MicroRNA-503-5p improves carotid artery stenosis by inhibiting the proliferation of vascular smooth muscle cells

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Abstract. Carotid artery stenosis (CAS) is a common arteriosclerotic vascular disease affected by vascular smooth muscle cells (VSMCs). The aim of the present study was to investigate the expression and diagnostic value of microRNA (miR)-503-5p in asymptomatic patients with CAS and to further explore the effect of miR-503-5p on VSMC proliferation. The levels of miR-503-5p in the serum of 62 asymptomatic patients with CAS and 60 healthy controls were detected by reverse transcription-quantitative PCR. The association between miR-503-5p and the clinical characteristics of the patients was analyzed using the χ^2 test. A receiver operating characteristic curve was drawn to evaluate the diagnostic value of miR-503-5p to distinguish asymptomatic patients with CAS from healthy controls. Finally, miR-503-5p inhibitors and mimics were transfected into VSMCs *in vitro* to detect the effect of miR-503-5p on the proliferation ability through Cell Counting Kit-8 assays. The serum levels of miR-503-5p in asymptomatic patients with CAS were significantly reduced as compared with those in healthy individuals. The expression levels of miR-503-5p were significantly associated with diabetes and arterial stenosis. Furthermore, the area under the ROC curve was 0.817, the specificity was 79.03% and the sensitivity was 83.30%, which proved that miR-503-5p had a high diagnostic accuracy in patients with CAS. Finally, the *in vitro* proliferation assay indicated that overexpression of miR-503-5p significantly inhibited the proliferation of VSMCs. In conclusion, miR-503-5p is a potential diagnostic biomarker for asymptomatic CAS and overexpression of miR-503-5p may inhibit the proliferation of VSMCs and improve CAS.

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Introduction

Carotid artery stenosis (CAS) is an important risk factor for ischemic neurological events. Nearly 80% of strokes occur in asymptomatic patients and the incidence increases with age (1,2). In addition, a number of cases were identified to suffer from coronary artery disease or peripheral atherosclerosis in asymptomatic patients with CAS (3). CAS is initially caused by abnormal proliferation of vascular smooth muscle cells (VSMCs), accompanied by intimal hyperplasia caused by matrix deposition of extracellular connective tissue to form plaques and eventually develops into symptomatic stenosis (4,5). There are various measures to prevent or treat CAS, including endarterectomy, endovascular stent placement (6) and medication (7). However, prophylactic surgery for CAS is controversial for patients with asymptomatic CAS (8). Statins and anti-platelet drugs are associated with a certain degree of stroke risk (9). Therefore, it is necessary to search for novel subclinical molecular biomarkers to reliably predict whether asymptomatic CAS is likely to develop into symptomatic CAS or remain stable.

MicroRNAs (miRNAs) are a group of highly conserved small non-coding RNA molecules that act as negative regulators of post-transcriptional regulation by inhibiting target gene expression. miRNAs have been indicated to be abnormally expressed in the physiological and pathological processes of various diseases. Numerous miRNAs have been reported to be abnormally expressed in CAS, including miR-330-5p (10) and miR-125a-3p (11), indicating that miRNAs have potential regulatory effects in CAS. In addition, certain miRNAs are considered potential markers of atherosclerosis and miRNAs also have an important role in VSMCs. Bi *et al* (12) reported that miR-503 inhibits the proliferation and migration of human aortic VSMCs induced by platelet-derived growth factors via targeting insulin receptors. Furthermore, Cremer *et al* (13) recently reported that miR-503-5p was associated with MALAT1 to reduce atherosclerosis in mice. In addition, miR-503-5p may regulate the differentiation of the mesenchymal stem cells into VSMCs, which has a certain effect in vascular tissue transplantation (14). However, the diagnostic value of miR-503-5p in CAS and the effect of miR-503-5p on the proliferation of VSMCs in CAS remains to be determined.

In the present study, the diagnostic value of miR-503-5p in patients with CAS was evaluated by detecting the expression

levels of miR-503-5p in patients with CAS. The effect of miR-503-5p on the proliferation of VSMCs in CAS was further investigated.

Materials and methods

Patient recruitment and sample collection. A total of 62 asymptomatic patients with CAS encountered at Anqiu People's Hospital (Weifang, China) between February 2010 and February 2014 were included. The selection criteria were an age of ≥18 years and asymptomatic CAS identified based on the patients' clinical data and the National Institutes of Health Stroke Scale (15). Exclusion criteria were a history of stroke, transient ischemic attack, coronary instability, congestive heart failure, chronic or acute inflammatory conditions, cancer and recent intracranial hemorrhage. From the health check-up center, 60 healthy controls with a similar age were selected as the control group. The inclusion criteria were no history of stroke and no mental illness. The patients' basic data and clinical characteristics were recorded accordingly (Table SI) and blood samples were obtained on the day of hospitalization. After taking blood samples, the serum samples were collected by centrifugation at 2,500 x g for 15 min at room temperature in a swinging-bucket centrifuge (Centrifuge 5810R; Eppendorf) and stored at -80˚C.

Cell culture and transfection. Human (h)VSMCs were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). The cells were cultured in an incubator at 37˚C with 5% CO_2. Cell transfection was performed when the cells were ~60% confluent. Transfected vectors were miR-503-5p mimics (cat. no. miR10002874-1-5; Guangzhou RiboBio Co., Ltd.), miR-503-5p inhibitor (cat. no. miR20002874-1-5; Guangzhou RiboBio Co., Ltd.), mimics negative control (mimics NC; cat. no. miR1N0000001-1-5; Guangzhou RiboBio Co., Ltd.) and inhibitor NC (cat. no. miR2N0000001-1-5; Guangzhou RiboBio Co., Ltd.), respectively. The transfection reagent was Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). TRIzol was used to extract total RNA from the subjects' serum and miRNA was isolated using the miRNApure Mini Kit (CWBiotech). The extracted RNA was reverse-transcribed into complementary (c)DNA according to the specifications of the Super cDNA First-Strand Synthesis Kit (CWBiotech). Finally, qPCR was performed with the Ultra SYBR Mixture and the ROX Assay kit (CWBiotech) in an ABI 7300 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mixtures were incubated at 95˚C for 10 min, followed by 40 cycles of 94˚C for 15 sec, 55˚C for 30 sec and 70˚C for 30 sec. Primer sequences used are as following: miR-503-5p forward 5'-CCTATTTCCCATGATTCCTTCATA-3' and reverse 5'-CTCGTTCGGCAGCACA-3'; and U6 forward 5'-AACGCTTCACGAATTTGCGT-3' and reverse 5'-CTCGTTCGGCAGCACA-3'. Using U6 RNA as the internal control, the relative expression of miR-503-5p was calculated using the $2^{-\Delta\Delta Cq}$ method (16). All of the experiments were repeated independently three times.

Cell proliferation assay. VSMCs at the exponential growth phase after transfection were seeded at $5x10^4$ cells per well in 96-well plates for continuous culture for 3 days and their proliferation capacity was determined at 0, 24, 48 or 72 h. Each time-point was performed in triplicate. Prior to detection, 10 μl Cell Counting kit 8 (CCK-8) reagent was added to each well, followed by further incubation for 1 h. The absorbance value was then detected at 490 nm using a Bio-Rad iMark plate reader (Bio-Rad Laboratories, Inc.). The effect of miR-503-5p on the proliferation ability of VSMCs was evaluated after 3 days of continuous detection.

Statistical analysis. All statistical data were processed and analyzed with SPSS 21.0 software (IBM Corp.) and GraphPad Prism 7.0 software. Student's t-test and one-way analysis of variance followed by Tukey's test were used to detect differences between groups. The χ^2 test was used to analyze the association between miR-503-5p expression and clinical characteristics of patients. A receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of miR-503-5p in CAS and calculate the area under the curve (AUC). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Serum levels of miR-503-5p in asymptomatic patients with CAS. The levels of miR-503-5p in the serum of all subjects were detected by RT-qPCR. The results indicated that the levels of miR-503-5p in asymptomatic patients with CAS was significantly lower than those in healthy controls ($P<0.001$; Fig. 1), suggesting that miR-503-5p may have a critical role in the development of asymptomatic CAS.

Association between miR-503-5p and clinicopathological features of patients. The association between the expression levels of miR-503-5p and the clinicopathological features of asymptomatic patients with CAS was then explored. According to the average expression levels of miR-503-5p in asymptomatic patients with CAS, all patients with asymptomatic CAS were divided into the high miR-503-5p expression group (n=24) and the low miR-503-5p expression group (n=38). As presented in Table I, the expression levels of miR-503-5p were not significantly associated with age, gender, body mass index (BMI), hypertension and dyslipidemia ($P>0.05$), but were significantly associated with diabetes ($P=0.034$) and carotid artery stenosis ($P=0.017$).

Diagnostic value of miR-503-5p in asymptomatic patients with CAS. A ROC curve was drawn according to the expression levels of miR-503-5p in asymptomatic patients with CAS and healthy controls to evaluate the diagnostic value of miR-503-5p for CAS. As indicated in Fig. 2, the AUC of the ROC curve was 0.817. The sensitivity was 83.30%, the specificity was 79.03% and the cut-off value was 0.810. The results suggested that miR-503-5p was of high diagnostic value in asymptomatic patients with CAS.

miR-503-5p regulates the proliferation of hVSMCs. The occurrence of CAS is linked to the abnormal proliferation

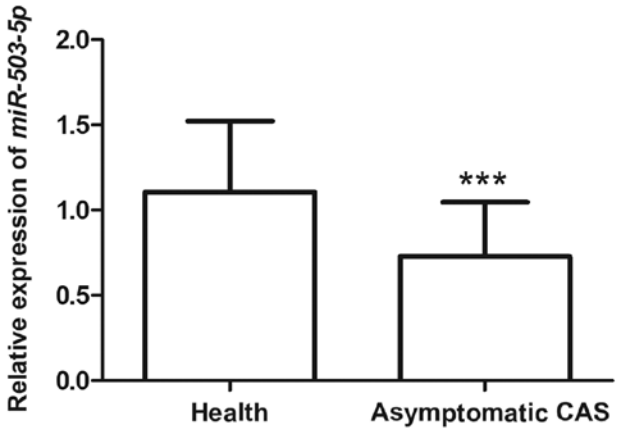

Figure 1. Expression of miR-503-5p in the serum of asymptomatic patients with CAS and the healthy control group detected by reverse transcription-quantitative PCR. Compared with that in the healthy control group, the expression of miR-503-5p in the serum of asymptomatic patients with CAS was significantly decreased. ***P<0.001. CAS, carotid artery stenosis; miR, microRNA.

of VSMCs. In the present study, the effect of miR-503-5p on the proliferation of hVSMCs was verified by transfection of miR-503-5p mimics and inhibitors. The transfection efficiency was verified by RT-qPCR analysis. As presented in Fig. 3A, the expression in the miR-503-5p mimics group was significantly higher than that in the control group (P<0.001), while the expression in the miR-503-5p inhibitor group was significantly lower (P<0.001). The results suggested that the overexpression and knockdown efficiency of miR-503-5p mimics and inhibitors in hVSMCs was higher.

A CCK-8 assay was used to further detect the effect of miR-503-5p on the proliferation ability of hVSMCs. As presented in Fig. 3B, the proliferation ability in the miR-503-5p mimics group was lower than that in the control group, while the proliferation ability in the miR-503-5p inhibitor group was significantly higher than that in the control group (P<0.01).

Discussion

The carotid artery is the major blood vessel in the neck that supplies blood from the heart to the brain and face. CAS is a pathological condition of vascular stenosis caused by atherosclerotic plaque (17). Asymptomatic CAS is defined as a patient without a history of ischemic stroke or transient ischemic attack in the ipsilateral carotid region and without focal neurological symptoms (18). At present, CAS is diagnosed by double ultrasound, CT angiography and MR angiography (19). Although traditional digital subtraction angiography is the gold standard for CAS diagnosis, its invasive nature carries the risk of stroke. Duplex ultrasound is non-radiative and non-invasive, but its sensitivity and specificity in diagnosing CAS are only moderate and require secondary verification (20,21). Therefore, it is urgent to develop novel sensitive, specific and non-invasive diagnostic markers for CAS.

In recent years, due to its high sensitivity, specificity and non-invasive nature, the detection of miRNAs as biomarkers has attracted an increasing amount of attention (22). In human

Table I. Association of miR-503-5p with the clinical parameters in asymptomatic patients with CAS (n=62).

		miR-503-5p expression		
Parameter	Total	Low (n=38)	High (n=24)	P-value
Age, years				0.586
≤58	22	15	7	
>58	40	23	17	
Gender				0.792
Male	36	23	13	
Female	26	15	11	
BMI				0.444
≤25	32	18	14	
>25	30	20	10	
Dyslipidemia				0.068
No	29	14	15	
Yes	33	24	9	
Hypertension				0.063
No	26	12	14	
Yes	36	26	10	
Diabetes				0.034
No	23	10	13	
Yes	39	28	11	
Degree of CAS, %				0.017
50-69	26	11	15	
70-99	36	27	9	

miR, microRNA; BMI, body mass index; CAS, carotid artery stenosis.

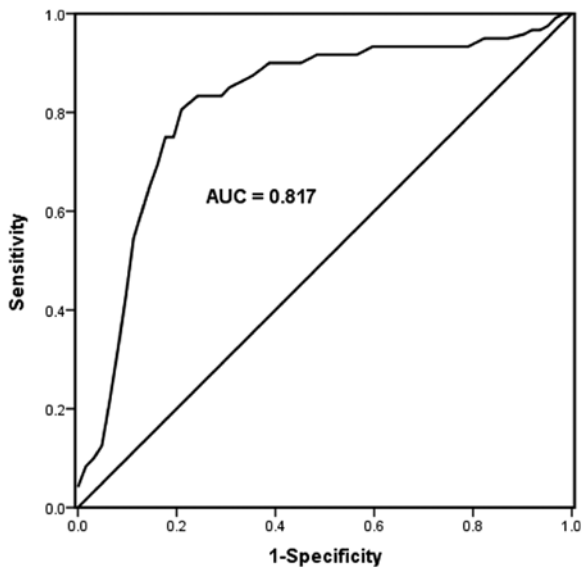

Figure 2. ROC curve for evaluating the diagnostic value of miR-503-5p for asymptomatic patients with carotid artery stenosis. The AUC was 0.826, the sensitivity was 85.30%, the specificity was 79.03% and the cut-off value was 0.826. AUC, area under the ROC curve; ROC, receiver operating characteristic.

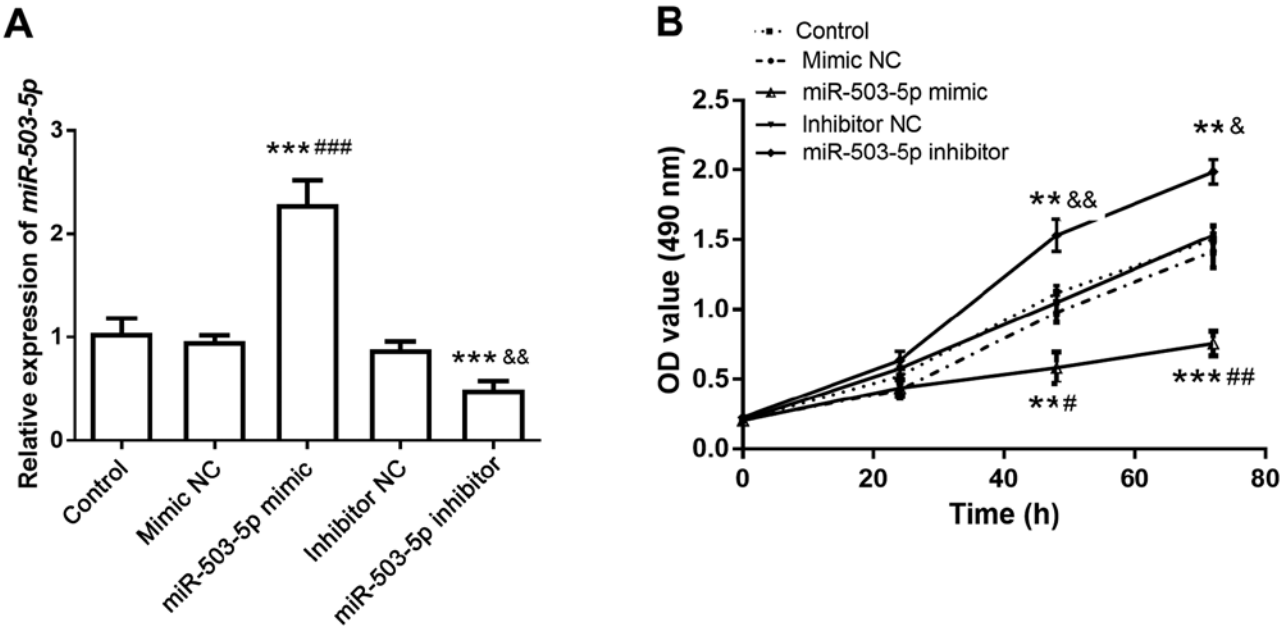

Figure 3. Detection of cell proliferation after transfection with miR-503-5p inhibitors and mimics. (A) The expression levels of miR-503-5p changed after transfection of miR-503-5p inhibitor and mimics, respectively. (B) After transfection with miR-503-5p inhibitors and mimics, the proliferation ability of cells was detected with a Cell Counting Kit-8. After transfection with miR-503-5p inhibitor, the proliferative ability of the cells was significantly promoted, whereas after transfection with miR-503-5p mimics, the proliferation ability was significantly decreased. **P<0.01, ***P<0.001, compared with control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, compared with mimic NC group; $^{\&}$P<0.05, $^{\&\&}$P<0.01, compared with inhibitor NC group. miR, microRNA; NC, negative control; OD, optical density.

cancer, certain serum miRNAs may be used as diagnostic and prognostic markers, including miR-1290 (23), miR-141 (24) and miR-193b (25). Disorder of miRNAs is also involved in the pathological processes of numerous cardiovascular diseases, including atherosclerosis and CAS. For instance, inhibition of miR-103 may reduce the inflammatory response and endoplasmic reticulum stress in patients with atherosclerosis (10). Serum miR-638 was significantly reduced in high-risk CAS patients undergoing carotid endarterectomy and was indicated to serve as a non-invasive biomarker associated with plaque vulnerability and ischemic stroke (26). In the present study, the expression of miR-503-5p was significantly downregulated in asymptomatic patients with CAS compared with that in healthy controls. This confirms that miR-503-5p has a crucial role in patients with CAS. miR-503 has been previously reported to be significantly downregulated in the serum of patients with coronary heart disease and to be a good prognostic marker of coronary heart disease (27). Ezetimibe inhibits PMA-induced monocyte/macrophage differentiation by upregulating the expression of miRNAs, including miR-503, and exerts an anti-atherosclerosis effect (28). These studies are consistent with the results of the present study.

To further confirm the potential role of miR-503-5p in asymptomatic CAS, its diagnostic value was examined. The experimental results indicate that miR-503-5p is highly specific and sensitive in distinguishing asymptomatic patients with CAS from healthy individuals. The clinical value of miR-503-5p has been widely reported in previous studies. Circular RNA (Circrna) 0000267 promotes gastric cancer progression through sponging miR-503-5p and regulation of high-mobility group AT-hook 2 expression (29). Low expression of miR-503 in gastric cancer tissues and serum may be used as a diagnostic marker for gastric cancer (30). In the present study, the promising diagnostic value of miR-503-5p in asymptomatic CAS patients was confirmed. Previous studies have reported that hypertension, dyslipidemia and diabetes are risk factors for the high prevalence of CAS (19). In addition, in patients with type II diabetes, hypertension and dyslipidemia have been indicated to have an accumulating effect on the carotid plaque burden (31). Recently, it has been reported that miR-503-5p is dysregulated in diabetic nephropathy (32). Therefore, in the present study, the clinicopathological features of patients with asymptomatic CAS and the expression of miR-503-5p were examined and it was indicated that the expression levels of miR-503-5p were associated with diabetes and carotid stenosis.

Studies have reported that plaque stability during the formation of CAS is associated with the function of VSMCs and endothelial cells (33,34). Furthermore, miR-145 was indicated to have a key role in CAS by regulating the function of VSMCs (35). At the same time, miR-503 inhibits the proliferation and migration of human aortic VSMCs induced by platelet-derived growth factors by targeting insulin receptors (12). Therefore, in the present study, miR-503-5p inhibitors and mimics were transfected into hVSMCs. After confirming the sufficient overexpression and knockdown efficiency, the proliferation ability of miR-503-5p on VSMCs was further tested. The results suggested that inhibition of miR-503-5p expression significantly promoted the proliferation of VSMCs. Studies have reported that fibroblast growth factor (FGF) expressed in 68% of cases of CAS (36). Circrna WD repeat domain 77 targets FGF-2 and regulates the proliferation and migration of VSMCs by stimulating miR-124 (37). At the same time, it has been reported that miR-503 inhibits tumor

angiogenesis and growth by targeting FGF2 and VEGFA (38). In pulmonary hypertension, miR-424- and miR-503-mediated endothelial cell apelin-FGF connections are destroyed (39). In addition, the relevant target of miR-503-5p was detected by TargetScan and it was indicated that epidermal growth factor 2 was the target of miR-503-5p. Therefore, it was speculated that miR-503-5p may regulate the functions of CAS and VSMCs by targeting FGF2. However, how miR-503-5p exerts its role in CAS remains to be further determined.

In conclusion, a series of experimental results have confirmed the low expression of miR-503-5p in asymptomatic patients with CAS and the low expression of miR-503-5p may be used as a potential diagnostic marker of asymptomatic CAS. At the same time, inhibition of miR-503-5p may promote the proliferation of VSMCs and may be used as a potential therapeutic target for CAS.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZY, HW, JL and XL designed the study. ZY, HW, JL and YL performed the experiments and interpreted the data. ZY, HW and YL drafted the manuscript. JL and XL revised it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiment was approved by the Anqiu People's Hospital Ethics Committee (Weifang, China) and written informed consent was obtained from all subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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