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Original Article

Predictive value of plasma microRNA-140-3p for restenosis in patients with lower extremity arterial occlusive disease undergoing interventional procedures

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Abstract: Objective: The purpose of this study is to explore the predictive value of plasma microRNA-140-3p (miRNA-140-3p) for restenosis in patients with lower extremity arterial occlusive disease (LEAOD) undergoing interventional procedures. Methods: Between January 2012 and April 2015, a total of 117 patients with LEAOD were recruited in this study. Patients were assigned into the restenosis and non-restenosis groups according to postoperative angiography. The miRNA-140-3p expression was measured using qRT-PCR. A receiver operating characteristic (ROC) curve was drawn for the predictive value of miRNA-140-3p expression in postoperative restenosis of patients with LEAOD. Logistic regression analysis was conducted to analyze the risk factors for postoperative restenosis in patients with LEAOD. Results: There were 19 and 98 patients in the restenosis and non-restenosis groups, respectively. The proportions of hypertension, diabetes, hyperlipidemia and smoking in the restenosis group were significantly higher than those in the non-restenosis group (all $P < 0.05$). Plasma miRNA-140-3p expression in the restenosis group was significantly lower than that in the non-restenosis group ($P < 0.05$). ROC curve analysis revealed that the area under the curve (AUC) of the predictive value of miRNA-140-3p expression to postoperative restenosis was 0.869, with the optimal threshold 0.255. The sensitivity and specificity were 84.2% and 82.7%, respectively. There were significant differences in hyperlipidemia, hypertension, coronary heart disease (CHD), diabetes and Fontaine stage between two groups (all $P < 0.05$). Logistic regression analysis demonstrated that low expression of miRNA-140-3p was an independent risk factor for the postoperative restenosis ($P < 0.05$). Conclusions: MiRNA-140-3p might be a promising tool for predicting there stenosis in LEAOD patients undergoing interventional procedures.

Keywords: MicroRNA-140-3p, restenosis, interventional treatment, lower extremity arterial occlusive disease, predictive value

Introduction

Lower extremity arterial occlusive disease (LEAOD) is a kind of peripheral arterial disease (PAD) which results from atherosclerotic occlusion in the arteries of legs, critically manifesting a sign of systemic atherosclerosis [1, 2]. Atherosclerosis obliterans (ASO) stems from atherosclerosis, which is induced by serious damage to endothelial cells from a range of stimuli, such as adhesion molecules, pro-inflammatory cytokines, sheer stress and oxidative stress [3, 4]. An increasing number of patients are suffering from ASO, and the possible risk factors include obesity, diabetes, hypertension, smoking and hypercholesteremia [4]. LEAOD, as one of the ASO, is the primary cause of adult limb loss in the whole world [2, 5]. Surgery is the most popular approach in the treatment of ASO [6]. However, a great number of patients suffered from restenosis within 1 year after surgery [7]. Percutaneous intervention has become the first treatment option for the most patients with arterial occlusive disease of the lower extremity [8]. Intermittent claudication and painful vascular ulcers, however, seriously reduce mobility and quality of life of these patients [9].

MicroRNAs (miRNAs), approximately 21 nucleotides in length, are a large family of highly conserved and post-transcriptional RNA molecules

that function as regulators of gene expression in eukaryotic organisms by translational inhibition and mRNA destabilization [10-12]. It was demonstrated that serum/plasma miRNAs are derived from multiple tissues/organs, which are stable and resistant to nuclease digestion [13]. Expressions of miRNAs in blood have been reported to be indicative and reproducible of disease state [13]. Thus, specific signatures of blood miRNAs could possibly be used as biomarkers for diagnosis, prognosis and even the etiology of a disease [4]. MiRNA-140 (including miRNA-140-3p and miRNA-140-5p) are enriched in human mesenchymal stem cells from many human tissues, including adipose, bone marrow and umbilical cord [14]. Previous studies have showed miRNA-140-3p is associated with poor prognosis in spinal chordoma, regulation in human airway smooth muscle cells, modulation of leydig cell numbers in the developing mouse testis [15-17]. MiRNA-140-3p has been confirmed as significantly different in human arteries, especially significantly down-regulated in ASO arteries [6]. Hence, we supposed that miRNA-140-3p may play a role in the pathogenesis of atherosclerosis. In this study, we target to investigate relationship between plasma miRNA-140-3p expression and restenosis in LEAOD patients undergoing interventional procedures.

Materials and methods

Ethic statement

This study was admitted by the Ethics Committee of Yantai Hospital of Traditional Chinese Medicine. The informed consents have been obtained from all patients. Our clinical trail registration number is ChiCTR-DDD-160-09809.

Study subjects

Between January 2012 to April 2015, a total of 117 patients (89 males and 28 females; mean age, 67.7 ± 7.9 years) who were received interventional treatment for LEAOD at Yantai Hospital of Traditional Chinese Medicine were recruited in our study. According to the computed tomography angiography (CTA) examination before and during the treatment, 77 cases (92 limbs) were diagnosed with arterystenosis and 40 cases (54 limbs) with artery occlusion. Clinical manifestations of all patients were evaluated according to Fontaine staging [18]. There was 1 patient in stage I, 40 patients in stage II, 54 patients in stage III and 22 patients in stage IV. Exclusion criteria: (1) patients without taking anti-platelet aggregation drugs after undergoing stent implantation or having renal inadequacy; (2) patients who had restenosis in two or more locations and in grafting vessels after revascularization which was given 1 month after the diagnosis of restenosis; (3) patients with a severe dysfunction in left ventricular whose ejection fraction is less than 40%; (4) patients with dysfunction in liver, kidney, lung, brain and other important organs; (5) patients with connective tissue disease, autoimmune diseases or malignancies; (6) patients with a history of acute myocardial infarction.

Interventional treatment

According to the results of preoperative CTA scan, an appropriate interventional treatment was selected. Anantegrade puncture in the ipsilateral femoral artery was the top priority when the stenosis or occlusion occurred in the middle or distal segment of superficial femoral artery or popliteal artery. When stenosis or occlusion occurred in the upper segment of common or superficial femoral artery, or in iliac artery, a retrograde puncture in the opposite femoral artery was preferred for intracavitary therapy. If the femoral artery was inappropriate for puncture in both sides, the puncture was performed in brachial artery. After local anesthesia, patients were planted with sheathing canal, through which angiography of the targeted arteries were carried out so as to draw out a further therapeutic regimen. For the patients whose vascular restenosis was shorter than 3 cm, percutaneous transluminal angioplasty (PTA) was conducted, while endovascular stent (ES) was performed for patients whose residual stenosis accounted for more than 30% after PTA with a long medical history. And for the patients with arterial restenosis more than 10 cm or with complete occlusion in artery, thrombus extraction-atherectomy was applied. In the process, the selection of the guide wire was based on the puncture approaches and treatment plans. The ev3 NanoCross and Bantam balloon were selected for PTA microballoon, and self-expandable metallic stents were used, such as ev3 protégé EverFlex and BARDLife-Stent XL.

Sample collection

After interventional treatment, all the patients underwent venous blood collection (10 mL) through venous puncture. The blood samples were stored in anticoagulant tubes containing sodium citrate and centrifuged to obtain plasma within 4 h in accordance with the standard Ficoll density gradient centrifugation. The centrifugal conditions: at room temperature; 1800 g for 10 min. The plasma was bottled in a 1-mL-sized nuclease-free Eppendorf (EP) tube and frozen at -80°C. The RNA sample (5 μL) was taken out for total RNA extractionin accordance with the manufacture manual of miRN easy Mini Kit (Qiagen Company, Hilden, Germany). The reagents kit was synthesized according to the first strand of TiangenmiRcute miRNA cDNA. Specifically, the miRNA was firstly modified by adding the poly (A) to the end of miRNA3, followed by centrifugation and reaction at 37°C for 60 min. Besides, general transcription primer oligo (DT)-Universal Tag was used for reverse transcription reaction at 37°C for 60 min after centrifugation. Finally, the first strand of cDNA corresponding to the miRNA was generated and restored at -20°C for further use.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The plasma miRNA-140-3p expression of each patient was detected by qRT-PCR. U6 was used as an internal control. All primers were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The total RNA (1 μg) was collected as template for reverse transcription. The primer sequence for miRNA-140-3p and U6 were 5'-TACCACAGGGTAGAACCACGG-3' and 5'-CTCGCTTCGGCAGCACA-3', respectively. PCR conditions: 40 cycles of predenaturation at 95°C for 20 s, denaturation at 95°C for 10 s, annealing at 60°C for 20 s and extension at 70°C for 20 s. The process was repeated 3 times for each sample, with the total reaction volume of 20 μL. The relative expression was calculated using $2^{-\Delta\Delta Ct}$ method.

Follow-up

All discharged patients were followed upperiodically by outpatient or telephone, during which patients were observed mainly for claudication symptoms and the arterial pulse. A CAT scan was conducted on the 117 LEAOD patients after the interventional treatment, and their ankle-brachial indexes (ABI) were measured. Restenosis after interventional treatment was judged referring to the efficacy of interventional treatment [19]. The patients were identified to have no restenosis when (1) stenosis of the diseased vessels was less than 30% by the postoperative CTA scan; (2) no obvious artery dissection or severe surgery related complications; and (3) continuous appeasing of claudication, rest pain and other symptoms in the lower limbs as well as a sound healing of ulcer. If failing those, the treatment would be otherwise regarded as invalid. According to the occurrence of restenosis, the patients were assigned into the restenosis group (n = 19; 14 males and 5 females; mean age, 68.6 ± 8.2 years) and the non-restenosis group (n = 98; 75 males and 23 females; mean age 67.5 ± 7.9 years).

Statistical analysis

SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation (SD). Comparisons of age, ABI, length of diseased vessel (DV), number of DV and miRNA-140-3p expression between two groups were analyzed using *t* test. Count data were expressed in percentage or rate. Comparisons of Gender, hyperlipidemia, hypertension, coronary heart disease (CHD), diabetes, Fontaine stage, smoking, drinking and miRNA-140-3p expression between two groups were analyzed using *chi*-square test. A Receiver operating characteristic (ROC) curve was drawn to evaluate the predictive value of miRNA-140-3p for restenosis in LEAOD patients after interventional treatment. Logistic regression analysis was conducted to analyze the independent risk factors for the postoperative restenosis. $P < 0.05$ was considered statistically significant.

Results

Comparisons of baseline data and miRNA-140-3p expression between the restenosis and non-restenosis groups

According to the periodic follow-up, the 117 LEAOD patients were assigned into the restenosis group (n = 19) and the non-restenosis group (n = 98). The baseline data of the two groups were presented in **Table 1**. The proportions of hypertension, diabetes, hyperlipidemia

Table 1. Comparison of baseline characteristics between the restenosis group and non-restenosis group

Index	Restenosis group (n = 19)	Non-restenosis group (n = 98)	*P*
Age	68.6 ± 8.2	67.5 ± 7.9	0.582
Gender (male/female)	14/5	75/23	0.790
Hyperlipidemia/n (%)	9 (47.37)	14 (14.29)	0.001
Hypertension/n (%)	11 (57.89)	27 (27.55)	0.010
CHD/n (%)	3 (15.79)	8 (8.16)	0.297
Diabetes/n (%)	9 (47.37)	18 (18.37)	0.006
ABI	0.39 ± 0.08	0.42 ± 0.09	0.179
Fontaine stage			0.163
Stage I-II	3 (21.06)	38 (38.78)	
Stage III-IV	16 (78.94)	60 (61.22)	
Length of DV/cm	6.71 ± 1.94	6.02 ± 1.81	0.136
Number of DVs	43 ± 10	39 ± 11	0.144
Smoking/n (%)	18 (94.74)	60 (61.22)	0.005
Drinking/n (%)	15 (78.95)	56 (57.14)	0.075
MiRNA-140-3p	0.22 ± 0.06	0.31 ± 0.08	< 0.001

Notes: comparisons of age, ABI, length of DV, number of DV and miRNA-140-3p between two groups were analyzed by *t* test; comparisons of gender, CHD, Fontaine stage, and drinking were analyzed by *chi*-square test; CHD, coronary heart disease; ABI, ankle-brachial index; DV, diseased vessel.

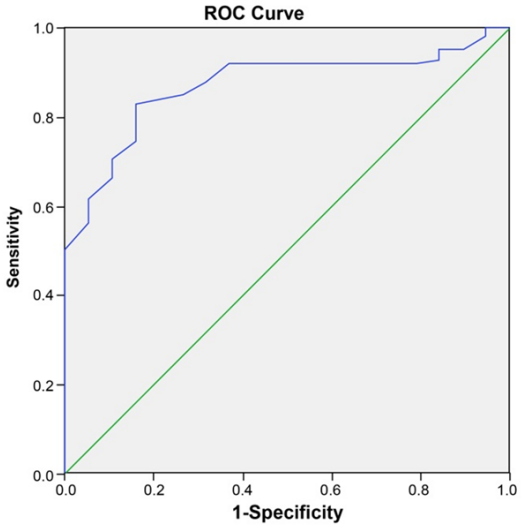

Figure 1. ROC curve analysis for the predictive value of miRNA-140-3p expression for restenosis in LEAOD patients undergoing interventional procedures. Note: ROC, receiver operating characteristic; LEAOD, lower extremity arterial occlusive disease.

and smoking were significantly higher in the restenosis group than those in the non-restenosis group (all $P < 0.05$). Compared with the non-restenosis group, the microRNA-140-3p expression was significantly decreased in the restenosis group ($P < 0.05$). There were no significant differences in age, gender, CHD, ABI, Fontaine stage, length of DV, number of DV and drinking between the two groups (all $P > 0.05$).

ROC curve analysis for the predictive value of miRNA-140-3p for restenosis in LEAOD patients

According to postoperative restenosis, the ROC curve was drawn to evaluate the predictive value of miRNA-140-3p for restenosis after interventional treatment in LEAOD patients. As shown in **Figure 1**, the area under the curve (AUC) was 0.869 (95% CI: 0.798~0.940, $P < 0.001$), and the optimal threshold value was 0.255. The sensitivity and specificity were 84.2% and 82.7%, respectively, indicating that miRNA-140-3p could be a promising tool for predicting postoperative restenosis. Based on the optimal threshold value, the miRNA-140-3p expression > 0.255 was defined as high expression, with miRNA-140-3p expression < 0.255 as low expression. There were significant differences in miRNA-140-3p high and low expressions between the two groups (both $P < 0.05$) (**Table 2**).

Association between miRNA-140-3p expression and the clinicopathological features in LEAOD patients

There were no significant differences in the age, gender, ABI, length of DV, number of DV, drinking and smoking between the miRNA-140-3p high and low expression (all $P > 0.05$), but significant differences were found in hyperlipidemia, hypertension, CHD, diabetes and Fontaine stage between the miRNA-140-3p high and low expression (all $P < 0.05$) (**Table 3**).

Logistic regression analysis of independent risk factors for restenosis in LEAOD patients

The postoperative restenosis was selected as the dependent variable, miRNA-140-3p, hyperlipidemia, hypertension, CHD, diabetes and Fontaine stage were selected as the independent variables. Logistic regression analysis in-

Table 2. Comparison of miRNA-140-3p expression in the restenosis group and non-restenosis group

Group	n	miRNA-140-3p		χ^2	*P*	Se	Sp	PPV	NPV
		Low	High						
Restenosis group	19	16	3	35.14	< 0.001	84.2%	82.7%	48.5%	96.4%
Non-restenosis group	98	17	81						

Note: Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

Table 3. Association between miRNA-140-3p expression and the clinicopathological features of LEAOD patients

Feature		n	MiRNA-140-3p		χ^2	*P*
			Low expression (n = 33)	High expression (n = 84)		
Age (years old)	> 65	74	25 (75.76)	49 (58.34)	3.09	0.079
	≤ 65	43	8 (24.24)	35 (41.66)		
Gender	Male	89	28 (84.85)	61 (72.62)	1.95	0.163
	Female	28	5 (15.15)	23 (27.38)		
Hyperlipidemia/n (%)	Without	94	21 (63.64)	73 (86.91)	8.12	0.004
	With	23	12 (36.37)	11 (13.09)		
Hypertension	Without	79	17 (51.52)	62 (73.81)	5.37	0.021
	With	38	16 (48.48)	22 (26.19)		
CHD	Without	106	27 (81.82)	79 (94.05)	4.16	0.041
	With	11	6 (18.18)	5 (5.95)		
Diabetes	Without	90	20 (60.61)	70 (83.34)	6.89	0.009
	With	27	13 (39.39)	14 (16.66)		
ABI	> 0.4	63	18 (54.55)	45 (53.58)	0.01	0.924
	≤ 0.4	54	15 (45.45)	39 (46.42)		
Fontaine stage	I-II	41	5 (15.15)	36 (42.18)	7.99	0.005
	III-IV	76	28 (84.85)	48 (57.82)		
Length of DV/cm	> 6.0	64	14 (42.43)	50 (59.52)	2.80	0.095
	≤ 6.0	53	19 (57.57)	34 (40.48)		
Number of DVs	> 40	57	17 (51.52)	40 (47.62)	0.14	0.704
	≤ 40	60	16 (48.48)	44 (52.38)		
Tobacco use	Without	39	7 (21.21)	32 (38.10)	3.04	0.081
	With	78	26 (78.79)	52 (61.90)		
Alcohol use	Without	46	9 (27.27)	37 (44.05)	2.79	0.095
	With	71	24 (72.73)	47 (55.95)		

Notes: Association between miRNA-140-3p expression and the clinicopathological features in LEAOD patients was analyzed by *chi*-square test; CHD, coronary heart disease; ABI, ankle-brachial index; DV, diseased vessel; LEAOD, lower extremity arterial occlusive disease.

dicated that low expression of miRNA-140-3p was an independent risk factor for postoperative restenosis ($P < 0.05$), but hyperlipidemia, hypertension, CHD, diabetes and Fontaine stage had no correlation with postoperative restenosis (all $P > 0.05$) (**Table 4**).

Discussion

The findings in the present study clearly supported the basic hypothesis that plasma miRNA-140-3p might be a promising tool for predicting restenosis after interventional treatment in LEAOD patients. Related results demonstrated that plasma miRNA-140-3p expression was significantly lower in the restenosis group than that in the non-restenosis group, and that the ROC curve with relative high sensitivity and specificity reflected the predictive value of miRNA-140-3p.

In our study, ABI was used to determine the efficacy of interventional treatment for the restenosis of LEAOD patients. ABI is the primary

Table 4. Logistic regression analysis of independent risk factors for restenosis in LEAOD patients

Factor	B	S.E.	Wald	Sig.	Exp (B)	95% CI
MiRNA-140-3p	2.94	0.72	16.67	< 0.001	18.92	4.61-77.60
Hyperlipidemia	0.90	0.81	1.23	0.268	2.46	0.50-12.05
Hypertension	0.45	0.81	0.32	0.572	1.58	0.32-7.64
CHD	-0.15	0.94	0.03	0.872	0.86	0.14-5.40
Diabetes	0.44	0.72	0.38	0.537	1.56	0.38-6.38
Fontaine stage	0.23	0.86	0.07	0.792	1.26	0.23-6.78

Notes: CHD, coronary heart disease; S.E., standard deviation; sig., *P* value; Exp (B): adjusted odds; CI: confidence interval; LEAOD, lower extremity arterial occlusive disease.

non-invasive test for diagnosing lower extremity artery disease (LEAD) [9]. The level of ABI is associated with LEAD severity; a reduced ABI indicates atherosclerosis and an increased risk of cardiovascular and cerebrovascular morbidity and mortality [20].

According to Wang M *et al*., miRNA-21 was upregulated mainly in ASO arteries with a less than 50% of stenosis, which indicated that miRNA-21 might serve as an indicator of early prediction for stenosis in ASO arteries [6]. In addition, it was also demonstrated that miRNA-130a, miRNA-27b and miRNA-210 could be serum markers for early-stage ASO [4]. As for the possible mechanism behind, we also shed light upon aberrant proliferation of vascular smooth muscle cells (VSMCs). VSMCs proliferation is a critical pathological process in various proliferative vascular diseases, including atherosclerosis and post-angioplasty restenosis [21]. Similarly, Owens GK *et al.* demonstrated that the transition of VSMCs from a differentiated phenotype to a dedifferentiated state has great significance in the pathogenesis of atherosclerosis [22]. Doran AC *et al.* also demonstrated that proliferation and migration of VSMCs were the major cellular processes and causes behind ASO formation and postoperative restenosis [23]. Therefore, we put forward a speculation that miRNA-140-3p might play a vital role in the restenosis of ASO by affecting the transition of VSMCs, although it needs to be validated by further experimental evidence.

The ROC curve serves as an effective marker or diagnostic test for discrimination between groups [24]. Sensitivity, specificity and the AUC are important components used to measure the validity of a diagnostic test [25]. The results of the ROC curve substantially upheld that miRNA-140-3p expression did have the predictive value for postoperative restenosis.

From our study, we also found that miRNA-140-3p expression was related with the factors including hyperlipidemia, hypertension, CHD, diabetes and Fontaine stage. Hyperlipidemia is a potential risk factor for various metabolic and cardiovascular disorders, and overproduction of lipoproteins can make a contribution to it [26]. Liu *et al.* reported that exogenously expressed miRNA-140 in mesenchymal stem cell line C3H10T1/2 cells increased adipocyte differentiation significantly, whereas the knockdown of miRNA-140 decreased adipocyte differentiation [27]. That may provide an explanation for the links between miRNA-140-3p and hyperlipidemia. Reduced primary patency rates of diabetic patients who received percutaneous treatment of lower extremity occlusive disease could be possibly explained by the advanced stage of disease [8]. A consistent study showed that compared with patients without diabetes, patients with diabetes had more occlusive disease in the infrapopliteal vessels and had a higher prevalence of obstruction in the posterior tibial, peroneal and plantar arteries [28].

To summarize, plasma miRNA-140-3p expression has a certain correlation with restenosis in LEAOD patients undergoing interventional procedures. Specifically, low expression of plasma miRNA-140-3p might suggest a higher risk of postoperative restenosis, suggesting that plasma miRNA-140-3p expression might be clinically referred for restenosis in LEAOD undergoing interventional procedures. However, there are also limitations in our study. First of all, the follow-up in this study was performed in a relative short time, therefore, we have no idea whether restenosis will occur and miRNA-140-3p expression will reduce in the following years or even decades. In addition, the sample size of the study was not large enough, which may cause other factors for restenosis to be missed. Therefore, further studies are demanded in the future based on a larger sample size and a longer follow-up.

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Disclosure of conflict of interest

None.

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