LINC01170 promotes the progression of endometrial carcinoma by activating the AKT pathway
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Summary

Purpose: To investigate the function of LINC01170 in the progression of endometrial carcinoma and its underlying mechanism.

Methods: The expression profiles and prognostic data of endometrial carcinoma were downloaded by GDC (genomic data commons) analysis tools. Differentially expressed long noncoding (lnc)RNAs were analyzed by the edgeR (empirical analysis of digital gene expression data in R) package. LncRNAs that were related to prognosis of endometrial carcinoma were calculated by the survival function. Moreover, the PHEAT map package was introduced to edit heatmaps of differentially expressed lncRNAs. Human endometrial carcinoma cell lines (Ishikawa, ECC and HEC-1A) were cultured. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expressions of lncRNAs and related genes. Cell proliferation was detected by MTT, and cell cycle and apoptosis were detected by flow cytometry. Additionally, Western blot was used to detect protein expressions of relative genes.

Results: Results showed that LINC01170 was a non-coding RNA. LINC01170 was overexpressed in endometrial carcinoma, which was a risk factor for prognosis of this disease. LINC01170 expressions in carcinoma and para-cancerous tissues of 50 patients with endometrial carcinoma were detected by qRT-PCR and found that the expression level of LINC01170 in endometrial carcinoma was remarkably increased than that of para-cancerous tissues. Moreover, the expression level of LINC01170 in advanced endometrial carcinoma was remarkably higher than that of early-stage disease. After interfering with LINC01170, the proliferation of both the Ishikawa and HEC-1A cells were remarkably decreased, and cell cycle was arrested at the G0/G1 phase. Meanwhile, apoptosis results showed a remarkable apoptosis rate after interfering with LINC01170. Western blot results also demonstrated the decreased activity of AKT pathway and phosphorylated expression of AKT protein after LINC01170 knockdown. In addition, expressions of CDK2, CDK4 and Bcl-2 were decreased after LINC01170 knockdown.

Conclusions: LINC01170 promotes the progression of endometrial carcinoma through stimulating proliferation, cell cycle transition and inhibiting apoptosis of endometrial carcinoma cells via AKT pathway.

Key words: AKT, endometrial carcinoma, LINC01170, LncRNA

Introduction

Endometrial carcinoma is a common malignant tumor of the female reproductive system [1]. Due to the biological characteristics of insidious early symptoms, easy postoperative recurrence and low sensitivity to radiotherapy and chemotherapy, endometrial carcinoma has become a major public health problem that endangers the health of females in China [2]. Pathological types of en-
endometrial carcinoma are complex, among which endometrial adenocarcinoma is one of the most common types [3]. In-depth research on the biological characteristics of endometrial adenocarcinoma is of great significance, which could provide new therapeutic targets and improve the diagnosis and treatment of this disease in our country.

Long non-coding RNAs (lncRNAs) are a class of RNAs with over 200 nucleotide (nt) in length. Functionally, lncRNAs cannot encode proteins, which was previously thought to be transcriptional noise without any biological function [4]. However, with the progress of molecular biology, it has been found that lncRNAs exert a crucial biological regulatory role in chromatin modification and remodeling, telomere maintenance, regulation of cell differentiation and cell cycle [5, 6].

Accumulating evidence has shown that many kinds of non-coding RNAs (ncRNAs), including miRNAs and lncRNAs, participate in the cellular physiological and pathological processes [7,8]. For example, lncRNA-GAS5 has a vital function in the development and occurrence of diseases. Epidemiological evidence has suggested that the expression of lncRNA-GAS5 in the circulating blood of patients with type 2 diabetes is obviously lower than that of healthy people. According to previous studies, the probability of diabetes in patients with lncRNA-GAS5<10 ng/µL in the circulating blood is about 12 times as much as that of other population [9]. In addition, the effect of lncRNA-GAS5 on the progression of chronic hepatitis is also significant [10-12]. Therefore, the study of lncRNA is helpful for providing a theoretical basis for treatment of various diseases.

A large number of studies have shown that lncRNAs can regulate the progression of endometrial carcinoma. For example, Inc-FER1L4 [13] and Inc-MEG3 [14] can inhibit the progress of endometrial carcinoma, whereas Inc-MALAT1 [15], Inc-HOTAIR [16,17] and Inc-OVAL [18] can promote the progression of disease. As a consequence, exploring the role of lncRNA in endometrial carcinoma can better investigate the pathogenesis of endometrial carcinoma and provide new directions for its treatment.

Methods

Data acquisition and collection

The expression profiles of endometrial carcinoma and para-cancerous tissues were downloaded from the TCGA (The Cancer Genome Atlas) website by GDC analysis tools. Differentially expressed lncRNAs were analyzed by edgeR package, and lncRNAs related to prognosis of endometrial carcinoma were calculated by survival analysis. Moreover, the PHEAT map package was introduced to edit heatmaps of differentially expressed IncRNAs in endometrial carcinoma.

Clinical sample collection

In total, 40 patients with endometrial carcinoma (observational group) treated in the Affiliated Hospital of Xuzhou Medical University from April 2009 to December 2015 were enrolled in this study. The enrolled patient age ranged between 37 and 65 years (median 55). All patients were subjected to hysterectomy. The surgical staging of disease was evaluated in accordance with the surgical staging standard of endometrial carcinoma revised by the International Union of Obstetrics and gynecology (FIGO) in 2009. No patient received radiotherapy or preoperative drug treatment. Meanwhile, 40 patients (control group) who received hysteroscopic curettage biopsy because of abnormal vaginal bleeding or those who were subjected to hysterectomy because of fibromyoma were selected as controls in this study. Patients in the control group were between 31 and 67 years old, with median age of 51 years. Similarly, normal endometrium tissues were collected during the operation. All the specimens were stored in liquid nitrogen for subsequent experiments. Informed consents were obtained from patients and family members before specimen collection. This study was approved by the ethics committee of the Affiliated Hospital of Xuzhou Medical University. Patients with severe diseases of important organs, such as heart, liver and kidney were excluded.

Cell culture

The human endometrial carcinoma cell lines (Ishikawa, ECC and HEC-1A) were purchased from the Institute of Biochemistry and Cell Biology (Beijing, China). These cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a 5% CO2 incubator at 37°C.

Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR)

TRIzol kit was purchased from Invitrogen (Carlsbad, CA, USA). The SYBRMixture (With Rox I) purchased from TaKaRa (Tokyo, Japan) was used for qRT-PCR. Primers of internal reference GAPDH and LINC001170 were purchased from Invitrogen. Total RNA of the cells was extracted by TRIzol kit according to the manufacturer’s instructions. The extracted mRNA then was reversely transcribed to cDNA according to the instructions of Revert Aid First strand complementary Deoxyribose Nucleic Acid (cDNA) Synthesis Kit, followed by qRT-PCR to detect the mRNA expressions of genes. The relative expression of LINC001170 was represented by 2^{-ΔΔCt}.

Cell transfection

According to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), cells were mixed with
si-NC or si-LINC01170, respectively. The mixture was incubated at room temperature for 20 min for the following experiments. qRt-PCR was performed to detect the interference efficiency of siRNAs.

Gene set enrichment analysis (GSEA)

GSEA 2.2.1 software was used for analysis. Enrolled patients were assigned into the high and low expression group based on the expression level of LINC01170. Datasets of the c2.cp.kegg.v5.1.symbols.gmt were obtained from the MsigDB database of the GSEA website. Subsequently, the enrichment analysis was carried out according to the method of default weighted enrichment statistics, and the number of random combinations was set to 1000 times.

MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

Transfected cells were digested and seeded into 96-well plates at a dose of 2×10³/mL. The cells were cultured in completed DMEM high glucose medium (Gibco, Grand Island, NY, USA) containing 10% FBS, and were maintained in a 5% CO2 incubator at 37°C. After cell adherence, serum-free medium was used for starvation for 24 hrs, followed by transfection of si-LINC01170 or si-NC, respectively. 20μl of MTT (5mg/mL) was added to each well. The cells were then cultured at 37°C for 4 hrs. The supernatant was discarded, and 150 μl of DMSO (dimethyl sulfoxide) was added to each well to dissolve the crystal violet. The proliferation ability was assessed with a microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated 6 times.

Cell apoptosis

Transfected cells were digested with EDTA-free trypsin and washed with PBS (phosphate buffered saline) twice. After centrifugation, 500 μl of binding buffer was added for cell suspension. Then, 5μL of Annexin V and 5 μl of PI (propidium iodide) were added for mixture. Subsequently, the cell apoptosis was analyzed by flow cytometry (Partec AG, Arlesheim, Switzerland). Early apoptotic cells were those stained only by Annexin V but without PI staining. The apoptosis rate was calculated according to the percentage of relevant cell number.

Cell cycle

Cells transfected with si-LINC0170 were collected and digested. For cell cycle detection, 500 μl of 1×binding buffer were added for re-suspension, and the concentration of the suspension was adjusted to 5×10⁵/mL. Then, 10 mL of PI was added for mixing. The mixture was incubated at room temperature for 15 min without exposure to light. Subsequently, cell cycle was analyzed by flow cytometry.

Western blot

Transfected Ishikawa and HEC-1A cells were collected at 72 hrs, and RIPA was used for cell lysate. Total proteins of the cells were extracted. The concentration of each protein sample was determined by a BCA (bicin-
choninic acid) kit (Beyotime, Shanghai, China). Briefly, 50 μg of total protein were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) under denaturing conditions and transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk, followed by incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000 overnight. After washing with PBS for three times, the membranes were incubated with the HRP (horseradish peroxidase) labeled sheep anti-rabbit secondary antibody (1:5000) at room temperature for 1 hr. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Figure 2. LINC01170 was overexpressed in endometrial carcinoma. A: LINC01170 was overexpressed in endometrial cancer tissues. B: LINC01170 was upregulated in advanced endometrial cancer tissues compared with early endometrial cancer tissues. ***p<0.001.

Figure 3. LINC01170 regulated cell cycle, apoptosis and AKT pathway. A: GSEA analysis revealed that LINC01170 regulates cell cycle, apoptosis and AKT pathway. B: LINC01170 expression was significantly upregulated in endometrial cancer cells. C,D: Transfection of si-LINC01170 1# and si-LINC01170 2# could significantly knock down LINC01170 in Ishikawa and HEC-1A cell lines. **p<0.01, ***p<0.001.
The role of LINC01170 in endometrial carcinoma

Statistics

SPSS 21.0 software package (IBM, Armonk, NY, USA) was used for statistical analyses. The quantitative data were presented as mean±standard deviation. T-test was used for comparing differences between the two groups. P<0.05 was considered statistically significant.

Results

LINC01170 was a risk factor for prognosis of endometrial carcinoma

To search for the lncRNAs that were related to prognosis of endometrial carcinoma, we downloaded the whole genome expression profiles of endometrial carcinoma and para-cancerous tissues from the TCGA database. Then the edgeR package was utilized to explore the differentially expressed lncRNAs (Figure 1A). Subsequently, we conducted prognostic analysis for these lncRNAs, and the results showed that LINC01170 was overexpressed in endometrial carcinoma. The expression of LINC01170 was negatively correlated with the prognosis of patients with endometrial carcinoma, which was considered as a risk factor for prognosis (Figures 1B,1C). To verify the above findings, we used qRT-PCR to detect LINC01170 expression in the endometrial carcinoma and para-cancerous tissues of 50 patients. The results indicated that the expression level of LINC01170 in endometrial carcinoma was remarkably increased in endometrial carcinoma tissues than that of para-cancerous tissues (Figure 2A). Moreover, higher expression level of LINC01170 was found in advanced endometrial carcinoma than that of early-stage endometrial carcinoma (Figure 2B). These results suggested that LINC01170 might contribute to the progression of endometrial carcinoma as an oncogene.

LINC01170 promoted the proliferation and cycle transition, and inhibited the apoptosis of endometrial carcinoma cells

To explore the role of LINC01170 in the progression of endometrial carcinoma we firstly conducted a GSEA analysis for LINC01170. The results demonstrated that the function of LINC01170 was mainly enriched in cell cycle and apoptosis via regulating the AKT pathway (Figure 3A). We then detected the expression level of LINC01170 in the endometrial carcinoma cell lines and normal endometrium cell lines, respectively and found that the expression level of LINC01170 was obviously upregulated in endometrial carcinoma cell lines, es-

![Figure 4](image-url)

**Figure 4.** Interference with LINC01170 could significantly inhibit proliferation and promote apoptosis and cell cycle arrest. A,B: Interference with LINC01170 significantly inhibited proliferation. C,D: Interference with LINC01170 could significantly promote apoptosis. E,F: Interference with LINC01170 could significantly promote cell cycle arrest in G0/G1 phase. *p<0.05, **p<0.01, ***p<0.001.
The role of LINC01170 in endometrial carcinoma

Interference with LINC01170 inhibited the progression of endometrial cancer. A, B: The expressions of CDK2, CDK4 and Bcl-2 were significantly decreased after LINC01170 knockdown, and the activity of AKT pathway was significantly decreased.

Figure 5. Interference with LINC01170 inhibited the progression of endometrial cancer. A, B: The expressions of CDK2, CDK4 and Bcl-2 were significantly decreased after LINC01170 knockdown, and the activity of AKT pathway was significantly decreased.

Discussion

Long non-coding RNAs exert their biological function at transcriptional and post-transcriptional level through cis- and trans-regulation [19,20]. For example, H19 may promote the protein expression of IGF1R by competitive binding to let-7 [21]. HOX transcript antisense intergenic RNA (HOTAIR) is located on the antisense chain of the HOXC gene cluster on chromosome 12. It can interact with the polycomb repressive complex 2 (PRC2) and results in the HOXD transcriptional silencing [22]. In addition, IncRNAs can regulate expressions of target genes by modulating protein phosphorylation [23], acetylation [24] and DNA methylation [25]. In this study, through differential and prognostic analysis, we found that LINC01170 was overexpressed in endometrial carcinoma compared with para-cancerous tissues. The underlying mechanism, however, is still not fully elucidated.

PI3K/AKT signaling pathway is an important tumor cell survival pathway, which exists widely in malignant tumors [26]. Activation of p-AKT can further phosphorylate a variety of downstream...
The role of LINC01170 in endometrial carcinoma

proteins, thereby regulating the survival, proliferation and apoptosis of tumor cells [27]. The phosphorylation level of AKT can reflect the activation of the PI3K/AKT pathway. In this study, we found decreased p-AKT expression after knockdown of LINC01170, suggesting that LINC01170 might affect the progression of endometrial carcinoma by regulating the AKT pathway.

Apoptosis is a typical pathway of programmed cell death. Under normal conditions, apoptosis can maintain the cellular homeostasis. However, apoptosis plays the role as a defense mechanism when a harmful substance exists. Apoptosis is mainly divided into two pathways. In the endogenous pathway, the apoptotic signal in cells triggers the intrinsic pathway, and the activated proteins bind to the anti-apoptotic proteins in the Bcl-2 family, thereby preventing its binding with the multi-domain pro-apoptotic proteins of Bax and Bak. Subsequently, Bax and/or Bak homodimers that are located on the outer membrane of mitochondria are capable of increasing the permeation of mitochondrial membrane and releasing cytochrome C and other apoptotic effectors. Cytochrome C binds to apoptotic protease activating factor-1 (APAF-1) and Caspase 9 to form a complex, that is, the apoptotic body. Apoptotic bodies further activate the downstream effect of caspase enzymes (Caspase 3 and Caspase 6), leading to apoptotic cell death [28]. The second pathway is the exogenous pathway. Briefly, a specific death receptor is activated and bound to its corresponding ligand. For example, tumor necrosis factor receptor (TNFR) binds with its ligand TNF. The binding of receptor and ligand leads to the trimeric reaction of these death receptors, resulting in the accumulation of death domains and the recruitment of FADD, Caspase 8 and Caspase 10. Meanwhile, death induction signal complex (DISC) is formed. Caspase 8/10 processed in DISC triggers a cascade reaction, which eventually leads to cell apoptosis [29]. In our study, increased cell apoptosis rate and decreased cell proliferation were found after knockdown of LINC01170. Anti-apoptotic molecules were downregulated as well.

Typically, cell cycle includes the G1, S, G2 and G0 (cell mitosis moratorium) phases. The proportion of cells in S and G2/M phases can reflect the capacity of cell proliferation. As one of the cyclin dependent kinases (CDKs), CDK2 regulates cell cycle mainly by orderly activation and inactivation. Studies have shown that CDK2-Cyclin A complex exhibits its activity in the S phase, which plays a major role in the transition from G1/S to G2/M [30,31]. In controllable cell cycle may lead to abnormal proliferation and differentiation, and can also destroy the balance of cell proliferation and apoptosis [32], thereby affecting the occurrence and development of tumors. Our results indicated that cell cycle was arrested in the G0/G1 phase after LINC01170 knockdown. Besides, expressions of CDK2 and CDK4 were remarkably decreased.

In this study, we found that LINC01170 was overexpressed in endometrial carcinoma and was considered as a risk factor for prognosis by analyzing clinical data of the TCGA database. GSEA analysis indicated that LINC01170 was mainly involved in the regulation of cell cycle, apoptosis, as well as the AKT pathway. Through cytology experiments, we found reduced proliferation, increased apoptosis and arrested cell cycle in the G0/G1 phase of endometrial carcinoma cells after LINC01170 knockdown. Finally, Western blot analysis demonstrated that the AKT pathway was significantly inhibited after knockdown of LINC01170. Meanwhile, expressions of cyclical molecules and apoptosis inhibitors were also obviously reduced.

Our study only verified the in vitro function of LINC0117, and we believe that further in vivo experiments should be carried out for in-depth investigations. In conclusion, this study elucidated the role of LINC01170 in endometrial carcinoma for the first time, which might provide new directions for the treatment and prevention of endometrial carcinoma.

Conclusions

LINC01170 promotes the progression of endometrial carcinoma through stimulating proliferation, cell cycle transition and inhibiting apoptosis of endometrial carcinoma cells via the AKT pathway.

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Conflict of interests

The authors declare no conflict of interests.
The role of LINC01170 in endometrial carcinoma

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