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Associations of serum expressions of miR-499 and sex determining region Y-box 6 with prognosis of acute myocardial infarction patients

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Abstract

***Background**: To explore the associations of serum expressions of miR-499 and sex determining region Y-box 6 (SOX6) with major adverse cardiovascular and cerebrovascular events (MACCE) and prognosis of acute myocardial infarction (AMI) patients undergoing percutaneous coronary intervention (PCI). **Methods**: The clinical data of 132 patients diagnosed from February 2016 to October 2019 were collected. Serum miR-499 and SOX6 expressions were detected by RT-qPCR. Optimal cut-off values were determined using receiver operating characteristic curves, based on which patients were divided into low and high miR-499 expression groups, and high and low SOX6 expression groups. Survival curves were plotted using Kaplan-Meier method, and the independent risk factors for MACCE were explored by multivariate logistic regression analysis. A nomogram model was established based on the factors and validated using internal data. **Results**: AMI group had higher miR-499 expression and lower SOX6 expression than those of control group (P<0.05). After PCI, miR-499 expression decreased and SOX6 expression increased (P<0.05). Low miR-499 expression group had higher 3-year survival and MACCE-free rates than those of high miR-499 expression group (P<0.05). Low SOX6 expression group had lower 3-year survival and MACCE-free rates than those of high SOX6 expression group (P<0.05). AMI history, LVEF, CK-MB, miR-499 and SOX6 expressions were independent risk factors for MACCE (P<0.05). The nomogram model had high accuracy for predicting overall survival, with a concordance index of 0.742 (95%CI=0.684-0.845). **Conclusions**: AMI patients have increased serum expression of miR-499 and decreased expression of SOX6. High miR-499 expression is an independent risk factor for poor prognosis. The established nomogram model can be used to predict the risk of MACCE after PCI.*

***Keywords**: miR-499; sex determining region Y-box 6; acute myocardial infarction; prognosis; major adverse cardiovascular and cerebrovascular events*

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Introduction

Acute myocardial infarction (AMI) usually refers to a process where secondary thrombosis occurs on the basis of rupture and erosion of vulnerable atherosclerotic plaque and endothelial injury, so that acute and continuous total coronary occlusion, and sharp decline or interruption of blood supply are caused, resulting in myocardial necrosis and inflammatory response (1), which is characterized by acute onset and high mortality (2). Clinically, AMI can lead to the incidence of major adverse cardiovascular and cerebrovascular events (MACCE) in patients in a short time period, thus worsening the patient's prognosis and increasing mortality (3). Percutaneous coronary intervention (PCI), emergency coronary artery bypass graft surgery (CABG) and antithrombotic drug therapy can be used to restore myocardial perfusion to save viable myocardium, which currently are the most effective treatment means for AMI. Although the prognosis can be greatly improved, reperfusion may lead to myocardial ischemia/ reperfusion injury (4).

Micro ribonucleic acids (miRNAs) are crucial players in the pathogenesis of a variety of cardiovascular diseases (5,6). Studies have shown that the expression of serum miR-1/-133a/-499 significantly rises in AMI patients, and miR-499 is expressed only in myocardial cells (7). In addition, the increased expression of miR-499 can be observed in AMI patients, while its expression declines in normal hearts (8). Xin *et al*. (9) found that miR-499 could serve as a potential predictive biomarker for AMI. One of the targets of miR-499 is sex determining region Y-box 6 (SOX6), a member of the SOX6 transcript family. However, whether miR-499 and SOX6 are valuable in prognostic evaluation of AMI has not been reported in the literature yet. In the present study, therefore, the expression levels of miR-499 and SOX6 in AMI were analyzed, and their associations with the prognosis of AMI patients were explored.

Materials and methods

General data

The clinical data of 132 patients diagnosed with AMI in the Department of Cardiology of our hospital from February 2016 to October 2019 were retrospectively analyzed. This study was approved by the Ethics Committee of our hospital (Ethic approval number: YCLL201512080923. Date: December 4th, 2015), and patients and their families gave the informed consent. There were 67 males and 65 females aged 34-76 years old, with a mean age of (56.32±6.84) years, and all of them were admitted to the hospital at 1-12 h after the onset. The diagnostic criteria for AMI were based on the 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology *(2017)*. All subjects were diagnosed with AMI by electrocardiography and coronary angiography (10), and they underwent PCI. The patients were followed up every 3 months for 3 years by telephone, outpatient clinic and medical record inquiry, with a follow-up rate of 100%. Additionally, 132 healthy people who received physical examination in our hospital during the same period were selected as controls, including 72 males and 60 females aged 35-71 years, with a mean age of (54.6±7.3) years. Exclusion criteria were as follows: patients complicated with cardiac insufficiency before the onset, or those with autoimmune deficiency diseases, malignancies, a history of major surgical trauma in the last 6 months, severe hepatic or renal dysfunction, or incomplete clinical data.

Main reagents and devices

RNA extraction kit (TRIzol® LS Reagent, Invitrogen, USA), RNA reverse transcription kit (SYBR Green, Sigma, USA), and miR-499

and SOX6 primers (synthesized by Invitrogen, Guangzhou) were used.

PRISM®7500 real-time quantitative polymerase chain reaction (RT-qPCR) system (ABI, USA), NanoDrop2000 ultra-micro nucleic acid analyzer (Thermo, USA), and low-temperature refrigerated centrifuge and Hema9600 gene amplification instrument (Zhuhai Hema Medical Instrument Co., Ltd.) were employed.

Collection of general data and serum

After enrollment, the general data such as age, gender, and medical history of subjects were surveyed by means of medical history inquiry and physical examination. The multi-vessel lesion status was judged by coronary angiography. The blood pressure of patients was measured in a quiet and resting state after admission, and both systolic blood pressure and diastolic blood pressure were recorded. Fasting (8-12 h) venous blood sample was collected in the morning the next day after admission and also collected 24 h after PCI, in which the total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein (HDL), white blood cell count (WBC), and creatine kinase-MB (CK-MB) were detected. At 24 h after the onset, color Doppler echocardiography was performed, and the left ventricular ejection fraction (LVEF) was measured and recorded. In addition, the patients underwent PCI within 12 h after admission, and 5 mL of cubital venous blood was collected again after 24 h. The blood was centrifuged with a high-speed refrigerated centrifuge at 3000 rpm for 15 min to separate the serum, and cryopreserved in an ultra-low temperature refrigerator at -80°C for later analysis of the expression levels of serum miR-499 and SOX6.

Detection of serum expression levels of miR-499 and SOX6 by RT-qPCR

The expression levels of serum miR-499 and SOX6 were determined using RT-qPCR. The total RNA was extracted from serum according to the instructions of protein extraction kits, and its concentration was detected using the NanoDrop2000 ultra-micro nucleic acid analyzer. Subsequently, 2 μg of eligible serum RNAs were reversely transcribed into cDNA (reverse transcription at 37°C for 15 min, and inactivation of reverse transcriptase at 85°C for 15 s). RT-qPCR was performed in a PRISM®7500 RT-qPCR system under the following conditions: pre-denaturation at 95°C for 10 min, (95°C for 15 s, 60°C for 15 s, 70°C for 20 s) × 40 cycles, extension at 72°C for 10 min. The primers of miR-499, SOX6 and GAPDH are shown in Table 1. With U6 as an internal reference, the relative mRNA expressions of miR-499 and SOX6 were calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

SPSS19.0 software was used for statistical analysis. The measurement data were expressed as mean ± standard deviation, and intergroup comparisons were performed by the t test. The intergroup comparisons of categorical data were conducted with the χ^2 test. $P<0.05$ was consid-

Table 1. Primer sequences of miR-499, SOX6 and GAPDH.

Gene	Primer sequence
miR-499	Forward: 5'-TTAAGACTTGCAGTGATGTTT-3'
	Reverse: 5'- GAACATGTCTGCGTATCTC-3'
SOX6	Forward: 5'-CACUUGUCAGUACCAUUCATT-3'
	Reverse: 5'-UGAAUGGUACUGACAAGUGTT-3'
GAPDH	Forward: 5'-CATCACTGCCACCCAGAAGACTG-3'
	Reverse: 5'-ATGCCAGTGAGCTTCCCGTTCAG-3'

ered statistically significant. The optimal cut-off value was determined using receiver operating characteristic (ROC) curve. The survival curve was plotted using the Kaplan-Meier method. The independent risk factors for MACCE were explored by multivariate logistic regression analysis. A nomogram model was established using rms package of R software and internally validated, and the concordance index (C-index) was calculated.

Results

Clinical characteristics of patients

The clinical characteristics of patients were compared between the AMI group and control group before PCI. The results showed that the proportion of patients with a history of AMI and the level of CK-MB were lower in the AMI group than those in the control group, while LVEF was higher in the AMI group than that in the control one (P<0.05). There were no significant differences in the age, gender, TC, TG, and HDL between the two groups (P>0.05) (Table 2).

At 24 h after PCI in AMI group, the serum was collected to analyze the changes in TC, TG, HDL, LDL, WBC, and CK-MB, and color Doppler echocardiography was conducted to measure LVEF. It was found that after PCI, serum TC, TG, HDL, LDL, and WBC had no significant differences in the AMI group compared with those before treatment (P>0.05), but CK-MB significantly declined and LVEF significantly rose (Table 3).

Expression levels of serum miR-499 and SOX6 in AMI patients

Before treatment, the AMI group had a higher relative expression level of serum miR-499 (P<0.05) and a lower expression level of SOX6 than the control group (P<0.05). After PCI, the relative expression level of serum miR-499 declined, while the expression level of serum

Table 2. Clinical characteristics of patients.

Item	AMI group (n=82)	Control group (n=82)	t/χ^2	P
Age (Y)	57.63±9.28	61.28±7.95	t=-1.081	0.264
Gender (n)/male	43	37		
Gender (n)/female	39	38	χ^2=0.089	0.675
History of AMI (n)	16	7	χ^2=5.149	0.024
Hypertension (n)	48	42	χ^2=2.575	0.112
Diabetes (n)	9	11	χ^2=2.791	0.089
Smoking (n)	34	24	χ^2=1.098	0.295
SBP (mmHg)	147.3±13.7	142.2±15.6	t=0.892	0.357
DBP (mmHg)	76.35±12.45	72.93±5.49	t=0.918	0.363
Heart rate (beats/min)	73.74±10.27	74.16±12.83	t=-0.273	0.793
TC (mmol/L)	4.26±0.84	4.32±0.68	t=0.243	0.812
TG (mmol/L)	1.68±0.64	1.52±0.38	t=0.924	0.438
HDL (mmol/L)	1.07±0.14	1.05±0.10	t=-1.233	0.181
LDL (mmol/L)	2.86±0.36	2.78±0.54	t=0.673	0.546
WBC ($\times 10^9$/L)	9.37±1.87	9.58±1.93	t=-0.367	0.711
LVEF (%)	56.87±3.59	76.35±6.12	t=15.234	0.000
Multi-vessel lesion (n)	49	50	χ^2=0.863	0.260
CK-MB (U/L)	143.26±12.56	124.87±15.66	t=-7.397	0.000

mmHg=0.133 kPa; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low-density lipoprotein; WBC: white blood cell count.

Table 3. Comparisons before and after PCI in AMI group.

Item	Before PCI	After PCI	t	P
TC (mmol/L)	4.26±0.84	4.30±0.74	t=0.243	0.545
TG (mmol/L)	1.68±0.64	1.57±0.61	t=0.876	0.354
HDL (mmol/L)	1.07±0.14	1.13±0.03	t=1.213	0.224
LDL (mmol/L)	2.86±0.36	2.70±0.35	t=-0.865	0.638
WBC ($\times 10^9$/L)	9.37±1.87	9.31±0.72	t=-0.521	0.425
LVEF (%)	56.87±3.59	70.97±2.03	t=10.375	0.000
CK-MB (U/L)	143.26±12.56	132.66±15.01	t=-5.894	0.000

SOX6 rose compared with those before treatment (P<0.05) (Table 4).

Risk factors for MACCE

Multivariate logistic regression analysis through the stepwise method was carried out by using the occurrence of MACCE during follow-up as the dependent variable, and the characteristic indices of patients with significantly different expression levels of serum miR-499 and SOX6 (P<0.05) as independent variables. The incidence of MACCE in AMI patients during follow-up, the expression levels of serum miR-499 and SOX6 and the statistically different indexes (P<0.05) were incorporated into the multivariate logistic regression analysis using the stepwise regression method. The results showed that the history of AMI, LVEF, CK-MB, miR-499, and SOX6 were independent risk factors for MACCE (P<0.05) (Table 5).

Establishment of the nomogram model

The nomogram model was established using independent risk factors in the multivariate logistic regression analysis as predictors, and validated using internal data. The validation results revealed that the C-index was 0.742 (95%CI=0.684-0.845) (Figure 1).

Table 4. Expression levels of serum miR-499 and SOX6.

Group	MiR-499	SOX6
AMI group		
Before treatment	1.24±0.15##	0.66±0.03#
After treatment	0.91±0.31*#	0.78±0.34*#
Control group	0.74±0.36	0.89±0.25

*P<0.05 before treatment vs. after treatment in AMI group. #P<0.05, ##P<0.05 vs. control group.

Accuracy and efficacy of the nomogram model

The calibration and efficacy of the nomogram model established were assessed. As shown in Figure 2, A is the reference curve, B is the fitting curve, and the shadow on both sides is 95%CI. It was found that the risk was underestimated by the model in the case of an incidence rate <35%, the risk was overestimated in the case of an incidence rate of 35-65%, the risk was underestimat-

Table 5. Multivariate logistic regression analysis results of risk factors for MACCE in AMI patients after PCI.

Variable	Partial regression coefficient	Standard error	χ^2	P	OR	95%CI
History of AMI (no= 0, yes =1)	1.072	0.146	4.251	0.005	0.142	1.221~1.833
LVEF (%)	0.393	0.049	4.624	0.042	1.469	1.318~1.538
CK-MB (U/L)	0.435	0.184	5.726	0.000	1.432	1.227~1.643
MiR-499	0.438	0.196	3.569	0.031	1.451	1.254~1.634
SOX6	0.447	0.251	4.689	0.017	1.670	1.015~1.377

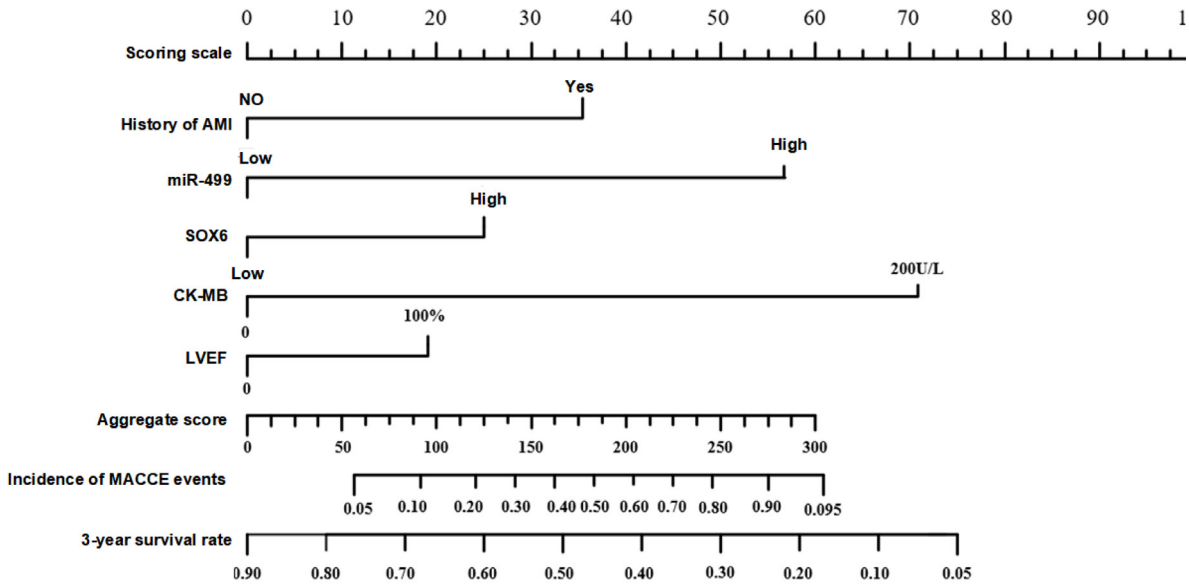

Fig. 1. Nomogram model for predicting MACCE in AMI patients after PCI.

ed in the case of an incidence rate of 65-100%, and the predicted value and the observed value were exactly the same in the case of an incidence rate of 35% and 65%. On the whole, the accuracy of the model was better. Moreover, the clinical decision curve illustrated that the nomogram model had a higher net benefit value, indicating the better efficacy of the model (Figure 2).

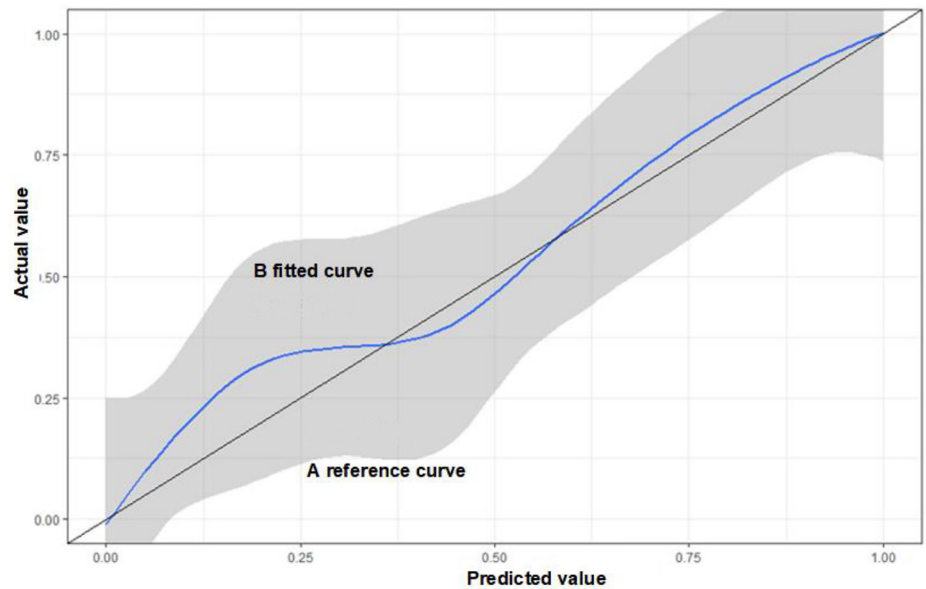

Fig. 2. Calibration curve of nomogram model for MACCE in AMI patients after PCI.

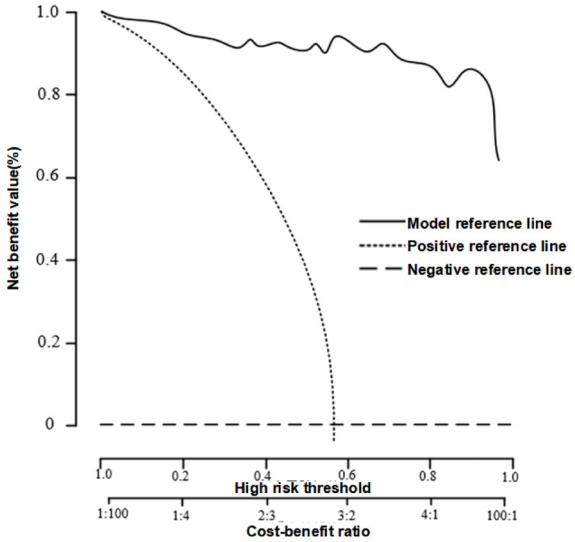

Fig. 3. Clinical decision curve of nomogram model for MACCE in AMI patients after PCI. Abscissa: threshold probability, ordinate: net benefit value, i.e. benefit (benefit of AMI patients after treatment) - harm (harm of MACCE in AMI patients after treatment). Negative reference line: no MACCE after operation in all patients, net benefit=0, positive reference line: MACCE after operation in all patients, net benefit= backslash of slope. When the probability predicted by the model was n for AMI patients after operation, the higher the corresponding net benefit value, the better, i.e. the closer the curve was to the X-axis and Y-axis, the better the efficacy.

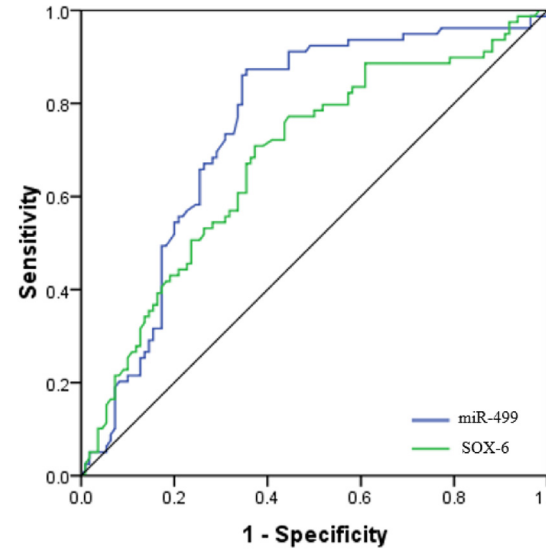

Fig. 4. ROC curve analysis results of serum miR-499 and SOX6 expressions for diagnosis of AMI.

ROC curve analysis results of serum miR-499 and SOX6 expressions

The results of ROC curve analysis showed that the optimal cut-off value of serum miR-499 expression in the diagnosis of AMI was 0.86, and its area under the curve (AUC), sensitivity, and specificity were 0.780, 78.25%, and 65.86%, respectively. The optimal cut-off value of serum SOX6 expression in the diagnosis of AMI was 0.75, and its AUC, sensitivity and specificity were 0.721, 78.87%, and 62.58%, respectively (Figure 4 and Table 6).

Associations of serum miR-499 and SOX6 expressions with poor prognosis of AMI patients

No AMI patients were lost to the 36-month follow-up, and the follow-up rate was 100% (132/132). The survival curve was plotted using the Kaplan-Meier method. Based on the optimal cut-off value of miR-499 in ROC curve analysis, the miR-499 level below the optimal cut-off value indicated the low expression, while that above the optimal cut-off value indicated the high expression. In this study, there were 61 cas-

Table 6. Sensitivity and specificity of serum miR-499 and SOX6 expressions for diagnosis of AMI.CI: Confidence interval.

Variable	AUC	Optimal cut-off value	Sensitivity (%)	Specificity (%)	P	95%CI
miR-499	0.780	0.86	78.25	78.87	<0.001	0.734-0.825
SOX6	0.721	0.75	65.86	62.58	0.009	0.702-0.793

es in the high miR-499 expression group and 71 cases in the low miR-499 expression group. At 1 year after PCI, there were 2 cases of MACCE and 2 cases of death in the high miR-499 expression group, and 8 cases of MACCE and 5 cases of death in the low miR-499 expression group, showing a significant difference in the 1-year MACCE rate (2.82% *vs*. 13.12%) (P<0.05), but not in the 1-year survival rate between high miR-499 expression group and low miR-499 expression group (97.18 *vs*. 91.80%) (P>0.05). Moreover, at 3 years after PCI, there were 30 cases of MACCE and 19 cases of death in the high miR-499 expression group, and 15 cases of MACCE and 10 cases of death in the low miR-499 expression group. The above results revealed that both 3-year MACCE rate and 3-year survival rate had significant differences between the two groups (49.18% *vs.*14.28%, 75.41% *vs.* 87.14%) (P<0.05) (Figure 5).

Based on the optimal cut-off value of SOX6 in ROC curve analysis, the SOX6 level below the optimal cut-off value indicated the low expression, while that above the optimal cut-off value indicated the high expression. In this study, there were 78 cases in High SOX6 expression group and 54 cases in Low SOX6 expression group. At 1 year after PCI, there were 6 cases of MACCE and 1 case of death in the High SOX6 expression group, and 4 cases of MACCE and 2 cases of death in the Low SOX6 expression group. It can be seen that neither 1-year MACCE rate nor 1-year survival rate had a significant difference between the High SOX6 expression group and the Low SOX6 expression group (7.69% *vs.*7.41%, 98.72% *vs.* 96.43%) (P>0.05). Moreover, at 3 years after PCI, there were 24 cases of MACCE and 14 cases of death in the High SOX6 expression group, and 27 cases of MACCE and 19 cases of death in the Low SOX6 expression group. The above results demonstrated that both 3-year MACCE-free rate and 3-year survival rate had significant differences between the two groups (50.00% *vs.* 30.77%, 82.05% *vs.* 64.81%) (P<0.05) (Figure 6).

Discussion

AMI is one of the most common cardiovascular emergencies in the clinic. According to the statistics in the *Outline of the report on cardiovascular disease in China (2010)* (11), there are approximately 290 million patients with cardiovascular disease in China currently, and more than 500 thousand new cases each year (12), and both morbidity and mortality rates of AMI have shown an increasing trend year by year (13).

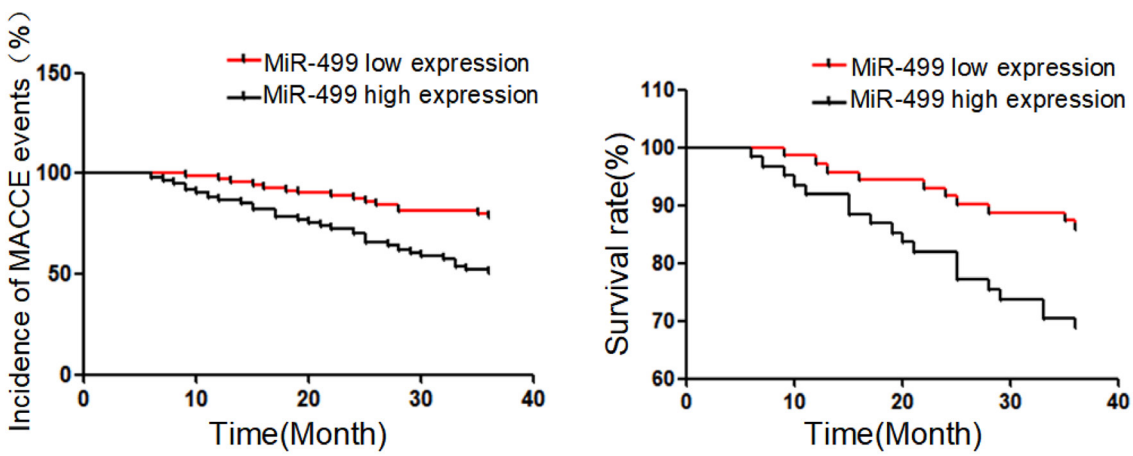

Fig. 5. MACCE-free and survival curve analysis results based on miR-499 expression.

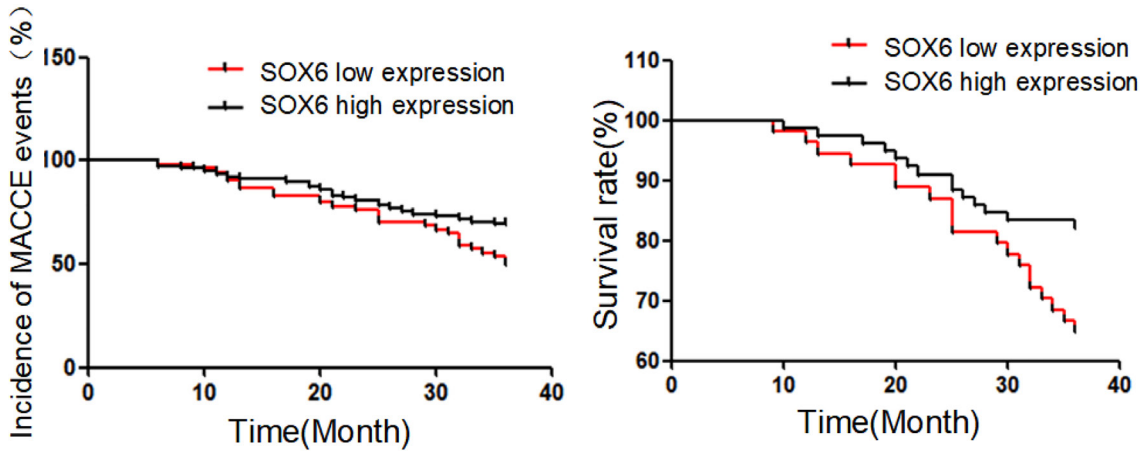

Fig. 6. MACCE-free and survival curve analysis results based on SOX6 expression.

AMI is characterized by acute onset and rapid changes, so the requirement for treatment timing is higher. Emergency revascularization is the major treatment method for MI, and irreversible lesions often occur in the ischemic myocardium after more than 12 h (14, 15). Therefore, it is of great importance to study indexes related to disease detection and outcome for developing therapeutic measures. Recent studies have shown that serological diagnostic indexes can offer references to clinical diagnosis of MI. MiRNAs are a class of small non-coding RNAs able to regulate the expression of transcriptional genes, which are involved in the signal transduction of myocardial and vascular smooth muscle cells, and related to the pathological process of MI, arrhythmia, cardiomyopathy, and vascular proliferation (16). MiR-499 is expressed in cardiac cells, and it is basically undetectable in human cardiac stem cells or human embryonic stem cells, but its expression can be found in myocardial cells after differentiation or mitosis and also in myocardial cells of fetuses, newborns, and adults (17). Li *et al*. (18) confirmed that SOX6 was the target of miR-499. It has been found (19) that miR-499 can target SOX6 through SOX6-3'UTR luciferase, and the SOX6 mRNA expression significantly declined after overexpression of miR-499 in myocardial cells of neonatal rats.

In the present study, the results showed that before treatment, the AMI group had a higher expression level of serum miR-499 and a lower expression level of SOX6 than the control group. After PCI, the expression level of serum miR-499 declined, while the expression level of serum SOX6 rose compared with those before treatment. The changes in the serum SOX6 level in AMI patients may be regulated by miR-499, indicating that there is a regulatory relation between miR-499 and SOX6 in AMI. Moreover, the optimal cut-off value and AUC of serum miR-499 and SOX6 in the diagnosis of AMI were 0.86 and 0.780, and 0.75 and 0.721, respectively, and both miR-499 and SOX6 had better sensitivity and specificity, suggesting that the expression levels of miR-499 and SOX6 are of certain value in the diagnosis of AMI.

In addition, the associations of the expression levels of serum miR-499 and SOX6 with the survival time and MACCE-free rate after PCI in AMI patients were explored in this study. The results revealed that the 3-year survival rate of patients in the low miR-499 expression group was significantly higher than that in the high miR-499 expression group (85.91% *vs*. 68.85%), and the 3-year MACCE-free rate in the low miR-499 ex-

pression group was significantly higher than that in the high miR-499 expression group (78.87% *vs.* 50.82%). The 3-year survival rate of patients in Low SOX6 expression group was lower than that in the High SOX6 expression group (64.81% *vs*. 82.05%), and the 3-year MACCE-free rate in the High SOX6 expression group was significantly higher than that in the Low SOX6 expression group (50.00% *vs.* 30.77%). To sum up, the low expression of miR-499 and the high expression of SOX6 are related to the prolonged survival time of AMI patients and the decreased incidence of MACCE.

Furthermore, according to multivariate logistic regression analysis, the history of AMI, LVEF, CK-MB, miR-499, and SOX6 were all independent risk factors for MACCE. Based on the above factors, the nomogram model for MACCE after PCI was established, and its accuracy was validated using C-index. C-index refers to the ability to accurately screen the risk of MACCE after operation, ranging from 0.5 (no predictive ability) to 1 (highest predictive ability) (20,21). In this study, the C-index of the nomogram model was 0.742 (95%CI=0.684-0.845), indicating that the model had high discrimination power and better predictive value, and it can be used as an auxiliary prediction tool after PCI in AMI patients to a certain extent. The sample size in this study was limited, so the clinical practice value of the nomogram model remains to be validated by more clinical data.

Conclusion

In conclusion, AMI patients have an increased expression of serum miR-499 and a decreased expression of SOX6. Highly-expressed serum miR-499 is an independent risk factor for the poor prognosis of AMI patients. The nomogram model established based on the above factors can be used to predict the risk of MACCE in AMI patients after PCI. The sample size in this study was small, so the predictive value of the nomogram model remains to be further validated.

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Authors' contributions

JM: Conceptualization, writing – review and editing and supervision; BW: Methodology, formal analysis and writing – original draft preparation; YL: Methodology, formal analysis and writing – original draft preparation; All authors have approved the submission and publication of this manuscript.

Conflict of interest

None to declare.

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