

Expression variations and clinical significance of MMP-1, MMP-2 and inflammatory factors in serum of patients with deep venous thrombosis of lower extremity

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Abstract. Expression levels and clinical significance of matrix metalloproteinase-1 (MMP1), MMP-2 and inflammatory factors in the serum of patients with deep venous thrombosis (DVT) of lower extremity were investigated. Fifty untreated DVT patients were selected as the DVT group, and 50 patients undergoing health examination were enrolled as the normal control group. Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of MMP-1, MMP-2, interleukin-6 (IL-6), IL-8 and tumor necrosis factor- α (TNF- α) in the serum. Western blotting was adopted to detect the expression levels of MMP-1 and MMP-2 proteins. Fluorescent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was applied to examine the messenger ribonucleic acid (mRNA) expression levels. Moreover, the circumferences of the patients were measured. The difference between the circumference of affected extremity and unaffected extremity was calculated. Correlation analysis was conducted separately for the levels of serum MMP-1, MMP-2, IL-6, IL-8 and TNF- α of patients in the DVT group. In the DVT group, the levels of MMP-1, MMP-2, IL-6, IL-8, and TNF- α at 7 days after treatment were significantly lower than those before treatment ($P < 0.01$). Compared with that before treatment, the circumference difference of the affected and unaffected extremities of the patients was reduced at 7 days after treatment ($P < 0.01$). The levels of IL-6, IL-8 and TNF- α were positively correlated with the levels of MMP-1 and MMP-2, respectively in the DVT group ($P < 0.05$ or $P < 0.01$). MMP-1, MMP-2 and inflammatory factors play an important role in the occurrence and development of DVT, of which the levels of IL-6, IL-8 and TNF- α are positively correlated with the levels of MMP-1 and

MMP-2, respectively. Therefore, monitoring the concentration of MMP-1, MMP-2 and inflammatory factors is of significant value for the diagnosis, progression and judgement of treatment effect of DVT in clinical practice.

Introduction

There is a high incidence rate of deep venous thrombosis (DVT) of lower extremity in China, and the pulmonary thromboembolism formed by detachment of thrombus is the leading cause for death of people (1). Studies have suggested that inflammatory reaction plays a key role in the occurrence and development of DVT, and that inflammatory cytokine is a bioactive peptide that not only acts as a signal transduction factor, but also performs as an effector molecule (2). Inflammatory factors can directly cause injuries of endothelial cells, and they can also promote the release of inflammatory factors by the blood coagulation system and further accelerate the inflammatory reaction and blood coagulation, thus having very close relationships with the blood coagulation, anti-coagulation and fibrinolysis processes (3).

Matrix metalloproteinases (MMPs) is a category of enzymes with zinc ion as the prosthetic group, which can degrade extracellular matrix proteins (4). Under the normal physiological status of tissues, the expression of MMPs is at a low level, but changes in the expression proportion of the MMPs to their inhibitory factors may lead to pathological responses such as inflammation, neovascularization and neoplasm metastasis (5). MMP-1 and MMP-2 belong to gelatinases, and their activation plays a vital role in the process of thromboembolism (4,6). Increasing the MMP-2 gene expression levels or activating enzyme activities may involve the venous thromboembolism (7). MMP-1, MMP-2 and activator of plasminogen are regarded as key factors for the occurrence and development of venous diseases of the lower extremities (8). The relative expression levels of MMP and its inhibitor can adjust inflammation and thrombosis (6).

Some scholars believe that MMP-1, MMP-2 and inflammatory factors may play key roles in the occurrence and development of DVT (9). To investigate the effects of MMP-1, MMP-2 and inflammation-associated factors on DVT, a total of 50 patients with DVT of lower extremity admitted and

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Key words: deep venous thrombosis of lower extremity, MMP-1, MMP-2, inflammatory factors

Table I. Comparison of general data.

Characteristics	DVT group (n=50)	Normal control group (n=50)	P-value
Age (years)	55.72±11.46	52.35±10.38	>0.05
Sex [n (male/female)]	27/23	28/22	>0.05
Weight (kg, mean ± SD)	61.56±9.05	64.84±10.53	>0.05

treated in the Department of Vascular Surgery of People's Hospital of Jiyang (Jinan, China) and another 50 volunteers receiving health examination were selected. The concentrations of MMP-1, MMP-2, interleukin-6 (IL-6), IL-8 and tumor necrosis factor- α (TNF- α) in the serum were tested, respectively, and the expression levels of MMP-1 and MMP-2 proteins as well as IL-6, IL-8 and TNF- α messenger ribonucleic acids (mRNAs) in peripheral blood mononuclear cells (PBMCs) of DVT patients were determined. In the meantime, the circumference at 15 cm above the knee and 10 cm below the knee of both extremities were measured before treatment and at 7 days after treatment, and the difference between the circumferences of unaffected and affected extremities was calculated. In addition, correlation analysis was conducted respectively for the levels of MMP-1, MMP-2, IL-6, IL-8 and TNF- α in the serum of patients in the DVT group, aiming to explore the functions of these factors in DVT.

Materials and methods

General data. Primer synthesis, reverse transcription kit and real-time fluorescent quantitative polymerase chain reaction (PCR) kit (Takara Biotechnology Co., Ltd., Dalian, China), TRIzol kit (Ambion; Thermo Fisher Scientific, Dallas, TX, USA). Rabbit anti-human MMP-1, MMP-2, glyceraldehyde-3-phosphate dehydrogenase (gAPDh) primary polyclonal antibodies and goat anti-rabbit horseradish peroxidase (hRP)-labeled secondary polyclonal antibody (cat nos. 10371-2-AP, 10373-2-AP, 10494-1-AP, SA00001-2; (Proteintech Group, Inc., Wuhan, China), bicinchoninic acid (BCA) protein assay kit and cell lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China).

A total of 50 DVT patients diagnosed in the Department of Vascular Surgery of People's Hospital of Jiyang from February 2016 to February 2017 were selected as the DVT group, including 27 males and 23 females aged 25-57 years, with an average age of 55.72±11.46 years. Inclusion criteria: i) patients who had DVT onset within 7 days, ii) patients who had similar disease conditions and iii) patients who had venous flow obstruction of the lower extremity which was confirmed by the antegrade venography of deep vein of lower extremity. Patients with the following conditions were excluded: pregnancy, infection, tumor, inherited anticoagulation factor deficiency, vascular abnormality or diabetes mellitus. Before treatment, all the patients had such clinical manifestations as high muscle tension and swelling in their affected extremities, and the DVT of unilateral lower extremity was verified via color Doppler ultrasound or venography examinations

Table II. Primer sequences of RT-qPCR.

Genes	Primer sequences
<i>IL-6</i>	F: 5'-CTGTTGTTGCTGTGGCTGAT-3' R: 5'-TCCGTCCACAAGCAATGAGT-3'
<i>IL-8</i>	F: 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' R: 5'-TCTCAGCCCTCTTCAAAAACCTTCTC-3'
<i>TNF-α</i>	F: 5'-TACTGAACCTTCGGGGTGATTGGTCC-3' R: 5'-CAGCCTTGTCCTTGAAGAGAACC-3'
<i>GAPDH</i>	F: 5'-ATGGCACCGTCAAGGCTGAG-3' R: 5'-GCAGTGATGGCATGGACTGT-3'

F, forward; R, reverse.

after admission to hospital. Another 50 healthy individuals receiving health examination in People's Hospital of Jiyang were enrolled as the normal control group which included 28 men and 22 women aged 26-58 years, with an average age of 52.35±10.38 years. They were all healthy adults without cardiac, hepatic, cerebral, renal and infectious diseases or diabetes mellitus. There were no statistically significant differences in the age, gender and weight between the two groups ($P>0.05$) (Table I).

The study was approved by the Ethics Committee of People's Hospital of Jiyang. Signed informed consents were obtained from the patients or guardians.

Clinical treatment. The patients with DVT of lower extremity were treated with continuous intravenous infusion of 200,000 units of urokinases for 5 days from the first day after the diagnosis was confirmed. Moreover, 4,000 units of low molecular weight heparin was injected subcutaneously twice daily, and 100 mg aspirin, an anti-platelet aggregation drug, was orally administered once daily.

Acquisition and detection of serum specimens. A total of 5 ml fasting venous blood was collected in the morning from each patient in the DVT group before treatment and at 7 days after treatment as well as each healthy person in the normal control group, respectively. The blood was added into a vacuum blood collection tube without any anticoagulant and centrifuged at 4,000 × g 4°C for 15 min. After that, the supernatant was absorbed carefully and then stored in a refrigerator at -80°C for standby use.

Enzyme-linked immunosorbent assay (ELISA) was performed to measure the levels of MMP-1, MMP-2, IL-6, IL-8 and TNF- α in the serum in accordance with the steps recommended in the kit instructions.

Isolation and culture of patient PBMCs. Before treatment and at 7 days after treatment, 10 ml fasting venous blood was drawn in the morning from each patient in the DVT group, and Ficoll-Paque PLUS was utilized to isolate the PBMCs. After the cells were cultured with Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% serum in an incubator with 5% CO₂ at 37°C for 2 h, the suspended cells were washed

Table III. Concentration of serum MMP-1, MMP-2 and inflammatory factors in the DVT and normal control groups before treatment.

Groups	MMP-1 ($\mu\text{mol/l}$)	MMP-2 ($\mu\text{mol/l}$)	IL-6 ($\mu\text{g/l}$)	IL-8 ($\mu\text{g/l}$)	TNF- α ($\mu\text{g/l}$)
DVT	3.255 \pm 0.572	4.073 \pm 0.546	0.152 \pm 0.038	0.797 \pm 0.155	54.37 \pm 11.48
Control group	0.642 \pm 0.127	0.926 \pm 0.283	0.094 \pm 0.012	0.344 \pm 0.087	16.21 \pm 3.772
P-value	<0.01	<0.01	<0.01	<0.01	<0.01

P<0.01 vs. the normal control group.

Table IV. Concentration of serum MMP-1, MMP-2 and inflammatory factors in patients of the DVT group before and after treatment.

Groups	MMP-1 ($\mu\text{mol/l}$)	MMP-2 ($\mu\text{mol/l}$)	IL-6 ($\mu\text{g/l}$)	IL-8 ($\mu\text{g/l}$)	TNF- α ($\mu\text{g/l}$)
Before treatment	3.255 \pm 0.572	4.073 \pm 0.546	0.152 \pm 0.038	0.797 \pm 0.155	54.37 \pm 11.48
After treatment	1.591 \pm 0.382	1.952 \pm 0.457	0.094 \pm 0.012	0.459 \pm 0.146	34.52 \pm 8.971
P-value	<0.01	<0.01	<0.01	<0.01	<0.01

P<0.01 vs. before treatment.

out, and the adherent cells obtained were the PBMCs, which were adopted for subsequent experiments.

Detection of MMP-1 and MMP-2 protein expression levels in patient PBMCs via western blotting. Patient PBMCs were digested with trypsin and then collected, which were lysed using the lysis buffer and centrifuged at 3,000 x g for 10 min at 4°C to collect the supernatant protein. The protein concentration was determined by virtue of the BCA protein assay kit, and 30 μg protein loading in each specimen was taken for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to a polyvinylidene fluoride (PVDF) membrane, sealing with blocking buffer for 2 h and addition of MMP-1, MMP-2 and GAPDH primary antibodies (dilution, 1:1,000) for incubation in the refrigerator at 4°C overnight. Next, the membrane was washed with Tris-buffered saline Tween-20 (TBST) 3 times, and then the secondary antibodies (dilution, 1:1,200) were added for incubation at room temperature for 2 h, followed by membrane washing with TBST 3 times. After that, the membrane was placed in enhanced chemiluminescence (ECL) liquid (MilliporeSigma, Burlington, MA, USA) for development in the dark, which was scanned and recorded by means of a gel imager for gray analysis. The densitometry was analyzed by Image J professional image analysis software (NIH, Bethesda, MD, USA).

Detection of mRNA expression levels of IL-6, IL-8 and TNF- α in patient PBMCs via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The PBMCs of the patients collected after trypsin digestion were applied to extract the total RNA of the cells via the TRIzol kit. Then the samples with an absorbance ratio (A_{260}/A_{280}) of 1.8-2.0 were selected for reverse transcription, followed by PCR with complementary deoxyribonucleic acid (cDNA) as the template. The primer sequences are shown in Table II, and the reaction conditions

are as follows: pre-denaturation at 94°C for 3 min and then at 95°C for 1 min, annealing at 50°C for 50 sec, extension at 72°C for 1 min, a total of 40 cycles of amplification and extension for 10 min. With GAPDH mRNA as the control, the experimental results were analyzed using the $2^{-\Delta\Delta C_q}$ method (10).

Measurement of circumference of the lower extremity. The circumferences at 15 cm above the knee and 10 cm below the knee of both extremities were measured while the blood samples of the DVT patients were collected. The difference between the circumference of affected extremity and unaffected extremity was calculated, and the circumference difference of the affected and unaffected extremities was compared before and after treatment.

Statistical analysis. Statistical Product and Service Solutions (SPSS) 17.0 software (IBM Corp., Armonk, NY, USA) was adopted for data processing. Measurement data were presented as mean \pm standard deviation, and t-test was used for inter-group comparison. Linear (Pearson's) correlation analysis was utilized to analyze correlations, and $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of levels of serum MMP-1, MMP-2 and inflammatory factors. The ELISA results indicated that the levels of MMP-1, MMP-2, IL-6, IL-8 and TNF- α in the serum of the DVT group were remarkably higher than those of the normal control group before treatment, and the differences were statistically significant ($P < 0.01$) (Table III).

After 7 days of treatment, the levels of serum MMP-1, MMP-2, IL-6, IL-8 and TNF- α in the DVT group were markedly lower compared with those before treatment, showing statistically significant differences ($P < 0.01$) (Table IV).

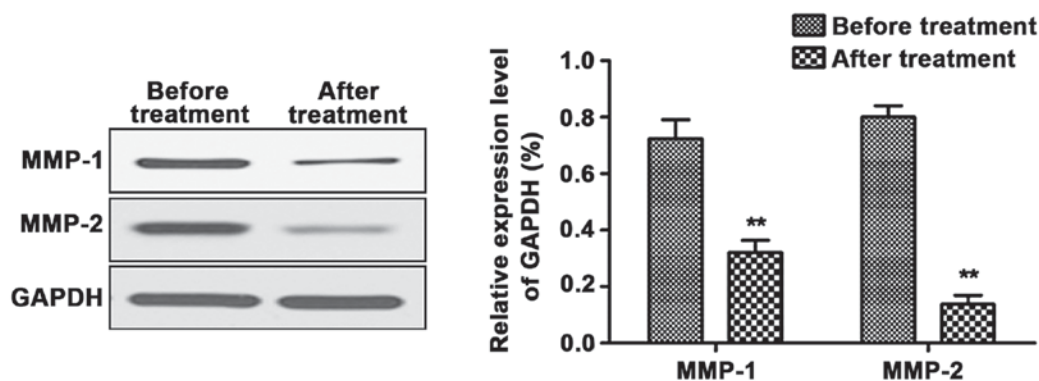


Figure 1. Expression levels of MMP-1 and MMP-2 proteins in PBMCs of the patients detected by western blotting. ** $P<0.01$ vs. before treatment.

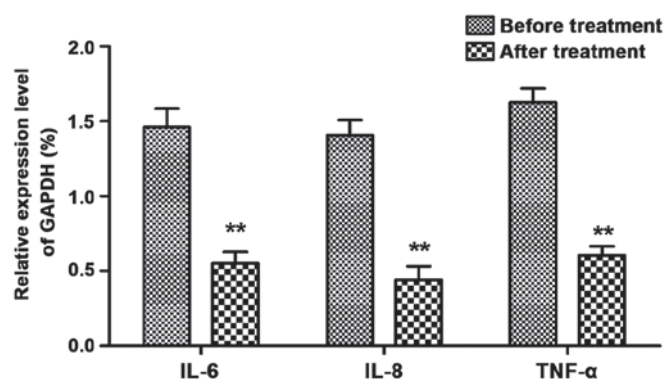


Figure 2. Expression levels of IL-6, IL-8 and TNF- α mRNAs in PBMCs of the patients detected by RT-qPCR. ** $P<0.01$ vs. before treatment.

Table V. Comparison of circumference of the affected and unaffected extremities of patients in the DVT group before and after treatment.

Groups	15 cm above the knee	10 cm below the knee
Before treatment	6.22 \pm 1.74	3.03 \pm 0.68
After treatment	2.84 \pm 0.76	3.16 \pm 0.92
P-value	<0.01	<0.01

$P<0.01$ vs. before treatment.

Expression levels of MMP-1 and MMP-2 proteins in PBMCs of the patients before and after treatment. The results of western blotting revealed that compared with those before treatment, the expression levels of MMP-1 and MMP-2 proteins in the PBMCs were decreased notably after treatment, and the differences were statistically significant ($P<0.01$) (Fig. 1).

Expression levels of IL-6, IL-8 and TNF- α mRNAs in PBMCs of the patients before and after treatment. As shown in Fig. 2, the RT-qPCR results manifested that the expression levels of IL-6, IL-8 and TNF- α mRNAs in the PBMCs declined compared with those before treatment, with statistically significant difference ($P<0.01$).

Table VI. Correlation of serum IL-6, IL-8 and TNF- α with MMP-1 and MMP-2 of patients in the DVT group.

Correlation coefficient	MMP-1	MMP-2
IL-6	0.551 ^b	0.638 ^b
IL-8	0.493 ^a	0.674 ^b
TNF- α	0.533 ^b	0.573 ^b

^a $P<0.05$, ^b $P<0.01$.

Comparison of circumference of the affected and unaffected extremities of patients in the DVT group before and after treatment. The degree of limb swelling of the DVT patients was alleviated obviously after treatment, and the difference between the circumferences of unaffected extremity and affected extremity of the patients was decreased remarkably at 7 days after treatment in comparison with that before treatment, displaying statistically significant difference ($P<0.01$) (Table V).

Correlation analysis of MMP-1 and MMP-2 with inflammatory factors in the serum of patients in the DVT group. The levels of IL-6, IL-8, TNF- α , MMP-1 and MMP-2 in the serum of DVT patients were recorded for Pearson's correlation analysis. It was indicated that the serum IL-6, IL-8 and TNF- α levels were positively correlated with MMP-1 and MMP-2 levels of patients in the DVT group, respectively ($P<0.05$ or $P<0.01$) (Table VI).

Discussion

Color Doppler ultrasound is a reliable and effective method for diagnosing DVT, with the advantages of noninvasive and repeatable examinations. However, imaging examinations still cannot determine whether fibrinolysis reaction exists in the human body. Therefore, studying thromboembolism at the molecular level and monitoring the expression levels of relevant molecules in the DVT progression have become new research hotspots (11).

In recent years, studies worldwide have demonstrated that inflammation participates in the venous thromboembolism. Inflammation can cause venous wall injury and induce

thrombosis which can further stimulate apparent inflammatory reactions on the venous wall (12). Inflammation is stimulated and regulated by cytokines to some extent, and relatively high levels of inflammatory cytokines in the blood are important risk factors for the venous thromboembolism (13). It is discovered in research that such inflammatory cytokines as IL-6, IL-8 and TNF- α are involved in the process of venous thromboembolism (14).

Studies on thrombus have revealed that IL-6 can significantly increase the expression levels of cell adhesion molecules [cluster of differentiation molecule 11b (CD11b)/CD18] and directly decrease the transcription and translation of L-selectin (CD62L) (15). IL-8 is a key factor for venous thromboembolism, and research has manifested that IL-8 is directly implicated in the thrombosis, accelerates thrombolysis and ultimately induces neovascularization (16). TNF- α is a type of inflammatory cytokine secreted by mononuclear macrophages and eosinophils. Studies have discovered that TNF- α is able to affect the adhesion and migration of leukocytes and influence the amount of thrombus generated at the same time (17). The content of serum TNF- α can indicate the degree of inflammation and tissue injuries in the body and regulate the growth and differentiation of multiple cells. Moreover, it can control the life activities of the cells by virtue of their autocrine (18).

Studies have suggested that inflammatory cytokines are capable of regulating the expression levels of MMP genes, whose major process is that they activate the activating transcription factor 2 and C-transcriptase via ceramide signaling pathway and then activate the activator protein-1 (AP-1), followed by binding to the AP-1 sites on the MMP genes, thus elevating the transcription levels of the MMP genes (19). It is found through research that blood stasis and venous hypertension can induce increase in MMP-2 expression, which is able to trigger degradation of the extracellular matrix, thus damaging the venous wall (20). In addition, platelets adhered to exposed collagens may lead to activation of MMP-1, thus directly lysing the protease-activated receptor type 1 (PAR1) of the platelets (21).

In order to investigate the roles of MMP-1, MMP-2 and inflammation-associated cytokines in DVT, a total of 50 patients with DVT of the lower extremity admitted and treated in the Department of Vascular Surgery of People's Hospital of Jiyang and 50 volunteers undergoing health examinations were selected to detect the concentrations of MMP-1, MMP-2, IL-6, IL-8 and TNF- α in their serum. The results indicated that before treatment, the levels of serum MMP-1, MMP-2, IL-6, IL-8 and TNF- α of the DVT group were remarkably higher than those of the normal control group, and that after treatment, the levels of serum MMP-1, MMP-2, IL-6, IL-8 and TNF- α in the DVT group were markedly lower compared with those before treatment. Meanwhile, the expression levels of MMP-1 and MMP-2 proteins as well as IL-6, IL-8 and TNF- α mRNAs in the PBMCs of the DVT patients were detected before and after treatment. It was manifested that the expression levels of MMP-1 and MMP-2 proteins as well as IL-6, IL-8 and TNF- α mRNAs in the PBMCs were decreased notably after treatment. Moreover, the circumference at 15 cm above the knee and 10 cm below the knee of both extremities of the patients were measured before treatment and at 7 days after treatment, and the results showed

that the difference between the circumferences of unaffected extremity and affected extremity of the patients was decreased remarkably after treatment in comparison with that before treatment. Pearson's correlation analysis demonstrated that the serum IL-6, IL-8 and TNF- α had positive correlation with MMP-1 and MMP-2 levels of patients in the DVT group, respectively.

In conclusion, MMP-1, MMP-2 and inflammatory factors play important roles in the occurrence and development of DVT, of which the levels of IL-6, IL-8 and TNF- α are positively correlated with the levels of MMP-1 and MMP-2, respectively. Therefore, determining the concentrations of MMP-1, MMP-2, IL-6, IL-8 and TNF- α in the peripheral blood is of significant value in clinical practice for the diagnosis, progression and treatment effect judgement of DVT.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TZ drafted the manuscript. TZ and QL were mainly devoted to ELISA. TZ and LW performed RT-qPCR. LW and GL were responsible for measurement of circumference of the lower extremity. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of People's Hospital of Jiyang (Jinan, China). Signed informed consents were obtained from the patients or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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