Original Article

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Elevated level of miR-17 along with decreased levels of TIMP-1 and IL-6 in plasma associated with the risk of in-stent restenosis

Feng Jiang[1,2,§], Xi Zhang[1,§], Ying-Min Lu[3], Yi-Gang Li[4], Xin Zhou[1,*], Yao-Sheng Wang[1,4,*]

[1] *Clinical Research Unit, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai, China;*
[2] *Scientific Research Center, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Chongming Branch, Shanghai, China;*
[3] *Cardiology, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Chongming Branch, Shanghai, China;*
[4] *Cardiology, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai, China.*

Summary **In-stent restenosis is highly related to the deposition of inflammatory extracellular matrix and the migration of endothelial and vascular smooth muscle cells. The miR-17/TIMP-1/interleukin pathway regulates vascular matrix remodeling and plays an important role in the inflammatory reaction. This study identified miR-17 and its related biomarkers in serum that potentially indicated susceptibility to in-stent restenosis (ISR) after coronary artery stenting. Subjects were 42 patients with single de novo coronary artery lesions who underwent regular coronary angiography one year after percutaneous coronary intervention. The clinical baseline information was recorded. Serum levels of biomarkers (including miR-17, TIMP-1, IL-6, IL-8, IL-2R, TNF-alpha, IL-10, and IL-1beta) were measured with real-time PCR or ELISA. Intergroup comparisons were used to compare patients with or without ISR. Compared to levels in the non-restenosis group, the serum miR-17 level was significantly higher (3.13 ± 0.22 *vs*. 1.06 ± 0.04, $p < 0.01$) and the serum TIMP-1 and IL-6 levels were significantly lower in the ISR group (TIMP-1: 0.33 ± 0.04 *vs*. 1.00 ± 0.05, $p < 0.01$; IL-6: 1.64 ± 0.18 *vs*. 3.52 ± 0.11, $p < 0.01$). Moreover, the levels of TIMP-1 and IL-6 decreased as the level of miR-17 increased. Spearman's correlation analysis indicated that the miR-17 level was inversely correlated with TIMP-1 and IL-6 levels. Findings suggest that an elevated level of miR-17 and decreased levels of TIMP-1 and IL-6 may be associated with the risk of ISR, which is in accordance with vascular matrix remodeling and an inflammatory reaction during the pathologic process of ISR. This study highlighted the potential for miR-17, TIMP-1, and IL-6 to serve as biomarkers for ISR.**

Keywords: miR-17, TIMP-1, IL-6, in-stent restenosis

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Dr. Yao-Sheng Wang, Cardiology, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Yangpu District, Shanghai 200092, China.
E-mail: wangyaosheng@xinhuamed.com.cn

Dr. Xin Zhou, Clinical Research Unit, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Yangpu District, Shanghai 200092, China.
E-mail: zhouxin@xinhuamed.com.cn

1. Introduction

Percutaneous coronary intervention (PCI) is currently the main method to treat coronary heart disease. Stent implantation is commonly used as for coronary intervention. There is a certain proportion of in-stent restenosis (ISR) after stent implantation that causes problems clinically. ISR involves many pathologic mechanisms, including intraplaque inflammation, lipid deposition, proliferation of vascular smooth muscle cells, endoluminal thrombus formation, and intraplaque

angiogenesis. These induce endothelial dysfunction and the instability of plaque. The recent incidence of ISR has decreased with the widespread use of drug-eluting stents (DESs). However, ISR still occurs in approximately 10% of patients receiving DESs, and ISR during the long-term period after stent implantation (more than 6 months) has not effectively improved (*1,2*). Given to the pathological characteristics of ISR after intracoronary stent implantation with PCI, potential biomarkers to indicate susceptibility to ISR need to be identified.

The inflammatory properties of the permanent coating applied on stent struts that allows controlled drug release are one of the key factors for ISR in patients receiving DESs. However, a nonselective anti-proliferative drug that is released on endothelial regeneration increases the risk of late and very late stent thrombosis (*3*).

MicroRNA (miRNA, miR) is highly conserved short non-coding RNA. miRNA can bind to the 3' end untranslated region (UTR) of the corresponding target mRNA and inhibit translation or promote degradation of mRNA at the post-transcriptional level (*4*). Recent studies have indicated that an imbalance in expression of miRNAs is closely related to the occurrence and development of diseases (*5*). To some extent, specific miRNAs can be considered as biomarkers of various cardiovascular diseases and may inspire effective ways to diagnose and evaluate cardiovascular diseases. Because of the stability of miRNAs in whole blood, plasma, or serum, numerous studies have speculated that specific miRNAs in peripheral circulating blood can be used as early warning indicators of various cardiovascular diseases (*6,7*). Basic studies have revealed significant changes in the expression of miRNAs that relate to endothelial growth and smooth muscle proliferation after balloon injury (*8*). miR-17, miR-21, miR-125, and miR-126 have been widely reported as regulators of inflammation and angiogenesis. Such miRNAs can be used as biomarkers and possible targets for interventions in the process of arterial injury and repair (*9-11*). Thus, ISR may also be accompanied by the expression of specific miRNAs. The molecular biological mechanism of ISR after PCI can be described at the level of identifying a series of miRNAs and corresponding downstream gene targets.

In light of these previous studies, the current study measured the levels of several key miRNAs, as well as corresponding specific targets, in patients with and without ISR. The aim of this study was to assess their value as potential molecular markers for ISR.

2. Materials and Methods

2.1. *Study subjects*

Subjects were 14 patients who underwent PCI one year earlier. Patients had single de novo coronary artery lesions and underwent regular coronary angiography (CAG) at Xinhua Hospital from October 2014 to October 2017. All 14 patients had ISR. Patients received standard dual therapy with aspirin 100 mg/day and clopidogrel 75 mg/day. Patients who suffered an acute myocardial infarction within one year of PCI were excluded. Twenty-eight patients without ISR who had single de novo coronary artery lesions and similar baseline characteristics served as the control group. ISR was defined as the presence of > 50% diameter stenosis in the stented segment. The study followed the principles outlined in the Declaration of Helsinki and it was approved by the ethics committee.

2.2. *Plasma collection*

Patients routinely underwent CAG one year after coronary artery PCI stent implantation. Some blood samples are left for clinical biochemical examination when blood vessels are punctured during CAG. The residual blood from clinical tests was collected at the time of routine follow-up. Plasma samples were collected by centrifugation (15 minutes at 1,200 × g) within 30 minutes, preserved in RNase-free tubes, and stored at -80°C for extraction of RNA.

2.3. *RNA isolation*

Total RNA was extracted using a Trizol-based miRNA isolation protocol (Tiangen, China). Plasma was lysed with Trizol at a 1:3 ratio. After 5 min, 0.8 mL of chloroform per 1 mL of sample was added. The three phases (aqueous, inter, and organic) were obtained by centrifugation at 4°C and 12,000 rpm for 15 min. The aqueous phase was then transferred to a fresh tube, and 2.0 mL of isopropanol per 1 mL of sample was added and incubated for 10 min. Total RNA was precipitated after samples were centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was removed, and the RNA pellet was washed with 1 mL of 75% ethanol and subsequently centrifuged at 7,500 × g for 5 min at 4°C. After the ethanol was removed, the RNA pellet was briefly air-dried and dissolved in RNase-free water. The RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and the RNA samples were stored at −80°C for future use.

2.4. *Detection and analysis of miRNAs and TIMP-1 mRNA by qRT-PCR*

A real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to determine the expression of miRNAs. Pure RNA (OD 1.8-2.2) was reverse-transcribed (RT) to cDNA at 42°C for 30 min using mirVana™ miRNA detection

Table 1. Nucleotide sequences of primers used for PCR amplification

Primer name	Forward primer(5'-3')	Reverse primer(5'-3')
miR-17	GCGGCCAAAGTGCTTACAGTG	CAGCCACAAAAGAGCACAAT
miR-21	ACACTCCAGCTGGGTAGCTTATCAGACTGA	TGGTGCGTGGAGTCG
miR-125	GTCCCTGAAGCCCTTTAACC	AACCTCACCTGTGACCCTG
miR-126	CATTATTACTTTTGGTACGC	GTCGTATCCAGTGCGTGTCGTG
TIMP-1	CTTCTGGCATCCTGTTGTTG	GGTATAAGGTGGTCTGGTTG
U6	GTGCTCGCTTCGGCAGCACATATAC	AAAAATATGGAACGCTTCACGAATTTG

Table 2. Characteristics of patients with or without restenosis

Items	With restenosis (n = 14)	Without restenosis (n = 28)	p value
Gender (% male)	50.00	64.29	0.374
Age (years)	66.29 ± 1.61	67.54 ± 1.72	0.645
BMI (kg/m^2)	24.22 ± 0.58	23.66 ± 0.48	0.490
Hypertension, %	71.43	75.00	0.804
Hypercholesterolemia, %	78.57	71.43	0.620
Diabetes, %	35.71	32.14	0.817
Current smoker, %	35.71	39.29	0.822
Current alcohol drinker, %	35.71	42.86	0.675
Family history of CVD, %	50.00	57.14	0.661

kits (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. cDNA (2 μL) was used as the template for qRT-PCR. Plasma expression of miRs was detected using SYBR Green miRNA qRT-PCR kits (Tiangen, China), according to the manufacturer's protocol with a Real-Time PCR System (Bio-Rad, USA). A melting curve analysis was performed at the end of the PCR cycle to validate the specificity of the expected PCR product. U6 was used as an internal control due to its prolonged and stable expression throughout all the evaluated samples. The relative level of expression of each miRNA was determined using the comparative CT method, which was defined as $2^{-\Delta Ct}$. For mRNA analysis, cDNA was synthesized using a cDNA Synthesis Kit (Bio-Rad, USA) and was subjected to 40 cycles of quantitative PCR with SYBR Premix (Takara, Japan) in the Real-Time PCR Detection System. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Each sample from each study subject was analyzed with PCR in triplicate. The primers for analysis are summarized in Table 1.

2.5. *Biochemical and clinical assays*

Levels of IL-6, IL-8, IL-2R, TNF-alpha, IL-10 and IL-1beta were measured using ELISA kits (IBL-America, USA). Related health parameters, clinical history, personal history, and family history were recorded.

2.6. *Statistical analysis*

Results are expressed as the mean ± SD. A two-tailed Student's t test was used to compare two groups. Two-way ANOVA was used to compare multiple groups, and analyses were performed using GraphPad Prism 6.0. Logistics regression analysis was performed to evaluate risk factors for ISR, and receiver operating characteristic (ROC) analysis was perform to access the efficiency of distinguishing patients with ISR from patients without ISR (using STATA 14.0). A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. *Clinical characteristics of subjects*

The general characteristics of patients with ISR (n = 14) and patients without ISR (n = 28), including gender, age, BMI, a family history of cardiovascular disease, a history of hypertension, hypercholesterolemia, or diabetes, and related risk factors are summarized in Table 2. More than half of the patients had hypertension, hypercholesterolemia, or a family history of cardiovascular disease. Similar characteristics were noted in patients with or without restenosis ($p > 0.05$ for all). The BMI of patients with restenosis (24.22 ± 0.58 kg/m^2) was slightly higher than that of patients without restenosis (23.66 ± 0.48 kg/m^2), and patients without restenosis were 1 year older than patients with restenosis; these differences were not significant.

3.2. *Circulating miRs and corresponding target gene levels in patients with or without ISR*

Real-time PCR analysis was used to measure plasma

levels of miR-17, miR-21, miR-125, and miR-126. The miR-17 level was significantly higher in patients with ISR compared to patients without ISR ($p < 0.01$), as shown in Figure 1. The average level of miR-17 was 3.13 ± 0.22 in patients with ISR and 1.06 ± 0.04 in patients without ISR. There was no significant difference in the levels of miR-21, miR-125, and miR-126 between the two groups. TIMP-1, as the downstream target of miR-17, was expressed at a lower level in patients with ISR compared to that in patients without ISR ($p < 0.05$). The TIMP-1 level was 0.33 ± 0.04 in patients with ISR and 1.00 ± 0.05 in patients without ISR. A logistic regression model indicated that miR-17 was significantly associated with a risk of ISR (odds ratio [OR]: 7.2254, $p < 0.01$, 95% confidence interval [CI]: 1.9825–26.3331), and TIMP-1 was also significantly related to ISR (OR: 0.002, $p < 0.01$, 95% CI: 0.00005–0.098).

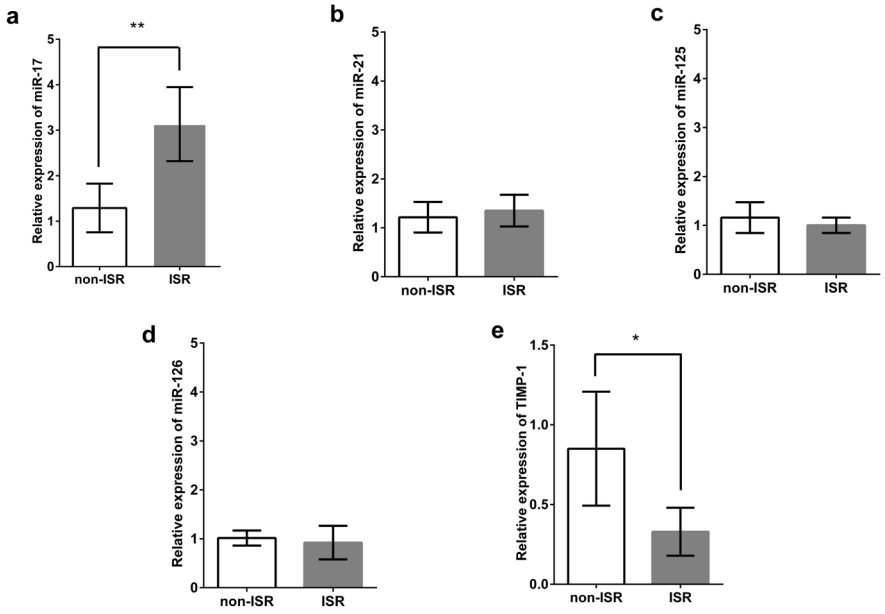

Figure 1. The expression of miR-17 (a), miR-21 (b), miR-125 (c), miR-126 (d), and TIMP-1(e) in plasma was evaluated with real-time PCR.

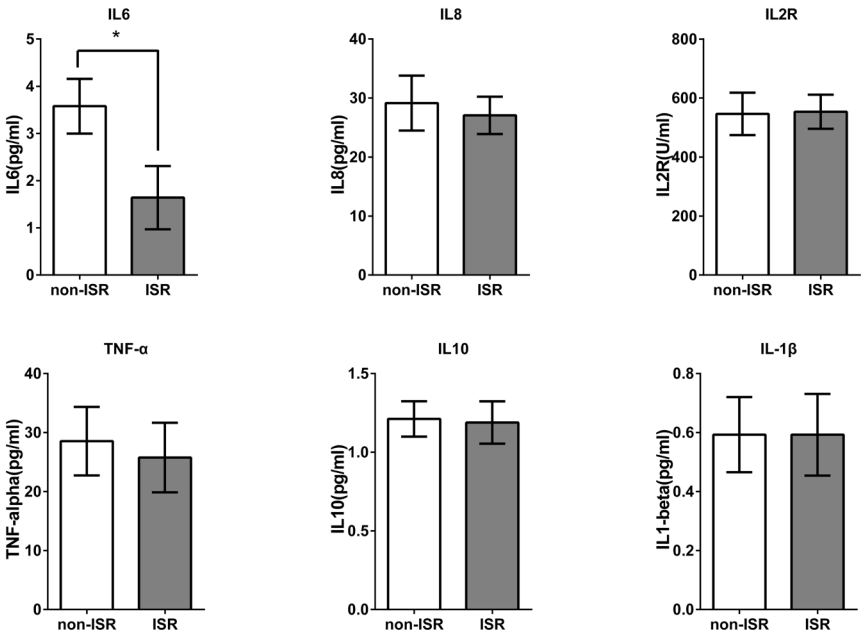

Figure 2. The plasma levels of IL-6 (a), IL-8 (b), IL-2R (c), TNF-alpha (d), IL-10 (e), and IL-1beta (f) were measured in patients with or without ISR.

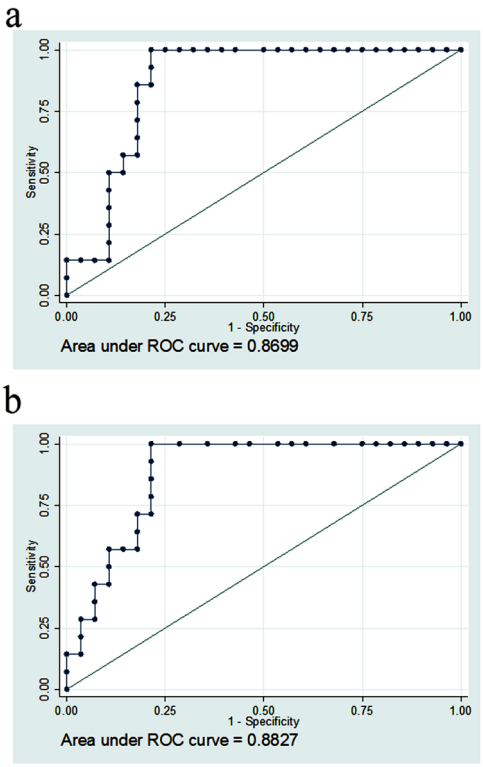

Figure 3. ROC curve analysis using plasma miR-17(a) and TIMP-1(b) to distinguish in-stent restenosis.

3.3. *Inflammatory marker levels in patients with or without ISR*

As shown in Figure 2, IL-6 levels were lower in patients with ISR than those in the patients without ISR ($p < 0.01$). The mean level of IL-6 in patients with ISR was 1.64 ± 0.18, while that in patients without ISR was 3.52 ± 0.11. However, significant differences in the levels of IL-8, IL-2R, TNF-alpha, IL-10, and IL-1beta were not noted in patients with or without ISR ($p > 0.05$ for all). The mean levels of IL-8, IL-2R, TNF-alpha, IL-10, and IL-1beta were 27.1 ± 3.17, 554 ± 57.9, 25.8 ± 5.9, 1.19 ± 0.13, and 0.50 ± 0.52 in patients with ISR and 29.1 ± 4.64, 546 ± 71.8, 28.5 ± 5.8, 1.21 ± 0.11, and 0.57 ± 0.50 in patients without ISR, respectively.

3.4. *ROC analysis of miR-17 and TIMP-1*

To investigate the relationship between the miR-17, TIMP-1, and ISR, ROC analyses were performed to evaluate the diagnostic ability of miR-17 and TIMP-1. As shown in Figure 3, the ROC curves for miR-17 and TIMP-1 reflected a strong distinction between patients with or without ISR, with an AUC of 0.8699 (95% CI: 0.75868–0.98112, $p < 0.001$) and 0.8827 (95% CI: 0.78159–0.98372, $p < 0.001$), respectively. miR-17 and TIMP-1 had a specificity of 78.57% and 78.57% in patients with ISR. miR-17 and TIMP-1 had a specificity of 85.71% and 92.86% in patients without ISR. miR-17 and TIMP-1 displayed acceptable sensitivity and specificity for the diagnosis of ISR.

4. Discussion

Recent studies have suggested that circulating miRNAs are useful biomarkers for the diagnosis of CVD. Several miRNAs have been found to take part in the pathogenesis of coronary artery disease (*12*) and atherosclerosis (*13*), but few studies focused on specific miRNAs related to ISR, which can be detected in circulating blood. miR-17, miR-21, miR-125, and miR-126 are commonly mentioned as corresponding to the pathologic process of ISR. One study found a higher level of expression of circulating miR-21 in patients with ISR (*14*). A point worth noting is that miR-21 and miR-126 are respectively related to ACS (*15*) and AMI (*16*), which may preclude their suitability for a specific diagnosis. On the basis of widely reported chip screening of miRNAs, the current study detected significantly higher plasma levels of miR-17 in patients with ISR *via* quantitative detection of real-time PCR. Given its clinical use, miR-17 is one biomarker with a higher specificity but not lower circulating expression, and it is more suitable for use as a diagnostic method.

Previous studies focused on the screening of miRs, since their levels of expression change in ISR, but little attention was paid to the corresponding downstream target gene levels or related effective factors, as a change at one point is usually not sufficient to support a potential diagnosis. The current study found that TIMP-1, a downstream target of miR-17, was expressed at lower levels in patients with ISR compared to levels in patients without ISR. Levels of expression of the inflammatory biochemical factor IL-6 were also lower in patients with ISR. These results indicated changes in the circulating cascade of miR-17/TIMP-1/IL-6 in patients with ISR, which closely coincide with the inflammatory response signaling pathway revealed by basic research. These observations indicate that miR-17 and its downstream factors are likely to allow predictive tests to diagnose ISR.

miR-17 has been widely studied. The coding area of miR-17 is located within an 800 base-pair region of human chromosome 13. miR-17 plays an essential role in the development of the human heart, lungs, and immune system (*17*). A study has revealed that miR-17 is involved in inflammation and oxidative stress, which are mechanisms relevant to macrophage polarization (*18*). miR-17 is also hypoxia-responsive; it may induce protective autophagy and counter apoptosis in vascular smooth muscle cells and contributes to vascular smooth muscle cell proliferation (*19,20*).

Based on bioinformatic analysis and experimental validation, TIMP-1 was identified as a target gene of miR-17. As previously reported, a functional study indicated that a decrease in TIMP-1 was responsible for

the upregulation of miR-17 (*21*).

ISR has been associated with the activation of matrix metalloproteinases (MMPs) and downregulation of their endogenous inhibitors (tissue inhibitor of matrix metalloproteinases [TIMPs]), which results in degradation of the artery wall matrix and decreased elasticity and which contributes to endothelial inflammation and vascular hyperpermeability (*22*). Expression of miR-17 is higher during ISR, resulting in decreased expression of its target-TIMPs and thus limiting potent inhibition of MMP-2 activity by TIMPs. Because TIMPs are essential to reducing inflammation, TIMP-1 (as a tissue inhibitor of MMPs) is expected to be lower in the pathologic process of ISR. The current study further demonstrated that these changes in levels of expression, according to the inflammatory pathway, can be readily detected in the circulation.

IL-6 is considered to be a pro-inflammatory lipocytokine. From molecular biology perspective, there is a negative correlation between TIMPs and IL-6. Mechanistic studies indicated that loss of TIMPs promoted production of IL-6, and recombinant adenovirus Ad-hTIMP-1 inhibited the inflammatory response and downregulated the expression of IL-6 (*23*). Blood levels of IL-6 were higher while blood levels of TIMP-1 were lower in individuals with unstable atherosclerotic plaque in their coronary arteries compared to individuals with stable atherosclerotic plaque (*24*). One year after PCI, IL-6 was lower than normal; this is because the inflammatory response in ISR was not merely equal to that in atherosclerotic injury. In the process of vascular wall repair after stent implantation, IL-6 changes nonlinearly, and only IL-6 plays an important role in the inflammatory response phase. IL-6 peaks 24 hours after drug-eluting stent implantation and then begins to decline (*25*). After restenosis occurs, the peak period of traumatic inflammation passes and the stable stage of an inflammatory reaction begins.

Previous studies on the primary mechanism underlying ISR mainly focused on the exaggerated neointimal proliferative response. Research has firmly established that proliferation and migration of VSMCs are critical cellular events responsible for the development of neointimal hyperplasia and that phenotypic modulation (transformation from a contractile to synthetic phenotype) of VSMCs plays an important role in this process. The current study verified the inflammatory cascade of ISR from another angle. Commonly, inflammatory-related factors are more widely distributed in the circulation. The current study quantitative measured key markers of the ISR cascade based on reported chip screening, and these findings are highly consistent with results of previous mechanistic studies.

The current study provides clinical evidence that circulating miR-17, TIMP-1, and IL-6 can serve as specific biomarkers for ISR. However, the small number of enrolled patients from a single center is a major limitation that must be considered. Further large-scale studies are needed to validate the clinical utility of miRs and their downstream target genes as practical biomarkers for ISR. Moreover, this study is a cross-sectional case-control study, and prospective follow-up studies should be conducted to better assess the predictive value of biomarkers for ISR.

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