tested (Figure 3C). The Bax and cleaved caspase-3 were up-regulated after exposure to CoCl2, while Bcl-2 was reduced. The over-expressed miR-204-5p reduced the Bax and cleaved caspase-3 expression, and increased Bcl-2 expression. The co-culture ofpcDNA3. 1-P4HB and miR-204-5p mimics further elevated the Bax and cleaved caspase-3 expression, and lowered Bcl-2 expression.

miR-204-5p affects the ROS and inflammation of CoCl2-injured HUVECs via

To explore the effect of miR-204-5p on inflammation in CoCl2-treated HUVECs, we first examined the production of ROS. It is observed from Figure 4A that exposure to CoCl2 substantially induced ROS production, and overexpression of miR-204-5p was effective in reducing this effect; however, further overexpression of P4HB counteracted the effect and increased ROS generation. The changes in MDA and LDH expression were consistent with SOD. In contrast, SOD and GSH were measured in the reverse direction, with CoCl2 decreasing their expression, overexpression of miR-204-5p elevating their expression, and overexpression of P4HB effectively inhibiting the production (Figure 4B). Consistently, TNF-α, IL- 1β and IL-6 expression raised due to CoCl2 stimulation, while overexpression of miR-204-5p effectively reduced them and continued overexpression of P4HB enhanced it (Figure 5).

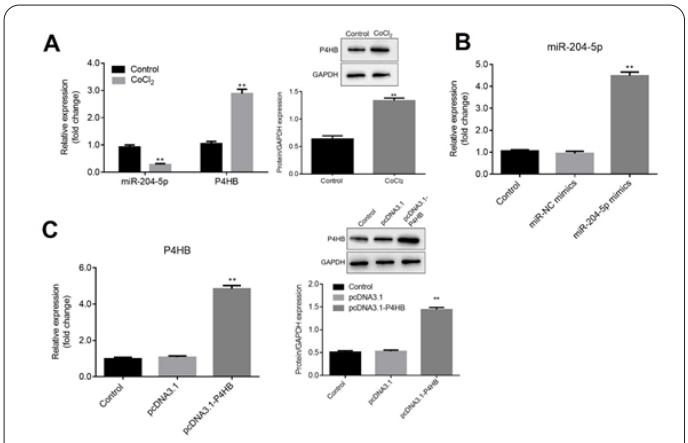

Figure 2. Expression of miR-204-5p and P4HB in normal cultured HUVEC and hypoxia/ischemia models. (A) Expression of miR-204-5p and P4HB in normal cultured HUVEC and hypoxia/ischemia models by qRT-PCR and WB. (B)Detection of transfection efficiency of miR-204-5p mimicked by qRT-PCR. (C) Transfection efficiency of pcDNA3. 1-P4HB detected by qRT-PCR and WB.

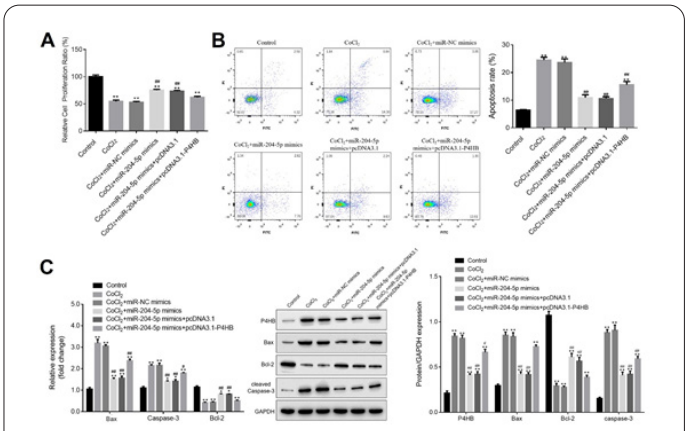

Figure 3. Effect of overexpression of miR-204-5p and P4HB on HUVEC apoptosis. (A) CCK-8 assay for cell viability. (B) Apoptosis detection by flow cytometry. (C) Western blot and qRT-PCR were performed to detect the expression of bcl2, bax, cleaved caspase3 and caspase3.

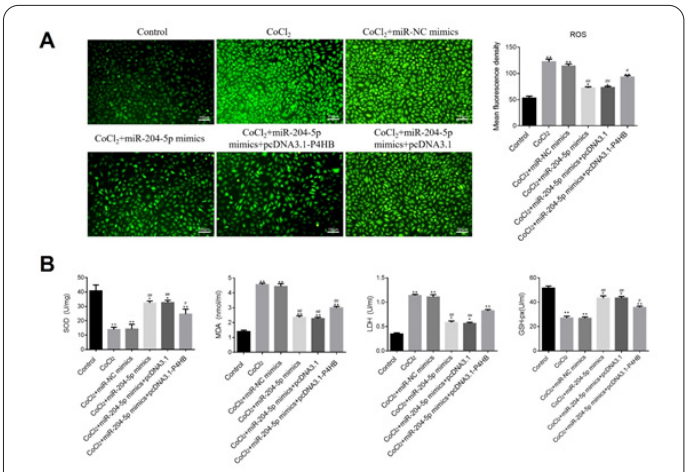

Figure 4. Effect of overexpression of miR-204-5p and P4HB on HUVEC reactive oxygen species. (A) ROS means fluorescence densitometry. (B) ELISA for the expression of MDA, SOD, LDH, GSH-px.

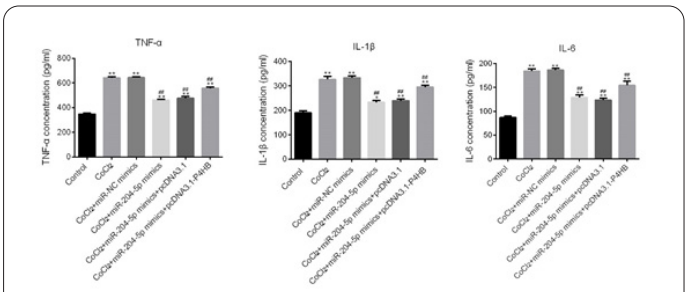

Figure 5. Effect of overexpression of miR-204-5p and P4HB on HUVEC inflammatory factors. ELISA for the expression of TNF-α, IL- 1β and IL-6.

Discussion

DVT is a type of clinically common peripheral vascular lesion (11). Both apoptosis of vascular endothelial cells and the production of inflammatory factors are considered in the development of DVT (32, 33). Mounting evidence has emerged that miRNAs, as microregulators, serve a vital role in modulating angiogenic signaling pathways(21, 34). In the present study, miR-204-5p was aberrantly low expressed in the serum of DVT patients and could affect apoptosis as well as inflammation in HUVEC through negative regulation of P4HB.

A previous study evaluating miRNA and mRNA profiles in rat models of DVT identified that miR-204-5p was expressed at low levels in patients with DVT (35). It is consistent with our findings. Nevertheless, whether miR-204-5p modulates apoptosis and cellular inflammation in HUVEC remains unknown. Therefore we explored the cellular model. The outcomes displayed that CoCl2-

stimulated modeling of simulated DVT markedly reduced cell viability and promoted apoptosis and inflammatory release, while overexpression of miR-204-5p alleviated apoptosis. Likewise, cellular inflammation resolves. Prior research reported that overexpression of miR-204-5p in atherosclerosis impaired the protective effect of C3G on damaged HUVECs through increased apoptosis and inflammation (8). Whereas in sepsis, it was determined that LPS impairs cell viability and promotes apoptosis, inflammatory response and oxidative stress in HUVECs by regulating the HULC/miR-204-5p/TRPM7 axis (9). These findings are somewhat different from our study, and the possible reasons and mechanisms for them require further exploration.

For a deeper exploration of the possible mechanisms involved, we predicted that P4HB is the target gene of miR-204-5p. Furthermore, the dual luciferase assay report proved that miR-204-5p negatively regulates P4HB. Assay data from clinical samples also supported the remarkably high expression of P4HB in the serum of DVT patients. Recently, P4HB was recognized to be a novel diagnostic and prognostic biomarker in several malignancies (24, 36). Differential protein analysis of the midbrain ischemia/reperfusion rat cerebral cortex based on i-TRAQ quantitative proteomic probing reveals that P4HB exerts a key role in oxidative stress, apoptosis and inflammatory responses (37). However, the effect of PAHB on apoptosis and inflammation in DVT remains unknown. Our results confirmed that overexpression of P4HB when cotransfected with miR-204-5p mimic effectively counteracted the effect of miR-204-5p overexpression and promoted apoptosis and inflammation generation.

Our comprehensive investigation has shed light on the significance of miR-204-5p in the context of deep vein thrombosis (DVT) and its potential therapeutic implications. Our findings have convincingly demonstrated that miR-204-5p exhibits aberrant downregulation in DVT, and its dysregulation is closely associated with the negative regulation of the P4HB gene. By experimentally manipulating the expression of miR-204-5p, we have successfully established its functional role in modulating various key aspects of endothelial cell physiology. Notably, the overexpression of miR-204-5p has been shown to effectively inhibit apoptosis in human umbilical vein endothelial cells (HUVECs), thereby counteracting the detrimental effects of DVT. Moreover, our results indicate that elevated levels of miR-204-5p can suppress reactive oxygen species (ROS) generation and reduce cellular inflammation in HUVECs, contributing to the preservation of endothelial homeostasis. The identification of P4HB as a direct target of miR-204-5p further strengthens the mechanistic understanding of its regulatory functions. By targeting P4HB, miR-204-5p exerts its protective effects on HUVECs, implicating the potential of this microRNA as a promising therapeutic target for DVT.

Conclusion

Overall, our study not only provides valuable insights into the molecular underpinnings of DVT but also offers a promising avenue for the development of novel biomarkers and therapeutic strategies. The dysregulation of miR-204-5p and its downstream target, P4HB, represents a potential diagnostic marker for DVT, enabling early detection and intervention. Furthermore, therapeutic approaches aimed at restoring miR-204-5p levels or targeting P4HB could hold great promise in attenuating the pathology associated with DVT and improving patient outcomes. Further research and clinical validation are warranted to fully harness the therapeutic potential of miR-204-5p in the context of DVT management.

Ethical Compliance

Permission for the study was obtained from the Ethics Committee of Suqian Hospital, Xuzhou Medical University, and informed consent from all participants was obtained

Disclosure statement

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

Acknowledgments

None.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

Jun Chen designed the study and wrote the manuscript; Jun Chen, Kaixin Zhang and Yi Yang performed the experiments; Yi Yang and Jianqiang Wu collected and analyzed the data. All authors read and approved the final manuscript.

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