Downregulated IncRNA HOTAIR inhibits the proliferation of granulosa cells in endometriosis by upregulating p21

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ABSTRACT

This study was carried out to elucidate the biological function of HOTAIR in granulosa cells of endometriosis and the underlying mechanism. Granulosa cells were extracted from endometriosis patients and subjects with fallopian tube factor alone who received IVF-ET. Relative levels of HOTAIR and p21 in the extracted granulosa cells were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Moreover, HOTAIR level in endometriosis patients in stage I-II or III-IV was determined. Regulatory effects of HOTAIR on the proliferation of KGN cells were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), 5-Ethynyl-2'-deoxyuridine (EdU) and colony formation assay. Flow cytometry was conducted to evaluate the potential influence of HOTAIR on apoptosis of KGN cells. The interaction between HOTAIR and EZH2, SUV12 was detected by RNA binding protein immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP) assay. Finally, the potential role of the HOTAIR/p21 axis in mediating cellular behaviors of KGN cells was explored. HOTAIR was downregulated in granulosa cells extracted from endometriosis patients relative to those with fallopian tube factor alone who received IVF-ET. Knockdown of HOTAIR suppressed the proliferative ability and induced apoptosis of KGN cells. RIP and ChIP assay showed that silence of HOTAIR released EZH2 to suppress the DNA methylation of p21. Knockdown of p21 could reverse the regulatory effect of HOTAIR on the proliferative change of KGN cells. Downregulated HOTAIR suppresses the proliferative ability and induces apoptosis of granulosa cells in endometriosis by upregulating p21.

Introduction

Endometriosis is a common disease in women of childbearing age. Endometriosis is the presence and growth of functional endometrial tissues outside the uterine cavity, which is closely related to pelvic pain and sterility (1). Periodic ischemia, necrosis, shedding and hemorrhage in endometriosis lesions are regulated by sex hormones, which are mainly occurred in the ovary, pelvic peritoneum and rectal vaginal septum (2, 3). About 10% of women of childbearing age suffer from endometriosis (4, 5). It is reported that 25%-50% of sterile women suffer from endometriosis, and the sterility rate in patients with endometriosis reaches 30%-50% (6).

Granulosa cells are an important source of ovarian steroid hormones, providing conditions for follicular development and maturation. Apoptosis of granulosa cells exerts a leading role in follicular atresia. Functional decline of granulosa cells remarkably affects follicular development and maturation (7-10). Therefore, granulosa cells can directly influence the developmental potential of the corresponding oocytes. The state of cumulus granulosa cells is closely related to the maturity of the corresponding ovum, fertilization ability and the quality of the corresponding embryo. A relevant study pointed out that the apoptotic rate of granulosa cells in non-pregnant women is higher than that of pregnant women in the in vitro fertilization-embryo transfer cycle (IVF-ET). It is indicated that the stimulated apoptosis of granulosa cells reduces the developmental potential of oocytes and influences pregnancy outcomes (11-13). Hence, improvement of the quality of ovarian granulosa cells in endometriosis patients contributes to improving the quality of the ovum.

Genome-wide sequencing studies have found that most genomes encode long non-coding RNAs (lncRNAs), a class of long, single-stranded RNAs without protein-encoding function (14). LncRNAs can regulate gene expressions at epigenetic, transcriptional and post-transcriptional levels. They are capable of regulating gene expressions during cell and embryo development, thus maintaining homeostasis (14). Abnormally expressed lncRNAs are associated with human diseases (15).

Accumulating evidences have identified the role of lncRNA in the female reproductive system, which is involved in the progression of various gynecologic tumors, including cervical, endometrial and ovarian tumors (16). However, the role of lncRNA in endometriosis is rarely studied. LncRNA HOTAIR locates on chromosome...
12q13.13.13, which is a 2158 bp IncRNA transcribed from the antisense strand of the C locus of the 12th chromosome. HOTAIR and chromatin-modifying enzymes synergistically regulate the silencing of related genes. HOTAIR has diverse biological functions in mediating cellular behaviors, tumor metastasis and drug resistance (17). The potential role of HOTAIR in endometriosis is rarely reported. This study elucidated the biological role in the progression of endometriosis, which provides novel ideas for clinical treatment of endometriosis.

Materials and Methods

Sample collection
Endometriosis patients and subjects with fallopian tube factor alone who received IVF-ET were enrolled. Follicular fluid with detected cumulus-oocyte complex was harvested on the day of oocyte pick-up. Sample collection for experiments was approved by the subjects and their spouses. All patients signed an informed consent document for diagnosis and research on tissue specimens before being enrolled in the project. This study was approved by the Ethics Committee of our hospital.

Cell culture and transfection
Human ovarian granulosa cell line KGN provided by American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in dulbecco’s modified eagle medium/F12 (DMEM/F12) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO, incubator. Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmid sequences were as follows: Si-HOTAIR1#: 5'-GGGCCUUCCUUAAGAUAUTT-3’ and si-HOTAIR2#: 5’-CCACAGUAGCGCCAGAGAUU-3'; negative control (NC): 5'-UUCUCCGAACGUGACAC-3’.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)
RNA was extracted from cells, reversely transcribed into complementary deoxyribose nucleic acid (cDNA) and subjected to qRT-PCR. Primer sequences of HOTAIR were: forward: 5’-CAGTGGGGAACTCTGACTCG-3'; reverse: 5’-GTGCCTGGTGCTCTCTTACC-3'; Sequences of glyceraldheyde 3-phosphate dehydrogenase (GAPDH) were: forward: 5’-GACTCATGACCAAGAGCTG-3'; reverse: 5’-AGAGGCCAGGATGATTCTGC-3’.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay
Transfected cells for 24-48 hours were inoculated into 96-well plates with 5000 cells per well. At the appointed time points, 20 μL of MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was applied in each well. Four hours later, the medium was replaced and 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added. The mixture was shaken at a low speed for 10 minutes to dissolve the crystals sufficiently. Absorbance was determined at 450 nm by an enzyme-linked immunosorbent detector. Each experiment was repeated in triplicate.

Colony formation assay
Cells were seeded in the 6-well plate with 100 cells per well and cultured for 2 weeks. Subsequently, cells were subjected to 15-min fixation in 4% paraformaldehyde and 30-min staining in 0.1% violet crystal. After removing the staining solution, colonies were air-dried and observed under a microscope.

Flow cytometry analysis
Transfected cells were subjected to incubation with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 10 μL of Propidium Iodide (PI) in the dark for 15 min following the protocols of Annexin V-FITC/PI apoptotic determination kit (Vazyme, Nanjing, China). Apoptosis was determined using the BD FACSCount II flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by FlowJo software.

5-Ethynyl-2’- deoxyuridine (EdU) assay
Cells seeded in the 96-well plate were labeled with 10 μmol/L EdU (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. Subsequently, cells were subjected to 15-min fixations in 4% paraformaldehyde and 20-min incubation in phosphate-buffered saline (PBS) containing 0.5% Triton-100. After washing with PBS containing 3% bovine serum albumin (BSA), 100 μL of the dying solution was applied per well for 30-min incubation in the dark. Subsequently, the cell nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) in the dark for 5 min. Apollo-positive cells and DAPI-positive cells were captured using a confocal laser scanning microscope.

Chromatin fractionation
Nuclear and cytoplasmic fractions were extracted using the PARIS Kit (Life Technologies, Gaithersburg, MD, USA). Subsequently, nuclear and cytoplasmic RNAs were subjected to qRT-PCR.

Chromatin immunoprecipitation (ChIP) assay
ChIP assay was performed using the Chromatin Immunoprecipitation Kit following the manufacturer’s instructions (Millipore, Billerica, MA, USA).

RNA binding protein immunoprecipitation (RIP) assay
RIP assay was performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions.

Western blot
Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The protein sample was loaded for electrophoresis. After transferring on a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical analysis
Statistical Product and Service Solutions (SPSS) 20.0
HOTAIR was downregulated in granulosa cells of endometriosis patients

Granulosa cells were extracted from endometriosis patients and subjects with fallopian tube factor alone who received IVF-ET. QRT-PCR data revealed a lower level of HOTAIR in granulosa cells extracted from endometriosis patients than those extracted from subjects with fallopian tube factor alone (Figure 1A). Moreover, Endometriosis patients in stage III-IV expressed a lower abundance of HOTAIR relative to those in stage I-II (Figure 1B). It is indicated that HOTAIR may exert an important role in the mature process of granulosa cells in endometriosis patients.

Knockdown of HOTAIR suppressed the proliferative ability and induced apoptosis of KGN cells

To explore the function of HOTAIR in granulosa cells, si-HOTAIR 1# and si-HOTAIR 2# were constructed. Transfection of both of them markedly downregulated HOTAIR levels in KGN cells (Figure 2A). MTT assay revealed a pronounced decline in the proliferative ability in KGN cells with HOTAIR knockdown (Figure 2B). Similarly, the number of EdU-positive cells was reduced after transfection of si-HOTAIR 1# or si-HOTAIR 2# in KGN cells, indicating a suppressed proliferative rate (Figure 3A). Fewer colonies were observed in KGN cells with HOTAIR knockdown relative to those transfected with si-NC (Figure S1A). Moreover, flow cytometry data revealed much more apoptotic cells after transfection of si-HOTAIR 1# or si-HOTAIR 2# (Figure 3B). Hence, silence of HOTAIR attenuated proliferative ability and induced apoptosis of KGN cells.

The regulatory effect of HOTAIR in the proliferation of KGN cells relied on p21 upregulation

Through correlation analysis, it is found that the HOTAIR level was negatively correlated to the p21 level (Figure 4A, R=-0.5072, P<0.001). The protein level of p21 was upregulated in granulosa cells extracted from endometriosis patients relative to those extracted from subjects with fallopian tube factor alone (Figure 4B). Furthermore, the p21 level was upregulated in KGN cells with HOTAIR knockdown, further suggesting their negative correlation (Figure 4C). Subsequently, it is revealed that HOTAIR was mainly enriched in the nucleus of KGN cells (Figure 4D). RIP assay showed a higher abundance of HOTAIR enriched in anti-EZH2 and anti-SUZ12 relative to anti-IgG (Figure 5A). Interestingly, both mRNA and protein levels of p21 were upregulated after transfection of si-EZH2 in

Results

software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation and the data were distributed normally (18). Intergroup differences were analyzed by the t-test. Correlation analysis was conducted by the Pearson test. P<0.05 was considered statistically significant. GraphPad Prism V6.0 software (Version X; La Jolla, CA, USA) was utilized for graph editing.

Figure 1. HOTAIR was downregulated in granulosa cells of endometriosis. (A) QRT-PCR data revealed a lower level of HOTAIR in granulosa cells extracted from endometriosis patients than those extracted from subjects with fallopian tube factor alone. (B) QRT-PCR data revealed a lower level of HOTAIR in endometriosis patients in stage III-IV relative to those in stage I-II.

Figure 2. Knockdown of HOTAIR suppressed the proliferative ability of KGN cells. (A) Transfection efficacy of si-HOTAIR 1# and si-HOTAIR 2#. (B) MTT assay showed decreased viability in KGN cells transfected with si-HOTAIR 1# or si-HOTAIR 2# at 6, 24, 48, 72 and 96 h.

Figure 3. Knockdown of HOTAIR suppressed the proliferative ability and induced apoptosis of KGN cells. (A) EdU assay showed fewer EdU-positive cells in KGN cells transfected with si-HOTAIR. (B) Flow cytometry showed an increased apoptotic rate in KGN cells transfected with si-HOTAIR.

Figure 4. P21 was downregulated in endometriosis. (A) A negative correlation between HOTAIR level and p21 level. (B) The protein level of p21 was upregulated in granulosa cells extracted from endometriosis patients. (C) Transfection of si-HOTAIR upregulated protein level of p21 in KGN cells. (D) HOTAIR was mainly expressed in the nucleus of KGN cells. GAPDH and U6 served as cytoplasmic and nuclear internal references.
KGN cells (Figure 5B, 5C). As the ChIP assay demonstrated, the knockdown of HOTAIR decreased the enrichment of p21 in EZH2 and H3K27me3 (Figure 5D).

**Knockdown of p21 reversed the regulatory effect of HOTAIR on KGN cells**

To further investigate the potential role of p21 in the HOTAIR-mediated progression of endometriosis, si-P21 was conducted. Transfection of si-P21 sufficiently downregulated the p21 level in KGN cells (Figure 6A). With the prolongation of cell culture, the knockdown of p21 gradually increased the viability in KGN cells (Figure 6B). Notably, the silence of p21 reversed the inhibited viability in KGN cells transfected with si-HOTAIR 1# (Figure 6C). The decreased clonality in KGN cells with HOTAIR knockdown was markedly improved after the co-transfection of si-P21 (Figure S1B). EdU assay also illustrated the elevated number of EdU-positive cells after co-transfection of si-P21 in KGN cells with HOTAIR knockdown (Figure 6D). It is concluded that the silence of p21 reversed the regulatory effect of HOTAIR on the proliferative ability of KGN cells.

**Discussion**

Endometriosis is a hormone-dependent disease characterized by extruterine growth of endometrial glands and stroma. Clinical signs and symptoms of endometriosis are diverse, including chronic pelvic pain, dysmenorrhea, sexual pain or sterility, etc. Accumulating evidences have uncovered a potential association between endometriosis and infertility. Nevertheless, a definite cause-effect relationship between them is still controversial. The prevalence of endometriosis increases dramatically in women with infertility, reaching as high as 25% to 50%. Such a prevalence is up to 30% to 50% in infertility women accompanied by endometriosis (19). The fecundity rate in healthy women of childbearing age is estimated to be around 15% to 20%, which is 2% to 10% in women with untreated endometriosis (20). Women with mild endometriosis have a significantly lower probability of pregnancy within 3 years than those with unexplained fertility (36% vs. 55%, respectively) (21). *In vitro* fertilization (IVF) studies have suggested that women with advanced endometriosis have poor ovarian reserve, low oocyte and embryo quality, as well as poor implantation (22). A recent study pointed out the pronounced apoptosis of ovarian granulosa cells in endometriosis patients, thus leading to the decline of oocyte quality and their developmental potential (23).

Most lncRNAs are transcribed by RNA polymerase II (RNA pol II) and have similar structures to those of mRNAs, such as 5′ caps and polyA tails (24). LncRNA is dynamically expressed during differentiation. Mature lncRNAs can be formed by polyadenylation and different alternative splicing so that the same gene can form one lncRNA with different transcripts (25).

It is now generally accepted that lncRNA exerts its biological functions mainly through regulating at epigenetic, transcriptional and post-transcriptional levels. LncRNA relies on its secondary structure to bind to proteins, thereafter leading to chromatin remodeling and dysregulated transcription factors. Meanwhile, lncRNAs mediate the upstream or downstream mRNAs through cis-regulation, and they could also directly influence the transcription, shearing and degradation processes (26). The latest research demonstrated that lncRNAs are capable of regulating DNA methylation, histone modification and RNA modification, and conversely, these biological processes in turn affect lncRNA expressions. HOX transcript antisense RNA (HOTAIR) was the first lncRNA found to have a trans- transcriptional regulatory effect. HOTAIR is 6232 bp long and is located on the homeobox C (HOXC) gene cluster of chromosome 12. It shuttles from chromosome 12 to chromosome 2 through Suz-12, thereby affecting the gene of chromosome 2 (27). HOTAIR can serve as a model scaffold to provide a binding surface for at least two different histone modification complexes. Subsequently, related histone-modifying enzymes are selectively aggregated to determine the modification pattern of the target genomic protein. HOTAIR is able to promote the binding of PRC2 to LSD1 and upregulate histone H3K27 methylation level, thus silencing gene expressions (28). Rinn et al. (29) confirmed that HOTAIR is necessary for PRC2 to silence the HOXD locus. The role of lncRNA in endometriosis has been highlighted. Downregulated lncRNA UCA1 serves as a diagnostic and prognostic biomarker...
for ovarian endometriosis (30). In addition, lncRNA-H19 regulates cell proliferation and invasion of ectopic endometrium by targeting ITGB3 via modulating miR-124-3p (31). However, the role of HOTAIR in endometriosis remains unclear. Our study mainly explored the role of HOTAIR in endometriosis. HOTAIR was found to be downregulated in endometriosis patients. Knockdown of HOTAIR markedly suppressed the proliferative ability and induced apoptosis of KGN cells.

P21 is a member of the cyclin-dependent kinase inhibitor (CLP) family. It is a cyclin-dependent kinase inhibitor downstream of the p53 gene. P21 is closely related to tumor suppression, which coordinates cell cycle and DNA replication by inhibiting the activities of cyclin-dependent kinase (CDKs) complexes. In this study, the knockdown of HOTAIR could release EZH2 to inhibit DNA methylation of p21, thereby upregulating p21 to suppress the proliferative ability of KGN cells. Our study provides an experimental reference for HOTAIR serving as a therapeutic target of endometriosis.

HOTAIR is identified to be down-regulated in endometriosis. Downregulated HOTAIR suppresses the proliferative ability and induces apoptosis of granulosa cells in endometriosis by upregulating p21.

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Conflict of interest
The authors declared no conflict of interest.

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