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lncRNA PCA3 Suppressed Carotid Artery Stenosis and Vascular Smooth Muscle Cell Function via Negatively Modulating the miR-124-3p/ITGB1 Axis

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Abstract

Background & objectives: Due to the hidden pathogen, carotid artery stenosis (CAS) always occurred at an advanced stage leading to serious sequelae and even deaths. The significance of long noncoding RNA (lncRNA) prostate cancer antigen 3 (PCA3) in CAS incidence and progression were evaluated aiming to explore a potential target for its therapy.

Materials and methods: Serum samples were collected from 83 asymptomatic CAS patients and 52 healthy individuals and PCA3 was compared using polymerase chain reaction (PCR). The PCA3 levels were compared between stable and unstable plaque in CAS patients. The effect of PCA3 on vascular smooth muscle cells (VSMCs) proliferation and motility was assessed by CCK8 and transwell assay.

Results: PCA3 was downregulated in CAS patients and their unstable plaque tissues compared with healthy individuals and stable plaque, respectively. Reduced PCA3 could discriminate CAS patients with relatively high sensitivity and specificity and were associated with higher total cholesterol level and stenosis degree, unstable plaque, and complications. PCA3 downregulation predicted the adverse outcomes of CAS patients. In VSMCs, overexpressing PCA3 significantly suppressed cell proliferation, migration, and invasion, which was alleviated by miR-124-3p/ITGB1 axis.

Conclusion: PCA3 served as a biomarker of CAS and regulates the function of VSMCs through sponging miR-124-3p/ITGB1 and indirectly influence the stability of plaque.

Keywords

atherosclerosis, plaque stability, ceRNA, vascular smooth muscle cell

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Introduction

With the improvement of living standards and the ageing structure of the population, the incidence of atherosclerosis is gradually increasing, and the related cerebrovascular events have also attracted special attention. Carotid artery stenosis (CAS) is one of the major adverse outcomes of atherosclerosis, which accounts for about 30% of ischemic strokes.[1] The onset of CAS is relatively hidden, which is often noticed after physical examination or clinical symptoms occurring. The lack of timely intervention results in hemiplegia, aphasia, and even death.[2] In the past decades, great progress has been made in the therapy of CAS, and the symptoms and prognosis of CAS patients have also been greatly improved. Previous studies suggested that the stenosis degree of the vascular lumen is the main factor associated with therapeutic strategy and patients' prognosis.[3,4] However, increasing evidence suggested that missed diagnoses caused by asymptomatic features and complications induced by plaque instability are adverse prognostic factors.[5–7] There is an urgent need for exploring

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effective biomarkers associated with the occurrence and development of CAS, especially the stability of plaque. Vascular smooth muscle cells (VSMCs) play critical roles in the integrity of fibrous caps, which significantly influences the stability of plaque.[8,9] The apoptosis and senescence of VSMCs and the reduced collagen and elastin would promote the thinning of the fibrous cap and the formation of atherogenesis, and further boost the instability of plaque.[10–12] Hence, the regulation of VSMCs' function is also closely correlated with CAS pathogen and progression.

Noncoding RNAs (ncRNAs) compromise over 98% of the human genome. Long ncRNAs (lncRNAs) has become the focus of recent studies, which are of complex biology but with various functions. With the deepening of the research, the role of lncRNAs in vascular diseases has been disclosed. There have been several lncRNAs revealed to mediate the occurrence and development of atherosclerosis mainly through regulating lipid metabolism, inflammation, cell proliferation, apoptosis, and motility.[13–15] LncRNA prostate cancer antigen 3 (PCA3) was located on chromosome 9q21-22, which was primarily and widely demonstrated to play roles in the progression and prognosis of prostate cancer. Recently, the effect of PCA3 on other human diseases has also attracted huge attention. It has been reported to regulate lipid accumulation and metabolism disorders.[16,17] Moreover, PCA3 could also aggravate the progression of atherosclerosis via promoting the efflux of cholesterol.[16] Therefore, PCA3 was speculated to be involved in the development of CAS, but there were little data available to confirm its function.

In this study, PCA3 was compared between CAS patients and healthy individuals to evaluate its potential in discriminating CAS patients. The effect of PCA3 on the biological function of VSMCs was also estimated to explore its regulatory effects on plaque stability exploring the potential regulating mechanism.

Materials and Methods

Study Subjects

This study enrolled 83 asymptomatic CAS patients and 52 healthy individuals from June 2019 to July 2021 according to the following criteria.

The asymptomatic CAS was defined as patients who had never suffered transient ischemia, stroke, or other related neurological symptoms caused by CAS in the past 6 months, and patients who had only mild dizziness or pain symptoms. CAS patients were diagnosed by Doppler color ultrasound or CT angiography at Taizhou Second People's Hospital and meet one of the following indications of operation: (1) the degree of stenosis $\geq 70\%$ (by color ultrasound) or 60% (by CT angiography); (2) the degree of stenosis $< 70\%$ by ultrasound but CT angiography indicated the unstable lesion.

Patients with one of the following terms were excluded: (1) patients who possess intracranial aneurysm, which cannot be managed in advance or simultaneously; (2) patients with coagulation dysfunction or contraindications to heparin and antiplatelet agents; (3) patients with serious dysfunction in heart, lung, liver, kidney, or other important organs.

Healthy individuals were enrolled from the group receiving a routine physical examination at our hospital without any indications of carotid artery abnormality. The study had been approved by the Ethics Committee of Taizhou Second People's Hospital and all participant or their families had signed informed consent. The age and gender composition of study subjects are matched. CAS patients were followed up for 1 to 6 months (median follow-up time = 108 d) to trace their disease development and status. The endpoints were defined as the occurrence of cerebral events and CAS-related deaths.

Sample Collection

Fasting venous blood samples were collected from each participant and were centrifugated to isolate serum. The carotid plaque samples were collected from CAS patients during performing carotid endarterectomy. The obtained carotid plaque specimens must be of good integrity, and the samples with severe damage were excluded. All samples were stored at $-80\,^{\circ}C$ for the following analyses.

Cell Culture and Transfection

Human vascular smooth muscle cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) culture medium with 10% fetal bovine serum (FBS) and 1% antibiotics at $37\,^{\circ}C$ reaching the logarithmic phase.

Cells were transfected with the overexpression vector of PCA3 established with the pcDH-CMV-MCS-EF1-copGFP lentivirus vector (System Biosciences, USA) using Lipofectamine 2000 (Invitrogen, USA). The expression of PCA3 was analyzed by real-time quantitative polymerase chain reaction (qPCR) to evaluate the transfection efficacy.

Real-Time qPCR

Cells and clinical samples were lysed with a Trizol reagent to isolate total RNA. Isolated RNA was assessed by NanoDrop 1000 and OD260/280 > 1.8 indicating the qualified purity. Complementary DNA (cDNA) was generated with the RevertAid^{TM} First Strand cDNA Synthesis Kit (Takara, Japan), and the reaction conditions were: room temperature for 10 min, $42\,^{\circ}C$ for 60 min, $95\,^{\circ}C$ for 5 min, and on ice for 5 min. The PCR reaction was conducted on the 7500 PCR system with SYBR Green reagent. The relative expression levels were calculated with the $2^{-\Delta\Delta ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (for PCA3) and cel-miR-39 (for miR-124-3p) ad internal references.

Luciferase Reporter Assay

The Luciferase reporter vector was established by cloning 3' untranslated regions (3'UTR) or mutant 3'UTR of PCA3 or

ITGB1 into the pGL3 vector (Promega, USA). The established vectors were co-transfected with miR-124-3p mimic, inhibitor, or negative controls using Lipofectamine 2000 (Invitrogen, USA) to evaluate the interaction between PCA3 or ITGB1 and miR-124-3p. The relative luciferase activity of PCA3 and ITGB1 was assessed by a dual luciferase reporter system (Promega, USA) normalized to Renila.

Cell Proliferation Assay

Cells were seeded into 96-well plates supplied with a completed culture medium. After adherent growth, the culture medium was replaced with fresh culture medium and incubated for 0, 24, 48, 72, and 96 h. CCK-8 reagent was added to each well and incubated for another 4 h and the absorbance at 450 nm was detected with a microplate reader. The OD_{450}-time curve was plotted to evaluate the proliferation of VSMCs.

Cell Motility Assay

Transfected cells were collected and resuspended in FBS-free culture medium and seeded onto the upper chamber of 24-well transwell plates. The bottom chamber was filled with a 10% FBS-containing culture medium. The transwell plates were incubated at 37 °C for 24 h and the culture medium was removed. After washing with PBS twice, cells on the subsurface of the upper chamber were fixed and stained for 30 min, respectively. Cells were counted under a microscope from 5 random fields.

Statistical Analysis

Receiver operating characteristic curve analysis was employed to evaluate the significance of serum PCA3 in discriminating CAS patients from healthy individuals. The association of PCA3 with patients' disease conditions was assessed by the chi-square test. The follow-up data were analyzed by Kaplan-Meier and Cox regression analysis to identify potential prognostic factors of CAS.

Cell experimental data were expressed as mean ± standard deviation (SD). (n = 3) and analyzed by student's t-test or one-way analysis of variance (ANOVA) using SPSS 26.0 software or GraphPad Prism 9.0 ($P < .05$).

Results

PCA3 was Downregulated in CAS Patients Especially in Unstable Plaque

Reduced serum PCA3 levels were observed in CAS patients compared with healthy individuals (Figure 1a). CAS patients could be differentiated from healthy individuals by PCA3 expression with the cutoff of 0.825, and the sensitivity and specificity were 83.13% and 86.54%, respectively (area under the curve = 0.889, Figure 1b). In collected plaque samples from CAS patients, the significant downregulation of PCA3 was also observed in the unstable plaque relative to the stable plaques (Figure 1c).

PCA3 was Correlated With Adverse Development and Outcomes of CAS Patients

CAS patients were divided into low-PCA3 and high-PCA3 groups according to the average serum PCA3 levels (0.72) in CAS patients. There were 45 patients in the low-PCA3 group, and most patients possessed a higher total cholesterol level and stenosis degree, unstable plaque, and complications of

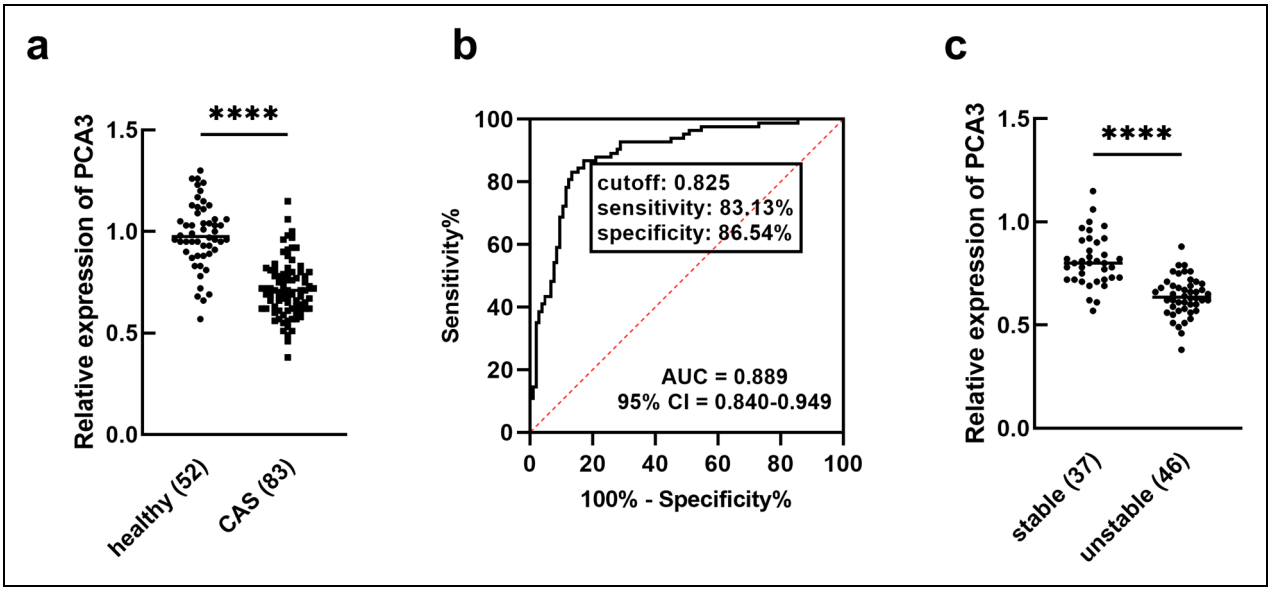

Figure 1. Expression of PCA3 in serum and plaque samples. PCA3 was significantly downregulated in CAS patients' serum (a), which showed sensitively and specifically diagnostic potential in CAS patients (b). (c) CAS patients with unstable plaque showed a lower expression of PCA3. ****$P < .0001$. Abbreviations: AUC, area under the curve; CAS, carotid artery stenosis; CI, confidence interval; PCA3, prostate cancer antigen 3.

Table 1. Association of PCA3 with Patients' Clinicopathological Features.

	Total (n = 83)	Low-PCA3	High-PCA3	*P*-value
Age				.734
≤ 60	42	22	20	
> 60	41	23	18	
BMI (kg/m^2)				.588
< 24	42	21	21	
≥ 24	41	24	17	
Gender				.865
Male	56	30	26	
Female	27	15	12	
Total cholesterol				.042
< 7.5	38	16	22	
≥ 7.5	45	29	16	
Low-density lipoprotein				.133
< 4.0	45	21	24	
≥ 4.0	38	24	14	
Stenosis degree				.021
50%–70%	17	5	12	
71%–99%	66	40	26	
Plaque stability				<.001
Stable	37	11	26	
Unstable	46	34	12	
Hypertension				.025
Yes	35	24	11	
No	48	21	27	
Smoking				.104
Yes	40	18	22	
No	43	27	16	
Diabetes				.002
Yes	46	32	14	
No	37	13	24	

Abbreviations: BMI, body mass index; PCA3, prostate cancer antigen 3.

hypertension and diabetes. A significant association of PCA3 with the total cholesterol ($P = .042$), stenosis degree ($P = .021$), plaque stability ($P < .001$), hypertension ($P = .025$), and diabetes ($P = .002$) was observed (Table 1).

Additionally, follow-up data showed that the 6-month development-free survival of the low-PCA3 group was poorer than that of the high-PCA3 group (Figure 2a). PCA3 was also identified as an independent prognostic biomarker of CAS with a hazard ratio (HR) of 9.359 (95% confidence interval [CI] = 2.225–39.367) together with low-density lipoprotein levels (HR = 3.826, 95% CI = 1.215–12.047), stenosis degree (HR = 8.805, 95% CI = 1.667–46.500) and plaque stability (HR = 4.691, 95% CI = 1.247–17.640, Figure 2b).

PCA3 Regulated the Function of VSMCs via Negatively Regulating miR-124-3p

PCA3-overexpressed VSMCs were established by cell transfection. The regulation of miR-124-3p showed no significant effect on PCA3 expression (Figure 3a), but it could alleviate the suppression by PCA3 overexpression (Figure 3b). Additionally, the luciferase reporter also confirmed that miR-124-3p could negatively regulate the luciferase activity of PCA3 through several binding sites (Figure 3c).

The overexpression of PCA3 significantly suppressed the proliferation (Figure 3d), migration (Figure 3e), and invasion (Figure 3f) of VSMCs, while the upregulation of miR-124-3p significantly attenuated the inhibitory effect of PCA3 overexpression.

The PCA3/miR-124-3p Axis Could Regulate ITGB1 in VSMCs

miR-124-3p was predicted to bind 3'UTR of ITGB1 with several binding sites. In VSMCs, miR-124-3p was found to negatively regulate the luciferase activity of WT-ITGB1, but showed no significant influence on the activity of MT-ITGB1 (Figure 4a). In transfected VSMCs, the overexpression of PCA3 significantly increased the mRNA level of ITGB1, while miR-124-3p overexpression reversed this elevation (Figure 4b).

Discussion

CAS and the formation of carotid plaque are the major inducing factors of stroke. Traditional views have considered stenosis degree as the main risk factor for the malignant development of CAS, however, cerebrovascular events also frequently occurred in some patients with minor stenosis.[18] Therefore, stenosis degree cannot be the unique criterion for the occurrence of cerebrovascular events. The stability of plaque has been included in the assessment of CAS development, but the mechanism that affected the stability and development of plaque remains unclear.[19,20] With the development of molecular mechanisms, the identification of CAS development-related molecules has been widely investigated. There have also been several lncRNAs suggested to indicate the onset and progression of CAS and regulate the stability of plaque. For instance, lncRNA non-coding RNA of nuclear factor of activated T cells (NRON) was demonstrated to regulate the growth and viability of VSMCs and further influence the stability of plaque stability.[21] Upregulated lncRNA rhabdomyosarcoma 2-associated transcript (RMST) was identified as a biomarker for CAS that screened the pathogen of CAS and predicts the occurrence of cerebral ischemic events.[22] In this study, the downregulation of PCA3 was observed in CAS patients' serum, which could discriminate CAS patients sensitively and specifically, indicating its diagnostic potential. In CAS patients with unstable plaque, the expression of PCA3 was much lower than that in the stable plaques, which is consistent with their significant association. Blood lipid level has been considered in the risk assessment of CAS. Here, the total cholesterol and low-density lipoprotein levels were included, and PCA3 was found to show a close association with CAS patients' total cholesterol levels. Moreover, reduced PCA3 level was also

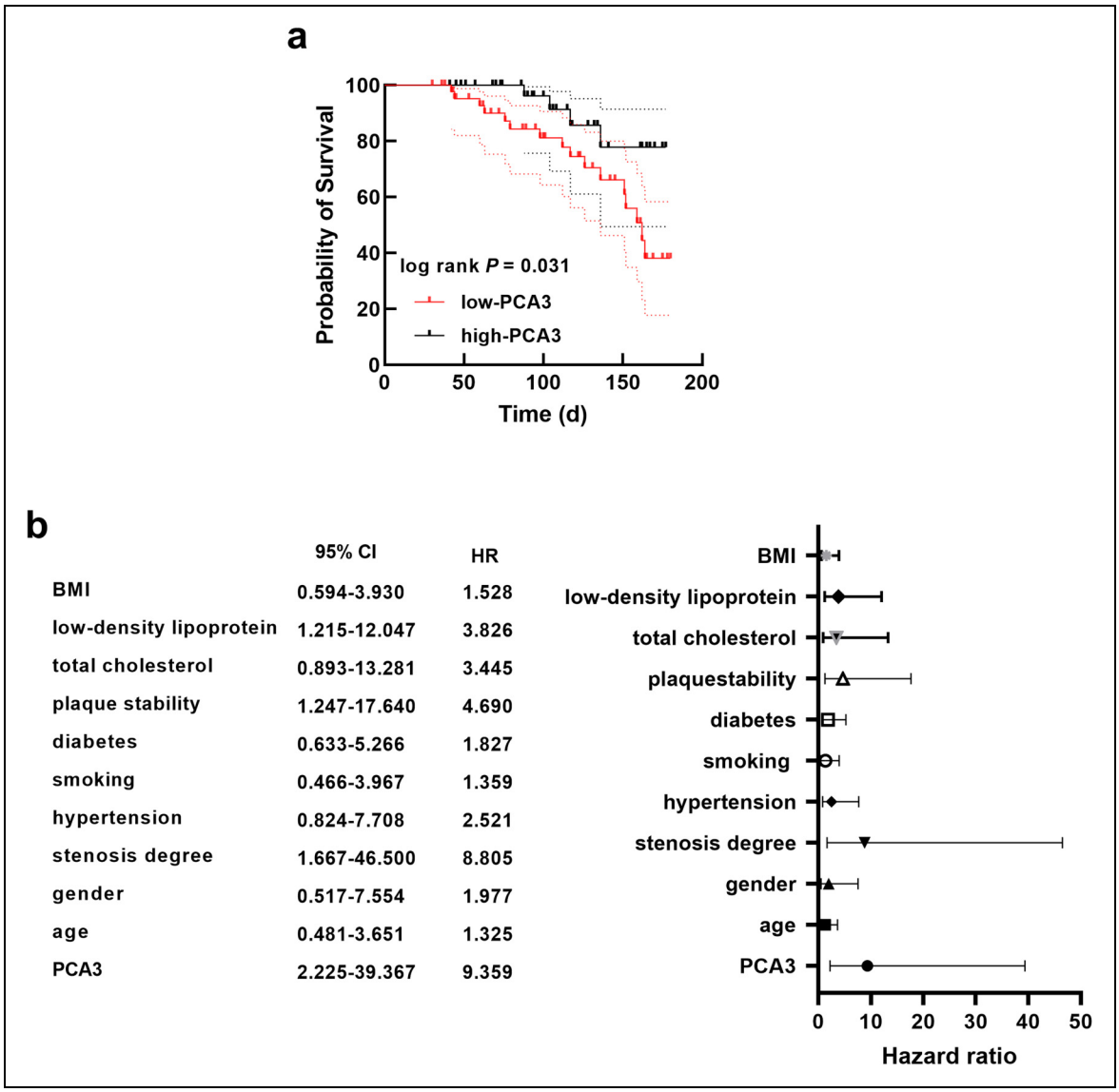

Figure 2. Prognostic significance of plaque PCA3 in CAS patients. The lower PCA3 levels were significantly associated with an adverse 6-month development-free survival of CAS patients (a) and were identified as an independent prognostic indicator together with low-density lipoprotein, stenosis degree, and plaque stability. Abbreviations: BMI, body mass index; CAS, carotid artery stenosis; CI, confidence interval; HR, hazard ratio; PCA3, prostate cancer antigen 3.

significantly associated with CAS patients' stenosis degree, plaque stability, and complications of hypertension and diabetes, which indicated the increasing severity and the advanced development of CAS. Meanwhile, PCA3 downregulation was also revealed to indicate the adverse outcomes of CAS patients and was demonstrated to act as an independent prognostic factor as well as low-density lipoprotein, stenosis degree, and plaque stability. However, the follow-up survey mainly focused on the 6-month prognosis of CAS patients in the present study. Longer follow-up time is needed in the future investigations to clear the significance of PCA3 in the long-term outcomes.

VSMCs are highly differentiated cells and are the major component of the vascular media. The proliferation of VSMCs after migration and its secretory collagen, elastin, and other extracellular matrix components would induce arterial lumen stenosis and stabilize atherosclerotic.[23] Therefore, the growth and motility of VSMCs have been considered the major pathological basis of CAS and other arterial diseases.[24,25] The molecular mechanism of VSMCs proliferation, migration, and phenotypic transformation remains unclear, but exploring related regulators could benefit the treatment of angiogenesis diseases.[26] The regulator effect of PCA3 was disclosed in prostate cancer, choriocarcinoma, and ovarian carcinoma cells.[27–29] Herein, overexpressing PCA3 was found to suppress the proliferation, migration, and invasion of VSMCs, indicating its potentials inhibitory effect on CAS development.

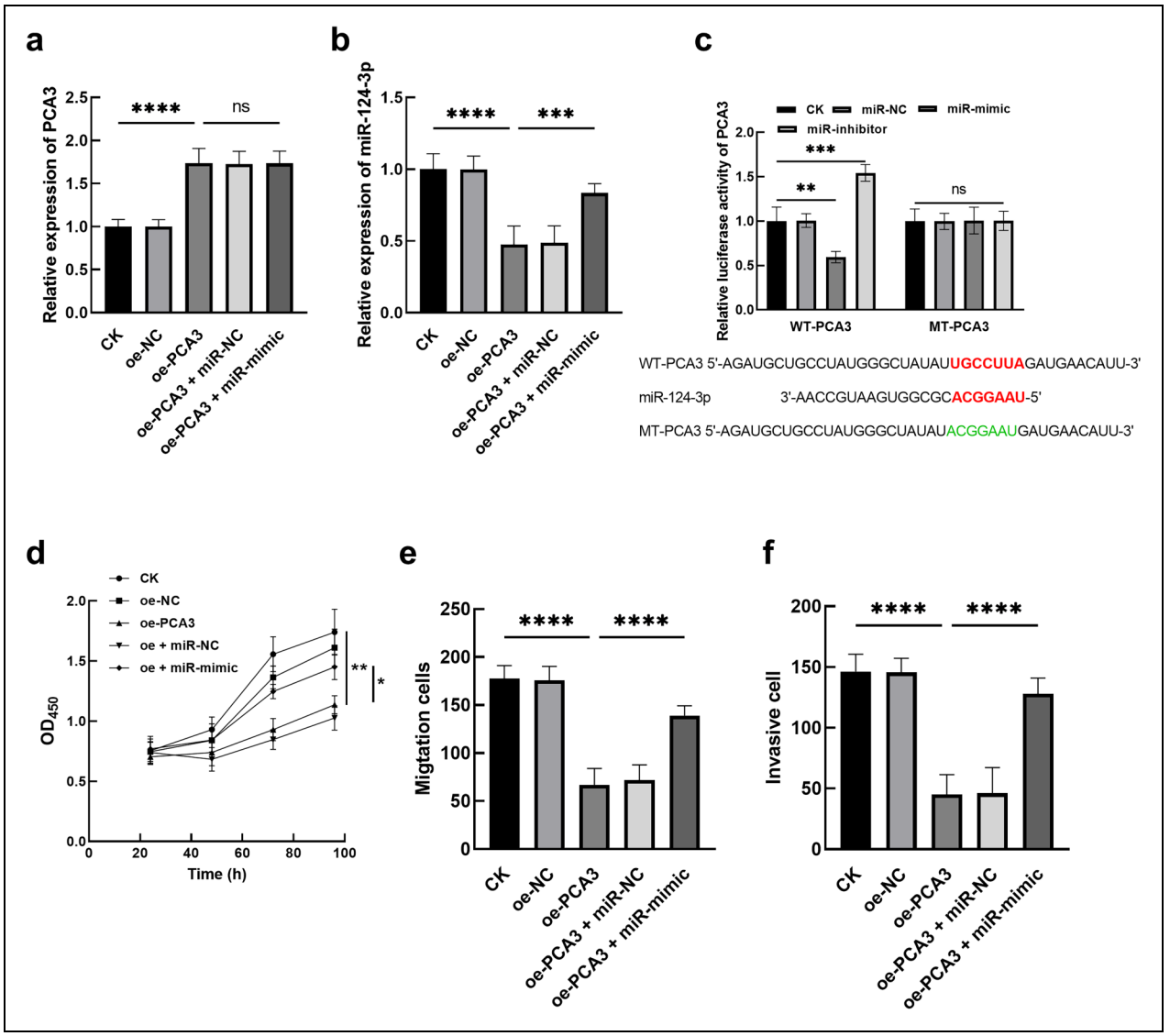

Figure 3. Effect of the PCA3/miR-124-3p axis on the function of VSMCs. miR-124-3p showed no significant effect on PCA3 expression (a) but could alleviate the suppressed effect of PCA3 overexpressionon miR-124-3p expression (b). (c) miR-124-3p could negatively regulate the luciferase activity of wild-type PCA3 in VSMCs. Overexpression of PCA3 dramatically suppressed the proliferation (d), migration (e), and invasion (f) of VSMCs, which was alleviated by miR-124-3p. $^{ns}P > .05$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. Abbreviations: PCA3, prostate cancer antigen 3; VSMC, vascular smooth muscle cell.

Competitive endogenous RNA (ceRNA) theory has been accepted as the major mechanism underlying the function of lncRNAs. In previous studies, PCA3 was illustrated to sponge miR-132-3p in regulating prostate cancer lipid metabolic disorder, and its regulatory effect on choriocarcinoma was suggested to result from the negative regulation of miR-106b.[17,28] miR-124-3p was predicted as a direct downstream ceRNA of PCA3, and the negative regulatory effect of PCA3 on miR-124-3p expression was confirmed in VSMCs. Previously, miR-124-3p was revealed to be significantly upregulated in CAS patients and was speculated to possess diagnostic potential.[30] Here, miR-124-3p was found to attenuate the inhibition of VSMCs growth and motility by PCA3 overexpression, indicating its involvement in VSMCs function. Additionally, ITGB1 was predicted as a direct target of miR-124-3p from online database. ITGB1 was also reported to be associated with the occurrence of coronary heart disease and was downregulated in advanced stage of atherosclerosis.[31] The negative regulation of ITGB1 was observed by miR-124-3p, and the regulatory effect of the PCA3/miR-124-3p axis was also demonstrated in VSMCs in the present study. Therefore, ITGB1 was speculated to mediate the regulatory effect of the PCA3/miR-124-3p axis on VSMCs.

Taken together, the downregulation of PCA3 in CAS could serve as a potential diagnostic and prognostic biomarker screening the occurrence and indicating the severe development of CAS. PCA3 inhibited the proliferation, migration, and invasion of VSMCs and further affected the stability of plaque via

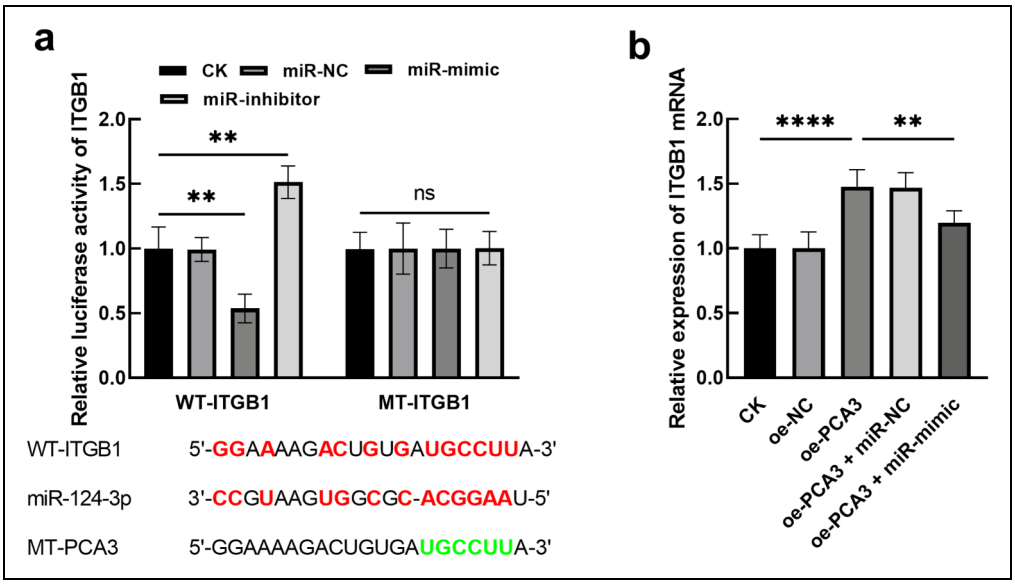

Figure 4. Regulatory effect of PCA3/miR-124-3p axis on ITGB1 in VSMCs. (a) miR-124-3p could negatively regulate the luciferase activity of ITGB1 by binding with several sites of ITGB1 3'UTR. (b) PCA3 overexpression significantly enhanced ITGB1 mRNA expression, which was reversed by miR-124-3p overexpression. $^{ns}P > .05$, $^{**}P < .01$, $^{****}P < .0001$. Abbreviations: mRNA, messenger RNA; PCA3, prostate cancer antigen 3; UTR, untranslated regions; VSMC, vascular smooth muscle cell.

negatively regulating the miR-124-3p/ITGB1 axis. However, except for the growth and motility of VSMCs, the secretion of collagen and the transformation of the VSMCs phenotype are also critical factors associated with plaque stability and CAS development.[32,33] Therefore, the effect of the PCA3/miR-124-3p axis on these processes needs further investigations to deeply declaim the regulatory mechanism. This study still provided a potential target for the therapy and drug development of CAS.

Declaration of Conflicting Interests

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