Peripheral Blood Levels of miR-448 and SIRT1 in Patients with Deep Venous Thrombosis and Their Relationship

Pu Wang, Jiao Dai, Dibing Li

First Department of Internal Medicine, Ningbo No. 6 Hospital, Ningbo, Zhejiang Province, China

SUMMARY

Background: The goal of this study was to investigate the changes in peripheral blood levels of miR-448 and silent information regulator 1 (SIRT1) in patients with deep venous thrombosis (DVT) and to analyze their relationship. Methods: A total of 112 patients treated from January 2019 to June 2020 were divided into DVT group (n = 40) and non-DVT group (n = 72). Fasting venous blood was extracted to separate serum and peripheral blood mononuclear cells (PBMCs). Enzyme-linked immunosorbent assay (ELISA) was employed to measure serum SIRT1 protein, and qPCR was utilized to detect miR-448 expression in PBMCs. The clinical data, serum indicators, and expressions of SIRT1 and miR-448 were compared, and the correlations of miR-448 and SIRT1 with DVT were analyzed using a multivariate Cox regression model. TargetScan Release 7.1 was used to predict the possibility of binding sites between miR-448 and SIRT1 mRNA 3'-untranslated region (3'-UTR), and dual-luciferase reporter assay was used to determine the targeting of miR-448 and SIRT1. HeLa cells were divided into overexpression, inhibition, and blank control groups. The cells were harvested 24 hours after transfection, followed by detection of SIRT1 mRNA expression by qPCR and measurement of supernatant SIRT1 protein expression by ELISA. Results: Serum SIRT1 protein level was lower and miR-448 expression in PBMCs was higher in DVT group than those in non-DVT group (p < 0.05). DVT group had a larger number of patients with vascular diseases and history of venous thrombosis than that of non-DVT group (p < 0.05). miR-448 was an independent risk factor for postoperative DVT, and SIRT1 was a protective factor (p < 0.01). There were potential complementary base binding sites between miR-448 and SIRT1 mRNA 3'-UTR. Dual-luciferase reporter assay verified the targeted regulation between miR-448 and SIRT1. HeLa cell SIRT1 mRNA expression and supernatant SIRT1 protein expression were lower in overexpression group while higher in inhibition group than those in blank control group (p < 0.05). Conclusions: Serum SIRT1 protein level decreases while miR-448 expression in PBMCs increases in patients with DVT, and miR-448 inhibits SIRT1 expression through binding to SIRT1 mRNA 3'-UTR with complementary bases, thus inducing inflammatory response to participate in the formation of DVT. Targeting miR-448 to regulate cytokine expression may become an effective target and approach for the treatment of DVT. miR-448 combined with SIRT1 has a high predictive value for the occurrence of DVT. (Clin. Lab. 2022;68:xx-xx. DOI: 10.7754/Clin.Lab.2021.210638)

KEY WORDS

deep venous thrombosis, miR-448, silent information regulator 1

INTRODUCTION

Deep venous thrombosis (DVT) is a peripheral vascular disease caused by venous intima injury, slow blood flow, and blood hypercoagulable state. Its incidence rate
accounts for 40% of peripheral vascular diseases [1]. DVT mostly occurs in the lower extremities, manifested by extensive limb swelling and pain, dark red skin, and dilated superficial veins. Thrombus shedding can cause pulmonary embolism, seriously threatening people's life and health [2,3]. According to the statistics, the incidence rate of DVT is 29 - 43% in neurosurgery without thrombus prevention [4]. Moreover, 90% of the emboli in pulmonary embolism in neurosurgery patients are derived from DVT in the lower extremities, with a mortality rate of up to 50% [5]. Hence, it is particularly important to effectively predict the occurrence of DVT in neurosurgery patients. Inflammatory cytokines play important roles in the pathogenesis of DVT; that is, they can cause vascular endothelial injury, thus participating in the formation and progression of DVT [6]. Silent information regulator 1 (SIRT1), a small molecule protein with anti-inflammatory and anti-oxidative stress effects, extensively exists in the body and is involved in various pathological processes of multiple diseases. SIRT1 can better regulate the body's inflammatory response and oxidative stress [7,8]. miRNAs are a class of non-coding, single-stranded RNA molecules with a length of about 22 nucleotides, which participate in the post-transcriptional regulation of gene expression and play essential roles in maintaining body homeostasis [9], but their influences in the pathogenesis of DVT remain unclear. In this study, therefore, the changes in serum SIRT1 level and miR-448 expression in the peripheral blood mononuclear cells (PBMCs) of DVT patients were detected to explore the predictive value of SIRT1 and miR-448 for the formation of DVT in the lower extremities of neurosurgery patients and to analyze whether miR-448 has a regulatory effect on SIRT1, providing new targets and ideas for the clinical treatment of DVT.

**MATERIALS AND METHODS**

**Baseline data**

Patients who underwent craniotomy hematoma removal surgery in our hospital from January 2019 to June 2020 were selected, and general data and treatment history were collected using questionnaires. The inclusion criteria were as follows: a) patients with definite diagnosis, successful operation process, and no obvious intraoperative complications, and b) those with good treatment compliance (active cooperation with treatment and inspection on time). The exclusion criteria involved: a) patients with previous neurological diseases or treatment history of such diseases, or b) those with systemic diseases such as hypertension, hyperlipidemia, diabetes or coagulation dysfunction. Finally, a total of 112 patients were enrolled in this study. They were followed up for 12 months after surgery. The patients were categorized into DVT group (n = 40) and non-DVT group (n = 72) according to the presence or absence of DVT. There were no statistically significant differences in the general data between the two groups.

**Diagnosis of DVT**

All patients received monthly DVT examinations for consecutive 12 months by the same ultrasound physician using a DC-8EXP B-ultrasound machine (Mindray, China) after surgery. In addition, B-ultrasound examination should be carried out immediately if the patient had clinical symptoms of DVT.

**Specimen collection and reagent preparation**

About 6 mL of fasting venous blood was extracted from each subject in the morning and anticoagulated with EDTA-Na2 to separate serum and PBMCs using human peripheral blood lymphocyte separation solution, followed by detection by enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR), respectively. Main reagents were as follows: Human Peripheral Blood Lymphocyte Separation Solution Kit was purchased from Solarbio Science & Technology Co., Ltd, Human SIRT1 ELISA kit was provided by Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd, TRIzol Reagent and UltraSYBR mixture were bought from Beijing Kangwei Century Biotechnology Co., Ltd, ReverTra Ace qPCR RT Kit was purchased from Toyobo Biotech Co., Ltd, miRNA 1st Strand cDNA Synthesis Kit was purchased by Nanjing Vazyme Biotech Co., Ltd. 293T cells and HeLa cells were bought from Wuhan Procell Life Science & Technology Co., Ltd. Besides, Lipofectamine™ 2000 Transfection Reagent was purchased from Invitrogen (USA), miR-488 overexpression (forward primer: 5’-UUAUUAUA CAACCUUGUAAAGUG-3’, reverse primer: 5’-CUU AUCCAGGUUUAAUUAUUU-3’), miR-488 inhibitor (5’-CAUUAGCCAGGUUUGUAUUAUU-3’), and negative control (forward primer: 5’-UUUCCGAACG UGUCAGU-3’, reverse primer: 5’-ACGUAGACAG UCGGGAGATT-3’), miR-488 internal reference U6 reverse transcription primers and qPCR primers were designed and synthesized by Shanghai Gene Pharma Co., Ltd, and internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and target gene SIRT1 qPCR primers were designed and synthesized by Shanghai Boshang Biotechnology Co., Ltd, (Table 1). Wild-type and mutant SIRT1 mRNA 3’-untranslated region (3’-UTR) vectors were purchased from Promega (USA).

**Measurement of serum SIRT1 protein**

The assay plate was soaked in the 1x wash solution for 30 seconds, and standard and sample solutions with different dilution multiples were added according to the instructions of ELISA kits. Subsequently, the detection antibody was added into each well, mixed well, and incubated at room temperature for 2 hours. Next, the plate was washed 6 times, then diluted horseradish peroxidase-labeled streptavidin was added to each well, and mixed well, followed by incubation at room temperature for 45 minutes. Later, the plate was washed 6 times, and...
then chromogenic substrate TMB was added to each well in the dark and incubated at room temperature for 15 minutes. Then, the color development was terminated by adding stop solution, and the optical density (OD) value was determined at 450 and 630 nm wavelengths using a microplate reader. After plotting the standard curve, SIRT1 protein concentration was calculated based on the OD_{450nm}-OD_{630nm} values.

Detection of miR-448 expression in PBMCs
The total RNA in each group of cells was extracted by TRIzol reagent, and the concentration and purity were determined using a UV spectrophotometer. Next, cDNA was synthesized according to the instructions of miRNA 1st Strand cDNA Synthesis Kit, followed by Ultra SYBR Mixture PCR amplification, with U6 as an internal reference. Based on the obtained data, the relative expression level of miR-448 was calculated by 2^{-ΔΔCt} method.

Bioinformatics prediction of the relationship between miR-448 and SIRT1
TargetScan Release 7.1 was used to predict the targeted regulatory sites between miR-448 and SIRT1 mRNA 3′-UTR. It was hypothesized that SIRT1 may be the target gene of miR-448. Hence, 293T cells were resuscitated and cultured routinely, pGL3-SIRT1-3′-UTR-mutant type (MUT) and pGL3-SIRT1-3′-UTR-wild type (WT) plasmids were constructed, and co-transfected separately using Lipofectamine™ 2000 Transfection Reagent. After the cells were incubated in an incubator at 37°C with 5% CO_2 for 48 hours, dual-luciferase reporter assay system was used to determine firefly luciferase activity/Renilla luciferase activity.

Observation of regulatory effect of miR-448 on expression of SIRT1
HeLa cells were divided into three groups, namely over-expression group and inhibition group (transfected with miR-448 mimics and inhibitor) as well as blank control group (transfected with negative control primers) according to the instructions of the Lipofectamine™ 2000 Transfection Reagent. Next, the cells were harvested after transfection for 24 hours, dual-luciferase reporter assay system was used to determine firefly luciferase activity/Renilla luciferase activity.

Comparisons of clinical data and hematology indicators
Hemoglobin, white blood cell counts, platelets, MPV, platelet distribution width (PDW), miR-448, SIRT1, amount of intraoperative bleeding, disease classification, postoperative awareness, mannitol use, and previous history of venous thrombosis were recorded. “Ultrasound diagnosis” was selected as the gold standard to evaluate the correlations of above indicators with DVT occurrence in the lower extremities of neurosurgery patients, and the diagnostic efficacy, including diagnostic accuracy, sensitivity, specificity, positive predictive value and negative predictive value.

Statistical analysis
SPSS 26.0 software was utilized for statistical analysis. Numerical data were expressed as percentage [n (%)] and compared using chi-square test. Measurement data were expressed as mean ± standard deviation (x ± s) and compared using t-test. Cox regression analysis was used to investigate the correlations of hematology indicators with DVT occurrence, and receiver operating characteristic (ROC) curves were plotted to evaluate the diagnostic efficacy of different indicators for predicting DVT. p < 0.05 indicated that there was a statistically significant difference.

RESULTS

General data
There were no statistically significant differences in the age, gender, body mass index (BMI), preoperative Glasgow coma score (GCS), preoperative bed rest duration, preoperative activity ability, and smoking and drinking history between the two groups (p > 0.05) (Table 2).

Clinical treatment data
There were statistically significant differences in the miR-448, SIRT1, disease classification and previous history of venous thrombosis between the two groups (p < 0.05). Among them, serum SIRT1 protein level was lower and miR-448 expression in PBMCs was higher in DVT group than those in non-DVT group (p < 0.05). The DVT group had a significantly larger number of patients with vascular diseases and previous history of venous thrombosis than non-DVT group (p < 0.05) (Table 3).

Cox regression analysis results of thrombosis
Multivariate Cox regression analysis indicated that miR-448 was an independent risk factor for postoperative DVT, while SIRT1 served as a protective factor (p < 0.01) (Table 4).

ROC curve analysis results of miR-448 and SIRT1 for predicting DVT
The area under the curve (AUC) of miR-448 and SIRT1 to predict DVT was 0.782 and 0.792, respectively, and
Table 1. Gene primer sequences and product fragments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Product fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>Forward: AAGCCCAAGCACAAGGCG</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Reverse: GATGACAGCGCGGTAG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: ACAACTTTGATCTGGAACG</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCCATCAGCCACAGTTTC</td>
<td></td>
</tr>
<tr>
<td>miR-488</td>
<td>Forward: GCCGGTTATAATACAACCTTGAGA</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Reverse: TATGGTTTCTCTGCTGTCTC</td>
<td></td>
</tr>
<tr>
<td>U6</td>
<td>Forward: CAGCATATACTAAAATTGGAACG</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACGAATTGCGTGTCATCC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. General data.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>DVT group (n = 40)</th>
<th>Non-DVT group (n = 72)</th>
<th>Statistic value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57.89 ± 5.43</td>
<td>58.02 ± 5.46</td>
<td>t = 0.121</td>
<td>0.904</td>
</tr>
<tr>
<td>Gender [n (%)]</td>
<td></td>
<td></td>
<td>χ² = 0.420</td>
<td>0.517</td>
</tr>
<tr>
<td>Male</td>
<td>22 (55.0)</td>
<td>35 (48.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>18 (45.0)</td>
<td>37 (51.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.43 ± 3.64</td>
<td>21.35 ± 3.72</td>
<td>t = 0.110</td>
<td>0.913</td>
</tr>
<tr>
<td>Preoperative GCS</td>
<td>8.72 ± 0.89</td>
<td>8.86 ± 0.92</td>
<td>t = 0.781</td>
<td>0.437</td>
</tr>
<tr>
<td>Preoperative bed rest duration (week)</td>
<td>2.34 ± 0.45</td>
<td>2.43 ± 0.43</td>
<td>t = 1.044</td>
<td>0.299</td>
</tr>
<tr>
<td>Preoperative activity ability [n (%)]</td>
<td></td>
<td></td>
<td>χ² = 0.023</td>
<td>0.989</td>
</tr>
<tr>
<td>Mildly restricted</td>
<td>16 (40.0)</td>
<td>29 (40.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderately restricted</td>
<td>13 (32.5)</td>
<td>22 (30.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severely restricted</td>
<td>11 (27.5)</td>
<td>19 (29.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking history [n (%)]</td>
<td>4 (10.0)</td>
<td>6 (8.3)</td>
<td>χ² = 0.063</td>
<td>0.802</td>
</tr>
<tr>
<td>Drinking history [n (%)]</td>
<td>5 (12.5)</td>
<td>7 (9.7)</td>
<td>χ² = 0.164</td>
<td>0.931</td>
</tr>
</tbody>
</table>

the AUC of combined diagnosis was 0.852. Furthermore, miR-448 combined with SIRT1 was significantly superior to miR-448 or SIRT1 alone in terms of diagnostic efficacy (Z value: 2.983 and 2.542, respectively, p < 0.05) (Figure 1 and Table 5).

Bioinformatics prediction and verification of relationship between miR-448 and SIRT1

TargetScan Release 7.1 predicted that there were potential complementary base binding sites between miR-448 and SIRT1 mRNA 3’-UTR, indicating that miR-448 regulates the expression of SIRT1 by binding to SIRT1 mRNA 3’-UTR (Figure 2). Dual-luciferase reporter assay system displayed that the luciferase activity of WT-SIRT1 was inhibited after transfection with miR-448 mimics (p < 0.05), while no significant change was observed in the luciferase activity of MUT-SIRT1 (p > 0.05), suggesting the targeted regulation between miR-448 and SIRT1.

Regulatory effect of miR-488 on expression of SIRT1

SIRT1 mRNA expression and supernatant SIRT1 protein expression levels were lower in overexpression group while they were higher in inhibition group than those in blank control group (p < 0.05) (Figure 3).
**DISCUSSION**

DVT is a common peripheral vascular disease, the incidence rate of which exhibits an increasing trend annually in recent years [10,11]. Thrombus shedding easily occurs in the acute stage of DVT, resulting in fatal pulmonary embolism, which is one of the major causes of sudden death. Moreover, delayed treatment or poor therapeutic effect may cause a protracted course of disease, progressing into post-thrombotic syndrome, which af-
Figure 1. ROC curve analysis results of miR-448 and SIRT1 for predicting DVT.

Figure 2. Bioinformatics prediction and verification of relationship between miR-448 and SIRT1.

ffects limb function for a long time and leads to venous ulcers [12]. At present, the pathogenesis of DVT remains unclear. A previous study indicated that inflammation-induced vascular endothelial cell injury due to imbalance of cytokine expression is involved in the occurrence and progression of DVT [1].
SIRT1 is an important member of the silent information regulator family, and an important small molecule protein with anti-inflammatory and anti-oxidative stress effects. It extensively exists in the body and takes part in various pathological processes of multiple diseases, which can better regulate the body's inflammatory response and oxidative stress. miRNAs are a research hotspot in the field of non-coding RNA, which can bind to the 3'-UTR of the target gene to inhibit gene transcription or translation. Previous studies have confirmed that miRNAs are involved in the regulation of physiological processes such as cell proliferation, differentiation, apoptosis and signal transduction, as well as the occurrence and progression of multiple diseases [13-15]. In recent years, the roles of miRNAs in vascular endothelial injury and progression of DVT have been paid more attention to. However, the role of miRNA in the formation of DVT has not yet been elucidated. A previous study indicated that miR-146a may play an important regulatory role in the imbalance of the fibrinolytic-anti-fibrinolytic system of DVT [16], let-7e-5p is reduced in the peripheral blood of DVT patients, while miR-483-3p and miR-195 are increased [17]. These miRNAs with differential expressions in DVT patients can be used as new targets for DVT treatment, providing a new basis for cell therapy of DVT. miR-448 is a member of the miRNA family and participates in the occurrence of various diseases such as coronary atherosclerosis, prostate carcinoma, and breast carcinoma. Li et al. [18] reported that overexpressed miR-448 could promote the proliferation and migration of vascular smooth muscle cells, and played an important role in multiple cardiovascular diseases, such as coronary heart disease, atherosclerosis and hypertension. In this study, therefore, the predictive value of miR-448 and SIRT1 for the formation of DVT was explored. Moreover, the analysis on clinical data showed that there were statistically significant differences in the miR-488, SIRT1, disease classification and previous history of venous thrombosis between the two groups of patients. miR-488 was significantly increased and SIRT1 was significantly decreased in patients with DVT, and the proportion of people with vascular diseases and previous history of venous thrombosis was significantly higher. Multivariate Cox regression analysis indicated that miR-448 was an independent risk factor for postoperative DVT, while SIRT1 served as a protective factor. In addition, the AUC of miR-448 and SIRT1 to predict DVT was 0.782 and 0.792, respectively, and the AUC of combined diagnosis was 0.852, suggesting that miR-448 combined with SIRT1 is significantly superior to miR-448 or SIRT1 alone in terms of diagnostic efficacy.

In this study, the results demonstrated that serum SIRT1 protein level was lower and miR-488 expression in PBMCs was higher in DVT patients than those in non-DVT patients, indicating that the changes in the expressions of the two participate in the occurrence and progression of DVT. A previous study reported that miR-488 is involved in inflammatory response, and it is highly expressed in obese people, but its role in DVT remains unclear [19]. Meanwhile, TargetScan predicted that there were potential complementary base binding sites between miR-448 and SIRT1 mRNA 3'-UTR, indi-
cating that miR-448 may inhibit the expression and function of SIRT1 gene by binding to SIRT1 mRNA 3'-UTR, leading to the body's homeostasis imbalance, then inflammation to induce vascular endothelial cell injury, and ultimately the formation of DVT. In order to further verify the regulatory effect of miR-448 on SIRT1, HeLa cells were transfected with miR-448 mimics to up-regulate the expression of miR-448, and the results indicated that after the cells were transfected with miR-448 inhibitor to down-regulate the expression of miR-448, SIRT1 mRNA and protein expression levels were reduced. Moreover, it was found that after the cells were transfected with miR-448 inhibitor to down-regulate the expression of miR-448, SIRT1 mRNA and protein expression levels were increased, further confirming that miR-448 has a negative regulatory effect on the expression and function of SIRT1. The present study elucidated the underlying pathogenesis of DVT from the perspective of epigenetic and immune regulation, providing new molecular biological indicators for the clinical diagnosis and treatment of DVT. In addition, targeting miR-448 to regulate cytokine expression may be an effective target and approach for the treatment of DVT.

CONCLUSION

In conclusion, serum SIRT1 protein level reduces while miR-448 expression in PBMCs increases in patients with DVT. miR-448 inhibits SIRT1 expression through binding to SIRT1 mRNA 3'-UTR with complementary bases, thus inducing inflammatory response to partici-pate in the formation of DVT. Targeting miR-448 to regulate cytokine expression may become an effective target and approach for the treatment of DVT. miR-448 combined with SIRT1 has a high predictive value for the occurrence of DVT.

Declaration of Interest:
The authors declare no conflicts of interest.

References: