

Relationship between serum microRNA-223 and microRNA-126 and plaque stability in patients with carotid atherosclerosis

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Abstract

Background

Numerous researches have demonstrated the essential functions of microRNAs (miRNAs) in cardiovascular disease. Herein, we want to probe into the roles of miR-126 and miR-223 in the prediction of plaque stability of carotid atherosclerosis (CA).

Methods

First, miRNA microarray was performed to analyze the differentially expressed miRNA in serum of normal controls and CA patients. Next, the differential expression of miR-223 and miR-126 in CA was verified and the correlations of their expression levels with plaque stability-related factors were analyzed. Then, the predictive efficacy of miR-223 and miR-126 on plaque stability was analyzed by receiver operating characteristic curve, then the targeting relationships of miR-223 and miR-126 with COX2 were verified by online prediction and luciferase activity assay. Finally, the relationship between COX2 expression and CA plaque stability was analyzed.

Results

Expression levels of miR-223 and miR-126 were decreased in the serum of CA patients and they had good diagnostic efficacy for CA. Additionally, we found that the expression levels of miR-223 and miR-126 in the serum of CA patients with unstable plaque were lower than those with stable plaque. miR-223 and miR-126 were negatively correlated with plaque instability-related indicators, while COX2, a direct target gene of miR-223 and miR-126, was positively related to plaque instability-related indicators.

Conclusion

This study indicates that miR-223 and miR-126 are lowly expressed in serum of CA patients, which can be used as a clinical indicator for predicting the plaque stability in CA patients.

Background

Carotid atherosclerosis (CA), known as the manifestation of carotid atherosclerotic disease, is correlated to elevated risk for cardiovascular diseases [1]. CA usually emerges in the onset and progression of cognitive dysfunction, which impairs the general cognitive function and reduces memory and motor function, visual perception, as well as executive function [2]. CA has been reported to be evaluated by different non-invasive methods, including B-mode ultrasound, Transcranial Doppler, computed tomography angiography and magnetic resonance imaging [3]. Carotid endarterectomy has been demonstrated to be useful in prevention of strokes for patients with CA before, while carotid stenting is

currently considered as an alternative therapeutic approach for these patients due to the high risk of receiving carotid endarterectomy [4]. Therefore, it is urgent to further understanding the onset and progression of CA.

MicroRNAs (miRNAs) refer to a group of versatile posttranscriptional modulators of gene expression, which functions in a plethora of both physiological and pathological processes [5]. In atherosclerosis, several miRNAs have been confirmed to be relevant to some pathological processes, such as modulation of lipoprotein metabolism and cholesterol efflux, cholesterol and lipid biosynthesis, immune responses, vascular function as well as endothelial cell biology [6]. MiR-223 is a cell-specific miRNA with hematopoietic lineage, and it functions in granulocytic differentiation and myeloid lineage development [7]. The preliminary findings of an article have suggested that miR-223 expression may be related to atherogenesis, and circulating miR-223 expression has a relatively certain value in the prediction of coronary atherosclerosis severity [8]. MiR-126 is enriched in endothelial cells and platelets, and it is of great significance in maintaining vascular integrity and endothelial homeostasis [9]. Indeed, evidence has shown that the miR-126 is considered to be a promising biomarker for cardiovascular therapy due to its profound function in vascular repair [10]. Moreover, each mRNA is often targeted through different miRNAs, thereby enabling a modulation of the expression of targeted mRNA [5]. Cyclooxygenase-2 (COX-2) is a pivotal modulator of inflammatory processes, which is expressed in the atherosclerotic lesions from both humans and animals [11]. A prior study has revealed that downregulated COX-2 may exacerbate the enhanced risk of atherosclerosis development [12]. In this present study, we aim to investigate miR-126 and miR-223 expression in the serum of CA patients and to analyze the relationship between their expression levels with plaque stability.

Methods

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki in 1964 and approved by the ethics committee of Affiliated hospital of north China university of science and technology. All the subjects signed the informed consent and enjoyed the right to know.

Clinical samples

Fifty-two patients with symptomatic CA who had been treated in the Vascular Surgery of Affiliated hospital of north China university of science and technology were selected from September 2016 to October 2018. If a patient had a transient ischemic attack or ischemic stroke in the vascular area due to moderate or severe CA or carotid artery obstruction within the first 4 weeks (these symptoms were attributed to CA), the patient was considered with symptomatic CA. In addition to those who refused to participate in the study, we also excluded patients with Ehlers-Danlos syndrome, mitral valve/aortic valve disease, Marfan syndrome, and connective tissue disease. In addition, 25 healthy volunteers who came to Affiliated hospital of north China university of science and technology for medical examination during the same period were selected for controls. The fasting peripheral blood of CA patients and healthy

volunteers were harvested, and the serum was separated and stored for future use. Meanwhile, the demographic characteristics of all subjects were collected and their blood biochemical indicators were measured.

Microarray-based analysis

Total RNA was extracted from the serum of 6 controls and 6 CA patients using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Small RNA (< 200 nt) for miRCURY™ array microarray (Exiqon, Vedbaek, Denmark) was isolated from total RNA using mirVana miRNA isolation kit (Ambion, Scoresby, Victoria, Australia), and miRNA microarray analysis was performed according to Illumina standard procedures. The screening conditions for differential miRNA were set with $|\text{LogFC}| > 2$ and $P < 0.05$.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

In the light of the manufacturer's instructions, total RNA was extracted from the serum using the miRNease Mini kit (Qiagen, Hilden, Germany), and 500 ng of total RNA was implemented to synthesize complementary DNA according to the PrimeScript RT kit (Qiagen). Based on these gene sequences published in GenBank, Primer 5.0 software was applied to design primers (Table 1), and checked by NCBI Blast and oligo 7. Then, LightCycler 480 SYBR Green I Master (Roche Diagnostics, Penzberg, Germany) was utilized for PCR reaction. Each SYBR Green reaction took 0.25 μL of primers and 2 μL of cDNA as templates, with a total volume of 20 μL . U6 and glyceraldehyde phosphate dehydrogenase were selected as internal references. $2^{-\Delta\Delta\text{Ct}}$ method was implemented to calculate the relative expression level.

Enzyme-linked immunosorbent assay (ELISA)

The serum concentrations of matrix metalloprotease (MMP)1, MMP9, interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1) protein in the subjects were determined by ELISA (Quantikine® ELISA, R&D Systems, Minneapolis, MN). The experimental results were calculated with reference to the standard curve. All operations are strictly carried out in the light of the instructions.

Luciferase activity assay

Based upon the prediction results of bioinformatics websites StarBase (<http://starbase.sysu.edu.cn/>) and TargetScan (<http://www.targetscan.org/>), miR-223 or miR-126 and COX2 3' untranslated region (UTR) binding site and its mutated sequence (Sangon, Shanghai, China) were inserted into the pmirGLO reporter vector (Promega, Madison, WI, USA). Then, in strict accordance with the requirements of Lipofectamine 2000 reagent (Invitrogen), the recombinant plasmid was co-transfected with miR-223/miR-126 mimic or mimic control to HEK293T cells (purchased from American Type Culture Collection, Manassas, VA, USA). Firefly and renilla luciferase activities were detected 48 hours post co-transfection.

Statistical analysis

SPSS 21.0 statistical software (SPSS, Inc, Chicago, IL, USA) was implemented for all the statistical calculations. The normally-distributed measurement data were expressed as mean \pm standard deviation. The t-test was conducted to compare two groups of independent samples, and one-way analysis of variance and Tukey's multiple comparison test were utilized to compare data of multiple groups. Enumeration data were analyzed by Fisher's exact test. Pearson's correlation coefficient test was conducted for correlation analysis. Receiver operating characteristic (ROC) curves were depicted to evaluate the predictive efficacy of 10 differentially expressed miRNAs in CA patients and the area under the curve (AUC) was used to estimate their diagnostic values. In addition, ROC curves were also implemented to evaluate the predictive power of serum miRNA expression levels for plaque stability in CA patients. All *P*-value less than 0.05 was considered as statistically significant.

Results

Baseline characteristics of controls and CA patients

We recruited a total of 52 CA patients, including 36 males, with an average age of (56.79 ± 6.81) years. In addition, 25 healthy volunteers were recruited as normal controls, including 15 males, with an average age of (54.23 ± 9.18) years. Table 2 lists the baseline characteristics of CA patients and controls. The comparisons indicated that there was no statistical difference in gender and age between the two groups (both $P > 0.05$), while the laboratory parameters (triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (HDL-C) and high-density lipoprotein cholesterol (HDL-C) were significantly different (all $P < 0.05$).

miR-126 and miR-223 are lowly expressed in the serum of CA patients

First, we analyzed the differential expression profiles of miRNA in the serum of 6 CA patients and 6 controls by miRNA microarray sequencing. With the setting screening conditions, we found that there were 430 differentially expressed miRNAs, of which 197 were up-regulated and 233 were down-regulated (Fig. 1A). The heatmap in Fig. 1B shows the first differentially expressed 30 miRNAs. Subsequently, we further performed RT-qPCR to detect the top 10 differentially expressed miRNAs (miR-384, miR-219a, miR-126, miR-101, miR-577, miR-135a, miR-223, miR-148, miR-137 and miR-149) in 52 CA patients and 25 healthy volunteers. The obtained results were consistent with the trend of microarray analysis (Fig. 1C). Afterwards, we analyzed the predictive efficacy of these 10 differentially expressed miRNAs in CA patients by ROC curve. We found that miR-126 and miR-223, which had lower expression in the serum of CA patients, had the largest AUC (Fig. 1D).

miR-223 and miR-126 have a good predictive effect on plaque instability in CA patients

Next, these 52 CA patients were allocated into plaque stable (PS, $n = 23$) and plaque unstable (PU, $n = 29$) based on contrast enhanced ultrasound test results, ultrasound test results, and clinical test history (symptomatic or asymptomatic). The comparison revealed that the low expression levels of miR-223 and miR-126 were detected in the serum of the PU group versus the PS group (both $P < 0.05$, Fig. 2A).

Meanwhile, the ROC curve was adopted to analyze the predictive power of the expression levels of miR-223 and miR-126 on the plaque stability of CA patients, and findings suggested that the expression levels of miR-223 and miR-126 had high predictive power (AUC: greater than 0.75) on instability plaque of CA patients (Fig. 2B).

miR-126 and miR-223 are negatively correlated with plaque instability factors in CA patients

Subsequently, we detected the serum levels of IL-6, MMP1, MMP9 and MCP1 in CA patients by ELISA, and results suggested that serum levels of IL-6, MMP1, MMP9 and MCP1 in PU patients were higher than those in PS patients (Fig. 3A). Further, the correlations between the expression levels of miR-223 and miR-126 in serum of CA patients and these factors were analyzed by Pearson's correlation analysis, we found that the serum levels of miR-126 and miR-223 were negatively correlated with IL-6, MMP1, MMP9 and MCP1 (Fig. 3B-E). In addition, we also found that the expression levels of miR-126 and miR-223 were negatively correlated with the plaque thickness in CA patients (both $P < 0.05$) (Fig. 3F). The results can preliminarily indicate that miR-126 and miR-223 have a certain effect on maintaining the stability of atherosclerotic plaque.

miR-126 and miR-223 target COX2

With the aim to explore the possible downstream molecular mechanisms of miR-126 and miR-223 in maintaining the stability of atherosclerotic plaques, we predicted the downstream targeting mRNAs of miR-126 and miR-223 through StarBase and TargetScan databases, respectively, and there existed an intersection: COX2 (Fig. 4A). Next, luciferase activity experiments indicated that co-transfection of miR-126 mimic or miR-223 mimic with COX2-WT could reduce the luciferase activity in HEK293T cells (Fig. 4B, C), confirming that COX2 was miR-126' and miR-223' direct target gene. Based on the aforementioned, we further examined the expression level of COX2 in the serum of CA patients and normal volunteers. Results revealed that high expression level of COX2 was found in the serum of CA patients relative to normal controls (Fig. 4D), and its level in the PU patients was also higher than that in the PS patients (both $P < 0.05$) (Fig. 4E). Further Pearson's correlation analysis suggested that the expression levels of miR-126 and miR-223 in serum of CA patients were negatively correlated with the expression level of COX2 (Fig. 4F).

COX2 expression level is positively correlated with plaque instability factors in CA patients

Furthermore, we analyzed the correlation between the expression level of COX2 and the plaque stability-related factors in the serum of CA patients through Pearson's correlation analysis. We found that the expression level of COX2 was positively correlated with levels of IL-6, MMP1, MMP9, MCP1 as well as plaque thickness (Fig. 5A-E). This result indicates that COX2 can promote the expression of IL-6, thereby promoting the secretion of MCP1 by macrophages, thus inducing carotid artery epithelial cells to analyze MMPs, thereby promoting the plaque instability of CA.

Discussion

As reported, miRNAs are indeed play a role in regulating gene expression in platelets, and platelets miRNAs is regarded as a diagnosis, prognosis as well as efficacy evaluation index in atherosclerosis [13]. Plaque rupture is a main vulnerable plaque type, and an elevated collagen-to-lipid ratio is a significant indicator of plaque rupture [11]. In our study, we strived to elucidate the function of miR-223 and miR-126 in the prediction of CA, and the corresponding results suggested that miR-223 and miR-126 could be used as useful biomarker for predicting CA.

Initially, the findings of this study indicated that miR-223 and miR-126 were lowly expressed in the serum of CA patients, which had good diagnostic efficacy for CA. In addition, we also found lower expression levels of miR-223 and miR-126 in the serum of CA patients with unstable plaque relative to those with stable plaque. In line with our findings, a study has elucidated that circulating miR-126 and miR-223 are important in the diagnosis of sarcoidosis in heart failure patients [14]. The results of another study have found that both miR-126 and miR-223 have shown a downward trend in animal platelets, suggesting that miR-126 and miR-223 have a close association with the occurrence of atherosclerotic plaques [13]. In the atherosclerotic mice models induced by ApoE^{-/-}, miR-223 upregulation has been found to abrogate atherosclerosis development [15]. Yu *et al.* have proposed that plasma miR-126 could be considered as a potent marker for the prediction of major adverse cardiovascular events [16]. Furthermore, the ability of miR-126 to differentiate patients with atherosclerosis patients from healthy people has been demonstrated via a ROC curve with high sensitivity and specificity [17].

Also, our study suggested that miR-223 and miR-126 were found to be negatively related to plaque instability-related indicators, and COX2, as a direct target gene of miR-223 and miR-126, was demonstrated to positively associated with plaque instability-related indicators. It is widely accepted that MMP system deregulation plays a role in atherosclerosis and vascular remodeling [18]. MCP-1 belongs to the chemokine family, which exerts functions in the onset of atherosclerosis. Absence of MCP-1 plays a protective role against atherosclerotic lesion formation in apolipoprotein B transgenic mice [11]. Inflammatory factor (eg. IL-6), lipid, MMP-9, adiponectin, neutrophil count and total bilirubin have the potentials to improve the plaque definition in complications, thereby choosing asymptomatic patients for the interventional therapies [19]. IL-6 is a proinflammatory cytokine that has a role in the development of atherosclerotic plaque, and miR-126 mimic has been found to degrade the secretion of inflammatory cytokines [20]. It has been indicated that COX-2-mediated prostaglandin production is able to promote atherosclerosis via a series of mechanisms, such as stimulated chemotaxis, synthesis of extracellular matrix, induction of vascular permeability, as well as propagation of inflammatory cytokine cascade [21]. COX-2 and MMPs, as an inflammatory mediator or its product, have pathobiological function in atherosclerosis, which could modulate cellular activation and extracellular matrix [22]. It has been revealed that an enhanced COX-2 and MMPs expression are implicated in the atherosclerotic plaques' destabilization [23]. All these evidences have confirmed our findings to some extent.

Conclusions

Altogether, the present findings in our study advance our understanding in the pathophysiology of CA and also offer useful information for developing novel therapeutic and diagnostic agents in CA. Nevertheless, further researches are warranted to delineate the precise molecular mechanisms of miR-233 and miR-126 in regulating the plaque stability in CA.

Abbreviations

CA :Carotid atherosclerosis ;miRNAs :MicroRNAs ;COX-2:Cyclooxygenase-2 ;RT-qPCR :Reverse transcription quantitative polymerase chain reaction ;ELISA :Enzyme-linked immunosorbent assay ;MMP :matrix metalloprotease ;IL :interleukin ;MCP-1:monocyte chemoattractant protein-1 ;UTR :untranslated region ;ROC :Receiver operating characteristic ;AUC :area under the curve ;TG :triglycerides ; TC :total cholesterol ; HDL-C :low-density lipoprotein cholesterol ; HDL-C :high-density lipoprotein cholesterol.

Declarations

Availability of data and materials

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

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki in 1964 and approved by the ethics committee of Affiliated hospital of north China university of science and technology. All the subjects signed the informed consent and enjoyed the right to know.

Competing interests

All authors declare that there is no conflict of interests in this study.

Funding

No funding received.

Authors' contributions

YZ is the guarantor of integrity of the entire study and contributed to the concepts and design of this study; JAL and JEL contributed to the definition of intellectual content and literature research of this study; LM contributed to the experimental studies and the data acquisition and analysis; SHZ took charge of the manuscript preparation and review. All authors read and approved the final manuscript.

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Not applicable.

Consent to publication

Not applicable.

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Tables

Table 1
Primer sequence for RT-qPCR

| Gene | Primer sequence |
|----------|---|
| U6 | F: 5'-CGCTTCGGCAGCACATATAC-3' R: 5'-AATATGGAACGCTTCACGA-3' |
| miR-223 | F: 5'-CCTGGCCTCCTGCAGTGCCA-3' R: 5'-CTGGTAAGCATGTGCCGCACT-3' |
| miR-126 | F: 5'-CGCTGGCGACGGGACATTATTA-3' R: 5'-TGCCGTGGACGGCGCATTATT-3' |
| miR-101 | F: 5'-TGCCCTGGCTCAGTTATCACAG-3' R: 5'-TGCCATCCTTCAGTTATCACAG-3' |
| miR-577 | F: 5'-TGGGGGAGTGAAGAGTAGATA-3' R: 5'-GTAAGTAGATATGGGGAAATGA-3' |
| miR-148 | F: 5'-GAGGCAAAGTTCTGAGACACTCC-3' R: 5'-GAGACAAAGTTCTGTAGTGCAG -3' |
| miR-135a | F: 5'-AGGCCTCGCTGTTCTCTATGGC-3' R: 5'-TGTCCCCGCCGTGCGCCACGGC-3' |
| miR-137 | F: 5'-GGTCCTCTGACTCTCTTCGGTGAC-3' R: 5'-TGCCGCTGGTACTCTCCTCGACTA -3' |
| miR-149 | F: 5'-GCCGGCGCCCGAGCTCTGGCT-3' R: 5'-TCCAGCTGCCCCAGCACAGCC-3' |
| miR-384 | F: 5'-TGTTAAATCAGGAATTTTAAAC-3' R: 5'-TGTTACAGGCATTATGAACAAT -3' |
| miR-219a | F: 5'-CCGCCCCGGGCCGCGGCTCCTG-3' R: 5'-CCCGCTCGAGGTTTGGGGGCGG-3' |
| GAPDH | F: 5'-GGGAGCCAAAAGGGTCAT-3' R: 5'-GAGTCCTTCCACGATACCAA-3' |
| COX2 | F: 5'-ATGGCACATGCAGCGCAAGTAGGT-3' |

Note: F, forward; R, reverse; miR, microRNA; GAPDH, glyceraldehyde phosphate dehydrogenase; COX2, cyclooxygenase-2.

| Gene | Primer sequence |
|---|----------------------------------|
| | R: 5'-CTATAGGGTAAATACGGGCCCTA-3' |
| Note: F, forward; R, reverse; miR, microRNA; GAPDH, glyceraldehyde phosphate dehydrogenase; COX2, cyclooxygenase-2. | |

Table 2
Baseline clinical characteristic in controls and patients with CA

| Characteristics | Controls (n = 25) | CA patients (n = 52) | P value |
|---|-------------------|----------------------|---------|
| Male (n, %) | 15 (60.0) | 36 (69.23) | .643 |
| Age (y, mean \pm SD) | 54.23 \pm 9.18 | 56.79 \pm 6.81 | .173 |
| TG (mmol/L, median, IQR) | 0.94 (0.69–1.21) | 1.41 (0.95–1.73) | .001 |
| TC (mmol/L, mean \pm SD) | 3.77 \pm 0.91 | 4.32 \pm 0.51 | .001 |
| LDL-C (mmol/L, mean \pm SD) | 2.50 \pm 0.44 | 2.74 \pm 0.42 | .023 |
| HDL-C (mmol/L, mean \pm SD) | 1.49 \pm 0.31 | 1.12 \pm 0.43 | .000 |
| Note: y, year; SD, standard deviation; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. | | | |

Figures

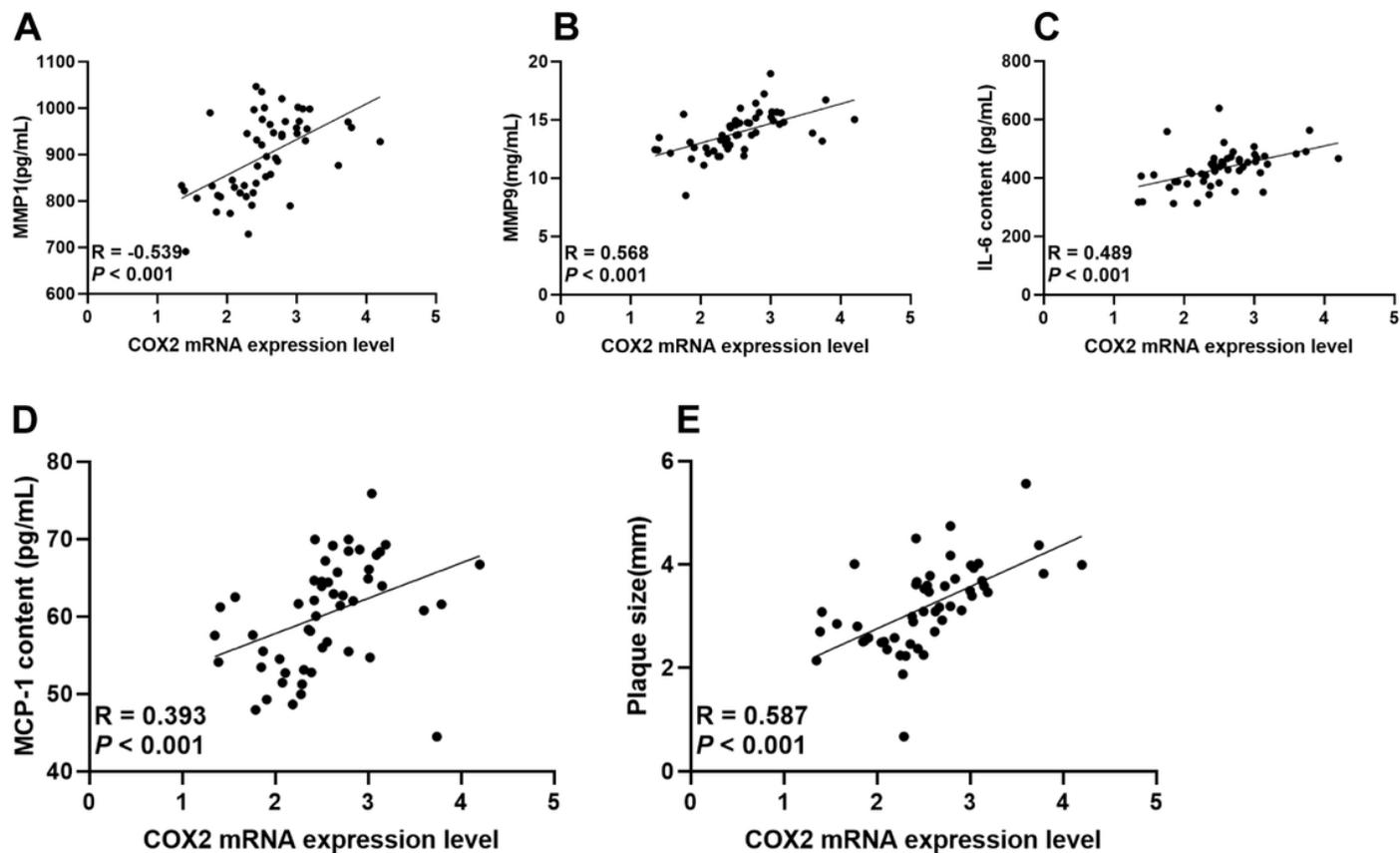


Figure 1

COX2 expression level is positively correlated with plaque instability factors in CA patients. A-E, Pearson's correlation analysis for the relationships of the expression level of COX2 with the serum levels of IL-6, MMP1, MMP9, MCP1 as well as plaque thickness in CA patients. Each dot represents a subject, and three independent repeated experiments were performed.

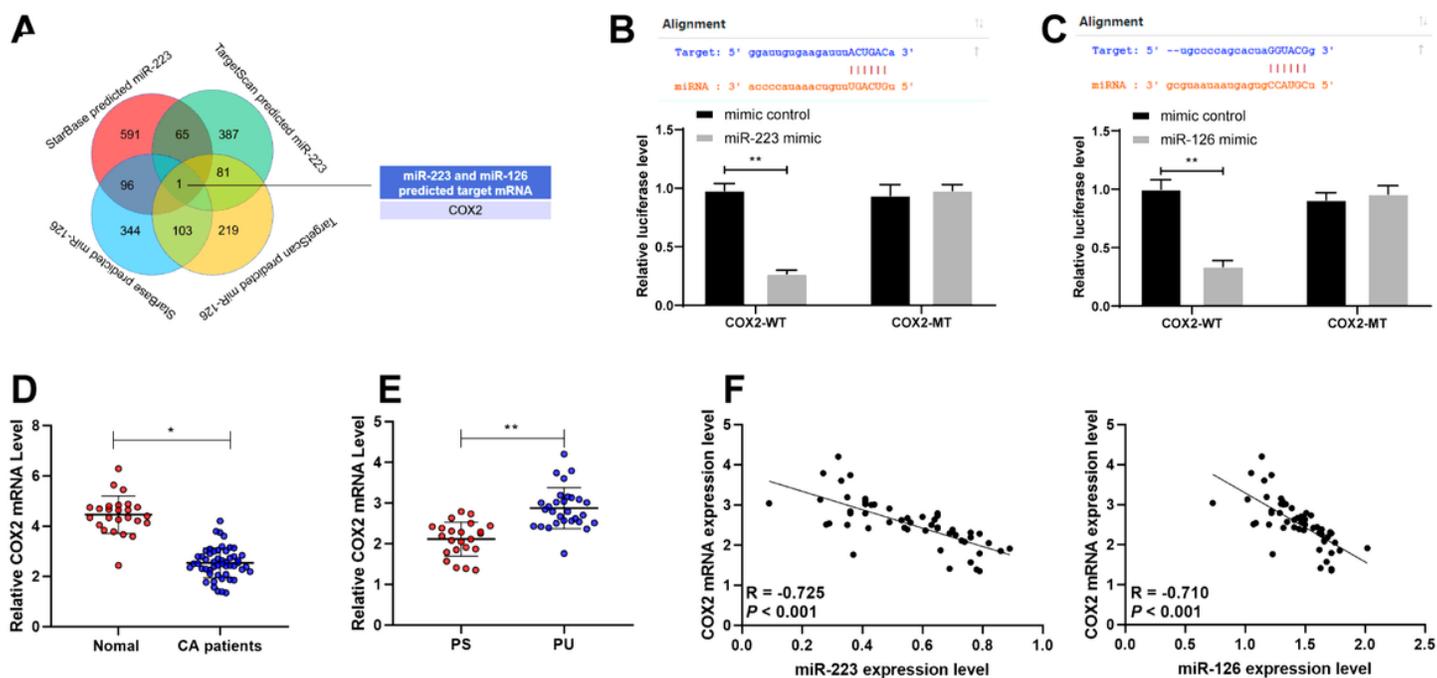


Figure 2

COX2 is a direct target gene of miR-126 and miR-233. A, StarBase and TargetScan predicting the downstream target gene COX2 of miR-126 and miR-223. B-C, Luciferase activity assay for detecting the target relationships of miR-223 and miR-126 with COX2. D, RT-qPCR for detection of COX2 mRNA expression in the serum of normal controls or CA patients. E, Expression levels of COX2 mRNA in serum of patients in the PS and the PU groups. F, Pearson's correlation analysis for the relationships of miR-223 and miR-126 expression levels with COX2 expression level. Each dot represents a subject, and three independent repeated experiments were performed. Two-way ANOVA and Tukey's multiple comparison test were utilized to analyze data in Panel B and C. The unpaired t-test was utilized to analyze data in Panel D-E. *P < 0.05; **P < 0.01.

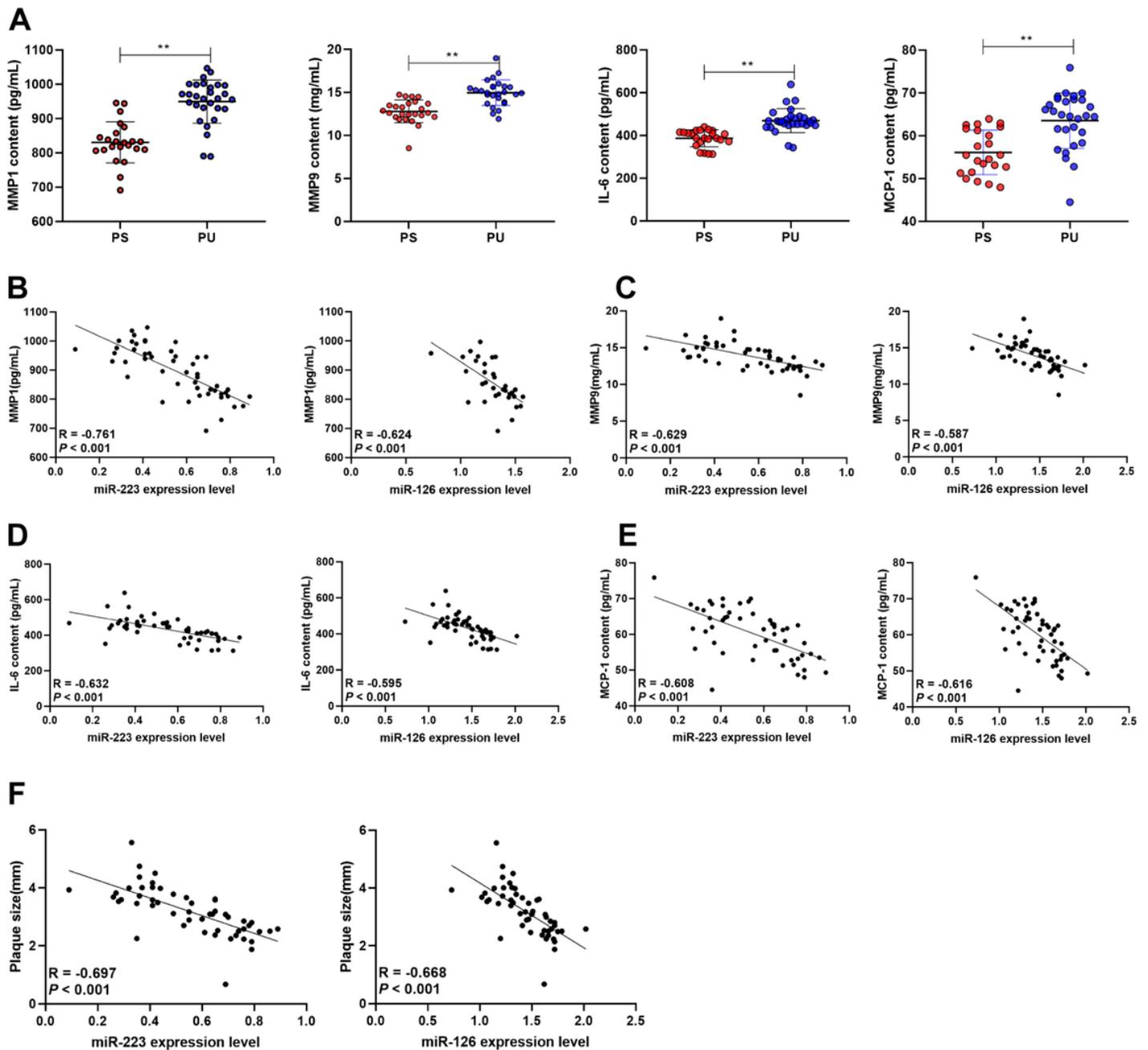


Figure 3

miR-126 and miR-223 are negatively associated with plaque instability factors in CA patients. A, ELISA for measuring the serum levels of IL-6, MMP1, MMP9 and MCP1 in CA patients. B-F, Pearson's correlation analysis for the relationships of miR-223 and miR-126 expression levels with IL-6, MMP1, MMP9, MCP1 levels, as well as plaque thickness in CA patients. Each dot represents a subject, and three independent repeated experiments were performed. The unpaired t-test was utilized to analyze data in Panel A. ** $P < 0.01$.

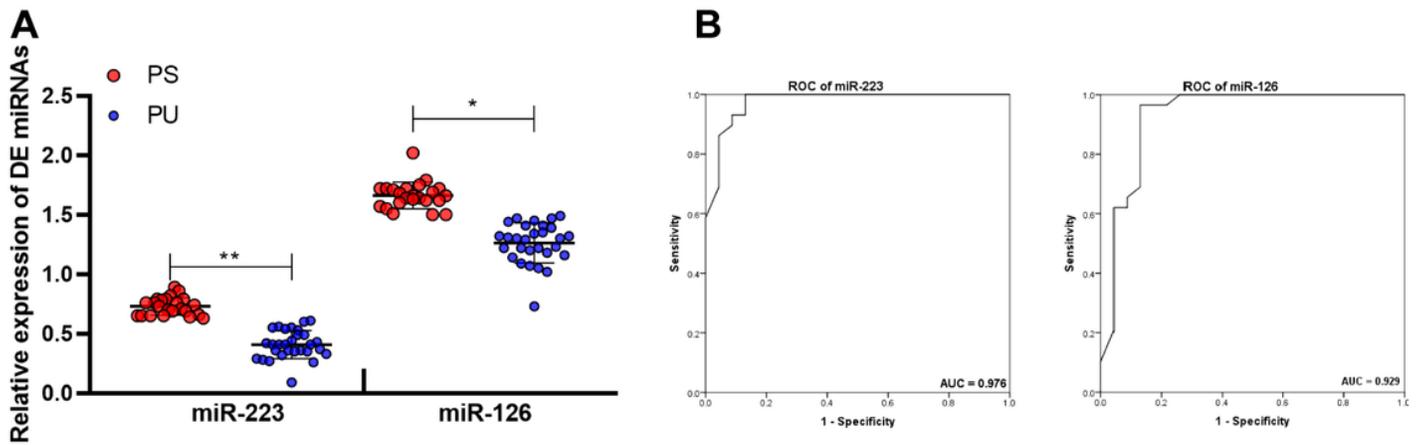


Figure 4

Both miR-223 and miR-126 have good predictive efficiency in instability plaque of CA patients. A, Expression levels of miR-223 and miR-126 in serum of CA patients in PS and PU groups. B, ROC curve analysis for the predictive power of miR-223 and miR-126 on plaque stability of CA patients. Each dot represents a subject, and three independent repeated experiments were performed. One-way ANOVA and Tukey's multiple comparison test were utilized to analyze data in Panel A. *P < 0.05; **P < 0.01.

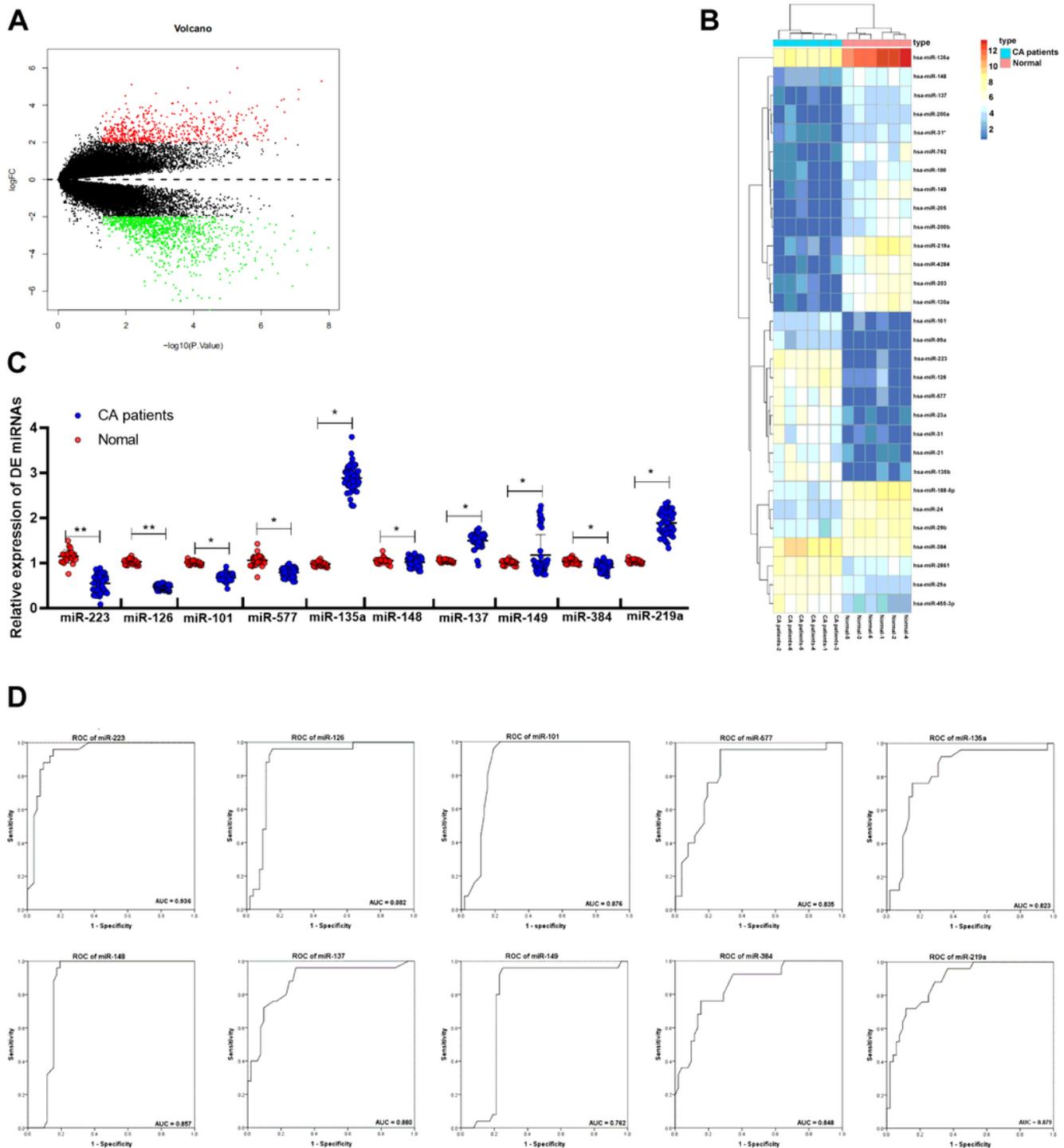


Figure 5

Lowly-expressed miR-126 and miR-223 are found in the serum of CA patients. A, Volcano graph showing differentially expressed miRNAs in the serum of 6 normal people and 6 CA patients. B, Heatmap showing the top 30 differentially expressed miRNAs in the results of microarray analysis. C, RT-qPCR for detecting the levels of the top 10 differentially expressed miRNAs in the serum of 25 normal people and 52 CA patients. D, ROC curve for the predictive power of the top 10 differentially expressed miRNAs in CA

patients. Three independent repeated experiments were performed. One-way ANOVA and Tukey's multiple comparison test were utilized to analyze data in Panel C. *P < 0.05; **P < 0.01.