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The Clinical Significance of Lncrna GAS5 And Mir-222-3p in Carotid Artery Stenosis

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ABSTRACT

Background: Carotid artery stenosis (CAS) is the major pathogen of cerebral infarction and brain death. Early detection and risk prediction could help the diagnosis and improve the outcome of patients. The clinical significance of lncRNA GAS5 (GAS5) and miR-222-3p in the diagnosis and prognosis of CAS was evaluated in this study to explore novel effective biomarkers of CAS.

Methods: A total of 72 CAS patients and 63 healthy individuals (control) were enrolled in this study. The expression levels of GAS5 and miR-222-3p in study subjects were detected using PCR. The ROC, Kaplan-Meier, and Cox regression analyses were carried out to estimate the diagnostic and prognostic value of GAS5 and miR-222-3p in CAS. The interaction between GAS5 and miR-222-3p was disclosed by the dual-luciferase reporter.

Results: The reduced expression of GAS5 and elevated expression of miR-222-3p were observed in CAS patients compared with the healthy controls, and a significant correlation between their expression levels in CAS was revealed. GAS5 and miR-222-3p could discriminate CAS patients from the healthy controls with high sensitivity and specificity. The GAS5 downregulation and miR-222-3p upregulation could predict the poor prognosis of CAS patients and may be associated with the severe development of patients. In human vascular smooth muscle cells, miR-222-3p could negatively regulate the luciferase activity of GAS5.

Conclusion: Both GAS5 and miR-222-3p served as the diagnostic and prognostic biomarkers of CAS. The function of GAS5 might result from the regulation of miR-222-3p, which needs further validation.

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INTRODUCTION

The carotid artery is one of the major vascular vessels responsible for the blood supply to the brain, delivering blood from the heart to the head and neck [Cobiella 2021]. Carotid artery stenosis (CAS) has been considered the killer of human health since CAS can induce cerebral ischemia, infarction, and even death [Arasu 2021]. The pathogen of CAS was diverse involving but not limited to atherosclerosis, carotid dissection, and inflammation- and immune-related vascular disease. The symptoms of CAS are always associated with cerebral ischemia, such as dizziness, loss of orientation and memory, and difficulty speaking and understanding [Bohnstedt 2013]. However, patients with mild-to-moderate CAS are always asymptomatic, which increases the embarrassment in the early diagnosis of CAS [Reiff 2021]. The detection of CAS is based on the clinical symptoms, physical, and imaging examination [Lee 2021; Netuka 2016]. There is an urgent need for the identification of effective biomarkers of CAS to improve its diagnosis and early intervention.

Recently, increasing research has evidenced the biomarker role of non-coding RNAs (ncRNAs) in various human diseases. Among the abundant subtypes of ncRNAs, the function of long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) widely have been reported, which are abnormally expressed in CAS patients and related to the early detection and risk prediction of CAS [Esteller 2011; Matsui 2017]. Dolz et al. appraised a series of dysregulated miRNAs in patients with asymptomatic CAS and considered them as the potential biomarkers of ACAS [Dolz 2017]. Among the identified miRNAs, miR-222-3p was evidenced to be a cardiovascular-related miRNA, which regulates the endothelial cell dysfunction in atherosclerosis and participates in the injury of human brain microvascular endothelial cells [Li 2021; Xue 2015], but its specific function in the progression of CAS remains unknown. The upstream sponge of miR-222-3p, lncRNA GAS5 (GAS5) has been demonstrated to regulate the function of miR-222-3p in the progression of various diseases [Yang L 2021; Yang Z 2021]. Moreover, GAS5 was illustrated to mediate the cell cycle arrest and apoptosis of vascular smooth muscle cells, which are closely correlated with the onset and development of CAS.

This study aimed to evaluate the significance of miR-222-3p and GAS5 in the early detection and prognosis prediction of CAS. Moreover, the regulatory effect of GAS5 on miR-222-3p was speculated to mediate the progression of CAS, which also was estimated in the present study.

MATERIALS AND METHODS

Study subjects and sample collection: Seventy-two CAS patients were enrolled in this study from January 2014 to December 2016 at Affiliated Hospital of Hebei University. Patients with reduced diameter of carotid artery over 50% were diagnosed with CAS, and patients with other comorbidities were excluded. Another 63 healthy individuals who received regular physical examination also were included as the control group. The study had been approved by the Ethics Committee of Affiliated Hospital of Hebei University, and all subjects signed informed consent before enrollment. A five-year follow-up survey was carried out on the CAS patients to evaluate the prognosis of the enrolled patients. Cardiovascular events, including ischemia, stroke, or correlated death were defined as the endpoint events, and unrelated deaths were excluded.

The fasting blood samples were collected from enrolled subjects. The collected samples were centrifugated at 4000 rpm for 10 min to obtain the serum, followed by centrifugation at 12000 rpm for 15 min to remove cell debris. The separated samples were preserved at -80°C for the following analyses.

RNA extraction and PCR: RNA extraction was performed with the help of the TRIzol reagent, followed by the purity and concentration evaluation using the NanoDrop-2000 (ThermoFisher, USA). The isolated RNA was used to generate cDNA using the MiScript Reverse Transcription Kit (Qiagen, Germany) and the High Capacity cDNA Reverse Transcription kit (Applied Biosystem, USA). The quantification of GAS5 and miR-222-3p was conducted on the 7300 Real-Time PCR system (Applied Biosystem, USA) with the SYBR Kit (Invitrogen, USA) and calculated by the $2^{-\Delta\Delta Ct}$ method normalized to GADPH (for GAS5) and U6 (for miR-222-3p).

Cell culture: Human vascular smooth muscle cells (HVSMCs) were obtained from ATCC and cultured in the DMEM culture medium supplied with 10% FBS. Cell culture was carried out at 37°C with 5% CO2 and 95% humidity. The culture medium was changed daily. The cultured cells were available after arriving at the logarithmic period.

Dual-luciferase reporter assay: The binding sites between GAS5 and miR-222-3p were predicted and cloned into the pGL3 reporter vector to establish the wild-type GAS5 vector (WT-GAS5). The mutant-type GAS5 vector (MT-GAS5) was established by the mutation in the binding sites. The HVSMC was transfected with WT-GAS5 or MT-GAS5 and miR-222-3p mimic or miR-222-3p inhibitor to assess the interaction between GAS5 and miR-222-3p. The relative luciferase activity was estimated with the Dual-Luciferase Reporter Assay System (Promega, USA). The results were normalized to the Renilla luciferase.

Statistical analysis: All the experiments were performed in triplicate with three independent determinations of each. The results were expressed as mean ± SD. The difference between groups was evaluated with the student's t-test or one-way ANOVA. The role of miR-222-3p and GAS5 in the development of CAS was assessed with the Chi-square test. The ROC analysis was conducted to assess the diagnostic value of miR-222-3p and GAS5 in CAS, while their prognostic value was estimated using the Kaplan-Meier and Cox regression analysis. The statistically significant difference was indicated by $P < 0.05$.

RESULTS

The general information of CAS patients and healthy controls: The enrolled healthy controls included 45 males and 18 females, with an average age of 62.35 ± 12.48 years old. The included CAS patients were composed of 46 males and 26 females, and the average age was 61.42 ± 8.45 years old. There were no significant differences in the age, gender composition, BMI, HDL, LDL, TC, TG, and FBG between the healthy controls and CAS patients ($P > 0.05$). (Table 1)

Table 1. General information of the study subjects

	Healthy control	CAS patients	*P*-value
Age (years)	62.35 ± 12.48	61.42 ± 8.45	0.994
Gender (M/F)	45/18	46/26	0.432
BMI (kg/m^2)	24.13 ± 2.98	23.32 ± 4.16	0.998
SBP (mmHg)	123.27 ± 8.39	144.44 ± 8.48	<0.001
DBP (mmHg)	83.83 ± 5.67	95.57 ± 6.72	<0.001
HDL (mg/dL)	50.41 ± 3.22	50.80 ± 4.31	>0.99
LDL (mg/dL)	111.03 ± 7.86	111.61 ± 7.22	0.991
TC (mg/dL)	187.62 ± 4.57	189.65 ± 6.08	0.584
TG (mg/dL)	126.19 ± 7.11	123.11 ± 3.43	0.093
FBG (mg/dL)	87.96 ± 8.99	89.66 ± 8.28	0.788

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; FBG, fasting blood glucose

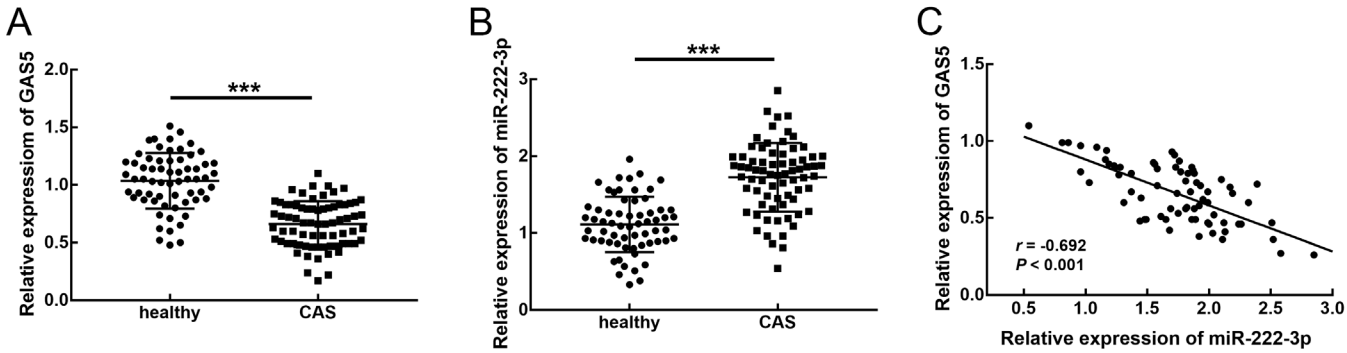

Figure 1. The expression of GAS5 and miR-222-3p in CAS. A) The downregulation of GAS5, and B) the upregulation of miR-222-3p were observed in CAS patients, compared with the healthy controls. C) The expression level of GAS5 was negatively correlated with the expression level of miR-222-3p in CAS with a coefficient of -0.692. ***$P < 0.001$

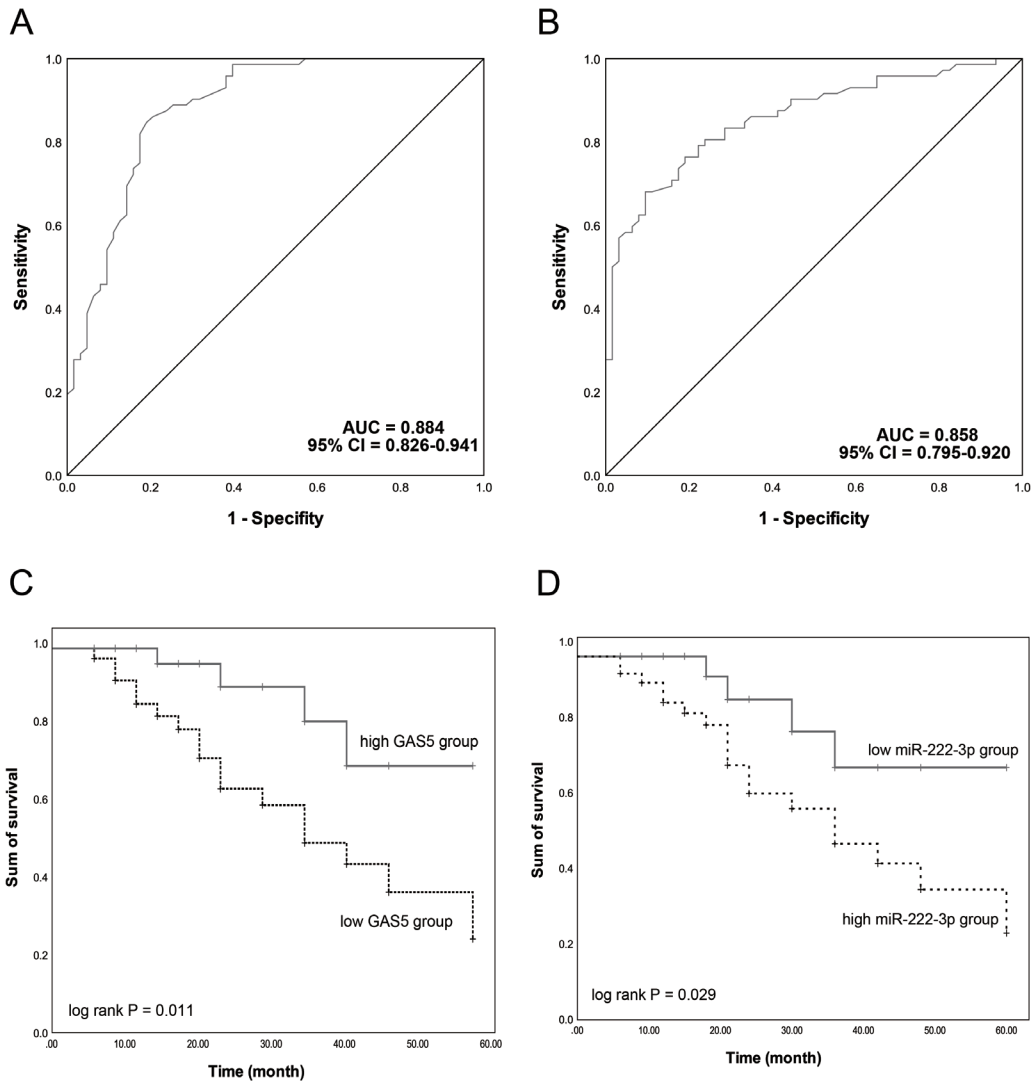

Figure 2. Evaluation of the diagnostic and prognostic value of GAS5 and miR-222-3p in CAS. A) GAS5, and B) miR-222-3p showed a significant diagnostic value of differentiating CAS patients from healthy individuals with the AUC of 0.884 and 0.858, respectively. C) The reduced GAS5 level, and D) the elevated level of miR-222-3p indicated a poorer prognosis of CAS patients.

CAS patients showed an increased SBP (144.44 ± 8.48 vs. 123.27 ± 8.39 mmHg) and DBP (95.57 ± 6.72 vs. 83.83 ± 5.67 mmHg) relative to the healthy controls ($P < 0.001$, Table 1).

The expression of miR-222-3p and GAS5 in CAS: In CAS patients, a reduced expression level of GAS5 (Figure 1A) and an elevated expression level of miR-222-3p (Figure 1B) was observed, compared with the healthy volunteers, and the difference was significant ($P < 0.001$), Figure 1). Moreover, the expression level of GAS5 showed a significantly negative correlation with miR-222-3p expression with r value of -0.692 (Figure 1C).

Diagnostic and prognostic value of miR-222-3p and GAS5 in CAS: The results of ROC revealed the significant diagnostic value of miR-222-3p and GAS5 in the distinction of CAS patients from the healthy controls with the AUC of 0.858 (Figure 2A) and 0.884 (Figure 2B), respectively. The sensitivity and specificity of GAS5 were obtained as 0.847 and 0.810, respectively, while the sensitivity and specificity of miR-222-3p were 0.681 and 0.905, respectively.

Based on the average expression levels in CAS patients, the patients were grouped as the high expression and the low expression group. Based on the expression of GAS5, CAS patients in the low expression group showed a significant poor prognosis relative to the patients in the high expression group (log rank $P = 0.011$, Figure 2C). While the patients with high miR-222-3p level were found to possess a worse outcome than that of patients in the low miR-222-3p group (log rank $P = 0.029$, Figure 2D). Moreover, both GAS5 (HR = 8.420, $P = 0.012$) and miR-222-3p (HR = 4.394, $P = 0.034$) served as two independent prognostic indicators as well as hypertension (HR = 4.899, $P = 0.034$) and CAS degree (HR = 3.529, $P = 0.042$). (Table 2)

Relationship of miR-222-3p and GAS5 with CAS patients' clinicopathological features: According to the average expression level of miR-222-3p and GAS5 in CAS patients, the association between patients' clinicopathological features and expression levels was estimated. The significant association of hypertension and CAS degree with GAS5 ($P = 0.038$ with hypertension, $P = 0.032$ with CAS degree) and miR-222-3p ($P = 0.019$ with hypertension, $P = 0.035$ with CAS degree) was observed, indicating their involvement in the development of CAS. (Table 3)

Evaluation on the interaction between miR-222-3p and GAS5: In HVSMC, the overexpression of miR-222-3p dramatically suppressed the luciferase activity of WT-GAS5, which was accelerated by miR-222-3p knockdown ($P < 0.001$). (Figure 3) While the luciferase activity of MT-GAS5 was not influenced by the regulation of miR-222-3p ($P > 0.05$, Figure 3).

DISCUSSION

CAS induces decreased blood perfusion, slows down the blood flow, resulting in the reduced blood supply to the brain and leading to cerebral infarction [Khan 2021]. Although great progress has been made in the clinical symptomatic treatment of CAS, the recurrence rate of CAS is still high [Dai 2017; Sprynger 2020]. Early detection is of great importance for patients to improve their quality of life and survival rate [Arasu 2021]. Previously, increasing research has focused on digging out sensitive and effective biomarkers that help screen CAS and benefit monitoring disease development. For instance, a clinical trait revealed that circulating miR-106b-5p could differentiate asymptomatic CAS from healthy individuals, and it also was able to predict the occurrence of the cerebral ischemic event [Jiang 2020]. LncRNA SNHG1 was demonstrated to indicate the risk of restenosis of CAS patients and regulate the biological processes of HVMSCs [Ma 2021]. miR-222-3p was identified as a dysregulated miRNA in CAS, which implies its potential in serving as a biomarker of CAS. GAS5 was disclosed to play roles in the progression of numerous human cancers and to mediate the apoptosis of vascular smooth muscle cells in the aortic aneurysm [Le 2021]. The interaction between GAS5 and miR-222-3p also has been reported to be involved in the progression of rheumatoid arthritis [Yang Z 2021]. Therefore, our study focused on the function GAS5 and miR-222-3p in the early detection and risk prediction of CAS, assessing their potential biomarker role.

The abnormal expression of both GAS5 and miR-222-3p was observed in CAS patients relative to healthy controls, which is consistent with previous reports [Li 2021; Xue 2015]. Meanwhile, a significant negative correlation between the expression of GAS5 and miR-222-3p in the serum of CAS patients also was heeded. The dysregulation of GAS5 and miR-222-3p was revealed to discriminate CAS patients from healthy individuals with relatively high sensitivity and specificity. Due to the fact that the clinical symptoms of CAS are always correlated with cerebral ischemia, the diagnosis of CAS utilizes the application of vascular and cerebral imaging, which are of great hysteresis [Bohnstedt 2013; Mortimer 2018].

Additionally, some CAS patients showed no typical symptoms, which makes the detection of CAS difficult [Krist 2021; Gaba 2018]. The significant diagnostic value of circulating GAS5 and miR-222-3p in CAS could benefit the screen of CAS and prevent patients from further deterioration.

A five-year follow-up survey was carried out and estimated the correlation of GAS5 and miR-222-3p expression with patients' prognosis. It was found that reduced GAS5 and increased miR-222-3p were always correlated with the poorer prognosis of CAS patients and showed obviously prognostic potential in CAS. Moreover, the different expressions of GAS5 and miR-222-3p in CAS showed close relationships with hypertension and CAS degree of patients, which are major risk factors of disease development, indicating their potential function in the progression of CAS. CAS could induce cerebral infarction and even brain death [Chen 2011; Ijäs 2014]. The involvement of GAS5 and miR-222-3p in CAS development provides a reliable prediction for the conditions of patients.

Sponging correlated functional miRNAs is the main molecular mechanism underlying the function of lncRNAs [Huang 2018; Paraskevopoulou 2016]. Herein, in vitro results showed that miR-222-3p could regulate the luciferase activity of GAS5 in HVSMC, which is consistent with their negative

Table 2. Multivariate Cox regression analysis evaluating the prognostic value of CAS patients' clinicopathological features and GAS5 and miR-222-3p

	HR factor	(95% CI)	*P*
GAS5	8.420	1.600-44.309	0.012
miR-222-3p	4.394	1.121-17.229	0.034
Age	2.476	0.774-7.915	0.261
Gender	2.433	0.661-8.957	0.281
Smoking	2.722	0.775-9.565	0.218
SBP	1.290	0.352-4.731	0.107
DBP	1.393	0.507-3.829	0.125
Hypertension	4.899	1.127-21.302	0.034
CAS degree	3.529	1.046-11.907	0.042

SBP, systolic blood pressure; DBP, diastolic blood pressure

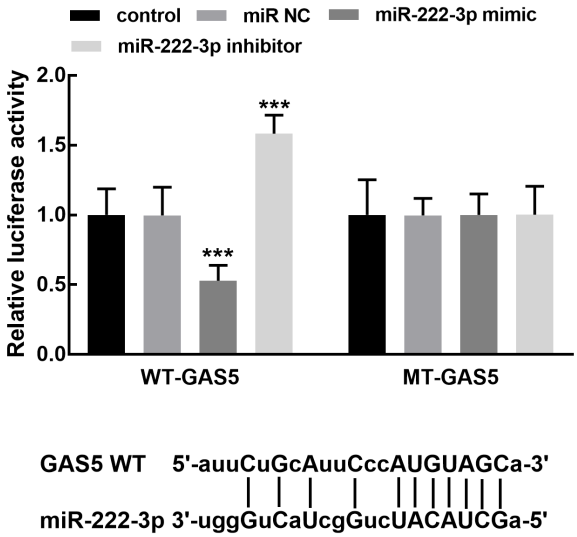

Figure 3. Assessment of the interaction between GAS5 and miR-222-3p in HVSMCs. miR-222-3p could suppressed the luciferase activity of GAS5, which was accelerated by miR-222-3p knockdown. ***$P < 0.001$

Table 3. Association between CAS patients' clinicopathological features and GAS5 or miR-222-3p expression levels

Variable	Total (N = 72)	GAS5 expression Low (*N* = 38)	GAS5 expression High (*N* = 34)	*P*	miR-222-3p expression Low (*N* = 30)	miR-222-3p expression High (*N* = 42)	*P*
Age							
< 60	42	21	21	0.576	19	23	0.467
≥ 60	30	17	13	-	11	19	
Gender							
Male	46	25	21	0.723	19	27	0.934
Female	26	13	13	-	11	15	
Smoking							
Present	38	22	16	0.358	17	21	0.576
Absent	34	16	18	-	13	21	
SBP (mmHg)							
< 145	32	14	18	0.170	15	17	0.423
≥ 145	40	24	16	-	15	25	
DBP (mmHg)							
< 85	33	15	18	0.252	17	16	0.119
≥ 85	39	23	16	-	13	26	
Hypertension							
Present	45	28	17	0.038	14	31	0.019
Absent	27	10	17	-	16	11	
CAS degree							
50-70%	37	15	22	0.032	17	20	0.035
71-99%	35	23	12	-	13	22	

SBP, systolic blood pressure; DBP, diastolic blood pressure

correlation in the clinical samples. Therefore, the function of GAS5 was speculated to be a result of the regulation of miR-222-3p, which needs further validation.

Taken together, both GAS5 and miR-222-3p are dysregulated in CAS, which showed a significantly negative correlation. GAS5 and miR-222-3p acted as diagnostic and prognostic biomarkers of CAS patients that distinguish CAS patients from healthy individuals and indicate disease development. The specific interaction mechanism between GAS5 and miR-222-3p needs further investigation.

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