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ORIGINAL RESEARCH

Study on hsa_circ_101209 in Plasma of Pregnant Women with Deep Venous Thrombosis

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Background: This study analyzed the expression and diagnostic value of hsa_circ_101209 in plasma of pregnant women with in deep vein thrombosis (DVT).

Methods: By circRNA microarray detection and GO/KEGG analysis, hsa_circ_14797 targeting miRNA-mRNA network was predicted. Sixty women with DVT were selected as the DVT group, and 60 women without DVT as the non-DVT group. hsa_circ_14797 in plasma was detected, as well as D-dimer (D-D) concentration and P-selectin expression. Target genes that may be regulated by hsa_circ_14797 were predicted, and GO analysis and KEGG pathway enrichment analysis were performed.

Results: hsa_circ_14797 was highly expressed in DVT. hsa_circ_14797 in plasma of DVT patients was positively correlated with D-D (r = 0.358, $P = 0.001$). The AUC of plasma hsa_circ_14797, D-D, and P-selectin for maternal DVT diagnosis were 0.787 (95% CI: 0.710–0.864), 0.882 (95% CI: 0.821–0.943), and 0.825 (95% CI: 0.754–0.895), respectively. The AUC of hsa_circ_14797 combined with D-D was 0.886 (95% CI: 0.828–0.944). The AUC of hsa_circ_14797 combined with P-selectin was 0.904 (95% CI: 0.853–0.954). The AUC of P-selectin combined with D-D was 0.935 (95% CI: 0.893–0.978). The AUC of hsa_circ_14797 combined with D-D and P-selectin was 0.953 (95% CI: 0.920–0.986). The functions of hsa_circ_14797 included the biological processes of angiogenesis, vascular development, and vascular morphology. The enrichment pathways included PI3K-Akt pathway, TGF-β pathway, and cytokine-cytokine receptor interaction.

Conclusion: hsa_circ_14797, D-D, and P-selectin in plasma of DVT pregnant patients are increased, and hsa_circ_14797 in plasma is positively correlated with D-D. hsa_circ_14797 combined with D-D and P-selectin can improve the accuracy of diagnosis of DVT and contribute to the early diagnosis of DVT.

Keywords: Deep vein thrombosis, circRNA, D-dimer, P-selectin, Biomarkers

Introduction

The term "deep vein thrombosis" (DVT) refers to abnormal clotting of blood in deep veins that primarily affects lower extremities.[1] During DVT, lower venous return pressure leads to blood clotting. After the onset of DVT, there is a high risk of embolism, which can be disabling or even life-threatening in severe cases.[2] Women in late pregnancy have a higher risk of DVT which will not only lead to diseases such as hypertension but also local vascular obstruction and necrotic lesions.[3] The increase of serum D-dimer (D-D) level during pregnancy may be related to continuous blood hypercoagulability during placental development, which is one of the reference indicators for clinical evaluation of coagulation function status, and D-D can also participate in embolic diseases.[4]

Clinically, clinical manifestations, laboratory testing, and imaging examinations, are mainly used for diagnosing DVT. Despite its high accuracy, venography is an invasive examination that some patients have an allergic reaction to. D-D, a degradation product of cross-linked fibrin, has been widely used in clinical diagnosis of venous thrombus embolism, with high sensitivity but low specificity.[5,6] Blood D-D levels are elevated during stroke, atrial fibrillation, inflammatory diseases, and disseminated intravascular coagulation.[7] P-selectin, a cell adhesion molecule in the selectin family, is located on the membrane of platelet α particles and endothelial Weibel-Palade bodies. Soluble P-selectin refers to

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P-selectin free in plasma, which can reflect the degree of platelet activation.[8] P-selectin level is not only a risk factor for DVT but also a reliable indicator for the diagnosis of DVT with high specificity.[9] However, the optimal threshold value of plasma P-selectin for the diagnosis of DVT in different laboratories has not been uniformly determined and needs further exploration.

CircRNA is widely found in human peripheral blood, exosomes, and even cell-free saliva. CircRNA is generated by the splicing receptor sites and donor sites of the upstream and downstream exons of pre-mRNA through the interaction of reverse splicing.[10,11] During the splicing process, the same gene can produce different circRNAs through selective cycling, resulting in the diversity of circRNAs. Studies of homology between different species indicate that circRNA has highly conserved properties and tissue specificity in species evolution. circRNA can act as a "sponge" for miRNA or RNA-binding proteins, participate in regulating intermolecular signal communication, and competitively bind to inhibit downstream gene expression.[12] CircRNA has high conservation, stability, tissue specificity, and universality in mammals, and has functions such as transcriptional regulation and protein translation.[13,14] CircRNA is associated with human diseases and important regulatory pathways, including angiogenesis related diseases, cancer, ischemic heart disease, degenerative diseases, and more.[15,16] To date, numerous studies have shown that circRNAs are closely associated with the diagnosis and treatment of cardiovascular diseases, and certain circRNAs have been identified as cardiovascular disease biomarkers.[17,18] Recently, circRNAs have been reported to accelerate lower extremity DVT via sponging miRNA, thus regulating mRNA expression.[19] Sun et al showed that differentially expressed circRNAs is dynamically involved in DVT development.[20] However, the role of circular RNA in DVT and its regulatory mechanism remain unclear.

This research delved into the impact and function of hsa_circ_101209 on pregnant women with DVT by examining their clinical traits, lab markers, and risk elements, seeking to establish a theoretical framework for DVT treatment in expectant mothers.

Materials and Methods

Patient Data

Fifty pregnant women with DVT who were admitted to The Affiliated Hospital of Yunnan University from May 2018 to October 2023 were selected as the study objects and set into the DVT group. Another 50 pregnant women who did not develop DVT during the same period were set into the non-DVT group for retrospective analysis. All pregnant women ranged in age from 22 to 42 years, with an average age of (31.67 ± 5.69) years. The average gestational age was (39.67 ± 1.37) weeks. BMI was 18~28 kg/m^2, with average BMI of (24.98 ± 2.35) kg/m^2. This study was approved by the medical Ethics Committee of The Affiliated Hospital of Yunnan University (No. 201708YN15).

Inclusion criteria: ① The pregnant women in the DVT group met the diagnostic criteria for DVT in the Guidelines for Diagnosis and Treatment of Deep Vein Thrombosis,[3] and were accompanied by sudden swelling of the affected limb, pain, increased soft tissue tension, and local tenderness; ② Gestational age > 28 weeks; ③ Regular pregnancy test; (4) Complete clinical data.

Exclusion criteria: ① Severe organ dysfunction at admission; ② A history of DVT; ③ Autoimmune diseases; ④ Uterine placental dysplasia.

Methods

Clinical data such as age, number of pregnancies, number of deliveries, BMI, body weight gain, gestational week of delivery, blood loss, and fetal weight were collected, and serum D-D and P-selectin levels were detected. On admission, 5 mL venous blood was taken from pregnant women and centrifuged at 3000 r/min for 10 min. After serum separation, D-D and P-selectin levels were detected by an automatic hemagglutination analyzer (CS-5100; Sysmex, Japan).

Outcome Measures

① The single factor of DVT in pregnant women in the third trimester. ② The value of serum D-D in predicting DVT in pregnant women in the third trimester. ③ Independent influencing factors of DVT in late pregnancy.

RT-qPCR

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR assay was performed by SYBR Premix Ex Taq (Takara, Japan) with ABI Prism 7900HT rapid real-time PCR system (Applied Biosystems, Life Technologies, USA). Using GAPDH as a loading control, hsa_circ_101209 expression was calculated by $2^{-\Delta\Delta Ct}$ method. The primer sequence (GenePharma, Shanghai, China) is shown in Table 1.

Statistical Analysis

SPSS22.0 statistical software was employed to process the data. Measured data are expressed as the mean ± standard deviation. Unpaired data with a normal distribution and homogeneity of variance between two groups were compared with unpaired *t*-tests, and multiple groups were compared with one-way analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered to indicate statistically significant difference.

Results

Basic Characteristics

DVT and non-DVT groups differed statistically significantly in age and blood loss, but not in other indicators ($P > 0.05$, As Shown in Table 2).

circRNA Expression Profile in the Plasma of Pregnant Women with DVT and Non-DVT

Using a high-throughput human circRNA chip, a box plot was constructed to compare circRNA expression profiles. The distribution of log2 ratios across all samples was similar, regardless of whether the samples were from patients with DVT or non-DVT controls (As Shown in Figure 1A). Based on hierarchical chemical clustering, the 3 DVT patients showed distinct circRNA expression profiles compared with the 3 non-DVT controls (As Shown in Figure 1B). This suggests that circRNA expression profile in patients with DVT is different from that in healthy controls. hsa_circ_101209 was selected for the follow-up experiment.

Table 1 qPCR Primers

Gene	Sequence (5'-3')
hsa_circ_101209	Forward: TTCTTCCCAAGCCTGGCATC Reverse: CACCTGAAAATGCGTCCACC
GAPDH	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA

Table 2 Comparison of Baseline Data Between DVT Group and Non-DVT Group (x ± s)

Indicators	DVT group	Non-DVT group	Z	P
Age	34.99. 11 ± 3.54	32.28 ± 4.66	−4. 00	0. 045
Number of pregnancies	2.62 ± 1.41	2.41 ± 1.41	−1. 13	0. 273
Number of deliveries	0.69 ± 0.67	0.58 ± 0.12	−1. 39	0. 181
BMI (kg/m^2)	23.79 ± 3.75	22.89 ± 4.01	−1. 76	0. 072
Body weight gain (kg)	12.01 ± 4.16	13.05 ± 3.98	−1. 57	0. 141
Gestational week of delivery	38.67 ± 1.11	39.04 ± 1.02	−1. 31	0. 211
Blood loss (mL)	367.99 ± 135.01	334. 89 ± 135. 12	−2. 43	0. 052
Fetal weight (g)	3341.65 ± 524. 01	3354.32 ± 455.01	−0. 14	0. 889

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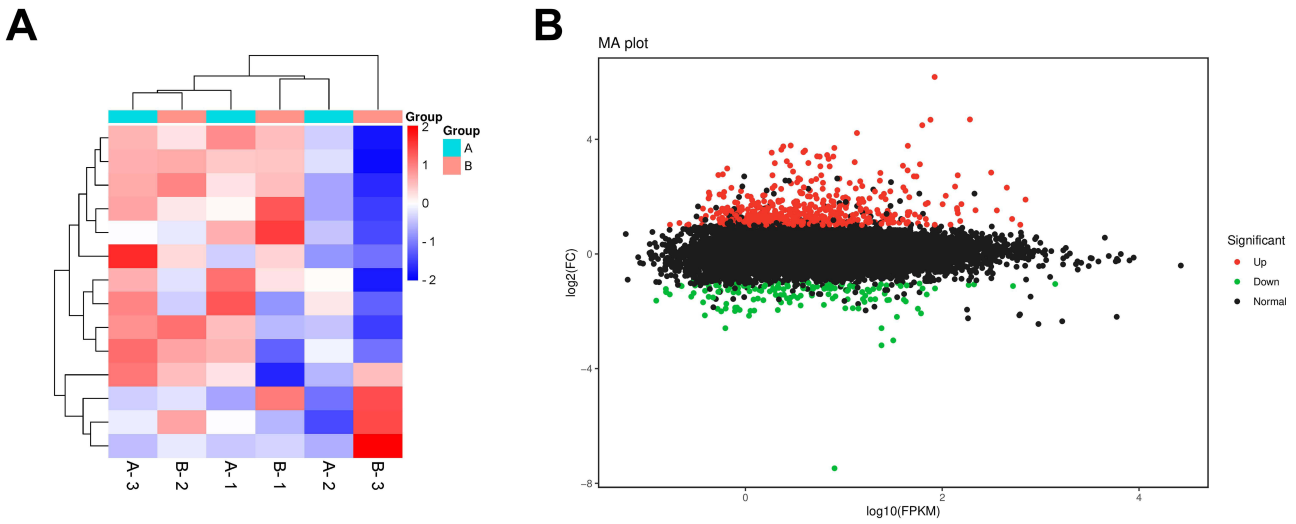

Figure 1 Hsa_circ_101209 in plasma of maternal patients with DVT and non-DVT patients. (**A**) Volcano maps showed that the differential expression of circular RNA had different P-values and folding changes. (**B**) Hierarchical cluster analysis showed that circRNA expression levels could be distinguished in related heat maps.

hsa_circ_101209 in the Plasma of Pregnant Women with DVT and Non-DVT Patients

RT-qPCR showed that plasma hsa_circ_101209 in the non-DVT group was 0.102 (0.082, 0.115). Plasma hsa_circ_101209 in the non-DVT group was 0.245 (0.145, 0.352), higher than the non-DVT group ($P < 0.001$). The change of hsa_circ_101209 in the plasma of DVT patients was 1.79 times compared with the non-DVT group (As Shown in Figure 2). Studies have shown that changes in circRNA multiples of more than 1.5 times may have a significant impact on cell biology. Therefore, plasma hsa_circ_101209 may be helpful for the diagnosis of DVT in pregnant women.

Diagnostic Value of Plasma hsa_circ_101209 in Maternal DVT Patients

ROC analysis and AUC calculation were performed on the diagnosis of maternal DVT by hsa_circ_101209. Youden index method determined the cutoff value. The AUC of plasma hsa_circ_101209 for diagnosis of DVT in pregnant women was 0.901 (95% CI: 0.842–0.963). With a cutoff value of 0.172, plasma hsa_circ_101209 maternal DVT had

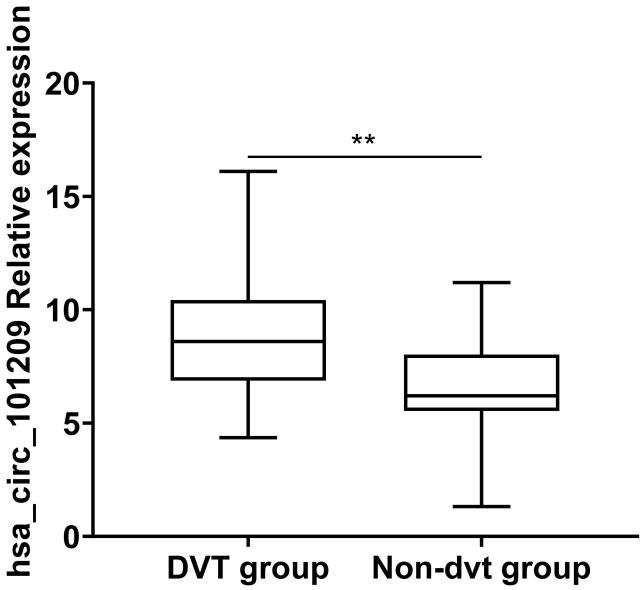

Figure 2 Hsa_circ_101209 in plasma of maternal patients with DVT and non-DVT patients. Data are expressed as mean ± SD Compared with non-DVT patients,* P<0.05, **P<0.01.

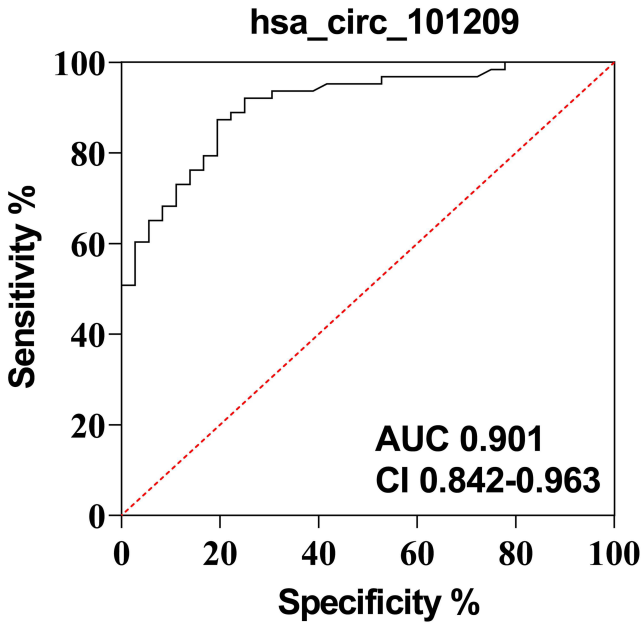

Figure 3 ROC curve analysis of hsa_circ_101209 in maternal DVT patients.

a sensitivity of 71.3% and specificity of 77.2% (As Shown in Figure 3). The results showed that hsa_circ_101209 could distinguish maternal DVT patients from non-DVT patients to some extent.

Plasma D-D in Pregnant Women with and without DVT

The plasma concentration of D-D in the non-DVT group was 0.455 (0.115, 0.753) mg/L, and 1.846 (0.942, 3.412) mg/L in maternal DVT patients. D-D plasma concentration was higher than that in the non-DVT group ($P < 0.001$) (As Shown in Figure 4A). Further analysis of the diagnostic value of plasma D-D in maternal DVT patients showed that AUC was 0.745 (95% CI: 00.647–0.889). A cutoff value of 0.508 mg/L yielded 94.8% sensitivity and 65.9% specificity for D-D (As Shown in Figure 4B).

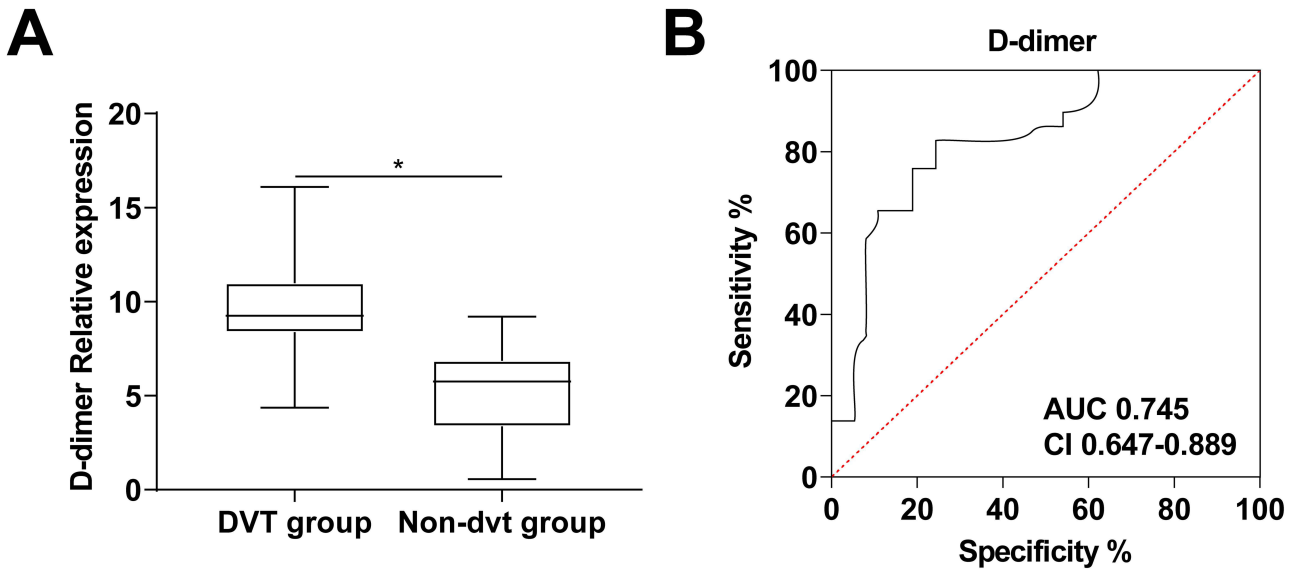

Figure 4 D-D in plasma of pregnant women with and without DVT. (**A**) D-D in plasma of pregnant women with and without DVT.Data are expressed as mean ± SD. Compared with non-DVT patients,* P<0.05,**P<0.01 (**B**) ROC curve analysis of D-D in maternal DVT patients.

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Plasma P-Selectin in Maternal DVT Patients and Non-DVT Patients

The concentration of soluble P-selectin in plasma of maternal patients with DVT was 50.431 ± 13.732 ng/mL, and that of non-DVT patients was 34.212 ± 10.245 ng/mL. Compared with the non-DVT group, soluble P-selectin was higher in the DVT group ($P < 0.001$) (As Shown in Figure 5A). The AUC of plasma P-selectin was 0.886 (95% CI: 0.822–0.970), and P-selectin concentration 37.02 ng/mL was used as the cutoff value for DVT diagnosis with 83.2% and 71.8% sensitivity and specificity, respectively (As Shown in Figure 5B).

Correlation Analysis of hsa_circ_101209 with D-D and P-Selectin

Correlation analysis of hsa_circ_101209, D-D, and P-selectin was carried out. hsa_circ_101209 in plasma was positively correlated with the concentration of D-D in the maternal DVT population (r = 0.358, $P = 0.001$) (As Shown in Figure 6A). hsa_circ_101209 levels and P-selectin levels had no statistically significant correlation (r = −0.072, $P = 0.524$) (As Shown in Figure 6B).

Diagnostic Value of hsa_circ_101209, D-D, and P-Selectin in Maternal DVT

In the diagnosis of maternal DVT, hsa_circ_101209 had high specificity. Binary Logistic regression was used to generate the new variable prediction probability of the joint index, and then the multivariable ROC curve was constructed. The combination of hsa_circ_101209 with D-D and P-selectin can improve the sensitivity and specificity of single index in the diagnosis of maternal DVT (Table 3). AUC reached 0.843 with a sensitivity and specificity of 89.3% and 91.2%, respectively, for DVT (As Shown in Figure 7A–D).

Target Gene and Function Prediction Analysis of hsa_circ_101209

Based on the gene regulation of hsa_circ_101209, the study of hsa_circ_101209 is helpful to explore the pathogenesis of DVT. Therefore, we conducted target gene prediction for hsa_circ_101209 in the database ENCOR1 (As Shown in Figure 8). GO studies revealed that the top 100 circRNAs with varied expression in DVT patients are intimately linked to protein transportation, ATP binding, and cytoplasmic activity. The KEGG study revealed key pathways of enrichment encompassing the thyroid hormone pathway, tumor proteoglycan, endocytosis, focal adhesion, FC-γR-mediated

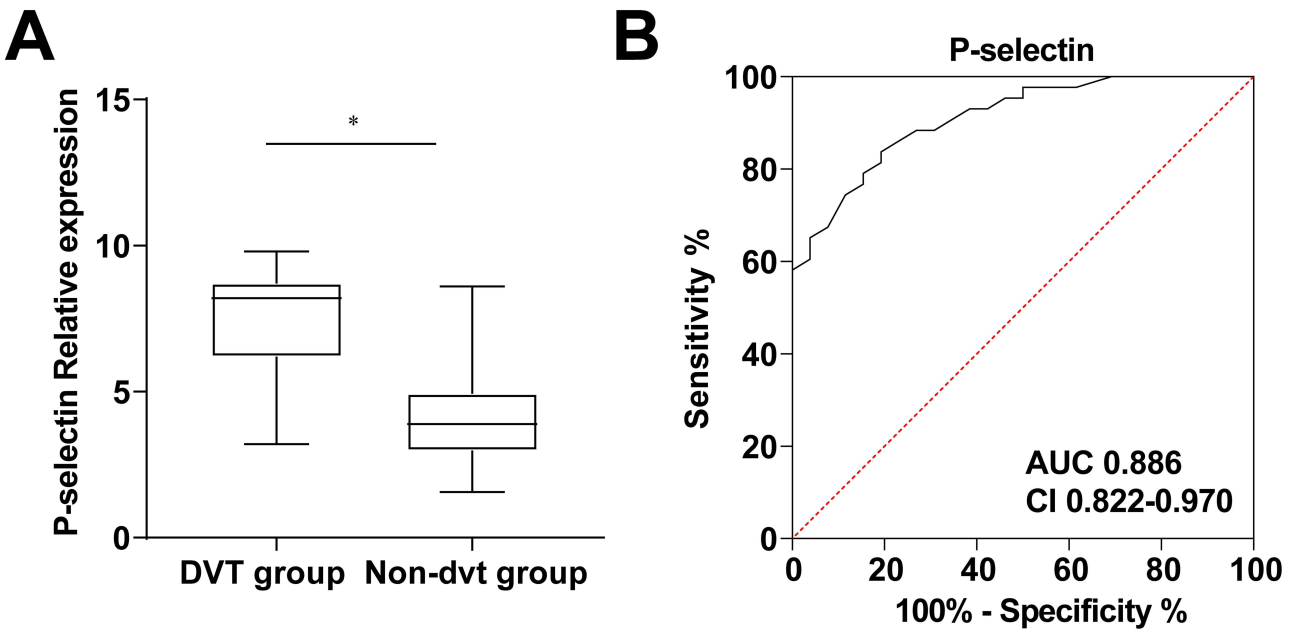

Figure 5 P-selectin in plasma of maternal DVT patients and non-DVT patients (**A**) P-selectin in plasma of pregnant women with and without DVT. Data are expressed as mean ± SD. Compared with non-DVT patients,* P<0.05,**P<0.01 (**B**) ROC curve analysis of P-selectin in maternal DVT patients.

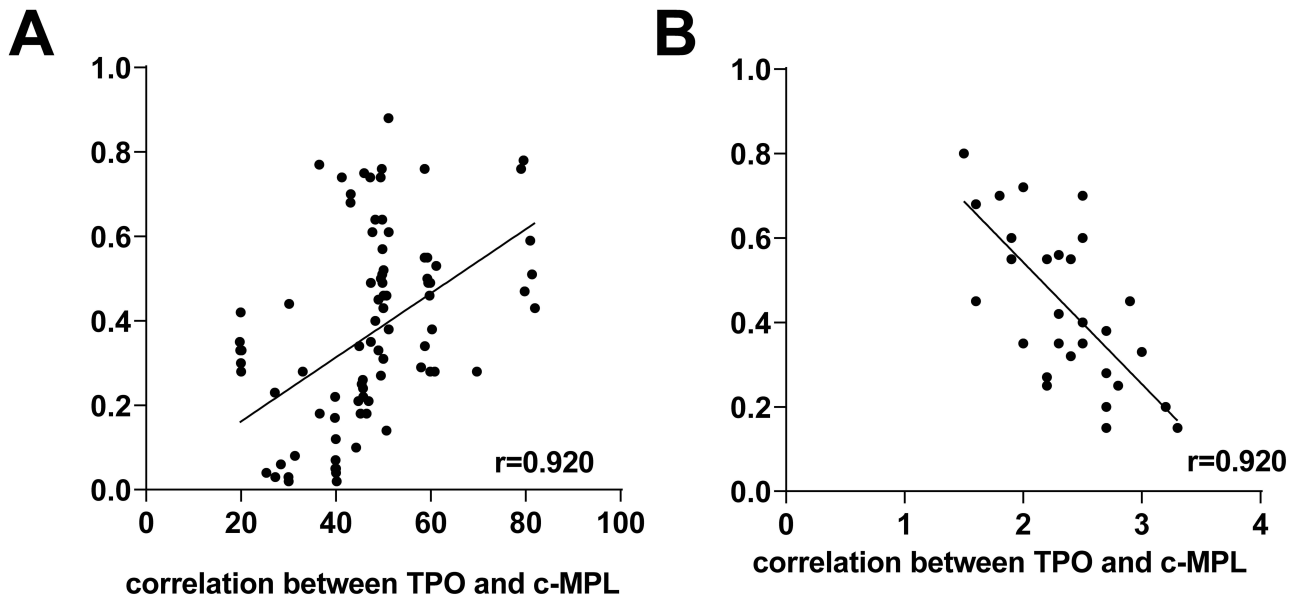

Figure 6 Correlation analysis of hsa_circ_101209 with D-D and P-selectin. (**A**) Correlation analysis between plasma hsa_circ_101209 and D-D. (**B**) Correlation analysis of hsa_circ_101209 and P-selectin in plasma.

phagocytosis, p53 pathway, insulin signaling pathway, antibiotic biosynthesis, AMP-activated protein kinase pathway, and bacterial invasion of epithelial cells.[19]

Discussion

In the third trimester of pregnancy, due to changes in the coagulation system and anticoagulation system in the body, the coagulation function is relatively hyperactive, resulting in a higher risk of DVT.[21] Pulmonary embolism and cerebral artery embolism caused by DVT may cause clinical death in pregnant women in the third trimester,[22] but there are few clinical studies on the influencing factors of DVT in the third trimester. Early diagnosis and treatment can improve DVT and reduce mortality. Therefore, it is of great significance to explore its clinical characteristics and influencing factors for targeted prevention and treatment to reduce the incidence of DVT.

circRNAs function as biomarkers for human diseases[23,24] and play crucially in various pathologic and physiological processes related to apoptosis, metabolism, and inflammation.[25,26] This study identified several circRNAs in DVT by using microarray technology. A total of 10 up-regulated target molecules were identified among the differentially expressed circRNAs based on the |average normalized fold change| > 2, the circRNA characteristics, and the parental genes, among which hsa_circ_101209 was the most significantly upregulated. hsa_circ_101209 was significantly upregulated in 50 maternal DVT patients.

D-D and P-selectin are the most widely studied molecular markers for the diagnosis of DVT. D-D reflects the overall activity of blood coagulation and fibrinolysis processes.[27] Clinically, clinicians use D-D as part of the diagnostic process

Table 3 Single and Combined Diagnostic Value of Hsa_circ_101209, P-Selectin, and D-Dimer in Maternal DVT

Indicators	Sensitivity	Specificity	Youden Index	AUC	95% CI	P value
circ_101209	71.30%	77.20%	0.445	0.905	0.842–0.963	< 0.001
D-dimer	94.80%	65.90%	0.612	0.745	0.647–0.889	< 0.001
P-selectin	83.20%	71.80%	0.534	0.886	0.822–0.970	< 0.001
circ_101209 + D-dimer	81.80%	85.10%	0.639	0.905	0.842–0.963	< 0.001
circ_101209 + P-selectin	82.50%	85.80%	0.679	0.842	0.834–0.977	< 0.001
D-dimer + P-selectin	85.30%	89.10%	0.748	0.912	0.812–0.975	< 0.001
circ_101209 + P-selectin + D-dimer	89.30%	91.20%	0.776	0.843	0.801–0.952	< 0.001

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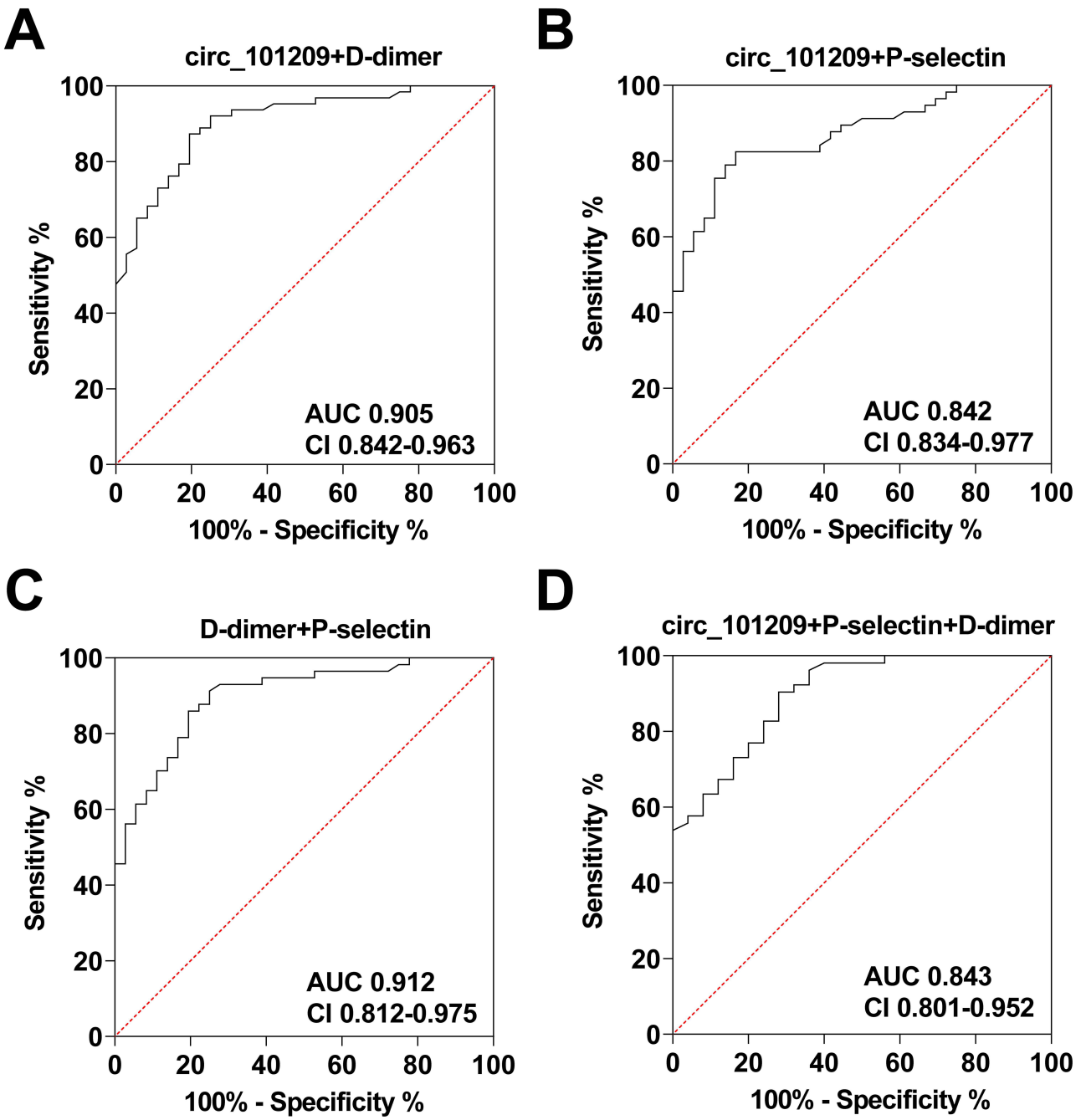

Figure 7 Comparison of diagnostic value of hsa_circ_101209, D-dimer and P-selectin in maternal DVT (**A**) hsa_circ_101209+D-dimer. (**B**) hsa_circ_101209+ p-selectin. (**C**) d-dimer + P-selectin. (**D**) sa_circ_101209 + D-dimer + P-selectin.

to rule out DVT or PE. Recent studies have found that the increase of plasma D-D concentration can not only be used for the diagnosis of the first onset of VTE, but also for predicting the risk of VTE and evaluating clinical prognosis.[28,29] VTE patients with low D-D levels at first diagnosis have a low risk of recurrence.[30] In a variety of tumor diseases, high concentrations of D-D are associated with secondary VTE.[31,32] It can also be employed to stratify patients at risk of acute PE based on D-D. Becattini et al[33] conducted a meta-analysis on the correlation between D-D concentration and mortality in acute PE patients and reported that patients with acute PE with high D-D concentrations were at greater risk of short-term and 3-month mortality.

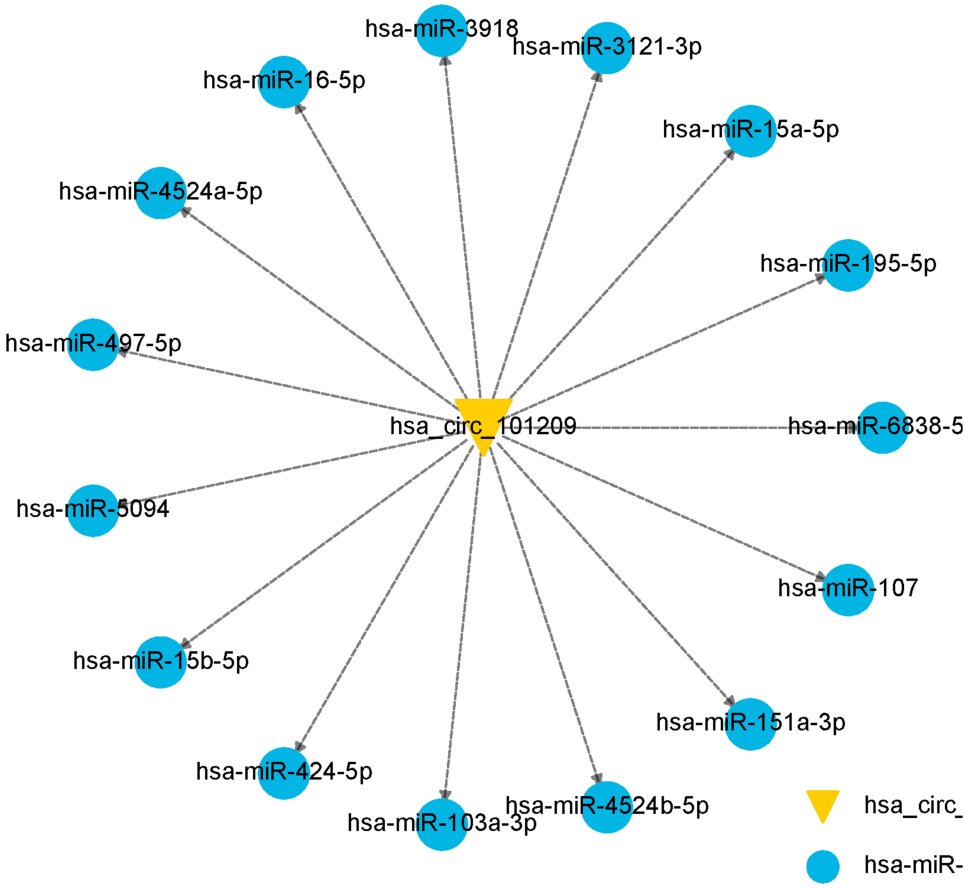

Figure 8 Target gene prediction of hsa_circ_101209 in ENCORI database.

Soluble P-selectin is involved in the regulation of thrombosis, hemostasis, and platelet-endothelial cell interaction by promoting the release of pro-coagulant particles, thromboxane A2, and matrix metalloproteinase-2.[34] In recent years, studies on P-selectin in VTE have confirmed that soluble P-selectin is elevated in plasma in patients with acute VTE.[35,36] Plasma concentrations of P-selectin and D-D in patients with acute DVT are elevated and greatly affected by vitamin K antagonist therapy, and the concentrations could increase again after the withdrawal of oral anticoagulants.[37] When P-selectin concentration shows high diagnostic specificity and sensitivity in DVT.[38] However, when different methods are used to detect P-selectin levels in different studies, the optimal critical value obtained is not uniform, and further exploration is needed.

hsa_circ_101209, P-selectin, and D-D were increased in plasma of maternal DVT patients compared with non-DVT patients, indicating that these indicators have certain diagnostic values for maternal DVT. hsa_circ_101209 was positively correlated with D-D levels in plasma of maternal DVT patients and had a certain diagnostic ability for maternal DVT patients. The combination of hsa_circ_101209 with D-D and P-selectin could improve the diagnosis accuracy of maternal DVT. Based on the gene regulation of hsa_circ_101209, the study of hsa_circ_101209 may provide new ideas for exploring maternal DVT.

In our study, some limitations were encountered. First, thrombosis is a dynamic process. Factors such as the time interval between sampling and onset, underlying disease, or anticoagulant therapy may affect thrombosis development. Therefore, it may be more meaningful to dynamically monitor the changes of plasma hsa_circ_101209 before and after the onset of disease and during treatment in the same population. Secondly, only one hsa_circ_101209 was selected for study in this experiment. Considering that hsa_circ_101209 can regulate multiple genes, and one gene can also be regulated by multiple hsa_circ_101209, studying the differential expression of a group of circRNAs in maternal DVT may be more helpful for disease diagnosis. Follow-up of maternal DVT patients and comparison of the changes of indicators before and after the onset of maternal DVT patients may provide more information for disease detection and prevention.

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Limitations of This Study

In this study, the sample size was insufficient, and in the future, it is necessary to expand the sample size to explore the role of hsa_circ_101209 in DVT. This study not only introduces new non-invasive biomarkers for the diagnosis of maternal DVT, but also conducts more in-depth research. It helps to further understand the value of hsa_circ_101209 in the pathogenesis, diagnosis, and prognosis evaluation of postpartum DVT.

Conclusion

Plasma hsa_circ_101209 has the potential to diagnose maternal DVT. The combined application of hsa_circ_101209, D-D, and P-selectin can improve the diagnostic accuracy of maternal DVT. hsa_circ_101209 is positively correlated with D-D level. The study of hsa_circ_101209 in DVT not only introduces a new non-invasive biomarker for the diagnosis of maternal DVT but also provides a new idea for exploring maternal DVT pathogenesis and treatment.

Data Sharing Statement

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

Ethics Approval

The present study was approved by the Ethics Committee of The Affiliated Hospital of Yunnan University (No. 201708YN15) and written informed consent was provided by all patients prior to the study start. All procedures were performed in accordance with the ethical standards of the Institutional Review Board and The Declaration of Helsinki, and its later amendments or comparable ethical standards.

Informed Consent

Written informed consent was obtained from each subject.

Funding

There is no funding to report.

Disclosure

The authors have no conflicts of interest to declare in this work.

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