

Original Article

miR-155 for diagnosing thromboangiitis obliterans and its effect on platelet function: a clinical study

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Abstract: Objective: To investigate the effect of miR-155 on the diagnosis of thromboangiitis obliterans and on platelet function. Method: A prospective study was conducted among 88 patients with thromboangiitis obliterans (TAO group) and 90 healthy subjects (control group). The relative expression levels of serum miR-155, P-selectin (CD62p) and platelet activated complex-1 (PAC-1) were measured. Results: The relative expression of miR-155 in the TAO group was significantly higher than that of the control group. Receiver operator characteristics analysis showed that the optimal diagnostic threshold of miR-155 was 22.82, with Yoden index of 79.6%, sensitivity of 81.8%, and specificity of 97.8%. As the stage of thromboangiitis obliterans got advanced, the serum level of miR-155 in patients became higher. The expression levels of miR-155 in patients at three stages were all higher than those in the healthy control subjects (all $P < 0.01$). As the stage of thromboangiitis obliterans got advanced, higher expression levels of CD62p and PAC-1 were detected in serum (both $P < 0.001$). Pearson correlation coefficients showed that serum miR-155 was positively correlated with CD62p and PAC-1 ($r = 0.637$, $r = 0.620$, both $P < 0.001$). Conclusion: The miR-155 is highly expressed in patients with thromboangiitis obliterans, so it is valuable for the diagnosis. Moreover, miR-155 can lead to the occurrence or progression of thromboangiitis obliterans, and may be related to the expressions of CD62p and PAC-1.

Keywords: Alteplase, acute cerebral infarction, clinical efficacy, inflammatory factors, neurological function

Introduction

Thromboangiitis obliterans, or Buerger's disease, is essentially non-suppurative inflammation and thrombotic lesion in the vascular lumen, which mostly occurred in the small and medium arteries of the extremities [1-3]. The pathogenesis of thromboangiitis obliterans is still unclear, and it is a difficult and complicated disease in the vascular surgery department [4]. Epidemiological study shows that thromboangiitis obliterans attacks people all over the world, with its favorite population of young and middle-aged male who smoke, but the morbidity in Middle East is 16-66% which is significantly higher than 0.5-5.6% in the European and American countries [5]. To date, there is no cure for this disease, because the existing drugs can only be applied to slow the progression, and surgical interventions or even amputation are necessary when the drug treatment does not function well [6, 7].

Thromboangiitis obliterans is an autoimmune disease closely related to smoking [8, 9]. With

the development of gene detection technology, it has been found that some non-coding RNAs (miRNAs) can up-regulate or down-regulate the transcriptional level of genes [10, 11]. The miRNAs are closely involved in the occurrence and progression of tumors, development of embryos, differentiation of cells and cerebral nerve, and occurrence of cardiac diseases [12-14]. The stable expression of miRNA, including miR-155, in human body fluid implies its enormous research potential [15]. Studies on miRNAs have also shown its close relevance with the occurrence and progression of autoimmune diseases [16, 17]. There was a correlation between miR-155 and rheumatoid arthritis and active systemic lupus erythematosus ($r = 0.652$ and $r = 0.781$) [18, 19]. Additionally, the average expression of miR-155 in peripheral blood of patients with thromboangiitis obliterans can reach 30.66, and the relative expression level of miR-155 in patients with thromboangiitis obliterans was 2.22 times higher than that of healthy subjects, which activated the platelet activity [20].

Thrombosis is prone to occur in the vascular lumen of patients with thromboangiitis obliterans, during which platelets play an important role [3]. After the activation of platelets, the release of various platelet factors and immunoinflammatory mediators PAC-1 and CD62-P can lead to platelet aggregation, thus promoting thrombosis [21, 22]. The platelet aggregation rate can directly indicate the platelet aggregation function, which is better than the conventional tests of blood routine and coagulation function [23]. CD62P, also known as P-selectin, is the main marker of platelet activation, which aggravates the hypercoagulability in patients, and plays an important role in the occurrence and progression of venous thrombosis [24]. PAC-1 only binds to activated platelets, not to resting platelets. After the activation of platelet, the excessive expression of PAC-1 on platelet membrane leads to the conformational change of PAC-1, and the enhanced binding affinity to fibrinogen and other ligands, which brings about platelet aggregation and thrombosis [25]. It has been found that platelets contain abundant and well-functioning miRNAs, a sort of multi-inclusion microsomes, which can be transported in cellular tissues and plays a regulatory role [26, 27]. The miRNAs are also involved in the regulation of platelet function, thereby affecting the occurrence and progression of platelet-related diseases [28]. Study found that miR-146a can promote the activation and aggregation of platelets, which could be a newly discovered mechanism for thromboangiitis obliterans [23]. It is possible to uncover the role that platelets play in the occurrence and progression of thromboangiitis obliterans by correlation study between miR-155 and platelet activity, which may provide novel idea and evidence for early diagnosis and treatment of thromboangiitis obliterans. Therefore, this study is focused on the correlation between expression level of miR-155 and platelet activity in patients with thromboangiitis obliterans.

Materials and methods

General information

A total of 88 patients with thromboangiitis obliterans admitted to Jiangsu Taizhou People's Hospital from January 2018 to July 2019 were enrolled as study subjects in TAO group. They were 18-50 years old, with an average age of 33.00 ± 4.91 years old, and 42 cases of them

were at stage I (ischemic stage), 32 cases at stage II (dystrophic stage), and 14 cases at stage III (tissue gangrene stage). In addition, 90 healthy physical examinees in Jiangsu Taizhou People's Hospital during the same period were enrolled in the control group, with an average age of 33.33 ± 4.65 years old. Written informed consent was obtained from all the subjects, and this study was approved by the Ethics Committee of Jiangsu Taizhou People's Hospital.

Inclusion and exclusion criteria

Patients were eligible if they met the *Diagnostic criteria for thromboangiitis obliterans* formulated by the Professional Committee on Peripheral Vascular Diseases of the Society of Integrated Traditional Chinese and Western Medicine in 2004 [29]; and aged between 18 and 50 years old.

Patients were excluded if they had incomplete clinical data; had serious heart, liver or kidney diseases; had mental disorders or cerebrovascular diseases; had difficulty or inconvenience in follow-up; or had other vascular diseases or tumors.

miRNA extraction and RT-PCR

Total RNA was extracted with the use of Trizol kit (Molecular Research Center, USA), and the concentration and integrity of RNA were detected. The forward and reverse primers were provided by Guangzhou Rainbow, China [30]. Then, the miRNA was reversely transcribed into the cDNA using reverse transcription kit (Fermentas, Canada), and was then used as template for DNA amplification. Finally, the expression of miR-155 in serum samples was determined by quantitative PCR with fluorescent probe [31]. Specific procedures were as follows. First, patients were fasted after 10 p.m. for the collection of 2 mL peripheral venous blood in the next morning. For patients with acute myocardial infarction, the 2 mL blood sample was collected before interventional surgery. The samples were added with ethylene diamine tetraacetic acid anticoagulant, gently mixed by shaking the tubes to keep the integrity of the cells, and made full contact of blood cells and anticoagulant. Second, the prepared sample were placed at 4°C for later plasma separation (within 2 hours). Third, the plasma was centri-

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Table 1. Comparison of general data

Items	TAO group (n=88)	Control group (n=90)	X ² /t	P
Sex (male/female)	71/17	64/26	2.224	0.136
Age (years old)	33.00±4.91	33.33±4.65	0.465	0.643
Body mass index (kg/m ²)	26.67±3.34	26.86±3.25	0.449	0.654

Note: TAO, thromboangiitis obliterans.

Table 2. Comparison of serum miR-155 levels

Group	n	Relative expression of miR-155
TAO group	88	30.32±9.77
Control group	90	16.04±5.56
t	-	11.944
P	-	<0.001

Note: TAO, Thromboangiitis obliterans.

fuged for 10 min using a high-speed centrifuge (Shanghai Shengwu, China) for the collection of supernatants (in Eppendorf tubes). Forth, the supernatant was centrifuged for another 10 min again. Fifth, twice-separated sample was placed at -80°C, and sub-packed into 500 µL. Sixth, total RNA of cells was extracted by Trizol kit, and reversely transcribed into cDNA by stem-loop reverse transcription and reverse transcriptase M-MLV. Last, miR-155 stem-loop primers were used. The forward and reverse primers sequences were as follows: 5'-GTGCA-GGGTCCGAGGTATT-3' (miR-155) and 5'-GCCG-CTTAATGCTAATCGTGATAG-3' (miR-155); reference primer U6 5'-TCCGCTCTTGGTGTGGTGTG-AGTCGC-3'. The cycling system contained a total of 25 µL: 12.5 µL of SYBR premix (2), 0.5 µL of forward and reverse primers for target gene respectively, 2.0 µL of DNA template, and 9.5 µL of ddH₂O. The reaction conditions were as follows: 4 min of pre-denaturation at 94°C, followed by 35 cycles of 40 s at 95°C, 30 s at 60°C, 30 s at 72°C, and another 1 min at 72°C. Amplified products of PCR were detected by agarose electrophoresis. The relative expression of miR-155 was determined by 2-ΔΔC(T) method with U6 snRNA as reference.

Detection of platelet function related factors

The 5 mL of blood samples collected from patients were stored in the sterile tube of ethylene diamine tetraacetic acid for 15 min at 4°C. Then, serum was separated by centrifuge 3300 rpm, placed in 40 µL phosphate buffer solution containing protease inhibitor, and stored at -80°C. Last, the CD62p and PAC-1 were detect-

ed by ELISA (Shanghai Mlbio, China) using a full-automatic and multi-functional micro-plate reader (Thermo, USA).

Statistical methods

SPSS 22.0 statistical software was used for statistical analysis in this study. The continuous variables were expressed as mean ± standard deviation. Paired t-test was applied for the within-group comparison of data conforming to normal distribution and homogeneity of variance, and rank sum test was applied for data not conforming to normal distribution and homogeneity of variance. One-way ANOVA was used for comparison among multiple groups, and for data with differences, Bonferroni post hoc test was then used for further pairwise comparison. Pearson product-moment correlation coefficients was utilized to analyze the linear correlation between miR-155 and CD62p, PAC-1. The receiver operating characteristic curve was plotted and the area under the curve and 95% confidence interval were calculated. P<0.05 was regarded as significant difference.

Results

Comparison of general data

There were no significant differences in gender, age and body mass index between the two groups (all P>0.05), as shown in **Table 1**.

Comparison of serum miR-155 levels

The relative expression of miR-155 in TAO group was higher than that in the healthy control group, with a statistical difference (P<0.001), as shown in **Table 2**. ROC diagnostic curve was plotted. The optimal diagnostic threshold of miR-155 was 22.82 obtained by Yoden index method. The Yoden index was 79.6%, the sensitivity was 81.8%, the specificity was 97.8%, and area under the ROC curve (AUROC) was 0.902, as shown in **Figure 1**.

Comparison of general data of patients at different stages

There were no significant differences in gender, age, lesion location and course of disease

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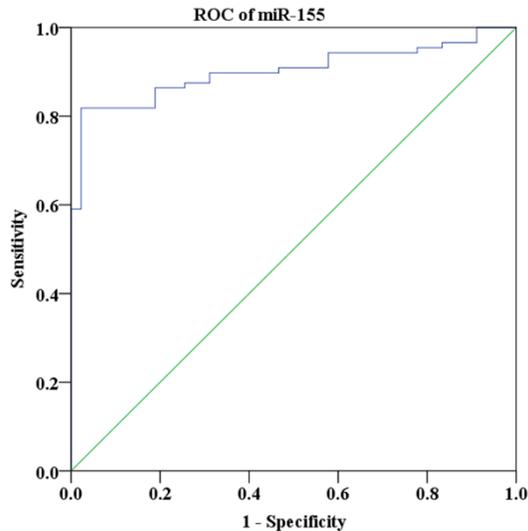


Figure 1. ROC curve of miR-155 for diagnosing thromboangiitis obliterans. ROC, receiver operating characteristic.

among patients at different stages (all $P > 0.05$), as shown in **Table 3**.

Comparison of serum miR-155 levels among patients at different stages

The level of serum miR-155 in patients at stage III was higher than that in patients at stage I and II, with statistical differences (both $P < 0.01$). The level of serum miR-155 in patients at stage II was higher than that in patients at stage I, with statistical difference ($P < 0.01$). The level of serum miR-155 in patients at stage I was higher than that in the healthy control group, with statistical difference ($P < 0.001$). See **Table 4**.

Comparison of serum CD62p and PAC-1 levels in patients at different stages

The levels of serum CD62p and PAC-1 in patients at stage III were higher than those in patients at stage I and II, with statistical differences (all $P < 0.001$), and the levels of serum CD62p and PAC-1 in patients at stage II were higher than those in patients at stage I, with statistical differences (both $P < 0.001$), as shown in **Table 5**.

Correlation between serum miR-155 and CD62p, PAC-1

The serum miR-155 was positively correlated with CD62p ($r = 0.637$, $P < 0.001$) and PAC-1 ($r = 0.620$, $P < 0.001$). See **Figure 2**.

Discussion

Local and systemic inflammatory reaction plays an important role in the occurrence and progression of thromboangiitis obliterans [32-34]. G protein-coupled receptor autoantibodies presented in the serum of 81% patients with thromboangiitis obliterans [35]. Moreover, the positive rates of anti-neutrophil cytoplasmic antibodies and anti-cardiolipin antibodies in the serum of patients with thromboangiitis obliterans were remarkably higher than those of healthy people [36, 37]. Increasing studies have suggested that the morbidity of thromboangiitis obliterans is closely related to the imbalance of autoimmune function.

The miRNAs are closely related to cell differentiation and activation, including immune cells, and the miR-155 is one of them, with MIR-155HG as its host gene [38, 39]. It was found that miR-155 was highly expressed in the urine of patients with systemic lupus erythematosus, and was closely related to lupus activity and proteinuria [19, 40]. Study also suggested that the expression of serum miR-155 in patients with thromboangiitis obliterans was 2.22 times higher than that in healthy people [20]. This study showed that the expression of miR-155 in patients with thromboangiitis obliterans was significantly higher than that in healthy people, and the expression of miR-155 was increased along with the progression of disease stages. Receiver operating characteristic curve showed that the optimal diagnostic threshold of miR-155 was at 22.82, with Yoden index of 79.6%, sensitivity of 81.8%, and specificity of 97.8%, indicating a valuable role of miR-155 in the diagnosis of thromboangiitis obliterans.

Platelet activation is the basis of thrombosis. Higher level of platelet activation results in higher incidence of thrombosis, which leads to hemadostenosis and ischemic symptoms [41]. Clinically, CD62p and PAC-1 are used to evaluate the level of platelet activation, and higher risk of thrombosis results in higher level of platelet activation [42]. This study found that the expression of CD62p and PAC-1 in serum was increased along with the progression of thromboangiitis obliterans stages. The levels of serum CD62p and PAC-1 in patients at stage III were higher than those in patients at stage I and II, suggesting higher risk of thrombosis in patients with advanced thromboangiitis obliterans.

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Table 3. Comparison of general data of patients at different stages

Items	Stage I	Stage II	Stage III	X ² /F	P
Sex (male/female)	34/8	25/7	12/2	0.364	0.834
Age (years old)	36.31±7.6	33.76±9.5	36.36±7.6	0.927	0.398
Lesion location				2.707	0.608
Left lower limb	21	18	6		
Right lower limb	19	13	6		
Both lower limbs	2	1	2		
Course of disease (years)	5.34±1.43	5.46±1.56	5.80±1.76	1.232	0.276
Body mass index (kg/m ²)	23.72±2.96	23.99±2.03	24.27±2.13	0.769	0.465

Table 4. Comparison of serum miR-155 among patients at different stages

Group	n	Relative expression of miR-155
Stage III	14	39.77±7.04 ^{***,###,&&}
Stage II	32	31.83±9.03 ^{***,##}
Stage I	42	26.03±8.62 ^{***}
Control group	90	16.04±5.56
F		23.393
P		<0.001

Note: compared with control group, ^{***}P<0.001; compared with patients at stage I, ^{##}P<0.01, ^{###}P<0.001; compared with patients at stage II, ^{&&}P<0.01.

Table 5. Comparison of serum CD62p and PAC-1 levels among patients at different stages

Group	n	CD62p (%)	PAC-1 (%)
Stage I	42	14.71±2.55	24.39±2.78
Stage II	32	16.80±1.52 ^{***}	26.69±2.12 ^{***}
Stage III	14	19.87±2.67 ^{***,###}	30.16±3.08 ^{***,###}
F		28.807	26.588
P		<0.001	<0.001

Note: CD62p, P-selectin; PAC-1, platelet activated complex-1. Compared with patients at stage I, ^{***}P<0.001; compared with patients at stage II, ^{###}P<0.001.

ans. Abnormal activation of CD62p and PAC-1 promotes the activation of platelets could be a factor that aggravates thromboangiitis obliterans, whereas, further animal experiments are needed to prove this theory, which might be a theoretical basis for the targeted therapy of thromboangiitis obliterans.

There are abundant well-functioning miRNAs in reticulated platelets [26]. Affected by the miRNAs, the reticulated platelets present increased volume, granularity and sensitivity, which leads to reduced reactivity of antiplatelet drugs [43]. However, deficiency of some miRNAs can also

lead to abnormal function of platelets, for instance, the deficiency of miR-233 can increase the expressions of platelet coagulation factor XIII and integrin β1, thereby increasing adsorption and adhesion of platelets, thus increasing the activity index CD62p, which most likely contributes to thrombosis [44]. Deficiency of miR-30c results in higher expression of plasminogen activator inhibitor, which affects platelet function and increases its activity [45]. Deficiency of miR-26b and miR-140 could increase expression of P-selectin, thereby increasing level of platelet activity indicators CD62p and PAC-1, and eventually leads to abnormal function of platelets [46]. There are great variety of miRNAs, which regulate platelet activity and biological function by involving in the expression of vesicle-associated membrane protein-8 [47]. Grouped observation based on adrenaline-induced platelet reactivity has revealed that miR-200b, miR-495 and miR-107 regulate the expression of platelet protein by affecting targeted mRNA, thus affecting platelet activity [48]. Platelets are mainly produced by megakaryocytes, while miR-155 can inhibit the differentiation of megakaryocyte-erythrocyte progenitor cell K562, thereby affecting the function of platelets [49]. This study found that the expression of serum miR-155 was positively correlated with the levels of CD62p and PAC-1 (r=0.637, r=0.620), indicating that the abnormal expression of miR-155 may increase the risk of thrombosis by affecting the levels of CD62p and PAC-1.

This is single-center study with a relatively small sample size. Therefore, we will expand the sample size and conduct multi-center surveys in the future to reduce sample bias. In addition, we will further explore the specific mechanism of the miR-155's effect on thromboangiitis obliterans through basic experiments

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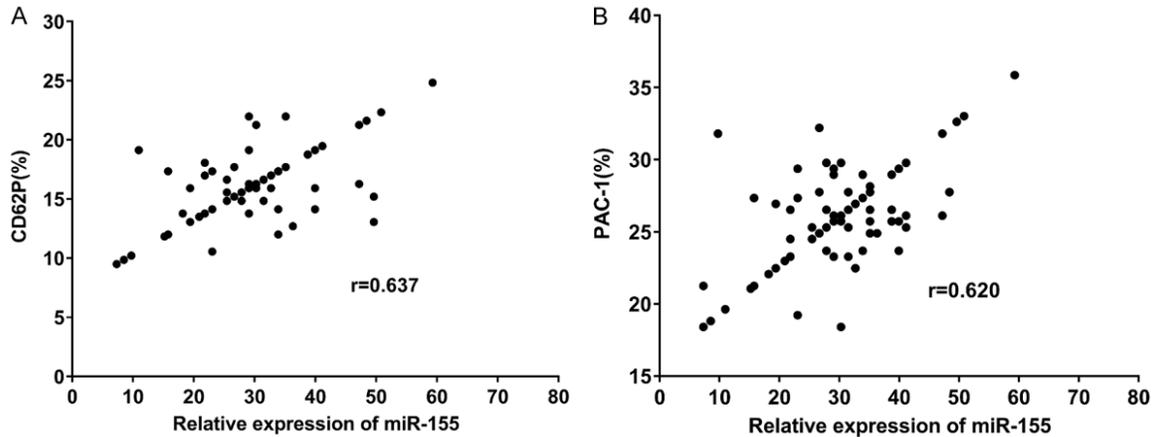


Figure 2. Correlation between serum miR-155 and CD62p, PAC-1. A. Correlation between the relative expression of serum miR-155 and CD62p; B. Correlation between the relative expression of serum miR-155 and PAC-1. CD62p, P-selectin; PAC-1, platelet activated complex-1.

in vitro and animal experiments, so as to provide a precise theoretical basis for the targeted therapy of thromboangiitis obliterans.

In conclusion, miR-155 is overexpressed in patients with thromboangiitis obliterans, with high diagnostic value. Besides, miR-155 can lead to the occurrence and progression of thromboangiitis obliterans, and may be related to the expression of CD62p and PAC-1.

Disclosure of conflict of interest

None.

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