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ORIGINAL ARTICLE

Effects of GATA6-AS/MMP9 on malignant progression of endometrial carcinoma

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Summary

Purpose: *Previous studies have shown that long non-coding RNA (lncRNA) GATA6-AS is a tumor suppressor gene. However, the role of GATA6-AS in endometrial cancer (EC) has not been reported. We aimed at investigating the expression characteristics of GATA6-AS in EC tissues and cell lines, and explored whether it inhibits the malignant progression of EC through modulating matrix metalloproteinase-9 (MMP9).*

Methods: *GATA6-AS expression in 17 pairs of EC tissues and adjacent ones was studied by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Meanwhile, GATA6-AS expression levels in EC cell lines were also evaluated by qRT-PCR assay. In addition, GATA6-AS overexpression model was constructed using lentivirus in EC cell lines KLE and HEC-1B. The impacts of GATA6-AS overexpression model was constructed using lentivirus in EC cell lines KLE and HEC-1B on the proliferation capacity and apoptosis of EC cells were assessed by cell counting kit-8 (CCK-8), 5-Ethynyl-2'- deoxyuridine (EdU), and flow cytometry experiments. Furthermore, we explored the interaction between GATA6-AS and MMP9 in EC cells via performing luciferase assay and cell reverse experiments.*

Results: *Our data showed that GATA6-AS expression in EC tissue specimens was remarkably lower than that in adjacent ones. In vitro cell experiments revealed that overexpression of GATA6-AS markedly attenuated the proliferation ability of EC cells while elevated their apoptosis. Meanwhile, luciferase assay confirmed the binding relationship between GATA6-AS and MMP9. In addition, cell reverse experiments further demonstrated the mutual regulation between GATA6-AS and MMP9, which was, overexpression of MMP9 reversed the inhibitory influence of upregulation of GATA6-AS on the malignant progression of EC.*

Conclusions: *lncRNA GATA6-AS, lowly expressed in EC tissue samples. Additionally, lncRNA GATA6-AS may suppress the malignant progression of EC through the modulation of regulating MMP9.*

Key words: *lncRNA GATA6-AS, MMP9, endometrial cancer, malignant progression*

Introduction

It has been reported that approximately 320,000 women are diagnosed with endometrial cancer every year, and 76,000 women die of EC-related diseases every year [1-3]. Endometrial carcinoma (EC) is regarded as one of the three most common malignant tumors of female genital tract. In western developed countries such as Europe and the United States, EC has ranked the first place among malignant tumors of female genital tract [4,5]. At present, although most of the patients are detected, diagnosed and treated in the early stage due to symptoms such as census or abnormal vaginal bleeding,

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some of the patients are still in the advanced stage at the time of treatment, which leads to a high recurrence rate and a poor prognosis [6-8]. Therefore, it is crucial to explore the pathogenesis of EC and find effective therapeutic targets to improve the prognosis of patients with EC, prolong their life length and improve their quality of life [9,10].

In recent years, studies have found that long non-coding RNA (lncRNA) plays a pivotal role in the metastasis and prognosis of tumors, and new advances and breakthroughs have been made in the study of its relationship with EC [11,12]. LncRNA, a class of RNA molecules with a length of more than 200 bases and no protein-coding function, has become a research hotspot in recent years [13,14]. In recent years, a great number of studies have found that in mammalian genomes, lncRNAs are transcribed from thousands of gene loci and processed into mature lncRNA molecules, which have the function of regulating animal embryonic development, gene expression and tumor progression [14]. Therefore, in-depth research on EC-related lncRNAs is of great significance for clarifying the pathogenesis of EC and exploring new diagnostic and therapeutic targets for EC [15,16]. Previous studies have suggested that lncRNA GATA6-AS is a tumor suppressor gene, however, the role of GATA6-AS in EC has not been reported [17-19].

In this study, we analyzed the effect of lncRNA GATA6-AS on the function of EC cells, and uncovered that lncRNA GATA6-AS can directly interact with MMP9 and thus regulate the malignant progression of EC.

Methods

Patients and EC samples

Specimens of tumor tissues and adjacent tissues from 17 EC patients were collected from our hospital. All specimens were located more than 5 cm away from the cancerous tissues. All subjects did not receive antitumor treatment such as radiotherapy or chemotherapy before surgery. This study was approved by the Ethics Committee of Lianyungang Hospital affiliated to Xuzhou Medical University, and all patients signed the informed consent form. All patients were followed up after discharge for general conditions, clinical symptoms and imaging examinations.

Cell lines and reagents

Human EC cell lines (HEC-1A, HEC-1B, KLE, Ishikawa) and human endometrial mesenchymal cell line (T-HESC) provided by American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in dulbecco's modified eagle medium (DMEM) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in an incubator at 37°C with 5% CO_2.

Transfection

Lentivirus transfection was performed with lentiviral vector containing lncRNA GATA6-AS overexpression sequence (GenePharma, Shanghai, China) according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8) assay

Cells were plated in 96-well plates (2×10^3 cells/well) in 100 ul culture medium. CCK-8 assay (Dojindo, Kumamoto, Japan) was performed based on the manufacturer's protocol.

5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay

After transfected for 24 hours, EDU test (RiboBio, Nanjing, China) was carried out according to the manufacturer's requirements.

Flow cytometry analysis

The combination of Annexin V-FITC (Merck, Billerica, MA, USA) and PI was used for flow cytometry analysis according to the manufacturer's instructions. The samples were stored on ice and protected from light, and immediately analyzed by flow cytometry (BD Company, Franklin Lakes, NJ, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) method was used to extract total RNA from tissue samples. RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent. QRT-PCR reaction was carried out using SYBR® Premix Ex Taq ™ (TaKaRa, Tokyo, Japan) kit. The following primers used are shown: lncRNA GATA6-AS: forward: 5'-ATGCGCTTTTTGCCCTGAAG-3', reverse: 5'-AGGTCAGCTGGGGAATGTTG-3'; matrix metalloproteinase-9 (MMP9): forward: 5'-TGTACCGCTATGGTTACACTCG-3', reverse: 5'-GGCAGGGACAGTTGCTTCT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western blot

Cells were lysed, shaken on ice for 30 minutes, and centrifuged at 4°C, 14000 × g for 15 minutes. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). The extracted proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was carried out based on standard procedures.

Dual-luciferase assay

Cells were seeded in 24-well plates and co-transfected with MMP9/NC and pMIR luciferase reporter plasmids. The plasmids were then introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the reporter luciferase activity of each group was normalized to the control firefly luciferase activity (Promega, Madison, WI, USA).

Statistics

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA). Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). P <0.05 was considered statistically significant.

Results

lncRNA GATA6-AS was lowly expressed in EC

QRT-PCR detected the expression of lncRNA GATA6-AS in 17 pairs of EC tumor tissue samples and their adjacent ones. Figure 1A shows that EC tumor samples contained a significant reduced expression of CLIC1 (Figure 1A). Meanwhile, in comparison to T-HESC, GATA6-AS was markedly lowly expressed in EC cell lines (Figure 1B).

lncRNA GATA6-AS inhibited cell proliferation and promoted the apoptosis of EC cell lines

To clarify the influence of GATA6-AS on the proliferation and apoptosis of EC cells, we constructed GATA6-AS overexpression models in EC cell lines KLE and HEC-1B and verified the transfection efficiency by qRT-PCR (Figure 2A and 2B). Subsequently, the results of CCK-8 test(Figure 2C and 2D), EDU experiments(Figure 2E and 2F) and

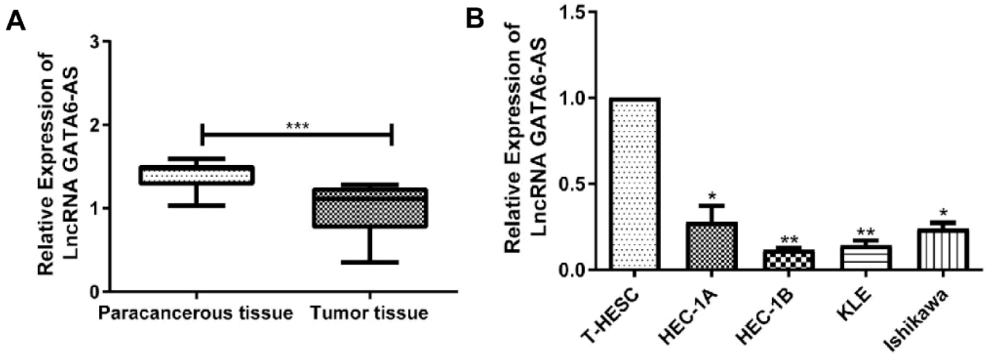

Figure 1. LncRNA GATA6-AS is underexpressed in endometrial cancer tissues and cell lines. **A:** qRT-PCR detected the expression of GATA6-AS in tumor tissues and adjacent tissues of endometrial cancer. **B:** qRT-PCR detected the expression of GATA6-AS in endometrial cancer cell lines. Data are average ± SD, *p<0.05, **p<0.01, ***p<0.001.

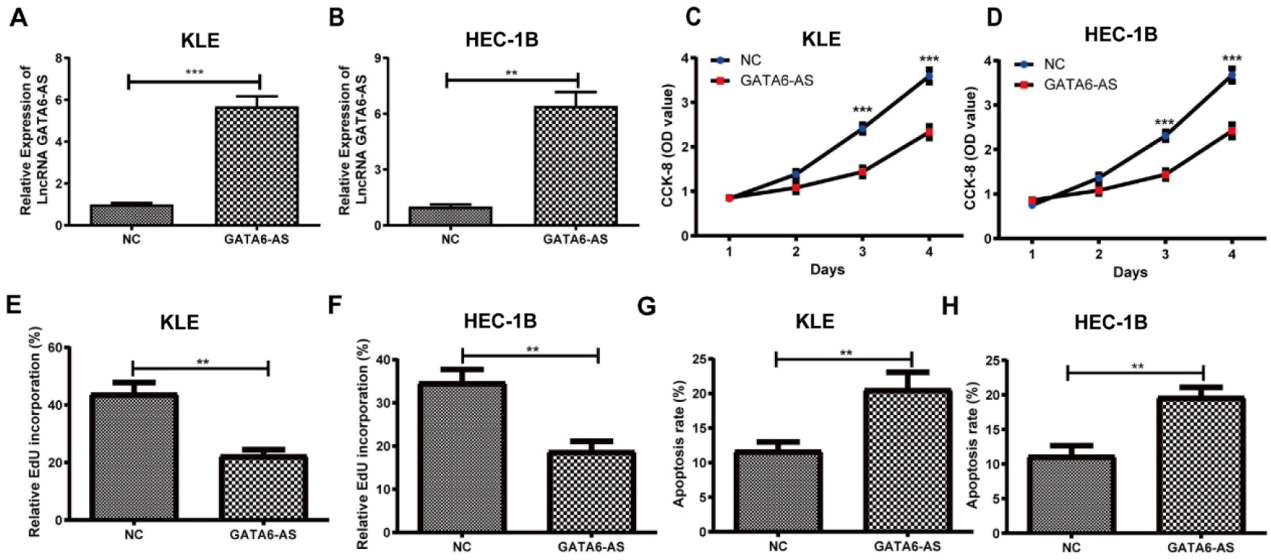

Figure 2. LncRNA GATA6-AS can inhibit the proliferation and promote apoptosis of endometrial cancer cells. **A and B:** qRT-PCR detected the transfection efficiency of GATA6-AS overexpression vector in KLE and HEC-1B endometrial cancer cell lines. **C and D:** CCK-8 detected cell proliferation after transfection with GATA6-AS overexpression vector in endometrial cancer cell lines. **E and F:** EDU test detected the ability of endometrial cancer cells to proliferate after transfection of GATA6-AS overexpression vector in KLE and HEC-1B endometrial cancer cell lines. **G and H:** Flow cytometry was performed for detection of cell apoptosis after transfection of GATA6-AS overexpression vector in KLE and HEC-1B endometrial cancer cell lines. Data are average ± SD, **p<0.01, ***p<0.001.

flow cytometry(Figure 2G and 2H) analysis revealed that overexpression of GATA6-AS markedly attenuated the cell proliferation capacity while enhanced apoptosis of EC cells.

LncRNA GATA6-AS directly binds to MMP9

Bioinformatics website predicted that there exists three potential target genes of GATA6-AS (Figure 3A). Hence, qRT-PCR was carried out to measure the expression of the three potential genes after transfection of GATA6-AS overexpression vector in EC cells, of which MMP9 expression was most remarkably reduced (Figure 3B). Luciferase assay then confirmed that GATA6-AS can combine with MMP9 through a specific binding site (Figure 3C). In addition, *in vitro* cell experiments revealed a significant decrease in MMP9 protein expression in EC cell lines after overexpression of GATA6-AS, measured by Western blotting assay (Figure 3D).

MMP9 modulated lncRNA GATA6-AS in EC

To further clarify by which GATA6-AS and MMP9 regulate the malignant progression of EC cells, we simultaneously overexpressing this two proteins in KLE and HEC-1B cell lines. As a result, in comparison to single transfection of GATA6-AS over-expression vector, co-transfection of MMP9 and GATA6-AS overexpression vectors enhanced the mRNA expression of MMP9 more significantly (Figure 4A). Meanwhile, it was found that this co-transfection reversed the role of upregulation of GATA6-AS alone in suppressing cell proliferation but promoting apoptosis of EC cells (Figures 4B, 4C, 4D).

Discussion

Current studies have confirmed that the progression of EC may be a chronic and persistent process caused by intracellular and epigenetic chang-

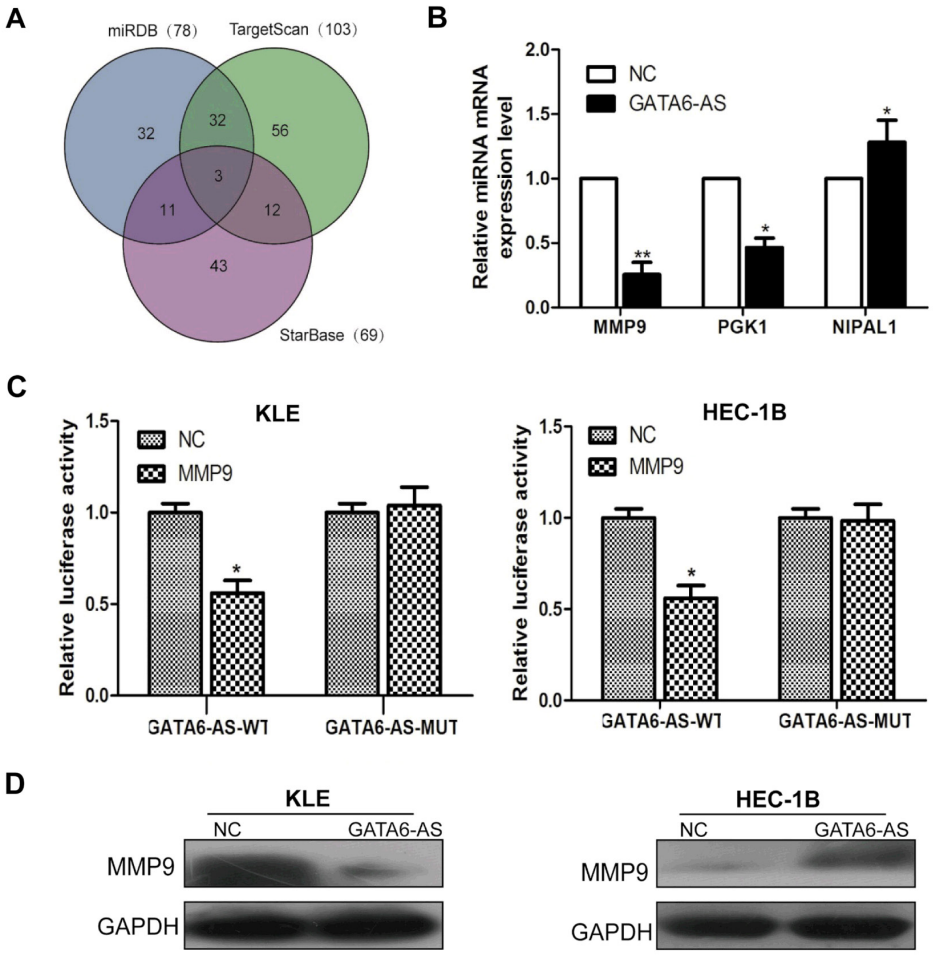

Figure 3. LncRNA GATA6-AS can bind to MMP9. **A:** Bioinformatics analysis predicted the target genes that LncRNA GATA6-AS may bind. **B:** qRT-PCR detected the expression levels of three potential target genes after over-expressing LncRNA GATA6-AS in endometrial cancer cell lines. **C:** The results of the dual luciferase reporter gene experiment show that LncRNA GATA6-AS can be targeted by MMP9 through specific binding sites. **D:** Western blotting detected the protein expression of MMP9 after overexpression of LncRNA GATA6-AS in endometrial cancer cell lines KLE and HEC-1B. Data are average ± SD, *p<0.05, **p<0.01.

es, activation of oncogenes, inactivation of tumor suppressor genes, changed in hormones and their receptors [4-6]. However, the specific pathogenesis of EC still remains elusive. So far, no breakthrough has been made in the early diagnosis and recurrence prediction of EC, nor is there any effective targeted therapy for EC. Hence, the research on the differential expression and important role of lncRNAs in various tumor tissues may open up some new directions for cancer therapy [9-12].

LncRNA can affect the apoptotic signaling pathway, tumor invasion and metastasis, and thus play a crucial part in tumor progression [13-15]. The long sequence and diverse spatial structure

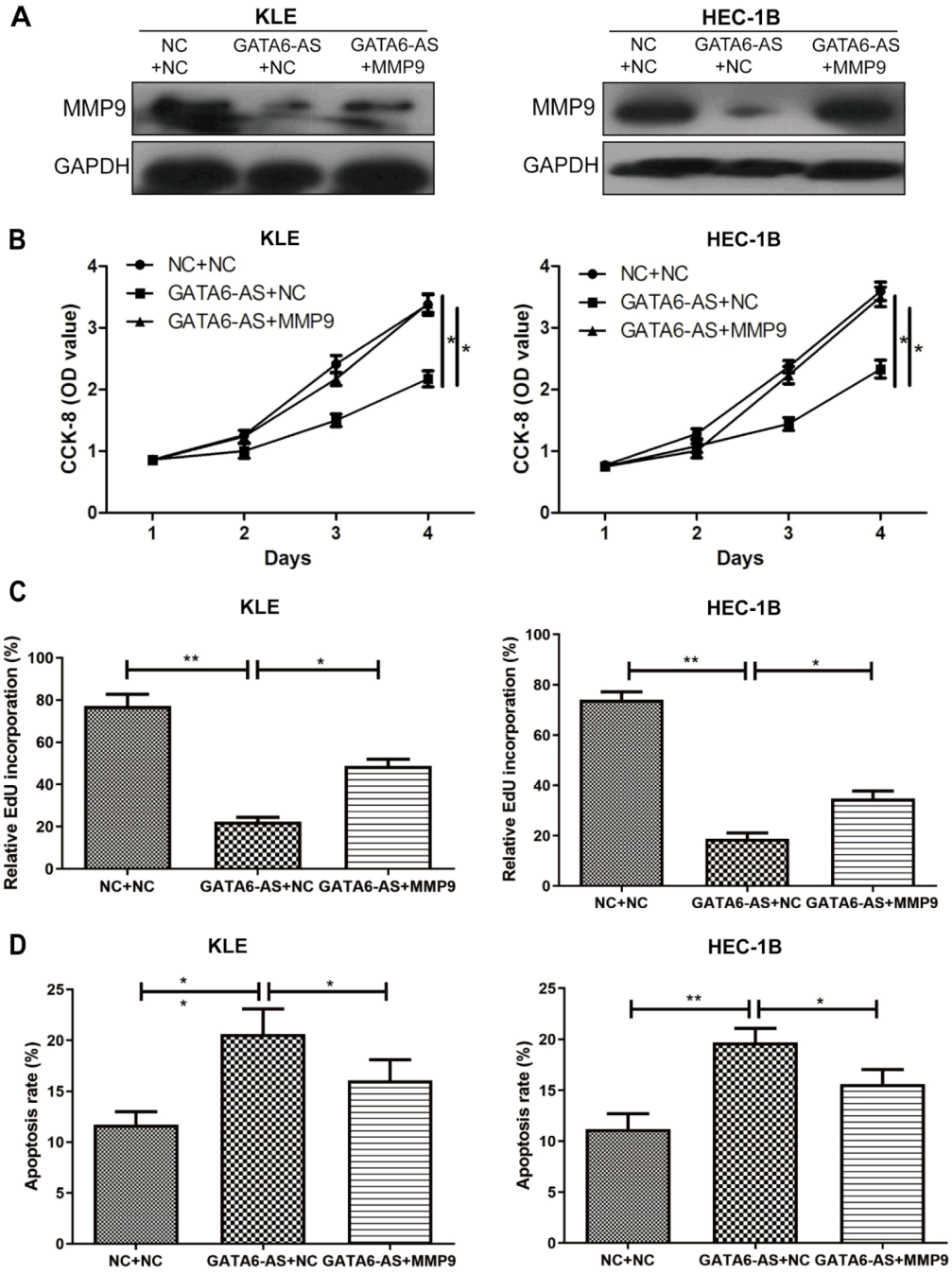

Figure 4. LncRNA GATA6-AS regulates the expression of MMP9 in endometrial cancer cell lines. **A:** Western blotting detected the protein expression of MMP9 in KLE and HEC-1B endometrial cancer cell lines after co-transfection of GATA6-AS and MMP9 overexpression vectors. **B:** CCK-8 assay detected cell proliferation after co-transfection of GATA6-AS and MMP9 overexpression vector in KLE and HEC-1B endometrial cancer cell lines. **C:** EDU test detected the cell proliferation ability after co-transfection of GATA6-AS and MMP9 overexpression vector in KLE and HEC-1B endometrial cancer cell lines. **D:** Flow cytometry assay detected apoptosis of endometrial cancer cells after co-transfection of GATA6-AS and MMP9 in KLE and HEC-1B endometrial cancer cell line. Data are average ± SD, * $p<0.05$, ** $p<0.01$.

result in a complicated function of LncRNA, which mainly regulates gene expression from three levels, namely epigenetic, transcriptional and post-transcriptional level. Epigenetics mainly include DNA methylation and demethylation, RNA interference, histone modification, chromosome remodeling, etc. It does not affect DNA sequence, so changes in gene function are reversible and heritable [13,15]. Meanwhile, lncRNA can regulate gene expression by inducing chromosomal remodeling by affecting the expression of neighboring genes, and can also bind to basic transcription factors to inactivate promoters and activate helper proteins to inactivate target genes [14-16]. It may also serve as a cofactor to modulate the activity of transcription factors, thereby affecting gene transcription. In addition, it can act as a small RNA precursor to target to form a double-stranded RNA complex with mRNA, affecting the processes of splicing, transport and degradation of mRNA, thereby achieving the goal of post-transcriptional regulation [16]. So far, a variety of lncRNAs have been found to be closely related to tumorigenesis. Among them, lncRNAs that promote tumorigenesis are usually up-regulated in tumor tissues to be involved in tumorigenesis and development [16]. In this study, we examined the expression levels of lncRNA GATA6-AS in tumor tissue specimens of 17 patients with intrauterine carcinoma. As a result, the expression of lncRNA GATA6-AS was remarkably down-regulated, while that of MMP9 showed an opposite result. Therefore, lncRNA GATA6-AS is believed to be a tumor-suppressing gene in EC. To further explore the effect of GATA6-AS on the function of EC cells, we constructed a GATA6-AS overexpression model and found that upregulation of this lncRNA markedly suppressed the proliferation while promote the apoptosis of EC cells. However, the specific molecular mechanism still remains unclear.

MMP9, as one of the molecules that play a critical role in MMPs, participates in tumor formation in many ways. MMP9 promotes tumors to break through the histological barrier by destroying and degrading various components, including type IV collagen, in the extracellular matrix adjacent to the tumor surface and the vascular wall basement membrane. Studies have found that MMP9 promotes the formation of blood vessels and lymphatic vessels by degrading ECM and vascular basement membranes, increasing the supply of nutrients for tumor growth, and participating in tumor immune regulation. The degradation products of ECM regulate the adhesion of tumor cells to surrounding tissues, and thus play an essential part in the process of tumor cells leaving the primary focus and transferring to new focus adhesion and growth [20,21]. In this research, we confirmed that lncRNA GATA6-AS can directly bind to MMP9 to modulate its expression. Furthemore, the results of cell proliferation test and flow cytometry analysis revealed that lncRNA GATA6-AS and MMP9 may form a mutually inhibiting feedback regulation in EC, and jointly affect the malignant progression of EC.

Conclusions

In summary, the expression of lncRNA GATA6-AS showed a significant reduction in EC tumor tissues and may inhibit the malignant progression of EC through modulating MMP9.

Conflict of interests

The authors declare no conflict of interests.

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