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Original Article

LncRNA RP11-395G23.3 suppresses the endometrial cancer progression via regulating microRNA-205-5p/PTEN axis

Weijuan Xin[1], Xiaodong Gao[2*], Shuting Zhao[2*], Peng Zhao[3], Hui Yu[4], Qianyu Wu[2], Keqin Hua[1]

[1]Department of Obstetrics and Gynecology, Obstetrics and Gynecology Hospital of Fudan University, 128 Shen-Yang Road, Shanghai 200090, China; [2]Department of Obstetrics and Gynecology, Shanghai East Hospital, Tongji University School of Medicine, 150 Jimo Road, Pudong New Area, Shanghai 200120, China; [3]Department of Internal Medicine, People's Hospital of Dezhou, 1751 Xinhu Street, Dezhou 253001, China; [4]Clinical Nursing Staff Room, Department of Medicine, Dezhou University, 566 West Collage Street, Dezhou 253023, China. [*]Equal contributors.

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Abstract: The focal point of this research was the functional role of RP11-395G23.3 in endometrial cancer (EC). The expression of RP11-395G23.3, microRNA (miRNA)-205-5p, and their target proteins were detected by quantitative real-time polymerase chain reaction and western-blot analyses. Flow cytometry and proliferation, Transwell, and wound healing assays were used to detect the effects of RP11-395G23.3 and miRNA-205-5p on tumor cell migration and proliferation in vitro. RP11-395G23.3 expression was negatively related to miRNA-205-5p, but positively related to phosphatase and tensin homolog (PTEN) expression in human EC tissues. We discovered that low RP11-395G23.3 expression was significantly related to advanced histological grade and lymphovascular space invasion in EC patients. In addition, overexpression of RP11-395G23.3 significantly inhibited the proliferation, invasion, migration, and induced apoptosis of Ishikawa and HEC-1A cells in vitro. Our results also showed that RP11-395G23.3 could directly bind to miRNA-205-5p through its miRNA response elements and eliminate the inhibitory effect of targeting gene PTEN, thus leading to the signaling pathway of phosphatidylinositol-3-kinase/AKT inactivation. We demonstrated for the first time that RP11-395G23.3 may inhibit the development and pathogenesis of EC by acting as a sponge for miRNA-205-5p and increasing PTEN expression. RP11-395G23.3 may be a target for the diagnosis and treatment of EC.

Keywords: lncRNA RP11-395G23.3, endometrial cancer, microRNA-205-5p, PTEN, ceRNAs

Introduction

Endometrial cancer (EC) is one of the most common female genital cancers, and its incidence is increasing [1, 2]. Although surgery, radiotherapy, chemotherapy, and hormone therapy can significantly prolong the survival of patients with early EC, distant metastasis and recurrence are still inevitable [3-6]. It is therefore urgent to identify and study new biomarkers to facilitate early EC diagnosis and timely interventions.

Long noncoding RNAs (lncRNAs) are approximately 200 nucleotides in length [7, 8]; they play critical roles in the development of cancer and may be used as prognostic markers and therapeutic targets [9, 10]. Some lncRNAs regulate cell growth, apoptosis, invasion, chromatin modification, and transcription [11-13]. As endogenous RNAs, lncRNAs play a part in regulating gene expression by competing for binding with microRNAs (miRNAs) [14, 15]. Our previous work demonstrated that lncRNA RP11-395G23.3, as a competitive endogenous RNA (ceRNA), participates in EC progression and is related to the miRNA-205-5p-phosphatase and tensin homolog (PTEN) network [16]. However, how the lncRNA RP11-395G23.3 and miRNA-205-5p-PTEN network participates in the occurrence and development of EC has not been elucidated.

miRNAs are noncoding single-stranded small RNAs with a length of approximately 22 nucleotides [17]. miRNAs participate in essential functions in various biological regulatory pathways including cell apoptosis, differentiation, and proliferation [18]. There are a variety of miRNA disorders in EC, such as significant upregulation of miRNA-205-5p, which is associated with advanced stage and myometrial invasion [19]. miRNA-205-5p is one of the miRNAs regulating different cell pathways for cell survival, apoptosis, angiogenesis, and metastasis [20]; miRNA-205-5p may affect the inactivated tumor suppressor PTEN in EC [21, 22]. Therefore, it is particularly important to study the mechanism of miRNA-205-5p.

PTEN is an inhibitory protein with phosphatase activity that negatively regulates the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway. PTEN can inhibit tumor growth by antagonizing the activity of phosphorylases such as tyrosine kinase and specifically dephosphorylating the third phosphate of inositol triphosphate [23, 24]. Loss of PTEN function leads to overactivation of the PI3K/AKT pathway, which may increase the proliferation, metastasis, and invasion of EC cells. PTEN gene mutations are common in EC and have been used as a disease marker [25, 26]. However, no studies have assessed whether miRNA-205-5p can target PTEN and the PI3K/AKT signaling pathway in EC.

Here, we show that lncRNA RP11-395G23.3 can be used as a ceRNA to modulate PTEN by acting on miRNA-205-5p in EC. Our findings have clinical and biological implications for the diagnosis and treatment of EC.

Materials and methods

Patient samples and cell culture

The study included 42 samples of EC, 15 of atypical proliferative endometrium (EAH), and 29 of normal endometrial tissue (NE) obtained from women who had undergone surgery at the Obstetrics and Gynecology Hospital of Fudan University between January, 2014 and February, 2016. None of the patients recruited in the present study had received chemotherapy, radiotherapy or hormone therapy prior to surgery. This research was approved by the Department of Obstetrics and Gynecology Hospital of Fudan University in China. All subjects provided written informed consent for sample collection and subsequent analyses. The research was done in accordance with the principles and guidelines expressed in the Helsinki Declaration. EC cell lines (HEC-1A and Ishikawa) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HEC-1A cells were cultured in McCoY's 5A medium (Thermo Fisher Scientific, Waltham, MA, USA), and Ishikawa cells were cultured in minimum Eagle's medium (Thermo Fisher Scientific). Culture conditions included a 37°C incubator, 10% fetal bovine serum (FBS), and 5% CO_2.

Luciferase reporter assay

The wild-type 3'-untranslated region (UTR) sequences of PTEN cDNA (pMIR-REPORT Luciferase-PTEN 3'UTR-wt) were synthesized into the luciferase reporter vector (Obio Technology, Shanghai, China). A mutant PTEN 3'-UTR vector (pMIR-REPORT Luciferase-PTEN 3'UTR-mut) that contained a mutation in the predicted miR-205-5p-binding sequence was also generated. The WT-PTEN or MUT-PTEN and the miRNA-205-5p mimic or miRNA-205-5p negative control (NC) were transfected into HEC-1A cells with Lipofectamine® 3000 reagent (Thermo Fisher Scientific). After 48 h of transfection, luciferase activity was measured with a Luciferase Reporter Assay System kit (Promega Corporation, Madison, Madison, USA).

Transfection

To overexpress miRNA-205-5p and lncRNA RP11-395G23.3, all vectors and mimics were transfected into Ishikawa and HEC-1A cells with Lipofectamine® 3000 Transfection Reagent. MirVana miRNA Mimic Negative Control #1 (Thermo Fisher Scientific) was used. The sequences were as follows: miR-205-5p mimic (5'-UCCUUCAUUCCACCGGAGUCUG-3'; 5'-GACUCCGGUGGAAUGAAGGAUU-3') and mimic NC (5'-UUCUCCGAACGUGUCACGUTT-3'; 5'-ACGUGACACGUUCGGAGAATT-3'). The lncRNA overexpression plasmid was pLenti-EF1a-EGFP-F2A-Puro-CMV-RP11-395G23.3 and the empty vector was used as a control (control group). The blank group was only treated with Lipofectamine® 3000 Transfection Reagent. The working concentration of miRNA mimics and NC were 50 nM. The concentration used for plasmids was 100 nM.

RNA and miRNA expression analysis

Total RNA was extracted from cells with TRIzol. Reverse transcription was performed with a PrimeScriptTMRT kit (TaKaRa, Beijing, China). SYBR Premix Ex TaqTM (TaKaRa) was used for quantitative real-time polymerase chain reaction (qRT-PCR). Glyceraldehyde 3-phosphate dehydrogenase was used as the internal reference RNA for lncRNA and mRNA expression analysis, while snRNA U6 was used for miRNA. All qRT-PCR reactions were performed in a PCR System 7500 (ABI, USA). The relative expression of lncRNA or miRNA were detected after normalizing to the internal control using the $2^{-\Delta\Delta Ct}$ method. The primers were designed using a primer designing software package (RP11-395G23.3 forward: TCCATCTCCAAGGCGTCACTCC; reverse: AGTCCGCAGGCAACAATCACAG).

Western blot analysis

Ishikawa and HEC-1A cells were lysed with the mixture of radioimmunoprecipitation buffer (KeyGEN, China), 1 nM benzenesulfonyl fluoride, and protease and phosphatase inhibitors. The concentrations of protein samples were determined with BCA protein kits (KeyGEN). Proteins were denatured and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore, USA). The following antibodies were used overnight at 4°C: pro-caspase-3 (1:1000, Abcam, UK; cat. no. ab32351), PTEN (1:1000, Abcam; cat. no. ab32199), phosphoinositide-dependent kinase-1 (PDK1; 1:1000, Abcam; cat. no. ab110025), AKT (1:1000, Cell Signaling Technology, USA; cat. no. #2920), Phospho-Akt (1:1000, Cell Signaling Technology; cat. no. #4060), and β-actin (1:1000, Proteintech Group, USA; cat. no. 66009-1-Ig). Membranes were cultured with secondary antibodies (Rockland, USA) at room temperature for 2 h. An enhanced chemiluminescence system (Bio-Rad, USA) was used for signal detection.

Cell proliferation and chemosensitivity assay

Cell proliferation was analyzed using a Cell Counting Kit-8 assay (CCK-8, Dojindo, Japan). Cells were seeded in 96-well plates (2,000 cells/well) following 24 h of transfection. After 24, 48, and 72 h incubation, the cells were cultured with CCK-8 solution at 37°C for 2 h. Cell viability was measured by absorbance at 450 nm using a spectrophotometer.

Cell apoptosis assay

Ishikawa and HEC-1A cells (1×10^6) were plated in 6-well plates and treated with cisplatin (10 μM/ml) for 24 h prior to harvesting. Cold phosphate-buffered saline-washed cells were resuspended in 1× binding buffer and stained with Annexin V-FITC and 7AAD viability dye (Annexin V-FITC/7AAD kit, BD Biosciences, USA). Cells were cultured in darkness at room temperature for 30 min and analyzed by flow cytometry.

Invasion assay

Culture inserts of 8-mm pore size (Transwell; Costar, USA) were seeded into 24-well culture plates for invasion assays. The upper surface of the membrane at the bottom of the Transwell chamber was coated with Matrigel (100 μg/well; BD Biosciences). In the bottom chamber, 500 μl medium containing 10% FBS was added, and 1×10^6 cells (excluding FBS) were inoculated into the top chamber. After 24 h, the cells that had invaded the pore were fixed with methanol and stained with 0.05% crystal purple. The invading cells were then quantitatively analyzed under a microscope (Olympus, Tokyo, Japan).

Wound-healing assay

Ishikawa and HEC-1A cells (1×10^6 cells/well) were incubated in 24-well plates. The cell monolayer was then wounded with a 100-μl pipette tip and cultured in serum-free medium. Cell invasion images were captured at 0 and 24 h.

Statistical analysis

Statistical analysis was carried out using SPSS 19.0 software and GraphPad Prism software 7.0. Comparisons of quantitative data were done with one-way analyses of variance and Student's t tests. Values are expressed as mean ± standard deviation (SD). Qualitative data are expressed by rate and were compared with chi-square tests. Spearman rank correlation analyses were used to calculate correlations, and R = 0 was used as the relevant standard. Differences were considered significant at $P<0.05$.

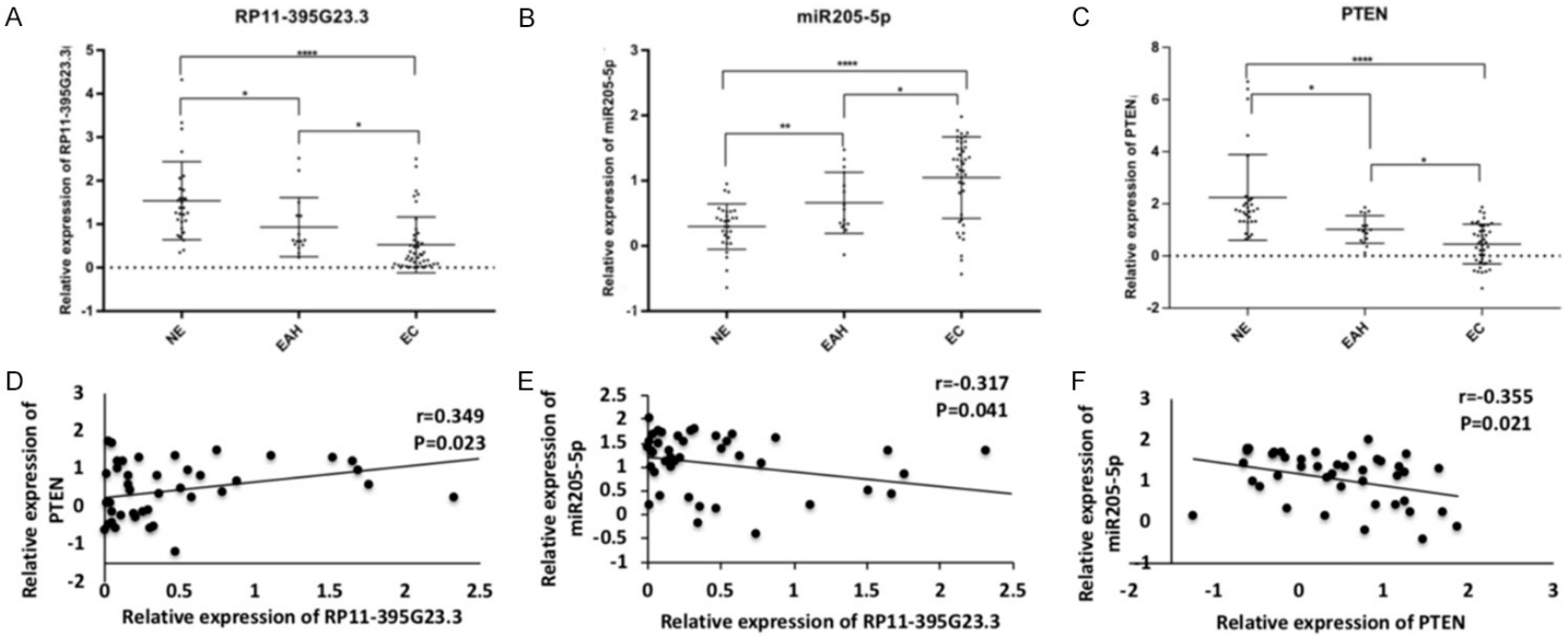

Figure 1. RP11-395G23.3, PTEN and microRNA-205-5p are aberrantly expressed in EC, EAH and NE. A-C. Expression levels of RP11-395G23.3, PTEN and microRNA-205-5p in EC, EAH and NE tissues examined by RT-qPCR. D-F. Correlation between RP11-395G23.3, microRNA-205-5p and PTEN in EC tissues. *P<0.05, **P<0.01, ****P<0.0001.

Table 1. Correlation of RP11-395G23.3 expression with clinicopathological characteristics of EC patients

Variables	Cases (N)	RP11-395G23.3		
		Low (n%)	High (n%)	P
Age (years)				0.686
<50	8	6 (14.3)	2 (4.8)	
≥50	34	23 (54.7)	11 (26.2)	
Histological subtype				0.06
Endometrioid	33	25 (59.5)	8 (19.1)	
Serous	7	4 (9.5)	3 (7.1)	
Clear cell	2	0 (0)	2 (4.8)	
Menstruation				0.637
Premenopausal	14	9 (21.4)	5 (11.9)	
Menopausal	28	20 (47.6)	8 (19.1)	
FIGO stage				0.101
I-II	32	20 (47.6)	12 (28.6)	
III-IV	10	9 (21.4)	1 (2.4)	
Histological grade				0.007*
G1	17	9 (27.3)	8 (24.2)	
G2	8	8 (24.2)	0 (0)	
G3	8	8 (24.2)	0 (0)	
Myometrial invasion				0.513
<1/2	26	17 (40.5)	9 (21.4)	
≥1/2	16	12 (28.6)	4 (9.5)	
Lymphovascular space invasion				0.045*
Present	12	11 (26.2)	1 (2.4)	
Absent	30	18 (42.8)	12 (28.6)	
Lymph node metastasis				0.146
Present	9	8 (19)	1 (2.4)	
Absent	33	21 (50)	12 (28.6)	

*P<0.05.

Results

RP11-395G23.3, miRNA-205-5p, and PTEN expression in the endometrium

The expression of RP11-395G23.3, PTEN, and miRNA-205-5p were detected by qRT-PCR in 42 EC, 15 EAH, and 29 NE samples. The relative expression of RP11-395G23.3 and PTEN in EC were dramatically decreased compared with EAH and NE (both P<0.05). The relative expression of RP11-395G23.3 and PTEN in the EAH group also decreased compared with NE (P<0.05) (**Figure 1A**, **1C**). As in our previous studies, relative miRNA-205-5p expression was markedly higher than that of EAH and NE (both P<0.05). The relative expression of miRNA-205-5p in the EAH group was significantly increased compared with NE (P<0.05, **Figure 1B**).

Expression and clinical significance of RP11-395G23.3 in human EC tissues

As the relative expression of RP11-395G23.3 in human EC tissues was dramatically downregulated compared to NE tissues, there could have some clinical significance. Next, we analyzed the clinicopathological significance of RP11-395G23.3. Using the mean as the cutoff value, RP11-395G23.3 expression in EC tissues was defined as high (≥mean) or low (<mean). As shown in **Table 1**, low RP11-395G23.3 expression was associated with advanced histological grade and lymphovascular space invasion (both P<0.05). The results indicate that low RP11-395G23.3 expression was related to the phenotype of invasive tumors, especially metastatic potential (**Table 1**).

Correlation between RP11-395G23.3, miRNA-205-5p, and PTEN expression in the endometrium

Correlation analyses revealed that RP11-395G23.3 expression was negatively related to miRNA-205-5p expression, but positively related to PTEN expression in human EC tissues, suggesting a possible interaction between RP11-395G23.3 and miRNA-205-5p in the EC context (**Figure 1D**, **1E**). Furthermore, PTEN was negatively correlated with miRNA-205-5p in human EC tissues (**Figure 1F**).

RP11-395G23.3, miRNA-205-5p, and PTEN interactions in EC cells

Our previous research showed that miRNA-205-5p targets RP11-395G23.3 with one conserved target site in EC. RP11-395G23.3 may inhibit both miRNA-205-5p expression and activity via this putative binding site at the post-

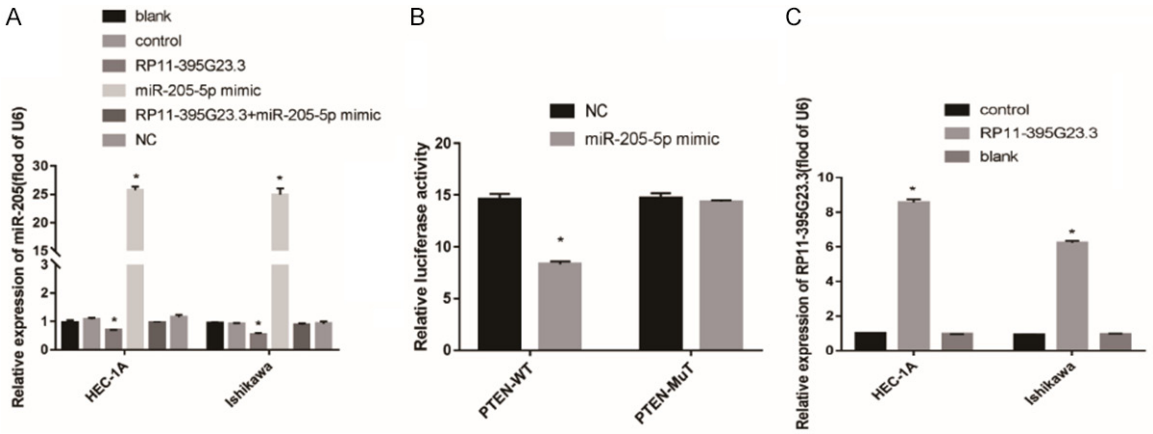

Figure 2. A. Relative expression levels of microRNA-205-5p in HEC-1A and Ishikawa cells, transfected with control, RP11-395G23.3, microRNA-205-5p mimic, and RP11-395G23.3+microRNA-205-5p mimic. B. Relative luciferase activity in HEC-1A cells transfected with microRNA-205-5p mimic and co-transfected with PTEN-WT or PTEN-MuT. C. Relative expression levels of RP11-395G23.3 in HEC-1A and Ishikawa cells, transfected with control, RP11-395G23.3 and blank were measured by RT-qPCR and normalized to U6. *P<0.05.

transcriptional level in both normal and cancer tissues [16]. An RP11-395G23.3 overexpression group was constructed in EC cell lines (**Figure 2C**). Compared with the control group, relative miRNA-205-5p expression was dramatically decreased (P<0.05). However, no significant changes were detected with overexpression of RP11-395G23.3 and miR-205-5p (**Figure 2A**). This confirmed that miRNA-205-5p was competitively inhibited by RP11-395G23.3 in EC cell lines. PTEN is a direct target of miRNA-205-5p (**Figure 2B**), as reported in previous studies [21]. Thus, the RP11-395G23.3-miRNA-205-5p-PTEN network was successfully identified in EC cells. In addition, we established an overexpression concentration gradient of RP11-395G23.3 that showed the optimum concentration of RP11-395G23.3 (1 mg/ml in EC cells) promoted the expression of PTEN (**Figure 3**).

RP11-395G23.3 affected the PTEN/PI3K/AKT pathway by antagonistic miRNA-205-5p in EC cells

To investigate the RP11-395g23.3 signaling pathway in EC, we analyzed PTEN expression and PI3K/AKT signaling in EC cell lines. The results revealed that the expression of PTEN was upregulated, while p-AKT/AKT, PDK1, and caspase-3 were downregulated when RP11-395G23.3 was overexpressed. Conversely, miRNA-205-5p overexpression resulted in a reduction of PTEN and increases of p-AKT/AKT, PDK1, and caspase-3 levels in EC cell lines. However, there was a significant difference compared with control when RP11-395G23.3 and miRNA-205-5p were co-overexpressed in EC cell lines (**Figure 4**). Taken together, the results indicate that RP11-395G23.3 may interfere with miRNA-205-5p-mediated inhibition of PTEN and play an important part in EC pathogenesis.

RP11-395G23.3 downregulated the viability, migration, and invasion of EC cells through the RP11-395G23.3 miRNA-205-5p/PTEN network

The regulatory effects of the RP11-395G23.3/miRNA-205-5p/PTEN network on the viability, invasion, and migration of EC cells was further determined. CCK-8 assays were performed to assess the proliferation of transfected EC cells at different times. Transwell and wound-healing assays were used to evaluate EC cell mobility. RP11-395G23.3 overexpression significantly decreased the viability, invasion, and migration of EC cells, while these parameters were markedly increased with miRNA-205-5p overexpression. However, there was no statistically significant difference compared with control when RP11-395G23.3 and miRNA-205-5p were co-overexpressed in EC cell lines (**Figure 5A-C**). This suggests that RP11-395G23.3 mediated the effects of miRNA-205-5p on EC cell viability, invasion, and migration.

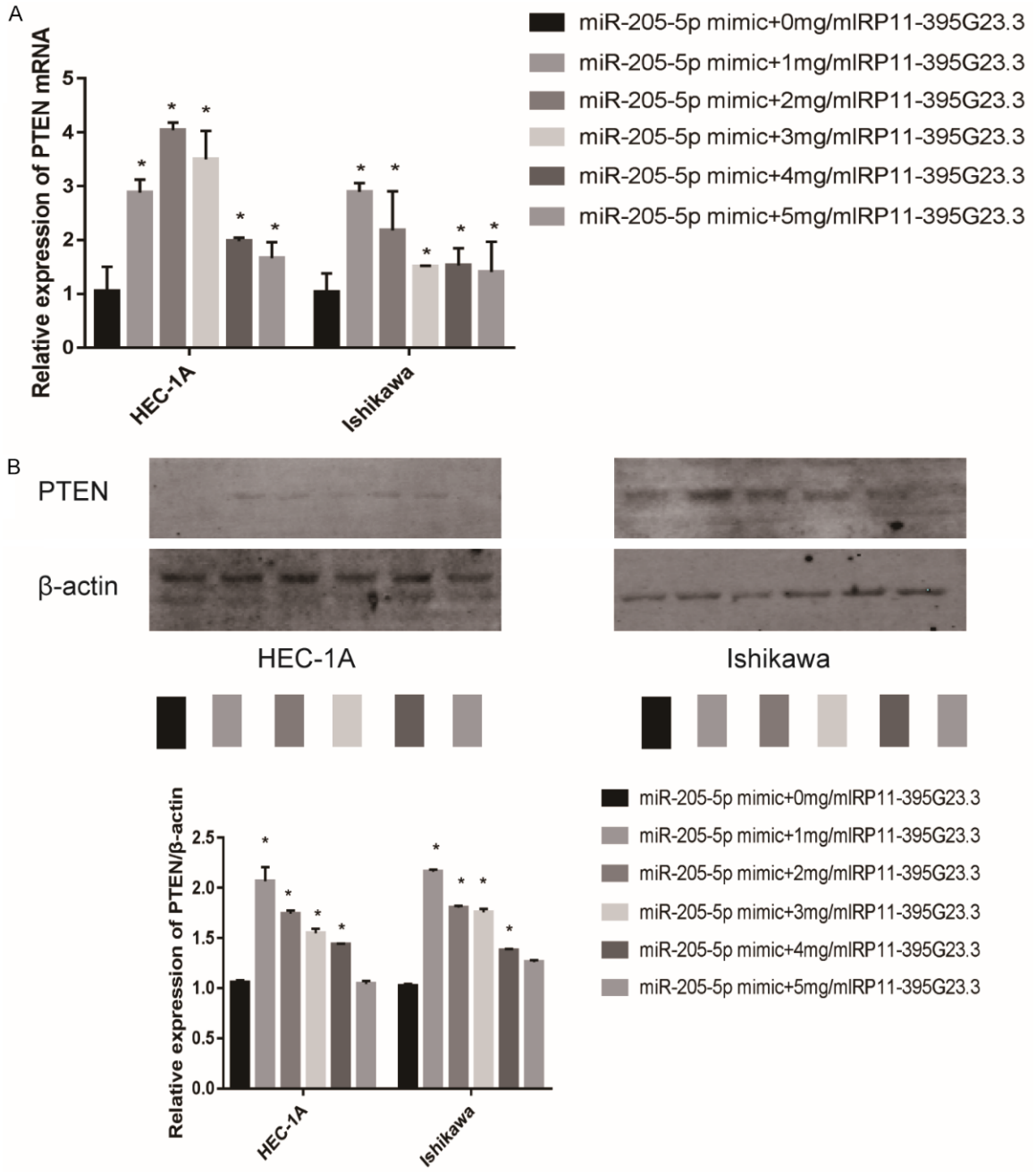

Figure 3. Regulation of PTEN by overexpression of RP11-395G23.3 and microRNA-205-5p in HEC-1A and Ishikawa cells. A. mRNA expression of PTEN examined by RT-qPCR; B. Protein expression of PTEN examined by western blot analysis. *P<0.05 vs. control.

RP11-395G23.3 induced EC cell apoptosis through the RP11-395G23.3/miRNA-205-5p/PTEN network

As RP11-395G23.3 downregulated the viability, migration, and invasion of EC cells, flow cytometry was used to detect apoptosis in both cell lines. The effect of RP11-395G23.3 overexpression on apoptosis was also determined. The results showed that overexpression of RP11-395G23.3 significantly induced EC cell apoptosis compared with control. miRNA-205-5p overexpression markedly inhibited apoptosis. There was no difference compared with the control when RP11-395G23.3 and miRNA-205-5p were co-overexpressed in HEC-

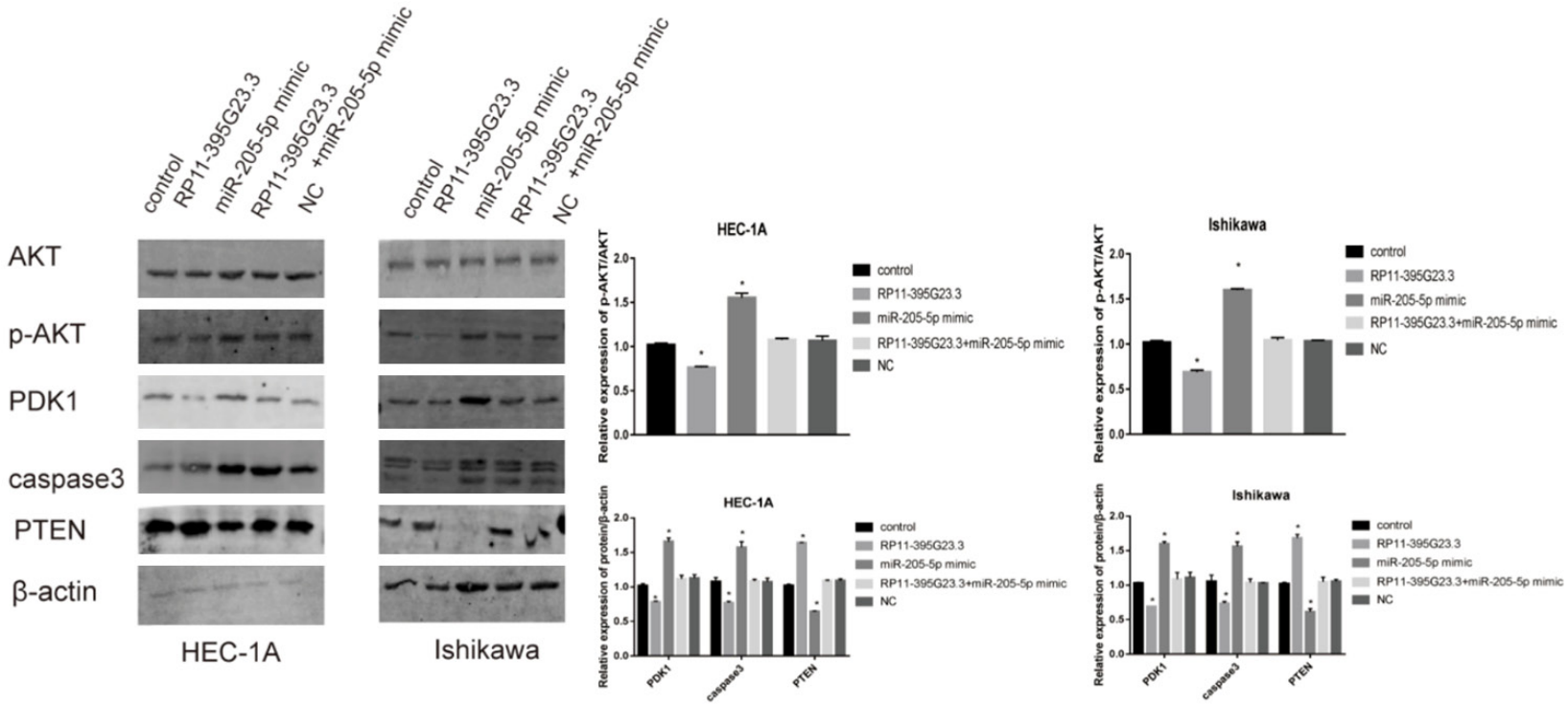

Figure 4. Effect of RP11-395G23.3 on PTEN and PI3K/Akt signaling pathway in HEC-1A and Ishikawa cells. The protein levels of AKT, p-AKT, PDK1, caspase 3 and PTEN were evaluated by Western blot. β-actin served as the loading control. *P<0.05.

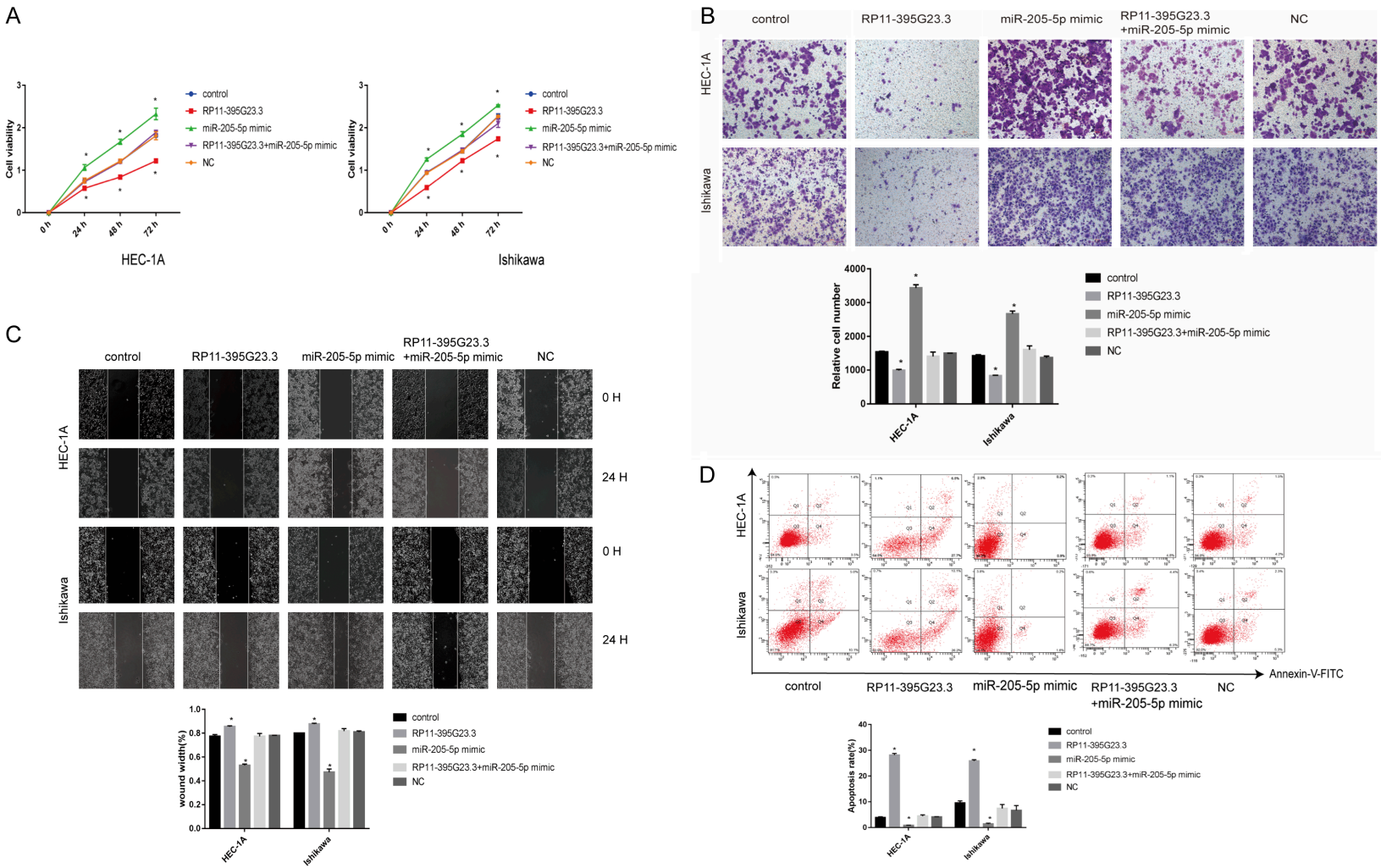

Figure 5. A. Role of RP11-395G23.3 and microRNA-205-5p in cell proliferation. B. Functions of RP11-395G23.3 and microRNA-205-5p in cell invasion. Scale bar = 100 μm. C. Functions of RP11-395G23.3 and microRNA-205-5p in cell migration. Scale bar = 100 μm. D. Functions of RP11-395G23.3 and microRNA-205-5p in cell apoptosis. HEC-1A and Ishikawa cells were transfected with control, RP11-395G23.3, microRNA-205-5p mimic, RP11-395G23.3+microRNA-205-5p mimic and NC groups. *P<0.05.

1A and Ishikawa cell lines (**Figure 5D**). These findings indicated that RP11-395G23.3 dysregulation may contribute to EC development and exert a tumor suppressor function.

Discussion

EC development and progression occur over continuous and complex steps involving the abnormal expression of several genes [27, 28]. Some lncRNAs function as oncogenes or tumor suppressor genes in EC [29]. It is therefore urgent to explore new lncRNAs to develop diagnostic markers and therapeutic targets. In this study, we investigated the effect of RP11-395G23.3 in EC development.

lncRNAs play important roles in cancer development and progression, but most have not been studied in detail in terms of their functions and mechanisms. RP11-395G23.3, a new type of lncRNA, has been implicated in the development of EC as a ceRNA [16]. However, the exact mechanism remains still unclear. We conducted an in-depth study on the role of RP11-395G23.3 in EC. To our knowledge, this is the first evidence that RP11-395G23.3 is downregulated in EC samples. In addition, low RP11-395G23.3 expression was associated with advanced histological grade and lymphovascular space invasion. Functional assays demonstrated that upregulation of RP11-395G23.3 significantly inhibited the proliferation, invasion, migration, and induced apoptosis of EC cells in vitro. The results demonstrate that RP11-395G23.3 functions as a tumor suppressor in EC progression.

lncRNAs, as ceRNAs, regulate tumor progression through small spongy RNAs [30-32]. Our previous studies revealed that that RP11-395G23.3, as a competitive endogenous gene, is associated with the miRNA-205-5p-PTEN network in EC [16]. Here, we showed that RP11-395G23.3 acted as a sponge to bind miRNA-205-5p, and the expression of miRNA-205-5p and RP11-395G23.3 were negatively correlated in EC cells. RP11-395G23.3 was further confirmed to be a miRNA-205-5p sponge in EC cells.

Previous studies have demonstrated that miRNA-205-5p can play a tumor-promoting role in EC [33-35]. In addition, the PTEN/PI3K/AKT pathway can be regulated to inhibit tumor growth [36]. As an antagonist regulator of the PI3K/AKT pathway, PTEN is involved in EC occurrence and progression. Interestingly, we found a negative correlation between miRNA-205-5p and PTEN in EC samples. RP11-395G23.3 activates PTEN expression, but inactivates the PI3K/AKT pathway, while RP11-395G23.3 may affect the PTEN/PI3K/AKT pathway via antagonism of miRNA-205-5p. These results suggest that RP11-395G23.3 can bind miRNA-205-5p to promote PTEN activation, blocking PI3K/AKT signaling, and inhibiting EC growth.

Conclusion

We demonstrated that RP11-395G23.3 is downregulated in EC and can regulate miRNA-205-5p through its function as a ceRNA, thereby inhibiting PTEN and activating PI3K/AKT signaling, thus contributing to EC pathogenesis and progression. Collectively, our results suggest that RP11-395G23.3 plays an inhibitory role in the development and progression of EC. The lncRNA RP11-395G23.3/miRNA-205-5p/PTEN network may be a new clinical marker and therapeutic target for EC.

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Disclosure of conflict of interest

None.

Address correspondence to: Keqin Hua, Department of Obstetrics and Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200090, China. E-mail: huakeqinjiaoshou1@126.com; Qianyu Wu, Department of Obstetrics and Gynecology, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200120, China. E-mail: 5185874@qq.com

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