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Dysregulation Serum miR-19a-3p is a Diagnostic Biomarker for Asymptomatic Carotid Artery Stenosis and a Promising Predictor of Cerebral Ischemia Events

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Abstract

This study aims to identify the diagnostic potential of microRNA-19a-3p (miR-19a-3p) for asymptomatic carotid artery stenosis (CAS) and clinical predictive potential for cerebral ischemia events (CIEs). Serum samples from 101 asymptomatic CAS patients and 98 healthy controls were collected. And it was found that serum miR-19a-3p in asymptomatic CAS patients was generally elevated ($P < .05$). Increased miR-19a-3p in asymptomatic CAS was associated with severe CAS (odds ratio = 3.920, 95% confidence interval [CI] = 1.482-10.372, $P < .01$). The area under the receiver operating characteristic (ROC) curve (AUC) was 0.905, indicating that the level of miR-19a-3p was statistically significant for the diagnosis of asymptomatic CAS. Furthermore, the level of serum miR-19a-3p (hazard ratio [HR] = 8.507, 95% confidence interval [CI] = 2.239-32.328, $P = .002$) and degree of artery stenosis (HR = 3.695, 95% CI = 1.127-12.109, $P = .031$) were independent predictors of occurrence of CIE. Moreover, patients with elevated miR-19a-3p levels were more likely to experience CIE than patients with low levels. Upregulated miR-19a-3p can be used as a diagnostic biomarker for asymptomatic CAS patients and as an independent predictor of CIE.

Keywords

miR-19a-3p, cerebral ischemia event, asymptomatic carotid artery stenosis

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Introduction

Ischemic stroke is considered one of the most serious diseases worldwide, threatening the health and life of aging populations.[1] The 5-year mortality rate of stroke exceeds 50% and there are 1.5 million new cases of stroke each year in China.[2] Carotid artery stenosis (CAS) is one of the main risks of ischemic stroke, accounting for one-fifth of its causes.[3] CAS gradually develops from the initial asymptomatic state to symptomatic CAS. And the annual risk of ischemic stroke in asymptomatic CAS is 2% to 5%.[4] However, current imaging has different deficiencies in the diagnosis of moderate stenosis (>50%) or severe stenosis (>70%) in asymptomatic CAS patients. For example, the preferred noninvasive diagnosis method of CAS in clinical practice is duplex ultrasound imaging, which is simple and safe, but it is easily affected by the level of operators, and it is expensive and not suitable for large-scale screening of the population[5-7] Therefore, the current priority is to identify and treat asymptomatic CAS patients as early as possible through noninvasive and low-cost methods to reduce the risk of stroke.

MicroRNAs (miRNAs) are small noncoding RNA molecules with a length of approximately 22 nucleotides. miRNAs are abundant and stable in serum, plasma, urine, saliva, milk, and cerebrospinal fluid.[8] Therefore, their dysregulation has

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Table 1. Clinical Data of the Study Population.

Variables	Healthy subjects ($n = 98$)	CAS patients ($n = 101$)	*P* value
Gender (male and female)	49/49	46/55	.529
Age (years)	62.86 ± 7.38	63.49 ± 7.19	.544
BMI (kg/m^2)	22.98 ± 2.94	23.16 ± 2.83	.656
FBG (mg/dL)	89.29 ± 17.17	91.90 ± 17.39	.288
TC (mg/dL)	192.53 ± 4.33	191.87 ± 3.89	.254
TG (mg/dL)	123.39 ± 12.71	124.88 ± 13.46	.423
HDL (mg/dL)	50.31 ± 3.99	49.22 ± 4.07	.057
LDL (mg/dL)	111.80 ± 6.75	112.42 ± 8.65	.573
SBP (mm Hg)	128.15 ± 16.01	132.34 ± 15.02	.059
DBP (mm Hg)	79.27 ± 12.01	83.17 ± 17.40	.068
Degree of carotid artery stenosis			
50%-69% (*N*, %)	–	67 (66.34%)	–
70%-79% (*N*, %)	–	21 (20.79%)	–
80%-99% (*N*, %)	–	13 (12.87%)	–

Abbreviations: CAS, carotid stenosis; BMI, body mass index; FBG, fasting blood glucose; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure. Data are expressed as n (%) or mean ± standard deviation.

become a potential clinical biomarker for the diagnosis, prognosis, and prediction of human diseases.[9] As one of many miRNAs, miR-19a-3p is considered to be a diagnostic biomarker for early noninvasive gastric cancer.[10] In cerebral ischemia-reperfusion injury, miR-19a-3p is markedly upregulated and is related to inflammation and apoptosis.[11] miR-19a-3p has been reported to be involved in atherosclerosis, and atherosclerosis is considered to be a common cause of CAS.[12] More importantly, in transient ischemic attack (TIA), a complication of CAS, glycine improves TIA by regulating miR-19a-3p to inhibit glucose metabolism, inflammation, and apoptosis.[13]

Although the function of miR-19a-3p in CAS-related diseases has been described in detail, the clinical potential of miR-19a-3p in asymptomatic CAS remains unknown. Therefore, according to the current research status of CAS, we examined the serum miR-19a-3p levels of recruited asymptomatic CAS patients and healthy subjects. This was done to evaluate miR-19a-3p diagnostic value for asymptomatic CAS patients and analyze its level of predictive potential for the occurrence of cerebral ischemia event (CIE). The present research aims to identify asymptomatic CAS patients through a new non-invasive diagnostic method and to treat them to prevent the occurrence of stroke.

Materials and Methods

Ethics Statement

All participants signed a written informed agreement, and the serum samples collection procedure was endorsed by the Ethics Review Committee of Baoji People's Hospital. All research strictly abides by the ethics of the Helsinki Declaration.

Serum Samples Collection

From June 2013 to June 2015, serum samples from 101 asymptomatic CAS patients (females 55, males 46, ages 63.49 ± 7.19 years) in Baoji People's Hospital were collected. Besides, the serum samples from 98 age-matched healthy subjects (females 49, males 49, ages 62.86 ± 7.38 years) were collected as the control group during the same period. The degree of asymptomatic CAS of the subjects was determined according to the European Carotid Surgery Trial (ECST).[14] Inclusion criteria for asymptomatic CAS patients were: (a) at least 1 CAS ≥ 50% was first found on Doppler ultrasound; and (b) patients without a history of ischemic stroke, focal neurological symptoms, TIA, or amaurosis.[15,16] Furthermore, patients with chronic cardiovascular disease, chronic liver diseases, endocrine and metabolic diseases, chronic inflammation, nervous system disease, tumors, and brain diseases such as cerebral hemorrhage, ischemic stroke, or stroke of uncertain caused were excluded. The control group came from the health examination center. In addition to excluding arterial disease, all participants underwent Doppler ultrasound to determine the degree of CAS. According to the results, patients were divided into moderate and severe CAS. The basic clinical information of the 2 groups of subjects was recorded in Table 1. The blood samples from the subjects were centrifuged to take the serum and stored at −80 °C.

Follow-up Plan

According to previous studies,[17] CIE is defined as ipsilateral stroke, TIA, amaurosis, or sudden death. We performed follow-up telephone interview with patients, relatives, or/and general practitioners during the 5 years' follow-up period to record the subject's occurrence of CIE. Meanwhile, the Kaplan–Meier curve was drawn to evaluate the predictive potential of miR-19a-3p in the happening of CIE.

RNA Isolation and Fluorescence Quantitative Reverse Transcription-Polymerase Chain Reaction

TRIzol reagent was added to the serum to isolate total RNA. The quality and concentration of the extracted RNA were identified and then the miRNA reverse transcription reaction was performed. Complementary DNA was synthesized with the all-in-one miRNA first strand cDNA synthesis kit. Using cDNA as a template, the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) reaction was performed on a Roche 480 real-time PCR system to evaluate the mRNA level of miR-19a-3p using the all-in-one miRNA qPCR assay kit. The expression of endogenous noncoding small nuclear RNA U6 was normalized, and the relative levels of miR-19a-3p were judged by $2^{-\Delta\Delta Ct}$.

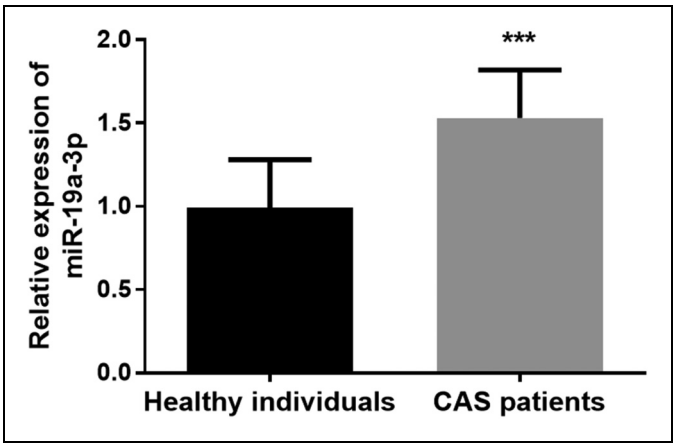

Figure 1. A comparison of indicated serum miR-19a-3p expression levels in asymptomatic CAS patients and healthy individuals (*** $P < .001$, compared with healthy individuals).

Receiver Operating Characteristic Curves Analysis

By calculating area under the curve (AUC) and 95% confidence interval, the ability of biomarkers to accurately identify asymptomatic CAS patients from healthy controls was then determined. When the AUC was >0.8, the receiver operating characteristic (ROC) curve was considered significant.

Analysis and Statistics

All the experimental results have been biologically repeated 3 or more times. Statistical analysis of the collected data was done through SPSS 23. And GraphPad Prism 8.0 was used to draw the figures. The differences between groups were compared by Students' tests and analysis of variance (ANOVA) analysis. $P < .05$ indicated statistically significant difference.

Results

Clinical Characteristics of Asymptomatic CAS Patients and Healthy Subjects

A total of 101 asymptomatic CAS patients and 98 healthy subjects participated in this study. For the rigor of the study, we tested the basic clinical characteristics of the study participants. The analysis showed that there were no significant differences between the 2 groups in age, gender, body mass index, fasting blood glucose, total cholesterol, triglycerides, high-density lipoprotein, low-density lipoprotein (LDL), systolic blood pressure, and diastolic blood pressure ($P > .05$, Table 1). The conclusions suggest that the participants included in this study are representative.

Serum miR-19a-3p was Upregulated in Asymptomatic CAS Patients

Then, we detected the serum miR-19a-3p levels of the subjects included in the study. The results confirmed that compared with the control group (1.01 ± 0.26), the mRNA expression of miR-19a-3p in asymptomatic CAS patients (1.48 ± 0.31) was significantly increased ($P < .001$, Figure 1). These data indicated that the regulation of miR-19a-3p may be crucial in the pathogenesis of asymptomatic CAS.

Elevated Serum miR-19a-3p was Associated with Severe CAS

To determine the potential of dysregulated miR-19a-3p as a biomarker for the diagnosis of asymptomatic CAS, we first used logistic regression analysis to predict the influencing factors of the degree of CAS. We divided the participants into moderate carotid stenosis ($n = 61$) and severe carotid stenosis ($n = 40$). The results showed that elevated serum miR-19a-3p level was an independent predictor of severity of asymptomatic CAS (odds ratio = 3.920, 95% CI = 1.482-10.372, $P < .05$; Table 2).

Diagnostic Potential of miR-19a-3p for Asymptomatic CAS

To determine the applicability and possibility of miR-19a-3p as a new potential biomarker for diagnosing asymptomatic CAS, we plotted a ROC curve based on the miR-19a-3p level of the subjects. The AUC was 0.905 (95% CI 0.864-0.946, $P < .001$, Figure 2), sensitivity was 80.2%, and specificity was 86.7%. The data show that miR-19a-3p has a high diagnostic value and can be used to identify asymptomatic CAS in healthy people.

The Potential Value of Serum miR-19a-3p in Predicting CIE

We further studied the predictive ability of miR-19a-3p on CIE. According to the average level of serum miR-19a-3p, asymptomatic CAS patients were divided into the high serum

Table 2. Logistic Regression Analysis of the Correlation Between Severe Carotid Artery Stenosis and Different Variables.

Variables	OR	95% CI	*P* value
MiR-19a-3p	3.920	1.482-10.372	.006
Gender (Male and female)	1.222	0.465-3.211	.685
Age (years)	0.998	0.408-2.440	.997
BMI (kg/m^2)	1.250	0.503-3.110	.631
FBG (mg/dL)	1.754	0.698-4.404	.232
TC (mg/dL)	1.315	0.532-3.248	.553
TG (mg/dL)	1.536	0.639-3.689	.337
HDL (mg/dL)	0.389	0.138-1.100	.075
LDL (mg/dL)	1.719	0.678-4.358	.254
SBP (mm Hg)	1.226	0.495-3.036	.659
DBP (mm Hg)	1.024	0.409-2.562	.960

Abbreviations: BMI, body mass index; FBG, fasting blood glucose; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; OR, odds ratio; CI, confidence interval.

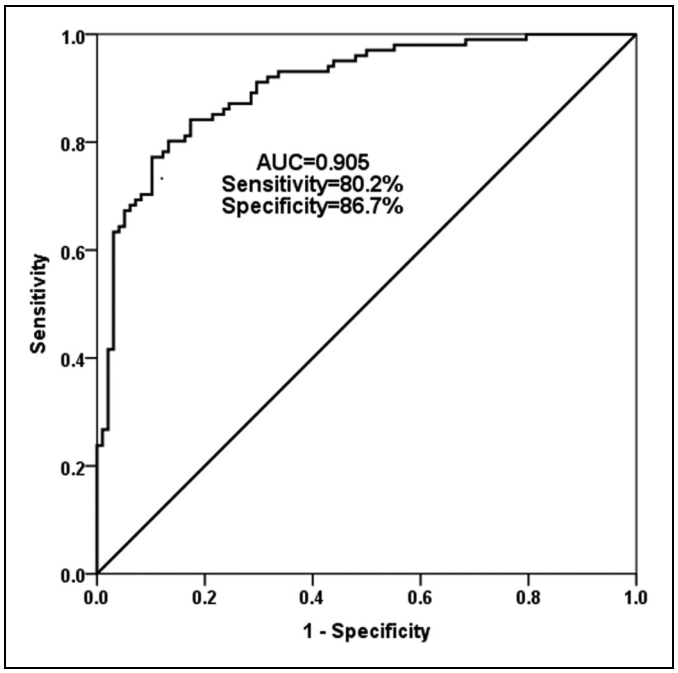

Figure 2. The diagnostic potential of serum miR-19a-3p for asymptomatic CAS. The receiver operating characteristic (ROC) curve showed that miR-19a-3p had a sensitivity of 80.2%, specificity of 86.7%, and an area under the curve (AUC) of 0.905 for the identification of asymptomatic CAS patients from healthy subjects.

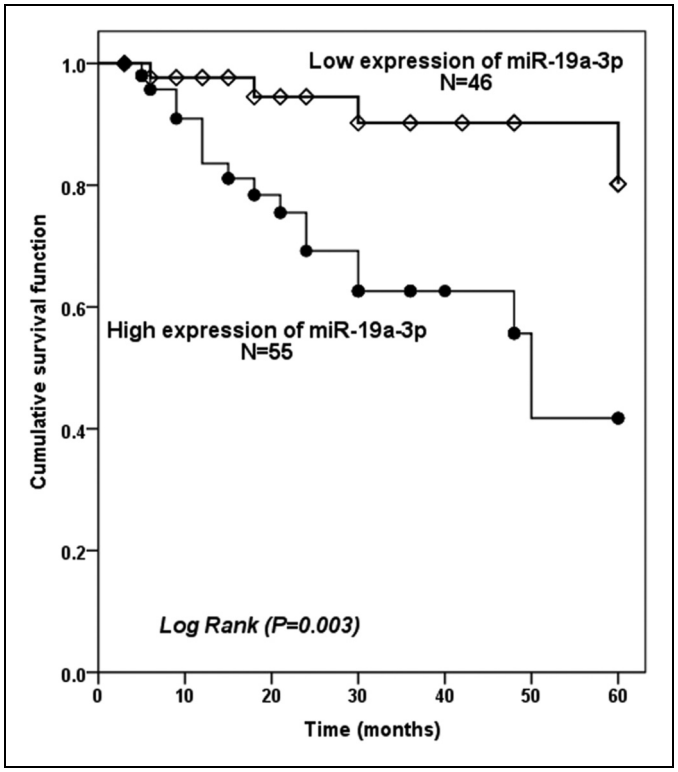

Figure 3. Kaplan–Meier plot showed that patients with high expression of miR-19a-3p experienced more CIE than those with low expression (Log-rank $P < .05$ was considered as statistically significant).

Table 3. Multivariate Cox Regression Analysis to Detect the Risk Factors of CIE in carotid artery stenosis Patients.

	Multivariate analysis		
Characteristics	HR	95% CI	*P*
MiR-19a-3p	8.507	2.239-32.328	.002
Gender (male and female)	1.227	0.428-3.515	.704
Age (years)	1.337	0.425-4.208	.619
BMI (kg/m^2)	1.087	0.399-2.967	.870
FBG (mg/dL)	1.062	0.411-2.749	.901
TC (mg/dL)	2.055	0.683-6.183	.200
TG (mg/dL)	2.481	0.865-7.119	.091
HDL (mg/dL)	0.601	0.225-1.608	.311
LDL (mg/dL)	1.536	0.516-4.567	.440
SBP (mm Hg)	1.110	0.419-2.944	.833
DBP (mm Hg)	1.711	0.625-4.686	.296
Degree of carotid artery stenosis	3.695	1.127-12.109	.031

Abbreviations: BMI, body mass index; FBG, fasting blood glucose; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, hazard ratio; CI, confidence interval.

miR-19a-3p group ($n = 55$) and low serum miR-19a-3p group ($n = 46$). Kaplan–Meier curve analysis found that asymptomatic CAS patients with high serum miR-19a-3p were more likely to develop CIE in the further 5 years (log-rank $P = .003$, Figure 3). Multivariate Cox analysis showed that serum miR-19a-3p (hazard ratio [HR] = 8.507, 95% CI = 2.239-32.328, $P = .002$) and degree of CAS (HR = 3.695, 95% CI = 1.127-12.109, $P = .031$, Table 3) were both high risk factors for CIE.

Discussion

The present study determined the potential value of serum miR-19a-3p as a diagnostic marker of asymptomatic CAS by detecting the expression level of serum miR-19a-3p in asymptomatic CAS patients. Its predictive value for the occurrence of CIEs was also evaluated. We found that the mean expression level of miR-19a-3p in serum of asymptomatic CAS patients was markedly higher than that in healthy subjects. Besides, miR-19a-3p was a strong predictor of the severity of CAS. Serum miR-19a-3p can significantly distinguish asymptomatic CAS patients from healthy subjects and has a high diagnostic value. Our research also shows that serum miR-19a-3p may be useful in predicting future CIE occurrence. As far as we know, this is the first time that miR-19a-3p has a good risk predictive effect on the occurrence of CIE in asymptomatic CAS patients.

In China, there are more than 1.5 million new stroke patients every year, and at least half of them are experiencing some degree of physical disability.[2] Therefore, CIE including stroke prevention has become a major goal of health strategies. And CAS can be one of the most common risk factors for CIE.[18] Therefore, it is necessary to diagnose asymptomatic patients with CAS early and take effective preventive or therapeutic measures in time to reduce the risk of CIE.

Nowadays, there are various diagnostic methods for asymptomatic CAS, but there are also certain limitations.[19] Magnetic resonance angiography (MRA) is a nonintrusive method to diagnose asymptomatic CAS. However, in addition to the high price, it also exaggerates the degree of stenosis and has limitations on important steel plates, which is not suitable for patients who use metal stents or pacemakers in vivo.[20] Computed tomography angiography (CTA) is quick and less invasive, whereas contrast agents and x-rays can be potentially harmful.[21] Digital subtracted angiography (DAS) is currently the gold standard for the diagnosis of asymptomatic CAS, which can understand the location, extent, and degree of lesions in detail, and determine the nature of lesions such as ulceration, calcification, or thrombosis.[22] However, due to its invasiveness, high cost, and many complications, it is generally used for preoperative detection. At present, the preferred noninvasive diagnosis method of CAS in clinical practice is duplex ultrasound imaging, which is simple and safe, but it is easily affected by the level of operators, and it is expensive and not suitable for large-scale screening of the population.[5] The search for new, noninvasive, and less costly methods for the screening diagnosis of asymptomatic CAS is still expected to be developed.

Currently, miRNA has attracted widespread attention. It not only participates in the pathogenesis of common diseases, but the dysregulation level can also be used as a biomarker for diagnostic, prognostic, and prediction of disease. Serum miRNAs are ideal biomarkers for disease diagnosis and prognosis due to their low cost, easy application, high stability, and minimal invasiveness. For example, miR-214 participates in CAS by regulating angiogenesis and senescence of vascular smooth muscle cells (VSMCs).[23] The level of miR-638 is generally decreased in patients with severe muscular artery stenosis and is associated with plate instability.[24] Circulating miR-3352 may be a biomarker for ischemic stroke in rats.[25] The elevation of serum miR-374 in atherosclerosis is its diagnostic biomarker.[26] MiR-19a-3p is upregulated in pancreatic cancer and can be used as a diagnostic marker.[27]

Notably, miR-19a-3p is upregulated in atherosclerosis.[12] In addition, it has been reported that miR-19a-3p has a dual role in ischemic diseases. It can improve myocardial ischemia by regulating angiogenesis and myocardial fibrosis,[28] but also can promote ischemic stroke by interfering with glucose metabolism and promoting neuronal apoptosis.[29] Furthermore, photochemically induces significant up-regulation of miR-19a-3p in brain tissue and plasma of rats with focal cerebral ischemia.[30] Atherosclerosis and ischemic brain injury are closely related to CAS. Given the above, we speculated that miR-19a-3p may also be related to asymptomatic CAS. To confirm our conjecture, we first included 2 groups of subjects with no significant difference in basic clinical information except in the degree of CAS. The study concluded that miR-19a-3p was generally higher in asymptomatic CAS patients than in healthy subjects. This is consistent with the results of miR-19a-3p in atherosclerosis and CIE, suggesting that the increase of miR-19a-3p may be involved in asymptomatic CAS. Subsequently, we analyzed the influencing factors of the degree of asymptomatic CAS by logistic regression, and the results confirmed that the increase of miR-19a-3p was an independent risk factor for severe CAS in asymptomatic CAS patients.

To identify the applicability and possibility of miR-19a-3p as a diagnostic biomarker of asymptomatic CAS, we draw the ROC curves based on the subject's serum levels. We considered that miR-19a-3p showed high accuracy in distinguishing between asymptomatic CAS patients and healthy subjects. As we all know, CIE is positively correlated with the degree of CAS,[31] and it is a recognized cause of CIE.[32] In addition, miR-19a-3p has also been reported as a CIE-related miRNA. Therefore, we evaluate its ability to predict the occurrence of CIE in the further 5 years. The research validated that patients with high miR-19a-3p experienced more CIE, and miR-19a-3p is an independent predictor of CIE.

Afterward, it must be noted that this study has certain limitations. Previous reports have confirmed that in hyperlipidemia, slightly oxidized LDL indirectly regulates miR-19a-3p and participates in the progression of atherosclerosis.[33] And abnormal LDL is an important factor in CAS.[34] However, in our present research, we have not been able to explore the correlation between miR-19a-3p and clinical indicators including LDL in time, which is a major defect of this study. We will continue to focus on this issue in the future. Furthermore, this study focused on CAS and failed to exclude other arterial diseases (aorta-frontal artery, subinguinal or cardiovascular atherosclerosis) and brain diseases in time. Therefore, this preliminary study requires larger samples and stricter sample inclusion for in-depth research.

In conclusion, we confirmed for the first time that the level of miR-19a-3p in the serum of asymptomatic CAS patients is elevated, which is a valuable and potential biomarker for the diagnosis of asymptomatic CAS. Besides, the high expression of miR-19a-3p has a certain predictive potential for the occurrence of CIE, and it can prevent the occurrence of CIE by early identification of asymptomatic CAS patients to prevent the occurrence of CIE.

Declaration of Conflicting Interests

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