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LncRNA H19 regulates the expression of its target gene HOXA10 in endometrial carcinoma through competing with miR-612

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Abstract. – OBJECTIVE: The role of long non-coding RNA (lncRNA) H19 in endometrial carcinoma was studied, and its mechanism was also explored.

PATIENTS AND METHODS: Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of lncRNA H19, miR-612, and HOXA10 in endometrial carcinoma, and the relationship between lncRNA H19 and survival time was analyzed. The high expression or knockdown of lncRNA H19 in endometrial cancer cells was completed by cell transfection experiments. Cell counting kit-8 (CCK-8) assay was used to detect changes in the viability of endometrial cancer cells. Dual luciferase reporter assay was performed to verify that miR-612 could bind to lncRNA H19 or HOXA10. QRT-PCR and Western Blot assays were used to detect changes in the expression of HOXA10 in endometrial cancer cells before and after overexpression or knockdown of lncRNA H19.

RESULTS: The expression of lncRNA H19 and HOXA10 was high in endometrial carcinoma and miR-612 was lowly expressed. The survival curve suggested that lncRNA H19 was negatively correlated with patient survival. The mRNA expression of lncRNA H19 in endometrial cancer cells, including HEC1-A, HEC1-B, AN-3CA, and Ishikawa, was detected by qRT-PCR. It was found that the expression of lncRNA H19 was highest in AN3CA and lowest in Ishikawa. The cell transfection experiments allowed Ishikawa cells to overexpress lncRNA H19 and AN-3CA cells to reduce lncRNA H19 expression. After overexpression of lncRNA H19, the viability of Ishikawa cells as well as the mRNA and protein levels of HOXA10 increased. However, after knocking down lncRNA H19, the viability of AN-3CA cells along with the mRNA and protein levels of HOXA10 decreased. The dual luciferase reporter assay results suggested that miR-612 could bind to lncRNA H19 and HOXA10.

CONCLUSIONS: The high expression of lncRNA H19 in endometrial carcinoma may regulate the expression level of its target gene HOXA10 by targeting miR-612, thus promoting cell proliferation to play a role in the development of endometrial carcinoma.

Key Words:
Endometrial cancer, LncRNA H19, MiR-612, HOXA10.

Introduction

Endometrial cancer is a common malignancy in female reproductive system[1]. In the past 10 years, the incidence of endometrial cancer was 25.1 per 100,000 women[2]. It has a high degree of malignancy and strong invasiveness. The 5-year survival rate of patients with endometrial carcinoma in different stages is 90%, while that of advanced patients with distant metastasis is less than 20%[3]. Studies have suggested that the mechanism of endometrial cancer is gene mutation in the process of endometrial hyperplasia, but the specific pathogenesis is still not completely clear[1].

Long non-coding RNAs (lncRNAs) are RNAs with a length of more than 200 nucleotides that are transcribed by RNA polymerase II with or without an open reading frame. Studies have found that a large number of lncRNAs are abnormally expressed in tumors, and can play a role in the proliferation, differentiation, and apoptosis of tumor cells through the regulation of different signaling pathways. Therefore, we can further understand the mechanism of tumorigenesis and development through the in-depth study of tumor-associated pathways, and achieve effective treatment of tumors through targeted interference with the signaling pathway[4]. LncRNAs can act as mRNA sponges to compete with endogenous mRNAs during gene expression, or bind to related proteins to regulate

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gene transcription and translation processes, thus inhibiting promoters and interacting with transcription factors[5,6]. They may have a catalytic role in the regulation of gene expression, for example, HOTAIR, XIST can mediate gene activation or inactivation through chromosome remodeling, or regulate cell function by changing the important complex of promoter and chromosome[7].

The lncRNA H19 was the first imprinted gene that was found to be the father's imprinted maternal expression and located on the human gene cluster of H19/IGF2 on chromosome 11p15.58. It plays a role in a variety of tumors. For example, in liver cancer, lncRNA H19 can affect the phenotype of vascular endothelial cells by affecting excretion secreted by CD90+ cells, thereby affecting the growth of liver tumors[9]. Meanwhile, lncRNA H19 can regulate metastasis through miR-675/TGF-β1 signaling pathway in prostate cancer[10]. In addition, lncRNA H19/ID2 in bladder cancer can antagonize p53-mediated cell cycle through the JB-D1 pathway and play a role in the regulation of bladder cancer cell proliferation[11,12]. In this investigation, we first explored the role of lncRNA H19 in endometrial cancer and discussed the mechanism.

Patients and Methods

Sample Collection

The fresh endometrial cancer tissues and normal tissues adjacent to them of 43 cases from July 2010 to July 2012 were collected. All surgical specimens were placed in sterile RNA-free cryotubes within 15 min and placed in liquid nitrogen and frozen for later use. All surgical patients did not receive any chemotherapy, radiotherapy, targeted therapy, biological therapy, or other treatment measures before surgery, and did not suffer from other diseases. All patients were followed up and their survival time was recorded. OS of patients with different lncRNA H19 level was calculated using Kaplan-Meier survival analysis. This study has been approved by the Ethics Committee of Dezhou People's Hospital and all patients have signed a paper version of informed consent.

Cell Culture and Transfection Experiments

Endometrial cancer cells, including HEC1-A, HEC1-B, AN3CA, and Ishikawa, were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS), and incubated in a constant temperature incubator at 37°C with 5% CO2 (Gibco, Rockville, MD, USA). LncRNA H19 overexpressing plasmid (pcDNA-H19) and knockdown sequence (si-H19-1, siH19-2) were constructed. One day before transfection, Ishikawa and AN3CA cells were seeded into a 6-well plate. When the cell density reached 60%-70% on the second day, transfection was performed according to the lipo 2000 instructions. The Ishikawa, AN3CA cells were transfected with pcDNA-H19 or its control plasmid and si-H19-1, si-H19-2 or its negative control, respectively. After 6 hours of transfection, complete medium was used to replace the medium without serum. After 48 hours, cells were collected for follow-up experiments.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Experiments

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA of cancer cells and cervical cancer tissue or its adjacent normal tissue. UV spectrophotometer was used to measure total RNA concentration, and A260/280 value of qualified sample was between 1.8 and 2.1. According to the reverse transcription kit instructions, 1 ug of total RNA was then taken for reverse transcription to obtain complementary Deoxyribose Nucleic Acid (cDNA). The qRT-PCR reaction solution was prepared according to the SYBR Fluorescent Quantitative Premix Kit operating instructions. The total system was 10 μL. The PCR reaction conditions were pre-denaturation at 95°C for 30s, 95°C for 5s, and 60°C for 30s for a total of 40 cycles. The experiment was repeated three times. U6 was used as the control of miR-612, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as the control of other genes. The primer sequences were as follows. LncRNA H19: F: GGTAGAAAAAGCAACCACGAAGC, R: ACATAAACCTCTGTCTGTGAGTGCC. MiR-612: F: GCTGGGCAGGGCTTCT, R: CAGTGCGTGTCGTGGAGT. HOXA10: F: GCCCCTTCCGAGAGCAGCAAAG, R: AGGTGGACGCTGCGGCTAATCTCTA. GAPDH F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC. U6: F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT.

Cell Counting Kit-8 (CCK-8) Experiment

After transfection for 48 h, Ishikawa and AN3CA cells were seeded in 96-well plates at 3. 5 × 103 cells per well. Three replicate wells

were set up. After incubated for 6, 24, 48, 72, and 96 hours, each well was added with 100 μL of fresh DMEM medium without FBS or penicillin-streptomycin solution and 10 μL of CCK8 (Dojindo, Kumamoto, Japan), which was then incubated at 37°C for 1 h. The microplate reader was subsequently used to measure the absorbance (OD value) at 450 nm. The experiment was repeated 3 times, and then the growth curve was drawn.

Western Blot Assay

The cells transfected for 48 hours were collected, and an appropriate amount of radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) was added to lyse the cells, which was then followed by sonication on ice, centrifugation, collection of the supernatant, and determination of protein concentration by bicinchoninic acid (BCA). After the protein was separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), wet transfer method was used to transfer the isolated protein to polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). After blocking in 5% skim milk at room temperature for 1 hour, the membrane was cut into protein bands, and corresponding primary antibody was added and incubated in a refrigerator at 4°C overnight. Tris-buffered saline-Tween (TBS-T) was used to wash the bands for 3 times, and the corresponding secondary antibody was added and incubated for 1 hour at room temperature. After washing for 3 times by TBS-T, the protein bands were developed using ECL solution.

Dual Luciferase Reporter Assay

Ishikawa, AN3CA cells with good growth state were seeded in 12-well plate, and then miR-612 mimics were co-transfected with HOXA10-WT, HOXA10-MUT, lncRNA H19-WT or lncRNA H19-MUT into Ishikawa or AN3CA cells. After 6 hours of transfection, the culture medium was replaced with complete medium. After 60 hours, the cells were lysed and dual luciferase activity was detected. The fluorescence value of renilla plasmid was used as internal reference.

Statistical Analysis

Data were analyzed using statistical product and service solutions (SPSS) 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Measured data were expressed as mean±standard deviation (x±s). The paired t-test was used for comparison between the two groups. The difference was statistically significant at $p<0.05$.

Results

High Expression of lncRNA H19 in Endometrial Carcinoma

QRT-PCR assay was performed to detect the expression of lncRNA H19 in endometrial carcinoma tissues and adjacent normal tissues. The results suggested that lncRNA H19 was highly expressed in endometrial carcinoma (Figure 1A), and the relationship between lncRNA H19 expression and patient survival was analyzed and found to be negatively correlated (Figure 1B). To further explore the role of lncRNA H19, bioinformatics analysis was

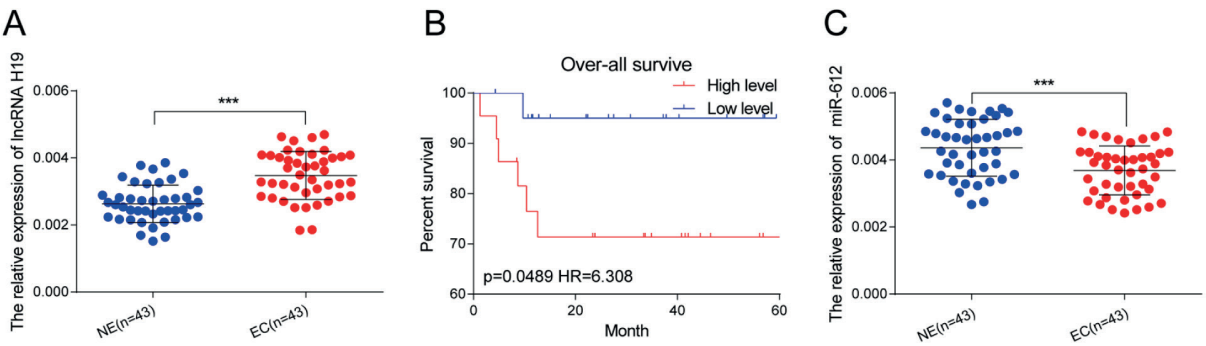

Figure 1. LncRNA H19 was highly expressed in endometrial cancer tissues. ***A,*** The expression of lncRNA H19 in endometrial cancer tissues was significantly higher than that in normal endometrium tissues. ***B,*** The overall survival rate of endometrial cancer patients with high expressed lncRNA H19 was significantly lower than that with lncRNA H19-low expression group. ***C,*** MiR-612 expression in endometrial cancer tissue was significantly lower than that in normal endometrium tissue.

performed and miR-612 was predicted to be able to bind to lncRNA H19, suggesting that lncRNA H19 might play a role through miRNA-612. In addition, it was found that miR-612 was lowly expressed in endometrial cancer tissues (Figure 1C).

lncRNA H19 Enhanced the Viability of Endometrial Cancer Cells

To further confirm the role of lncRNAs in endometrial cancer, firstly qRT-PCR was performed to detect lncRNA H19 expression in endometrial cancer cells including HEC1-A, HEC1-B, AN-3CA, and Ishikawa. The result indicated that lncRNA H19 was the highest in AN3CA cells and lowest in Ishikawa cells (Figure 2A), so these two cells were selected for subsequent experiments. We predicted the binding of miRNA-612 to HOXA10 using TargetScan software, which suggested that HOXA10 might be the target gene of miR-612. HOXA10 is an important transcription factor during embryonic development and plays a role in regulating the proliferation and differentiation of endometrial cancer cells13-15. The results showed that HOXA10 was highly expressed in endometrial cancer tissues (Figure 2B). By cell transfection experiments, lncRNA H19 was overexpressed in Ishikawa cells (Figure 2C) and knocked down in AN3CA cells (Figure 2D). Further CCK8 assay was used to detect changes in cell viability before and after transfection of both cells. The results suggested that the cell viability increased after Ishikawa cells overexpressed lncRNA H19 (Figure 2E), whereas the cell viability of AN3CA cells decreased after knockdown of lncRNA H19 (Figure 2F), indicating that lncRNA H19 could promote endometrial cancer growth.

lncRNA H19 as Competitive Endogenous RNA Competed With miR-612 to Regulate the Expression of Its Target Gene HOXA10

Predicted result of bioinformatics analysis showed that miR-612 can bind to lncRNA H19 3'UTR and HOXA10 3'UTR, and the base pairings were shown in Figures 3A and 3B, respectively. A luciferase reporter vector containing a wild-type or mutant lncRNA H19 3'UTR or HOXA10 3'UTR was co-transfected with miR-612 mimics or control in AN3CA and Ishikawa cells, and the luciferase activity of each group of cells was measured 48 hours later. The results indicated that the luciferase activity of the group of lncRNA H19-WT or HOXA10-WT co-transfected with miR-612 mimics was significantly lower than that of the other three groups (Figure 3A, 3B). It is suggested that lncRNA H19 might compete with miR-612 as a competitive endogenous RNA to regulate the expression of its target gene HOXA10.

lncRNA H19 Promoted the Expression of HOXA10

To further validate our conjecture, we designed in vitro cell experiments to verify. After overexpression of lncRNA H19 in Ishikawa or knockdown of lncRNA H19 in AN3CA cells, we used qRT-PCR and Western Blot assays to detect the expression of HOXA10 at mRNA and protein levels. It was found that after overexpression of lncRNA H19 in Ishikawa cells, both mRNA (Figure 4A) and protein levels (Figure 4C) of HOXA10 were up-regulated. However, knocking down lncRNA H19 in AN3CA cells down-regulated HO3A10 mRNA (Figure 4B) and protein levels (Figure 4D). This demonstrated that lncRNA could regulate the expression of HOXA10, which was possible through endogenously competing with miRNA-612.

Discussion

HOXA10 is an important member of homeobox gene (HOX) family and can be expressed in endometrial gland epithelium and stromal cells of normal people. It plays a regulatory role in cell transcription, differentiation, and embryonic development. Therefore, it is indispensable for embryo implantation and endometrial degeneration[16]. Studies[17-20] have shown that HOXA10 is involved in the development of a variety of gynecological diseases. Its abnormal expression was found in the endometrium of patients with ovarian cancer, endometrial cancer, endometriosis and other gynecological diseases.

In recent years, the expression and regulation mechanism of HOXA10 in endometrial cancer has gradually become a research hotspot. A study found that HOXA10 expression was abnormal in endometrial carcinoma, which was lower than that in normal endometrium. However, there was no significant difference of its expression between normal endometrium and proliferative endometrium. The expression of HOXA10 is regulated by sex hormones. Estrogen and progesterone alone or in combination can significantly upregulate the expression of HOXA10 in endometrial cancer cell lines. Progestin antagonists can significantly inhibit the expression of HOXA10 induced by pro-

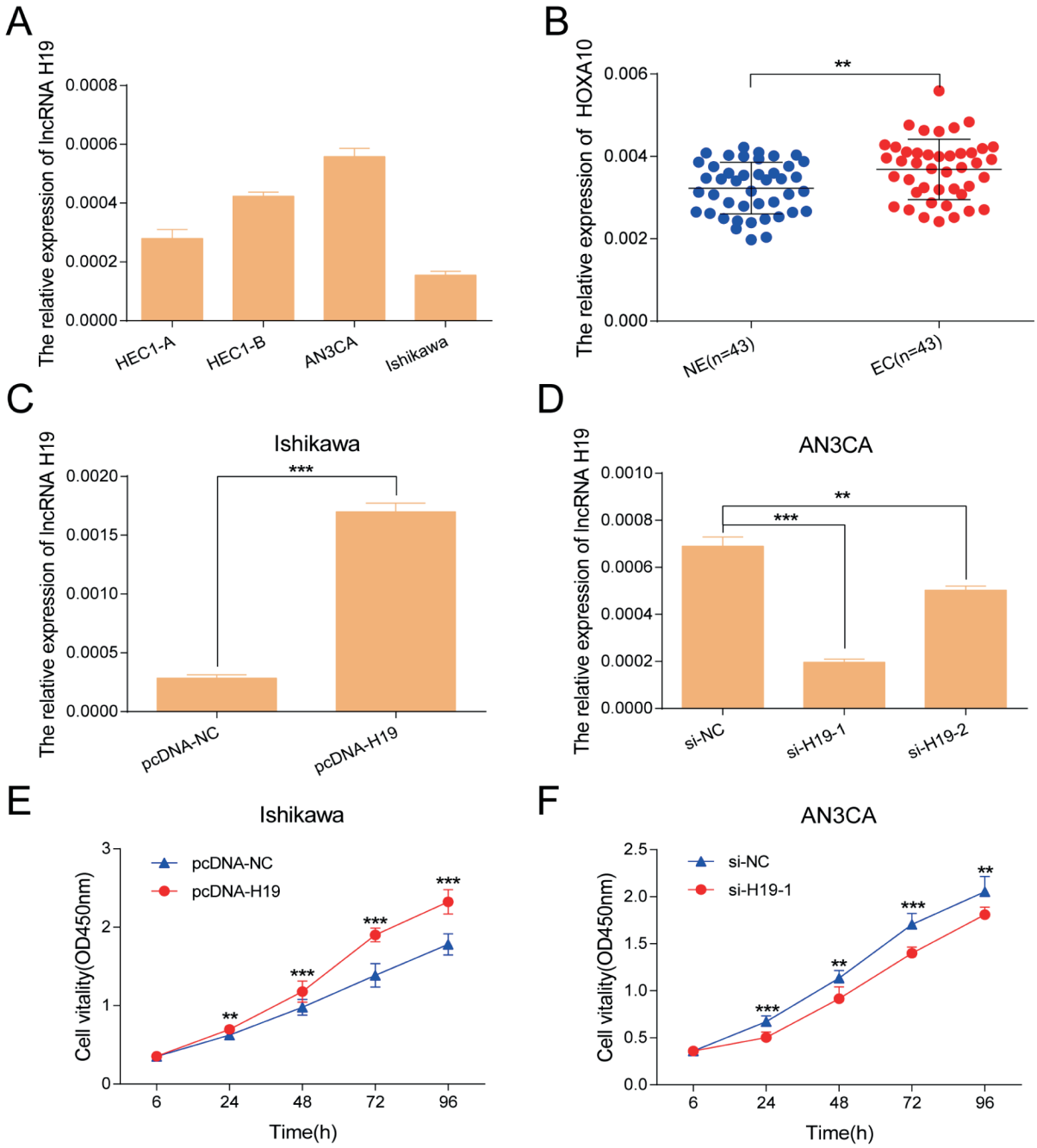

Figure 2. LncRNA H19 enhanced the viability of endometrial cancer cells. ***A,*** Expression of lncRNA H19 in endometrial cancer cells was shown. ***B,*** The expression of HOXA10 in endometrial carcinoma was significantly higher than that in normal endometrium. ***C,*** After overexpressing lncRNA H19, the expression level of lncRNA H19 in Ishikawa cells was significantly increased. D, After knockdown of lncRNA H19, the expression level of lncRNA H19 in AN3CA cells was significantly decreased, with the most significant interference efficiency with si-H19-1. ***E-F***, In Ishikawa and AN3CA cells, lncRNA H19 enhanced the viability of endometrial cancer cells.

gesterone[18]. HOXA10 can also induce E-cadherin expression in endometrial cancer cells by down-regulating Snail gene, so as to promote cell adhesion and inhibit metastasis, thus participating in the metastasis regulation of Ishikawa, which is an endometrial cancer cell line. It has been suggested that elevated CpG5' cytosine methylation of CpG island in HOXA10 promoter is associated with decreased expression in endometrial cancer cells. In addition, HOXA10 was considered to promote the

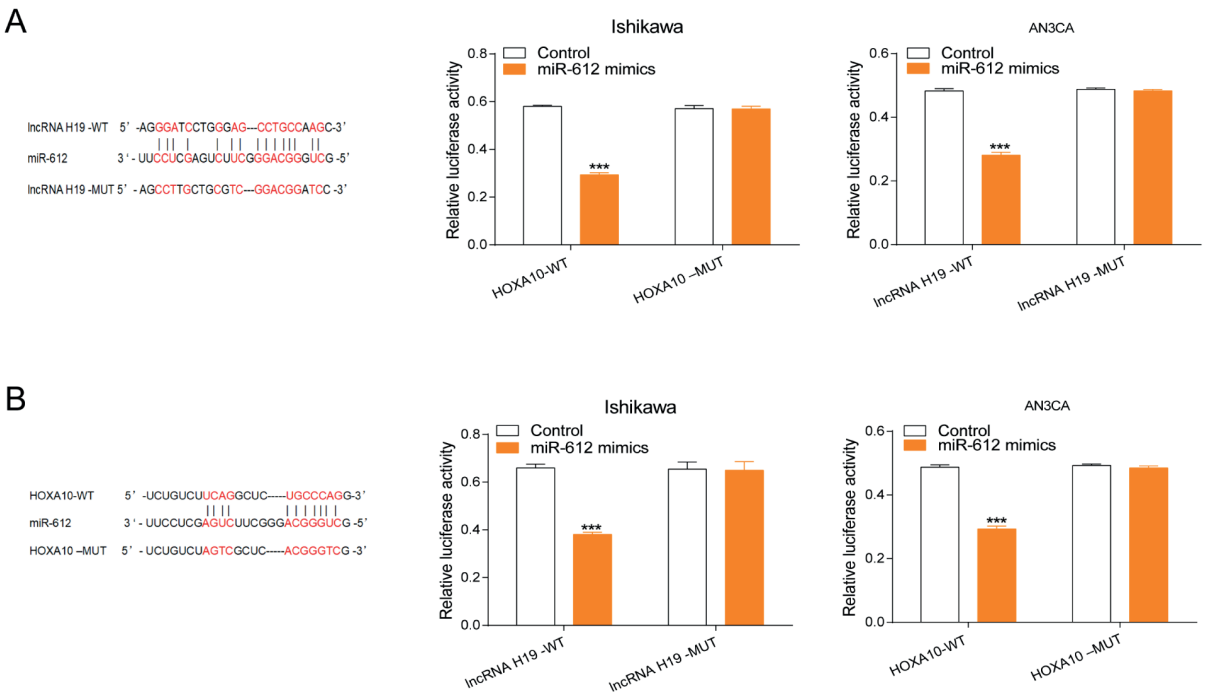

Figure 3. LncRNA H19 competed with miR-612 as a competitive endogenous RNA to regulate its target gene HOXA10 expression. ***A,*** Sequences of lncRNA H19-WT and lncRNA H19-MUT were shown. In Ishikawa and AN3CA cells, the luciferase activity was decreased in the lncRNA H19-WT and miR-612 mimic treated groups. ***B,*** Sequences of HOXA10-WT and HOXA10-MUT were shown. In Ishikawa and AN3CA cells, the luciferase activity was decreased in the HOXA10-WT and miR-612 mimic treated groups.

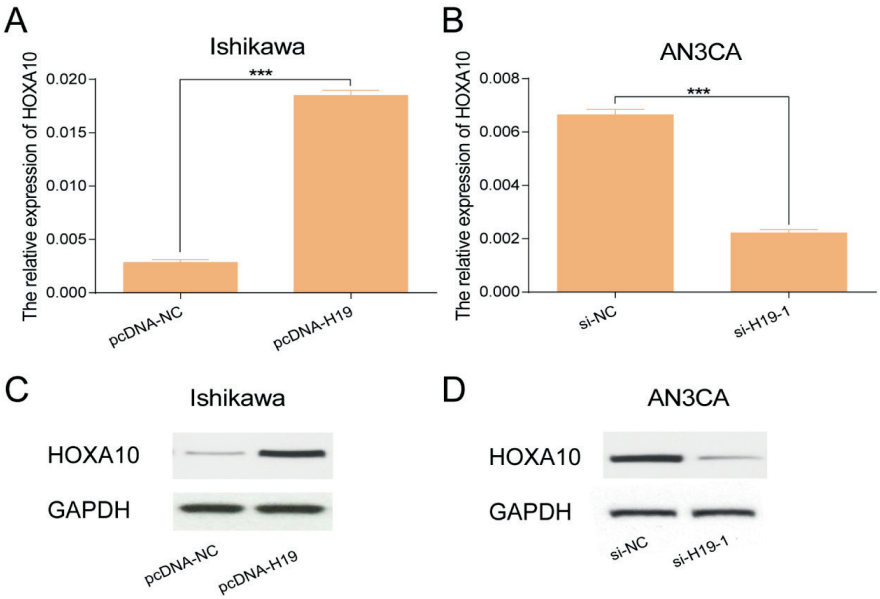

Figure 4. LncRNA H19 promoted HOXA10 expression. ***A,*** After overexpression of lncRNA H19, the mRNA expression level of HOXA10 in Ishikawa cells was significantly increased. ***B,*** HOXA10 mRNA expression levels in AN3CA cells were significantly reduced after inhibiting lncRNA H19 expression. ***C-D***, After knockdown of lncRNA H19, HOXA10 protein expression was down-regulated in Ishikawa and AN3CA cells. After overexpression of lncRNA H19, HOXA10 protein expression was up-regulated in Ishikawa and AN3CA cells.

progression of endometrial cancer by enhancing endothelial interstitial transformation[21].

However, the regulatory mechanism of HOXA10 is still not clear, and the role of lncRNA in tumors has been increasingly valued. Currently known mechanism of lncRNA in the regulation of tumors includes the following points. First, it can act as a "molecular sponge" to competitively combine with other sequences or structures (miRNAs, transcription factors, or RNA-binding proteins). Second, as a backbone, it has the ability to bind multiple proteins to make each protein spatially close, which allows proteins to interact. Third, as molecular signal, it can activate or silence other genes. In addition, lncRNA is capable of acting as a molecular guide to bind specific proteins by direct or indirect action and transport them to specific target sequences[22-24].

In this work, we detected the high expression of lncRNA H19 in endometrial cancer tissues, which can regulate cell viability of endometrial cancer cells, such as Ishikawa and AN3CA. Additionally, miR-612 was found to interact with lncRNA H19 3' UTR and HOXA10 3' UTR. Therefore, we speculated lncRNA H19 may play the role of "molecular sponge" and compete with HOXA10 for binding to miRNA-612. Cell experiments confirmed that overexpression of lncRNA H19 could promote the expression of HOXA10, while knocking down lncRNA H19 inhibited its expression, further verifying our hypothesis.

Conclusions

We showed that lncRNA H19 was highly expressed in endometrial cancer cells, and its high level enhanced the expression of HOXA10 by competing with miR-612 to bind to HOXA10, thus promoting the development of endometrial cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Kanekura K, Nishi H, Isaka K, Kuroda M. MicroRNA and gynecologic cancers. J Obstet Gynaecol Res 2016; 42: 612-617.
2) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.
3) Davidson BA, Foote J, Clark LH, Broadwater G, Ehrisman J, Gehrig P, Graybill W, Alvarez SA, Havrilesky LJ. Tumor grade and chemotherapy response in endometrioid endometrial cancer. Gynecol Oncol Rep 2016; 17: 3-6.
4) Niu G, Zhuang H, Li B, Cao G. Long noncoding RNA linc-UBC1 promotes tumor invasion and metastasis by regulating EZH2 and repressing E-cadherin in esophageal squamous cell carcinoma. J BUON 2018; 23: 157-162.
5) Wang Q, Yang L, Hu X, Jiang Y, Hu Y, Liu Z, Liu J, Wen T, Ma Y, An G, Feng G. Upregulated NNT-AS1, a long noncoding RNA, contributes to proliferation and migration of colorectal cancer cells in vitro and in vivo. Oncotarget 2017; 8: 3441-3453.
6) Lang ZQ, Wu YQ, Pan XB, Qu GM, Zhang TG. The identification of multifocal breast cancer-associated long non-coding RNAs. Eur Rev Med Pharmacol Sci 2017; 21: 5648-5654.
7) Musahl AS, Huang X, Rusakiewicz S, Ntini E, Marsico A, Kroemer G, Kepp O, Orom UA. A long non-coding RNA links calreticulin-mediated immunogenic cell removal to RB1 transcription. Oncogene 2015; 34: 5046-5054.
8) Brannan CI, Dees EC, Ingram RS, Tilghman SM. The product of the H19 gene may function as an RNA. Mol Cell Biol 1990; 10: 28-36.
9) Conigliaro A, Costa V, Lo DA, Saieva L, Buccheri S, Dieli F, Manno M, Raccosta S, Mancone C, Tripodi M, De Leo G, Alessandro R. CD90+ liver cancer cells modulate endothelial cell phenotype through the release of exosomes containing H19 lncRNA. Mol Cancer 2015; 14: 155.
10) Zhu M, Chen Q, Liu X, Sun Q, Zhao X, Deng R, Wang Y, Huang J, Xu M, Yan J, Yu J. LncRNA H19/miR-675 axis represses prostate cancer metastasis by targeting TGFBI. FEBS J 2014; 281: 3766-3775.
11) Wang C, Chen Q, Hamajima Y, Sun W, Zheng YQ, Hu XH, Ondrey FG, Lin JZ. Id2 regulates the proliferation of squamous cell carcinoma in vitro via the NF-kappaB/Cyclin D1 pathway. Chin J Cancer 2012; 31: 430-439.
12) Paolella BR, Havrda MC, Mantani A, Wray CM, Zhang Z, Israel MA. P53 directly represses Id2 to inhibit the proliferation of neural progenitor cells. Stem Cells 2011; 29: 1090-1101.
13) Donahoe PK, Clarke T, Teixeira J, Maheswaran S, MacLaughlin DT. Enhanced purification and production of Mullerian inhibiting substance for therapeutic applications. Mol Cell Endocrinol 2003; 211: 37-42.
14) Daftary GS, Taylor HS. Implantation in the human: the role of HOX genes. Semin Reprod Med 2000; 18: 311-320.
15) Zhao Y, Potter SS. Functional comparison of the Hoxa 4, Hoxa 10, and Hoxa 11 homeoboxes. Dev Biol 2002; 244: 21-36.
16) Szczepanska M, Wirstlein P, Luczak M, Jagodzinski P, Skrzypczak J. Expression of HOXA-10 and HOXA-11 in the endometria of women with idiopathic infertility. Folia Histochem Cytobiol 2011; 49: 111-118.
17) Szczepanska M, Wirstlein P, Luczak M, Jagodzinski PP, Skrzypczak J. Reduced expression of HOXA10 in

the mid luteal endometrium from infertile women with minimal endometriosis. Biomed Pharmacother 2010; 64: 697-705.

18) Zhong G, Wang Y, Liu X. Expression of HOXA10 in endometrial hyperplasia and adenocarcinoma and regulation by sex hormones *in vitro*. Int J Gynecol Cancer 2011; 21: 800-805.

19) Cheng W, Jiang Y, Liu C, Shen O, Tang W, Wang X. Identification of aberrant promoter hypomethylation of HOXA10 in ovarian cancer. J Cancer Res Clin Oncol 2010; 136: 1221-1227.

20) Fiegl H, Windbichler G, Mueller-Holzner E, Goebel G, Lechner M, Jacobs IJ, Widschwendter M. HOXA11 DNA methylation–a novel prognostic biomarker in ovarian cancer. Int J Cancer 2008; 123: 725-729.

21) Yoshida H, Broaddus R, Cheng W, Xie S, Naora H. Deregulation of the HOXA10 homeobox gene in endometrial carcinoma: role in epithelial-mesenchymal transition. Cancer Res 2006; 66: 889-897.

22) Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. Mol Cell 2011; 43: 904-914.

23) Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet 2016; 17: 47-62.

24) Hrdlickova B, de Almeida RC, Borek Z, Withoff S. Genetic variation in the non-coding genome: involvement of micro-RNAs and long non-coding RNAs in disease. Biochim Biophys Acta 2014; 1842: 1910-1922.