Carotid contrast-enhanced ultrasonography combined with sirtuin-3 in the diagnosis of plaques in carotid atherosclerosis

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Abstract

Background. Carotid atherosclerosis (CAS) is one of the main causes of ischemic stroke. Currently, the clinical evidence for contrast-enhanced ultrasonography (CEUS) as a method for diagnosing CAS is still inadequate. Sirtuin-3 (SIRT3) is associated with the inflammation response; however, few studies have evaluated SIRT3 in CAS.

Objectives. To investigate the role of SIRT3 in CAS patients and its diagnostic value for unstable plaques when combined with CEUS.

Materials and methods. This is a prospective observational study including 517 CAS patients who were admitted to our hospital from January 2015 to December 2020. All patients received a normal Doppler ultrasound, CEUS and magnetic resonance imaging (MRI). The latter was used as the gold standard in evaluating plaque conditions. Serum SIRT3 levels were measured using an enzyme-linked immunosorbent assay (ELISA). Serum levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-ch), low-density lipoprotein cholesterol (LDL-ch), C-reactive protein (CRP), and interleukin (IL)-6 levels were measured and recorded.

Results. Patients with severe CAS showed significantly higher levels of CRP, IL-6, TC, and LDL-ch, a higher frequency of unstable plaques, as well as a lower level of HDL-ch. In patients with severe CAS and CAS patients with stable plaques, the levels of SIRT3 were markedly lower. Patients with a high expression of SIRT3 showed significantly lower levels of CRP, IL-6, TC and LDL-ch, and higher levels of HDL-ch, as well as a lower frequency of unstable plaques. Receiver operating characteristic (ROC) curves showed that the combination of CEUS and SIRT3 could achieve high sensitivity and specificity in the diagnosis of unstable plaques. High levels of C-reactive protein, IL-6, TC, TG and LDL-ch, as well as low levels of SIRT3 and HDL-ch, and current smoking were risk factors of unstable plaques in CAS patients.

Conclusions. A low expression of SIRT3 predicted a higher risk for unstable plaques in CAS patients. The combination of CEUS and SIRT3 is a potential strategy for diagnosing unstable plaques.

Key words: plaques, contrast-enhanced ultrasonography, carotid atherosclerosis, SIRT3

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Background

Carotid atherosclerosis (CAS) is one of the main causes of ischemic stroke.[1,2] As reported in a recent study, in 2020, the global prevalence of increased carotid intima-media thickness (IMT) was approx. 27.6% and the prevalence of carotid plaques was approx. 21.1% in people aged 30–79 years.[3] In rural northeast China, the prevalence of CAS and carotid plaques can be as high as 33.1% and 31.5%, respectively.[4] Since CAS can remain asymptomatic for many years, the early diagnosis is very important in preventing severe consequences, such as stroke.[5,6]

Currently, imaging studies are the main strategy for diagnosing CAS, including computed tomography (CT) scans, Doppler ultrasounds, magnetic resonance imaging (MRI), etc.[7–9] Among these methods, contrast-enhanced ultrasonography (CEUS) was developed in the recent decade. It utilizes resonated ultrasound waves from circulating microbubbles to produce superior angiography-like images for vascular visualization.[10] In recent years, some studies have shown the application of CEUS in diagnosing CAS plaques.[11,12] However, the clinical evidence is still inadequate.

Except for imaging methods, serum biomarkers are also important indices for the diagnosis and prediction of CAS.[13] Biomarkers such as inflammatory factors (C-reactive protein (CRP), interleukins (ILs)), lipid-related factors (high-density lipoprotein cholesterol (HDL-ch) and low-density lipoprotein cholesterol (LDL-ch)), endothelial and cell adhesion factors (VCAM-1, ICAM-1), etc., have all been widely studied.[14,15] Sirtuin-3 (SIRT3) belongs to the sirtuin family. Studies have found SIRT3 to be associated with inflammation and correlated with the development of cardiovascular diseases, such as myocardial ischemia-reperfusion injury.[16,17] However, up to now, few studies have focused on the clinical significance of SIRT3 in CAS patients and its potential value in predicting the condition of plaques.

Objectives

We performed an observational study to investigate the role of SIRT3 in CAS patients and its diagnostic value for unstable plaques when combined with CEUS. Our research might provide new insights into diagnosing unstable plaques and identifying biomarkers in CAS patients.

Materials and methods

Subjects

This is a prospective observational study including 517 CAS patients who were admitted to our hospital from January 2015 to December 2020. All patients diagnosed with CAS underwent a MRI to confirm the diagnosis. All patients also had a normal Doppler ultrasound and CEUS to evaluate the plaques. The exclusion criteria were as follows: 1) patients with stroke, coronary syndrome, and other severe cardiovascular or cerebrovascular diseases, such as cardiogenic cerebral embolism; 2) patients who were allergic to the contrast agent; 3) patients with cancer or dysfunction of the kidney or liver. All patients were allocated into the mild-moderate CAS or the severe CAS group according to the IMT, plaque condition and degree of arterial stenosis. Patients with an IMT > 1.0 mm with arterial stenosis <70% were allocated to the mild-moderate CAS group, while patients with multiple plaques and arterial stenosis ≥70% to the severe CAS group. Written informed consent was obtained from all patients. The present study was approved by the ethics committee at the First Affiliated Hospital of Nanchang University, China (approval No. NCDXYY20150612).

Imaging measurements for plaques

All patients received a normal Doppler ultrasound, CEUS and MRI. The IMT was measured using an Acuson-Aspen Color Doppler ultrasound diagnostic instrument (GE Healthcare, Boston, USA) with a probe frequency of 7.5 MHz. The IMT of bilateral common carotid arteries was measured proximally and distally to the bifurcation as well as at the bifurcation. Plaques were observed and classified as stable (strong echo or medium echo) or unstable (mixed echo or low echo) plaques based on the echo condition.

Contrast-enhanced ultrasonography was used to further evaluate the conditions of the plaques using the Toshiba Aplio500 instrument (Toshiba, Tokyo, Japan) with a probe frequency of 5–14 MHz and a mechanical index (MI) of 0.08 MHz. SonoVue (Bracco, Geneva, Switzerland) was used as the contrast agent. Briefly, after the observation of the plaque, 1.0 mL of SonoVue suspension (dissolved in normal saline) was injected into a peripheral vein by rapid bolus injection, followed by an injection of 5 mL of normal saline. According to the measurement of teh plaques using CEUS, patients were described using 5 grades: grade 0 – no enhancement; grade 1 – an enhancement for adventitia but not the inner plaque; grade 2 – a small amount of scattered punctate enhancement within the plaquel; grade 3 – a linear enhancement extending into the plaque; and grade 4 – an intraplaque diffusion enhancement.

Magnetic resonance imaging was used as the gold standard to evaluate plaque conditions using a GE Discovery MR750w 3.0T MRI scanner (GE Healthcare). Briefly, after the full exposure of the neck blood vessels, the exact location of the carotid bifurcation and the plaques was obtained using 2D-TOF scanning. Then, axial scanning of the plaques was performed using T1WI, T2WI, 3D-T0F, and enhanced T1WI. For measurement of plaques, the signal-to-noise ratio (SNR) and imaging conditions

were analyzed and plaques were divided into different types according to the American Heart Association (AHA) classification. Types I, II, III, VII, and VIII were considered stable while all other types were regarded as unstable.[18]

Measurements of serum SIRT3 and other laboratory indices

The measurement of serum SIRT3 was conducted using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Lengton Bioscience, Shanghai, China), according to the manufacturer's instructions. Serum levels of total cholesterol (TC), triglycerides (TG), HDL-ch, LDL-ch, CRP, and interleukin (IL)-6 were measured using an automatic biochemical analyzer (Hitachi 7600; Hitachi Corporation, Tokyo, Japan).

Statistical analyses

The distribution of the data was analyzed using the Kolmogorov–Smirnov method. Non-normally distributed data were expressed as a median, interquartile range (IQR) and range. All continuous data included in this study had a non-normal distribution. The comparison between the 2 groups was conducted using the Mann–Whitney U test. Rates were analyzed using the χ^2 test. A receiver operating characteristic (ROC) curve was used for the diagnostic analysis, and Youden's index was used for selecting the cutoff value. Logistic regression was used to analyze the risk of unstable plaques. The Hosmer–Lemeshow test was used to measure the model's goodness-of-fit. A value of $p < 0.05$ was considered statistically significant. All calculations were performed using SPSS v. 18.0 (SPSS Inc., Chicago, USA).

Results

Basic characteristics of all patients

The study included a total of 517 CAS patients, with 311 (60.15%) cases of mild-moderate CAS and 206 (39.85%) cases of severe CAS. A total of 293 cases (56.67%) had stable plaques and 224 cases (43.33%) had unstable plaques. As shown in Table 1, severe CAS patients showed a significantly higher expression of CRP, IL-6, TC, and LDL-ch (all with a p-value <0.001), while the expression of HDL-ch was markedly lower in severe CAS patients ($p < 0.001$). Additionally, severe CAS patients showed a higher frequency of unstable plaques compared to mild-moderate patients ($p < 0.001$). The ratio of patients with a history of coronary heart disease was significantly higher in severe CAS patients ($p = 0.008$). The typical imaging results were shown in Fig. 1. All the statistical calculation results for continuous data and logistic regressions are listed in the Supplementary Data (Table S1–S10).

Table 1. Basic characteristics of the patients

Characteristics	All CAS (n = 517)	Mild-moderate (n = 311)	Severe (n = 206)	U or χ^2	p-value*
Age [years]	54 (13, 37–70)	54 (13, 37–68)	54 (14, 39–70)	31871.50	0.923
BMI [kg/m^2]	27.03 (6.47, 20.28–33.72)	27.29 (7.07, 20.28–33.71)	26.45 (5.82, 20.30–33.72)	30079.00	0.240
Female sex (%)	252 (48.74)	152 (48.87)	100 (48.54)	0.002	0.963
Complications, n (%)					
Diabetes	108 (20.89)	57 (18.33)	51 (24.76)	1.233	0.269
Hypertension	90 (17.41)	50 (16.08)	40 (19.42)	0.382	0.537
History of coronary heart disease	67 (12.96)	24 (7.72)	43 (20.87)	7.057	0.008
History of stroke	67 (12.96)	35 (11.25)	32 (15.53)	0.790	0.374
Current smoker	169 (32.69)	100 (32.15)	69 (33.50)	0.041	0.839
Plaque, n (%)					
Stable	293 (56.67)	231 (74.28)	62 (30.10)	39.112	<0.001
Unstable	224 (43.33)	80 (25.72)	144 (69.90)		
CRP [mg/L]	8.37 (6.40, 2.17–23.71)	6.44 (3.48, 2.17–15.34)	12.96 (8.25, 6.16–23.71)	7026.50	<0.001
IL-6 [pg/mL]	10.05 (7.57, 3.92–26.24)	9.24 (4.60, 3.92–26.02)	12.55 (10.24, 4.13–26.24)	20053.50	<0.001
TC [mmol/L]	4.24 (0.67, 3.25–5.38)	4.12 (0.59, 3.25–5.38)	4.45 (0.71, 3.26–5.38)	19848.50	<0.001
TG [mmol/L]	1.44 (0.42, 0.93–2.02)	1.43 (0.41, 0.93–1.99)	1.45 (0.42, 0.97–2.02)	29956.50	0.212
LDL-ch [mmol/L]	2.93 (0.53, 2.17–3.80)	2.86 (0.53, 2.17–3.79)	3.02 (0.54, 2.20–3.80)	23236.50	<0.001
HDL-ch [mmol/L]	1.11 (0.09, 0.96–1.24)	1.13 (0.10, 0.97–1.24)	1.09 (0.09, 0.96–1.24)	23555.50	<0.001

BMI – body mass index; CAS – carotid atherosclerosis; IL-6 – interleukin 6; TC – total cholesterol; TG – triglycerides; LDL-ch – low-density lipoprotein cholesterol; HDL-ch – high-density lipoprotein cholesterol; * p-value was obtained by comparing the mild-moderate and severe CAS groups. All continuous data presented non-normal distribution (BMI, CRP, IL-6, TC, TG, LDL-ch and HDL-ch, and SIRT3), and were expressed by median (interquartile range (IQR)). The comparison between the 2 groups was conducted using Mann–Whitney U test. Rates were analyzed with χ^2 test.

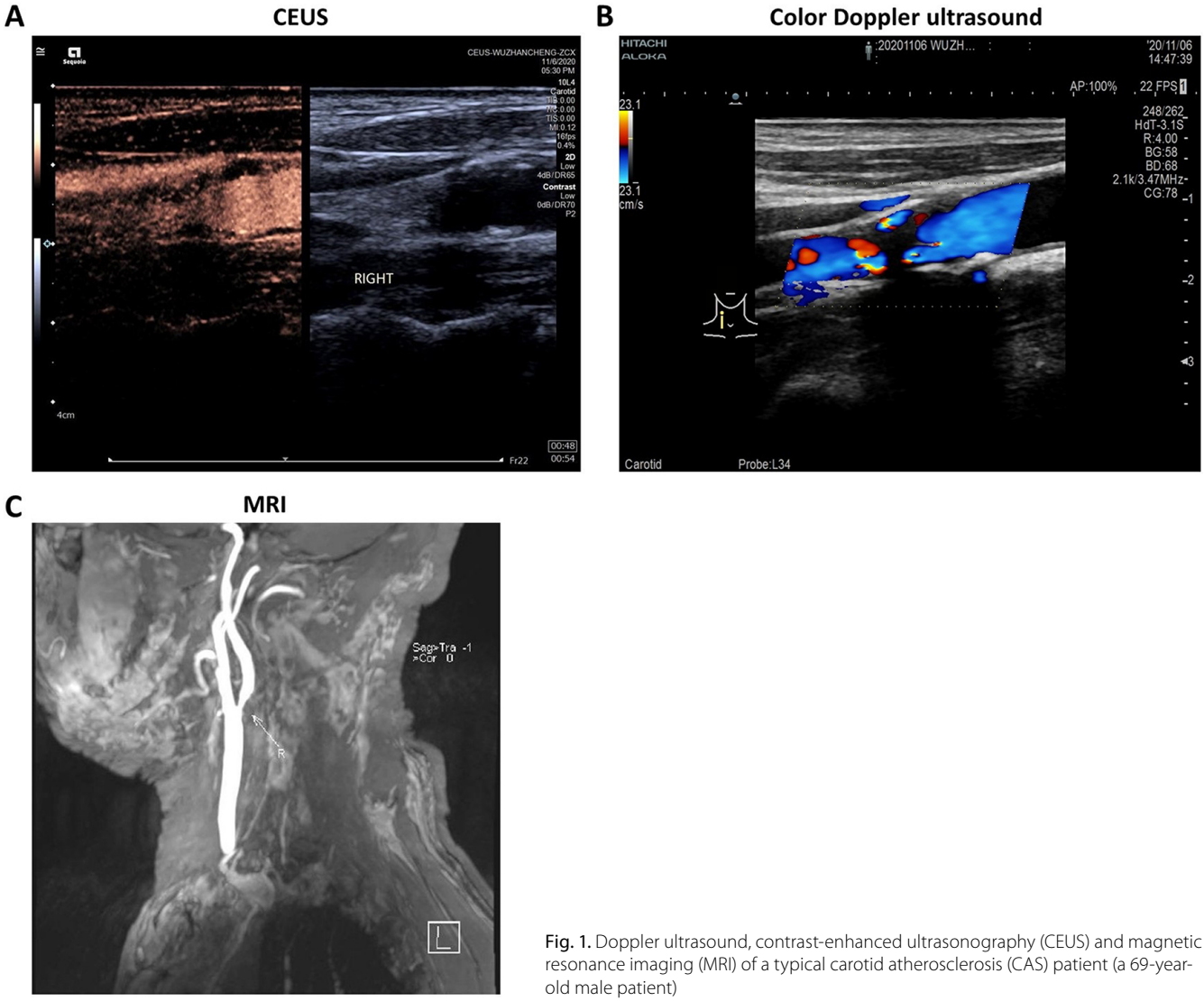

Fig. 1. Doppler ultrasound, contrast-enhanced ultrasonography (CEUS) and magnetic resonance imaging (MRI) of a typical carotid atherosclerosis (CAS) patient (a 69-year-old male patient)

SIRT3 was associated with the clinical outcomes of CAS patients

To further investigate the role of SIRT3 in CAS, serum SIRT3 levels were evaluated and compared. It was found that in severe CAS patients, the levels of SIRT3 were markedly lower than that of mild-moderate patients ($U = 22220.00$, $p < 0.001$, Fig. 2) Meanwhile, patients with unstable plaques showed a remarkably lower expression of SIRT3 compared to the patients with stable plaques ($U = 7899.00$, $p < 0.001$). Patients were further allocated into high or low SIRT3 expression groups, according to their median value (17.98 ng/mL). As shown in Table 2, patients with a high expression of SIRT3 showed significantly lower levels of CRP, IL-6, TC and LDL-ch, and higher levels of HDL-ch (all with a p-value < 0.001). The percentage of patients with unstable plaques was also lower in patients with higher SIRT3 levels ($p < 0.001$). These results indicate that SIRT3 might be associated with the clinical severity and plaque condition of CAS patients.

Diagnostic value of SIRT3 and CEUS for unstable plaques in CAS patients

Receiver operating characteristic curves were used to evaluate the diagnostic value of SIRT3 and CEUS for unstable plaques in CAS patients. Using a cutoff value of 17.14 ng/mL, SIRT3 showed the area under the curve (AUC) of 0.880 (95% confidence interval (95% CI): 0.852–0.907), with a sensitivity of 80.2% and specificity of 72.3% for diagnosis of unstable plaques. Meanwhile, CEUS grades with a cutoff value of 1.50 showed an AUC of 0.846 (95% CI: 0.810–0.882), achieving a sensitivity of 81.3% and specificity of 85.7% in diagnosing of unstable plaques. This result indicated that grades 0 and 1 were regarded as stable plaques, while unstable plaques were defined as grades 2–4. The cutoff values of SIRT3 and CEUS were combined and used for the diagnosis of unstable plaques. It was found that the combination of CEUS and SIRT3 could achieve a sensitivity of 94.20%, specificity of 69.28% and accuracy of 80.08% (Table 3, Fig. 3).

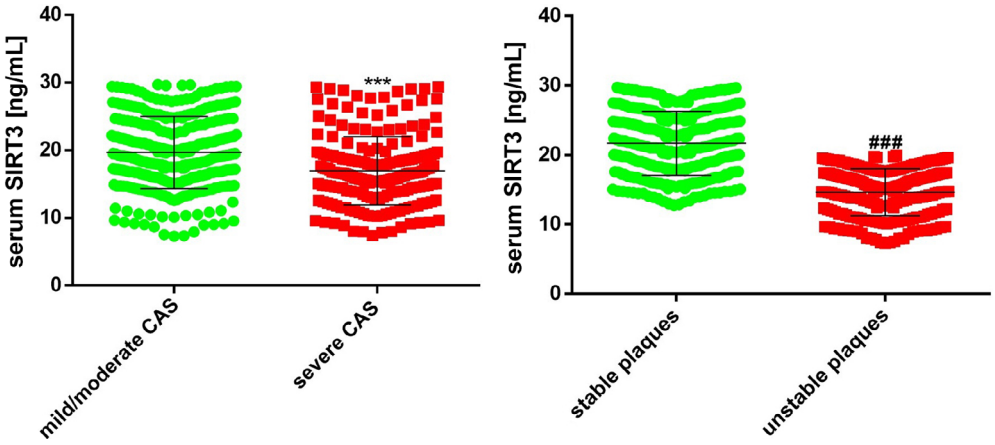

Fig. 2. Serum sirtuin-3 (SIRT3) levels in carotid atherosclerosis (CAS) patients with different severity and plaque conditions

*** $p < 0.001$ compared to mild/moderate CAS; ### $p < 0.001$ compared to stable plaques.

Table 2. Clinical characteristics of CAS patients with different expression of SIRT3

Characteristics	High SIRT3 (n = 257)	Low SIRT3 (n = 260)	U or χ^2	p-value*
Age [years]	54 (12.75, 37–69)	54 (13.00, 37–70)	32810.00	0.724
Female sex (%)	127 (49.42)	125 (48.08)	0.85	0.850
BMI [kg/m^2]	26.51 (6.36, 20.28–33.71)	27.08 (6.70, 20.30–33.72)	31614.00	0.290
Complications, n (%)				
Diabetes	49 (19.07)	59 (22.69)	0.397	0.529
Hypertension	48 (18.68)	42 (16.15)	0.223	0.637
History of coronary heart disease	26 (10.12)	41 (15.77)	1.416	0.234
History of stroke	29 (11.28)	38 (14.62)	0.495	0.482
Current smoker	73 (28.40)	96 (36.92)	1.650	0.199
Plaque, n (%)				
Stable	218 (84.82)	75 (28.85)	63.846	<0.001
Unstable	39 (12.54)	185 (89.81)		
CRP [mg/L]	6.67 (7.58, 2.17–23.07)	11.68 (4.22, 2.23–23.71)	16691.50	<0.001
IL-6 [pg/mL]	8.61 (9.79, 3.92–26.24)	13.11 (3.96, 4.25–26.23)	16276.50	<0.001
TC [mmol/L]	4.08 (0.74, 3.25–5.30)	4.47 (0.56, 3.26–5.38)	17177.00	<0.001
TG [mmol/L]	1.43 (0.44, 0.93–1.99)	1.45 (0.41, 0.96–2.02)	31918.00	0.380
LDL-ch [mmol/L]	2.79 (0.56, 2.17–3.80)	3.06 (0.47, 2.20–3.79)	19790.50	<0.001
HDL-ch [mmol/L]	1.13 (0.09, 0.96–1.24)	1.10 (0.10, 0.96–1.24)	24039.50	<0.001

SIRT3 – sirtuin-3; BMI – body mass index; CAS – carotid atherosclerosis; CRP – C-reactive protein; IL-6 – interleukin 6; TC – total cholesterol; TG – triglycerides; LDL-ch – low-density lipoprotein cholesterol; HDL-ch – high-density lipoprotein cholesterol; * p-value was obtained by comparison between SIRT3 high/low CAS groups. All continuous data presented non-normal distribution (BMI, CRP, IL-6, TC, TG, LDL-ch and HDL-ch, and SIRT3), and were expressed by median (interquartile range (IQR)). The comparison between the 2 groups was conducted using Mann–Whitney U test. Rates were analyzed using χ^2 test.

Risk factors for unstable plaques using logistic regression analysis

Binary logistic regression was employed to analyze risk factors for unstable plaques using a back-step method. After adjusting the model, we conducted multivariate logistic regressions evaluating age, body mass index (BMI), CRP, IL-6, and SIRT3 (model 1), sex and medical history (model 2), or the lipid metabolism factors – TC, TG, LDL-ch, and HDL-ch (model 3), using the entry method. The p-values of the Hosmer–Lemeshow tests for the 3 models were 0.983, 0.783 and 0.911, respectively. The results of each model are listed in Table 4. It was found that high levels of CRP, IL-6, TC, TG and LDL-ch, as well as low levels of SIRT3 and HDL-ch, and current smoking were all risk factors for unstable plaques in CAS patients (Table 4).

Discussion

Since CAS is closely associated with and is a main inducer of stroke, the early diagnosis of CAS is of great significance. We conducted an observational study to demonstrate

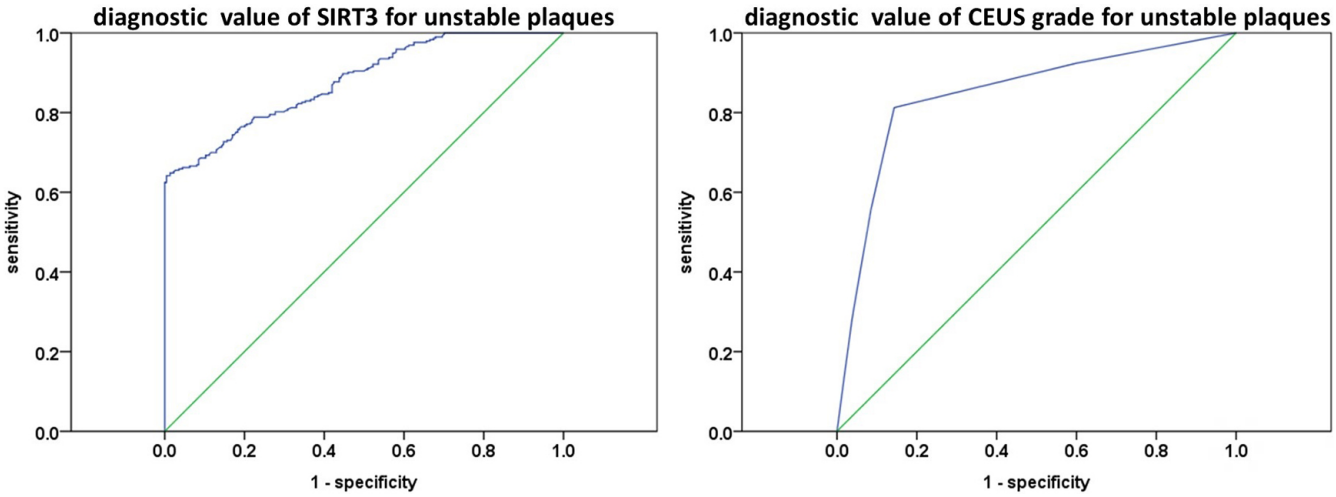

Fig. 3. Receiver operating characteristic (ROC) curve for the diagnostic value of sirtuin-3 (SIRT3) and contrast-enhanced ultrasonography (CEUS) (grade) in the diagnosis of unstable plaques

Table 3. Diagnostic value of SIRT3 and CEUS for the diagnosis of unstable plaques in CAS patients

Methods	True positive	False positive	True negative	False negative	Sensitivity	Specificity	Accuracy
Doppler ultrasound	150	77	216	74	66.96%	73.72%	70.79%
CEUS	182	42	251	42	81.25%	85.67%	83.75%
SIRT3	162	58	235	62	72.32%	80.20%	76.79%
CEUS+SIRT3	211	90	203	13	94.20%	69.28%	80.08%

SIRT3 – sirtuin-3; CEUS – contrast-enhanced ultrasonography; CAS – carotid atherosclerosis; * sensitivity = true positive/(true positive + false negative) × 100%; specificity = true negative/(true negative + false positive) × 100%; accuracy = (true positive + true negative)/(true positive + false negative + false positive + true negative) × 100%.

Table 4. Logistic regression for risk factors of unstable plaques

Variables	Hosmer– Lemeshow test	Wald	OR	95% CI	p-value
Model 1					
Age	0.983	3.603	0.925	0.854–1.003	0.058
BMI		0.222	1.037	0.893–1.204	0.637
CRP		39.226	2.648	1.952–3.592	<0.001
IL-6		24.906	1.682	1.371–2.063	<0.001
SIRT3		25.056	0.554	0.440–0.698	<0.001
Model 2					
Sex	0.783	0.177	1.078	0.758–1.533	0.674
Diabetes		0.979	0.804	0.522–1.238	0.322
Hypertension		0.758	1.232	0.770–1.972	0.384
History of coronary heart disease		1.892	0.694	0.413–1.168	0.169
History of stroke		0.001	1.010	0.583–1.752	0.971
Current smoker		5.293	0.629	0.424–0.934	0.021
Model 3					
TC	0.911	61.080	1.008	1.006–1.010	<0.001
TG		5.050	1.002	1.000–1.003	0.025
LDL-ch		42.322	1.004	1.003–1.006	<0.001
HDL-ch		30.696	0.138	0.068–0.278	<0.001

OR – odds ratio; 95% CI – 95% confidence interval; SIRT3 – sirtuin-3; BMI – body mass index; CRP – C-reactive protein; IL-6 – interleukin 6; TC – total cholesterol; TG – triglycerides; LDL-ch – low-density lipoprotein cholesterol; HDL-ch – high-density lipoprotein cholesterol.

that serum levels of SIRT3 were correlated with clinical outcomes, especially for severity and plaque stability. The combination of CEUS and SIRT3 might also be a potential diagnostic method for unstable plaques.

The application of CEUS is now widely accepted for the diagnosis of many cardiovascular and cerebrovascular diseases, including CAS.[19] Hamada et al. demonstrated that by using CEUS, physicians could observe a higher percentage of ulcerated findings in patients with plaque rupture.[20] Oura et al. showed that both CEUS and superb microvascular imaging had high sensitivity and specificity in the diagnosis of intraplaque neovascularization.[21] In other research, it was found that CEUS and multi-detector computed tomography angiography were the most accurate in the diagnosis of CAS, in which CEUS had a sensitivity and specificity of 94.1% and 97.95%, respectively, for the diagnosis of plaque ulceration.[22] A recent study reported that the combination of CEUS and erythrocyte sedimentation rate (ESR) in the diagnosis of Takayasu's arteritis could achieve a sensitivity and specificity of 81.1% and 81.5%, respectively.[23] In the present study, we found that CEUS could be used in the diagnosis of unstable plaques with a sensitivity of 81.25% and specificity of 85.67%, which was consistent with the above studies. We also found that the combination of CEUS and SIRT3 could achieve a sensitivity of 94.20%, a specificity of 69.28%, and an accuracy of 80.8% in the diagnosis of unstable plaques.

Sirtuin-3 is a member of the sirtuin family which has been associated with the inflammatory response in several studies.[24,25] Recently, researchers found that SIRT3 plays an important role in cardiovascular diseases. A recent study showed that SIRT3 could prevent myocardial ischemia-reperfusion injury by reducing oxidative stress and cell apoptosis.[26] Eid et al. demonstrated that the upregulation of both SIRT1 and SIRT3 was associated with improvements in myocardial ischemia-reperfusion injury.[27] In another study, Gaul et al. found that a deficiency of SIRT3 promoted arterial thrombosis, which is the main cause of plaque formation.[28] All of these studies indicated that SIRT3 is a key factor in the development of CAS. However, up to now, no clinical studies focused on the role of SIRT3 in CAS patients have been conducted. In our research, we found a low expression of SIRT3 in severe CAS patients and CAS patients with unstable plaques. In addition, a lower expression of SIRT3 predicted a higher risk for unstable plaques in CAS patients.

Limitations

Our study had some limitations. We obtained a limited sample size from a single center, which may limit the scientific value of this research. We did not consider how SIRT3 influences the formation of plaques; this issue requires more in vivo and in vitro studies.

Conclusions

In conclusion, this study demonstrated that SIRT3 was downregulated in severe CAS patients and CAS patients with unstable plaques. A low expression of SIRT3 predicted a higher risk for unstable plaques in CAS patients. The combination of CEUS and SIRT3 showed good sensitivity and specificity for the diagnosis of unstable plaques and might be a potential strategy in the clinic.

Supplementary data

The original statistical data of data distribution, Mann–Whitney U test and logistic regression are shown in Supplementary tables (https://doi.org/10.5281/zenodo.6993618).

The Supplementary material includes:

Table S1. Data distribution analysis for all continuous data in this study.

Table S2. Results of Mann–Whitney U test in table 1 and the left panel of figure 2.

Table S3. Results of Mann–Whitney U test in the right panel of figure 2.

Table S4. Results of Mann–Whitney U test in table 2.

Table S5. Hosmer–Lemeshow test of logistic regression model 1.

Table S6. Results of logistic regression model 1.

Table S7. Hosmer–Lemeshow test of logistic regression model 2.

Table S8. Results of logistic regression model 2.

Table S9. Hosmer–Lemeshow test of logistic regression model 3.

Table S10. Results of logistic regression model 3.

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