Role of microRNA-26a in the diagnosis of lower extremity deep vein thrombosis in patients with bone trauma

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Abstract. The present study aimed to investigate the role and mechanism of action of microRNA (miR)-26a in deep vein thrombosis (DVT). Peripheral blood was collected from 45 patients with DVT and 40 healthy controls. Levels of miR-26a, chemokine C-C motif ligand (CCL)2 mRNA and CCL7 mRNA were detected using reverse transcription-quantitative polymerase chain reaction and the value of miR-26a in the clinical diagnosis of DVT was assessed using receiver operating characteristic curve analysis. The correlation of miR-26a with CCL2 and CCL7 levels was analyzed using Spearman's rank correlation. In addition, miR-26a and protein kinase C δ (PRKCD) were overexpressed in human umbilical vein endothelial cells (HUVECs) and PRKCD expression was knocked down by small interfering (si)RNA. Western blotting was conducted to detect the expression of PRKCD and p65. Furthermore, a dual-luciferase reporter gene assay was performed. The results of the current study demonstrated that the expression of miR-26a was significantly downregulated in the peripheral blood of patients with DVT compared with healthy controls ($P<0.05$) and negatively correlated with CCL2 and CCL7 levels ($P<0.05$). Furthermore, it was demonstrated that miR-26a markedly inhibited the expression of PRKCD, significantly decreased levels of CCL2 and CCL7 mRNA ($P<0.05$) and inhibited activation of the NF-κB signaling pathway. Overexpression of PRKCD in HUVECs inhibited the effects of miR-26a and markedly upregulated the phosphorylation of p65. The present study indicated that miR-26a directly targets PRKCD mRNA and that miR-26a may be a useful biomarker in the clinical diagnosis of DVT. Thus, the present findings suggest that miR-26a regulates the NF-κB signaling pathway by binding to PRKCD mRNA, inhibits the expression of CCL2 and CCL7 and reduces the risk of DVT.

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Introduction

Deep vein thrombosis (DVT) is a type of venous thromboembolism disease that is common in the clinic and typically occurs following surgery for lower extremity bone trauma and other diseases, including diabetes and gestational hypertension (1,2). The incidence of DVT is >0.1% and has increased in recent years (3). The primary pathological characteristic of lower extremity venous thrombosis is abnormal blood coagulation in the lower extremity deep vein that obstructs the lumen and causes venous reflux obstacles (4). Lesions in the limb induce different degrees of swelling and pain and in some severe cases may cause limb gangrene. Following the detachment of thrombi, patients may experience pulmonary embolism and respiratory distress (5,6). Furthermore, DVT may progress to post-thrombotic syndrome, which seriously affects patient quality of life and may cause pain (7).

At present, early diagnosis of DVT is clinically significant for the treatment and prognosis of patients. The current gold standard for the clinical diagnosis of DVT is lower extremity venous imaging. However, this is a type of invasive examination and increases the risk of complications occurring, including thrombus detachment, allergic reactions and increased thrombosis severity, which limits its application in the clinic (8). Recent studies have demonstrated that biomarkers including C-reactive protein (CRP), D-dimer and interleukin (IL)-6, exhibit beneficial clinical value in the early diagnosis of DVT; however, these biomarkers are still not a substitute for lower extremity venous imaging (9-11). At present, the mechanism of DVT is unclear, which greatly limits screening for DVT diagnostic markers. Therefore, it is important to determine the mechanism of DVT in order to identify potential biomarkers.

Micro (mi)RNAs are endogenous, small noncoding RNAs that are approximately 20-22 nucleotides long and found in eukaryotes (12). Mature miRNA binds to the 3'-untranslated (UTR) region of its target mRNA, forming silencing complexes and inducing the degradation of target mRNA or inhibiting its translation (13,14). miRNAs are stable molecules that may be developed as biomarkers for various diseases and their levels are high in the peripheral blood (15). In addition, miRNAs are involved in almost all pathological and physiological processes in eukaryotic cells, including cancer, cardiovascular disease, Alzheimer's disease and inflammation (16-18).

In the present study, the diagnosis value of microRNA (miR)-26a in DVT was assessed. The role and mechanism of miR-26a in DVT was also analyzed and discussed.

Patients and methods

Patients. A total of 45 patients (27 males and 18 females; mean age, 53±8.63 years old) who had suffered from bone trauma were diagnosed with lower extremity DVT between May 2013 and April 2015 at the Xiangya Second Hospital of Central South University (Xiangya, China) and were enrolled in the present study. All patients were diagnosed by lower extremity venous imaging (19). Patients had experienced DVT for between 2 and 15 years. Clinical symptoms included pain and swelling of the lower limbs and difficulty walking. Among the 45 patients, 6 patients also had pulmonary embolism (PTE). For comparison, 40 healthy individuals were also enrolled. Peripheral whole blood was collected from the upper limb veins of 45 patients and 40 healthy individuals to extract total DNA. Prior written and informed consent was obtained from all individuals recruited in the current study and ethical approval was granted by the Ethics Review Board of Central South University.

Cell culture and reagents. Human umbilical vein endothelial cells (HUVECs) (Sciencell Research Laboratories, Inc., San Diego, CA, USA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (BD Biosciences, Franklin Lakes, NJ, USA) and cultured at 37˚C in an atmosphere containing 0.5% CO_2. TRIzol LS reagent and Lipofectamine 2000 transfection reagent were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Mouse anti-human GAPDH monoclonal antibody (AG019) and enhanced chemiluminescence substrate were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-human protein kinase C δ (PRKCD) polyclonal antibody (ab86800) and rabbit anti-nuclear factor (NF)-κB polyclonal antibody (ab16502) were purchased from Abcam (Cambridge, UK). The Takara PrimeScript RT and Regent SYBR PrimeScript RT-PCR kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from whole blood cells was extracted using TRIzol LS reagent. Reverse transcription was performed using a Takara PrimeScript RT kit according to the manufacturer's instructions. qPCR was performed to measure the levels of miR-26a, CCL2 mRNA and CCL7 mRNA and the Regent SYBR PrimeScript RT-PCR kit was used to perform qPCR following the manufacturer's instructions. U6 was used as a reference gene for miR-26a and GAPDH was used as a reference gene for CCL2 and CCL7. Primer sequences are listed in Table I. The reaction system consisted of the following: cDNA (2 μl), Buffer Mix (containing enzymes, 10 μl), forward primer (0.5 μl), reverse primer (0.5 μl) and 17 μl H_2O. To detect miR-26a, CCL2 and CCL7, PCR was performed under the following cycling conditions: 95˚C for 10 min, followed by 40 cycles of denaturing at 95˚C for 30 sec and primer annealing at 60˚C for 30 sec. Levels of miR-26a, CCL2 mRNA and CCL7 mRNA were calculated using the $2^{-\Delta\Delta Cq}$ method (20).

Transfection in HUVECs. HUVECs were seeded in 24-well plates at a density of $2x10^5$ cells/well 24 h at 37˚C prior to transfection. Cells were transfected with 0.5 μg of GV227-PRKCD-Neo-EGFP and 25 pmol of miR-26a mimics (Hanbio, Shanghai, China). Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions and cells were harvested 48 h later.

PRKCD small interfering (si)RNA was purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). PRKCD siRNA was transfected into HUVECs using Lipofectamine 2000 according to the manufacturer's instructions. Follow-up experiments were performed 48 h later. Cells were observed using an inverted fluorescent microscope (IX 73; Olympus Corp., Tokyo, Japan).

Western blot analysis. HUVECs were lysed in radio immunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Protein was determined by BCA assay, and 20 μg protein was separated by 12.5% SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20 for 1 h at room temperature and subsequently incubated with antibodies against PRKCD (1:800) or GAPDH (1:5,000) overnight at 4˚C. Subsequently, membranes were incubated with a secondary antibody horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig)G antibody (cat. no. BS12478; 1:5,000; Bioworld Technology, Inc., St. Louis Park, MN, USA) for 1 h at room temperature and washed with PBST 5 min three times. The protein chemiluminescence was then detected using enhanced chemiluminescence substrate and analyzed by Quantity one V 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Dual-luciferase reporter gene assay. The target gene of miR-26a was predicted using TargetScan (www.targetscan.org/). The miR-26a binding sequence and mutation region sequence in 3'-UTR of PRKCD were synthesized according to bioinformatic analysis (21) and then cloned into pMIR-REPORT plasmids (Thermo Fisher Scientific, Inc.). The two types of constructed plasmids and miR-26a mimics were respectively co-transfected into HEK293 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). The fluorescence value of each group was detected 24 h later using the dual-luciferase reporter gene system (Promega Corporation; Madison, WI, USA) according to the manufacturer's instructions and was compared with *Renilla* luciferase activity for normalization.

Statistical analysis. Data were expressed as the mean + standard deviation. Statistical analyses were performed using the two-tailed Student's t-test (paired) and SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The clinical value of miR-26a in the diagnosis of DVT was evaluated using a receiver operating characteristic (ROC) curve. In addition, Spearman's rank correlation analysis was conducted to determine the correlation between miR-26a and CCL2 and CCL7 levels. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

Primer	Direction	Primer sequences (5'-3')
miR-26a	Forward	CAAGUAAUCCAGGAUAGG
	Reverse	GGCCAACCGCGAGAAGATGTTTTTTTTT
CCL2	Forward	CCA ACTCCTGCCTCCGCTCTA
	Reverse	TGCAGATCTGGGTTGTGGAG
CCL7	Forward	CTGACCCCACACAGAAGTGG
	Reverse	CCCCATGAGGTAGAGAAGGGA
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
GAPDH	Forward	CGGAGTCAACGGATTTGGTCGTAT
	Reverse	AGCCTTCTCCATGGTGGTGAAGAC

miR-26a, microRNA-26a; CCL, chemokine C-C motif ligand.

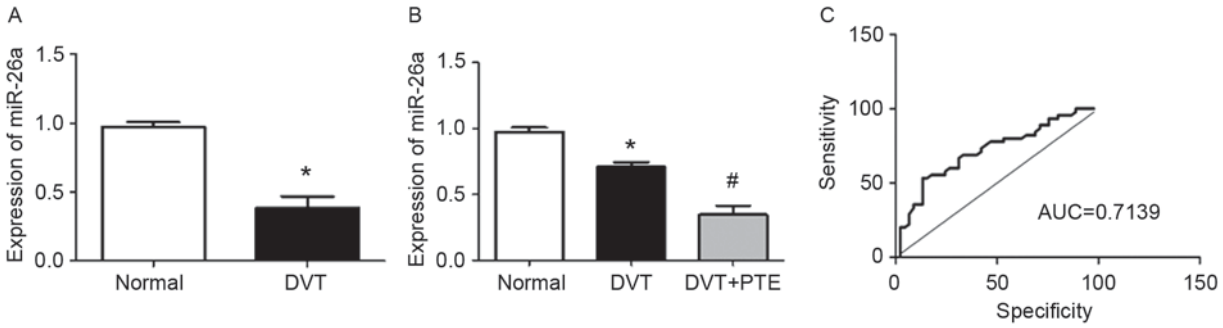

Figure 1. Levels of miR-26 in the peripheral blood of patients with DVT. The expression of miR-26 was detected by reverse transcription-quantitative polymerase chain reaction. (A) Levels of miR-26 in the peripheral blood of patients with DVT compared with normal controls. (B) Levels of miR-26 in the peripheral blood of patients with DVT complicated by PTE, patients with DVT alone and normal controls. *P<0.05 vs. normal group; #P<0.05 vs. DVT group. (C) Receiver operating characteristic curve indicated the value of miR-26a in the clinical diagnosis of DVT. miR-26, microRNA-26; DVT, deep vein thrombosis; PTE, pulmonary thromboembolism.

Results

Expression of miR-26 in the peripheral blood of patients with DVT. The results of RT-qPCR indicated that levels of miR-26a were significantly downregulated in the peripheral blood of patients with DVT compared with normal controls (P<0.05; Fig. 1A). In addition, levels of miR-26a in the peripheral blood of patients with DVT complicated with pulmonary embolism (DVT + PTE) were significantly lower than in patients with DVT alone (P<0.05; Fig. 1B). These results demonstrate that the downregulated expression of miR-26a may be associated with DVT.

Analysis of ROC curve. To determine the role of miR-26a in the diagnosis of DVT, a ROC curve was generated. The results of the ROC curve analysis revealed that the largest area under the curve (AUC) was 0.7136 (P<0.01; Fig. 1C), suggesting that miR-26a may be useful in the clinical diagnosis of DVT.

Correlation of CCL2 and CCL7 mRNA levels with miR-26a. CCL2 and CCL7 are important inflammatory chemokines that recruit monocytes to specified lesions, promote the release of local inflammatory factors and aggravate the inflammatory response (22,23). In the present study, the results from RT-qPCR suggested that levels of CCL2 and CCL7 mRNA were significantly increased in the peripheral blood of patients with DVT compared with the controls (P<0.05; Fig. 2A). Spearman's rank correlation analysis results indicated that the expression of miR-26a was negatively correlated with levels of CCL2 and CCL7 mRNA in the peripheral blood of patients with DVT (P<0.05; Fig. 2B and C, respectively). These results indicate that miR-26a may be involved in the regulation of CCL2 and CCL7 mRNA levels.

miR-26a-PRKCD-NF-κB signaling pathway regulates the expression of CCL2 and CCL7. Bioinformatics analysis revealed that there was a conservative binding domain of miR-26a in the 3'-UTR of PRKCD mRNA, indicating that miR-26a may regulate the expression of PRKCD. It has been demonstrated that PRKCD activates the NF-κB signaling pathway, promotes the expression and release of CCL2 and CCL7 and increases the local inflammatory response (24). Furthermore, it has been demonstrated that the inflammatory response is associated with the occurrence of DVT (25). Therefore, the effects of miR-26a on PRKCD, NF-κB-related protein and levels of CCL2 and CCL7 mRNA were examined

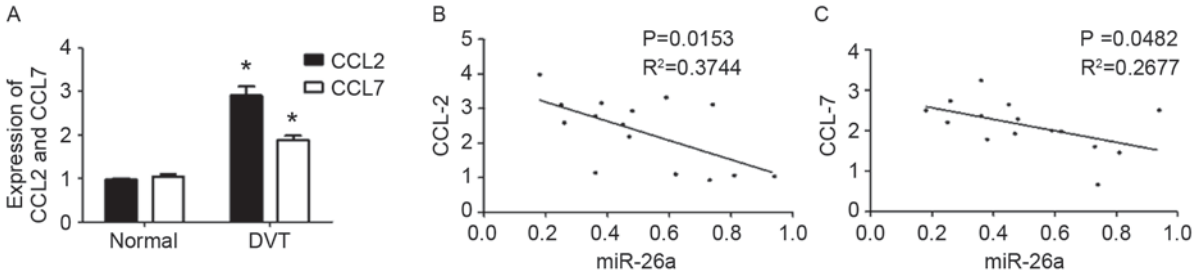

Figure 2. Levels of CCL2 and CCL7 mRNA and their correlation with miR-26a. (A) Levels of CCL2 and CCL7 mRNA in the peripheral blood of patients with DVT and normal individuals were measured using reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. corresponding normal group. (B) Correlation of CCL-2 mRNA with miR-26. (C) Correlation of CCL-7 mRNA with miR-26. DVT, deep vein thrombosis; miR-26, microRNA-26; CCL, chemokine C-C motif ligand 2.

in the current study (Fig. 3). The results determined that the expression of PRKCD was markedly decreased following the overexpression of miR-26a in HUVECs (Fig. 3A). In addition, levels of CCL2 and CCL7 mRNA were significantly decreased in miR-26a-overexpressed HUVECs compared with the control (P<0.05; Fig. 3B). The phosphorylation of p65 protein was markedly decreased in miR-26a-overexpressed HUVECs compared with the control (Fig. 3A), suggesting that the NF-κB signaling pathway was inactivated. However, overexpression of PRKCD in HUVECs attenuated the effects of miR-26a on p65 phosphorylation (Fig. 3D). Green fluorescence indicated the transfection efficiency (Fig. 3C). These results suggest that miR-26a may suppress the expression of PRKCD and inhibit the NF-κB signaling pathway, thus inhibiting CCL2 and CCL7 expression.

siR-PRKCD inhibits the activation of NF-κB. To further determine the effect of PRKCD on the NF-κB signaling pathway, PRKCD expression was reduced by siRNA. Western blotting indicated that the expression of PRKCD was markedly decreased following the transfection of siR-PRKCD (Fig. 3E). Similarly, the phosphorylation of p65 was markedly downregulated following a decrease in the expression of PRKCD (Fig. 3E), suggesting that the NF-κB signaling pathway was inhibited. Furthermore, levels of CCL2 and CCL7 mRNA were significantly decreased in siR-PRKCD-treated HUVECs compared with the control (P<0.05; Fig. 3F). These results suggest that PRKCD regulates the NF-κB signaling pathway and decreases the expression of CCL2 and CCL7.

Dual-luciferase reporter gene assay. The results of the dual-luciferase reporter gene assay (Fig. 4) demonstrated that fluorescence was significantly reduced following co-transfection of miR-26a mimic and luciferase reporter gene plasmid pMIR-REPORT-wild-type (P<0.01; Fig. 4B). However, there was no difference in fluorescence following co-transfection of miR-26a mimic and pMIR-REPORT-mutant (Fig. 4B). These results suggest that miR-26a is able to bind the specific domain in the 3'-UTR of PRKCD mRNA.

Discussion

In the present study, the expression of miR-26a was significantly downregulated in the peripheral blood of patients with DVT. The AUC of the ROC curve was 0.7136, suggesting that miR-26a may be valuable in the clinical diagnosis of DVT. Furthermore, the results of the present study indicated that miR-26a targeted PRKCD mRNA and inhibited the activation of the NF-κB signaling pathway, thus downregulating the expression of CCL2 and CCL7 mRNA and attenuating the local inflammatory response. Therefore, it was hypothesized that the reduction of miR-26a levels may increase levels of inflammatory cytokines in the peripheral blood of patients with bone trauma and increase the risk of DVT.

DVT is a common clinical disease and its incidence, morbidity and mortality rates have increased in recent years (26). Clinical manifestations of DVT are primarily lower limb swelling, ulceration and necrosis, which may seriously limit patient quality of life (27). Detachment of thrombi may cause pulmonary embolism and lead to patient mortality (21). Inflammation is a major of the risk factors of DVT (28,29). Various studies have demonstrated that levels of inflammatory cytokines and chemokines are increased in the peripheral blood of patients with DVT. Du and Tan (10) identified that certain inflammatory factors including IL-6, CRP and NF-κB are closely associated with DVT. Krieger *et al* (30) suggested that changes in CRP levels and the erythrocyte sedimentation coefficient may induce acute lower extremity DVT. miRNAs are post-transcription regulating factors and therefore may serve an important role in inflammation. It was reported by Sawant *et al* (31) that miR-21 targets B-cell lymphoma 6 mRNA and promotes the differentiation of T helper 2 cells. Lu *et al* (32) demonstrated that miR-376b regulates the expression of IL-6. Furthermore, it was determined that miR-26a is involved in the differentiation of T cells and regulation of the NF-κB signaling pathway (33). The present study indicated that levels of miR-26a were significantly downregulated in the peripheral blood of patients with DVT and negatively correlated with CCL2 and CCL7 expression. Additionally, bioinformatics analysis identified that miR-26a regulated the expression of PRKCD. Previous results demonstrated that PRKCD activates the NF-κB signal pathway and promotes the expression and release of inflammatory cytokines (23). Therefore, it was hypothesized that the downregulation of miR-26a in the peripheral blood of patients with DVT may be associated with inflammation.

To investigate this hypothesis, miR-26a was overexpressed in HUVECs and the expression of PRKCD was markedly

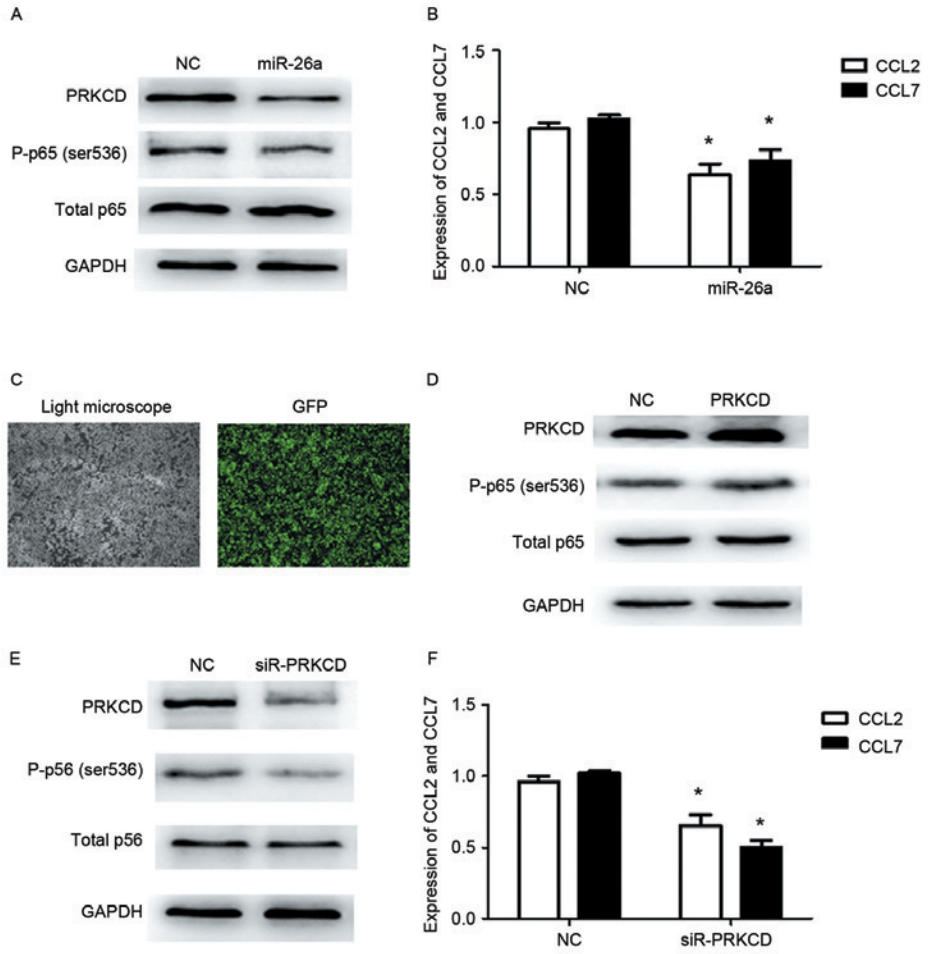

Figure 3. miR-26a regulates the expression of PRKCD, CCL2, CCL7 and p65. (A) Effect of miR-26a on the expression of PRKCD and p-p65 was assessed using western blotting. GAPDH was used as an internal control. (B) The effect of miR-26a on the levels of CCL2 and CCL7 mRNA was detected by RT-qPCR. (C) Fluorescence was detected following transfection with PRKCD expression vector. Magnification, x400. (D) Effect of PRKCD overexpression on the phosphorylation of p65 was detected by western blotting. (E) Effect of PPKCD knockdown on the expression of p65 and p-p65 was assessed by western blotting. GAPDH was used as an internal control. (F) Effect of PRKCD knockdown on the levels of CCL2 and CCL7 mRNA was detected by RT-par. *P<0.05 vs. NC. NC, negative control; p-p65, phosphorylated p65; miR-26, microRNA-26; CCL, chemokine C-C motif ligand 2; PRKCD, protein kinase C δ; GFP, green fluorescent protein; siR, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

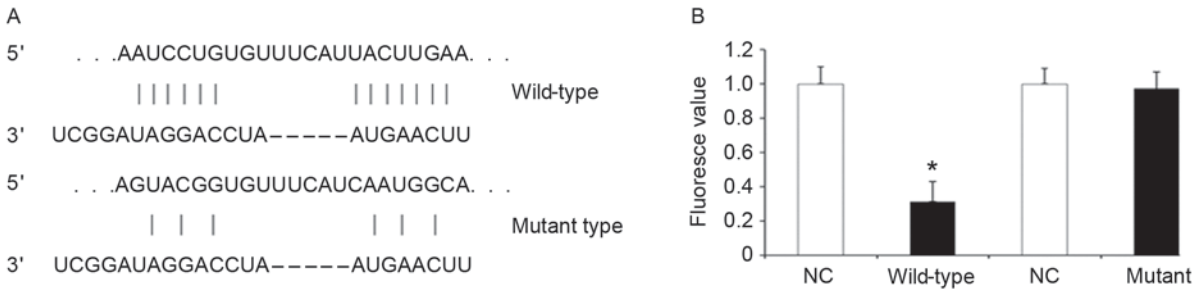

Figure 4. Identification of the miR-26a target gene using a dual-luciferase reporter gene assay. (A) Wild-type and mutant PRKCD sequences. (B) Fluorescence intensity changed when miR-26a mimic cells were co-transfected with pMIR-REPORT-wild-type or pMIR-REPORT-mutant type. *P<0.05 vs. NC. PRKCD, protein kinase C δ; NC, negative control.

decreased. In addition, levels of CCL2 and CCL7 mRNA were significantly decreased. The results from western blotting suggested that miR-26a was able to inhibit activation of the NF-κB signaling pathway. Reduction of PRKCD expression by siRNA also attenuated activation of the NF-κB signaling pathway. A previous study demonstrated that activation of NF-κB promoted the expression and release of CCL2 and CCL7 (34). CCL2 and CCL7 are able to recruit inflammatory cells to specific lesions, promote the release of local inflammatory factors and aggravate the inflammatory response (35). The results of the dual-luciferase reporter gene assay conducted in the current study indicated that miR-26a was able to bind to the 3'-UTR of PRKCD, demonstrating that PRKCD is a target gene of miR-26a. This suggests that the downregulation of

miR-26a increased PRKCD expression, activating the NF-κB signaling pathway and promoting the expression of CCL2 and CCL7.

In conclusion, the current study demonstrated that miR-26a may be valuable in the clinical diagnosis of DVT as novel biomarker. miR-26a may inactivate the NF-κB signaling pathway by binding PRKCD mRNA and may reduce the risk of DVT by inhibiting the inflammatory response.

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