Up-Regulation of LINC00158 Contributes to Endometrial Carcinoma Progression by Interacting with HMGB2

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Abstract

**Background:** Increasing evidence has reported IncRNAs exhibit significant biological functions in regulating endometrial carcinoma (EC) progression. LINC00158 has been identified as a crucial modulator in various diseases. Nevertheless, the specific molecular mechanism of LINC00158 in EC remains to be investigated.

**Methods:** q-PCR and Western blot analysis were performed to determine the expression of LINC00158 and HMGB2 in EC tissues and cells. Cell viability assay, EdU assay, flow cytometry, wound healing assay and transwell invasion assay were used to evaluate their effects on EC cell proliferation, migration and invasion. RIP assay was used to verify the interactions between LINC00158 and HMGB2. Xenograft tumor model was established to confirm the effects of LINC00158 in vivo.

**Results:** LINC00158 was obviously up-regulated in EC tissues in comparison to the normal endometrium. LINC00158 was greatly increased in EC cells than in the immortalized endometrial fibroblast cell T-HESCs. Cell viability of EC cells was reduced as indicated using CCK-8 assay and EdU experiments by knockdown of LINC00158. Loss of LINC00158 obviously induced EC cell apoptosis and blocked cell cycle, repressed EC cell migration and invasion capacity. HMGB2 was remarkably increased in EC tissues and it was positively correlated with LINC00158 expression. RIP assay was carried out and confirmed the direct binding relationship between LINC00158 and HMGB2. Decrease of LINC00158 was able to reduced HMGB2 expression in AN3CA and HEC1-A cells. LINC00158 activated HMGB2 to promote EC progression in vivo.

**Conclusions:** Our findings implied high expression of LINC00158 promoted EC progression via interacting with HMGB2.

Background

Endometrial carcinoma (EC) is a frequent gynecologic malignant tumor among women. It morbidity ranks at the sixth and mortality at the third all over the world with about 320,000 new cases diagnosed and 76,000 deaths [1]. Epidemiological researches have proved that most EC can occur after menopause. 40% incidences are correlated with obesity, estrogen exposure, diabetes and high blood pressure [2]. At present, abdominal hysterectomy remains the main treatment for EC [3, 4]. Radiotherapy, chemotherapy, hormone therapy are applied for more advanced disease stages [4]. Its overall prognosis is kind of favorable since the five-year survival rate is more than 80%.

LncRNA is kind of a transcript with over 200 oligonucleotides without protein-coding capacity [5, 6]. LncRNA possess multiple biological functions, including transcription, transcript splicing and translation [7-9]. The significance of IncRNA in various diseases has been focused on [10, 11]. For example, in breast cancer, IncRNA CERS6-AS1 can function as a malignancy promoter through binding to IGF2BP3 [12]. LncRNA FLVCR1-AS1 can contribute to glioma development through targeting miR-4731-5p and
E2F2 [13]. LncRNA FAM83A-AS1 can facilitate hepatocellular carcinoma via binding with NOP58 and inducing FAM83A mRNA stability [14].

In addition, in several recently published reviews, the functioning of IncRNAs in EC progression has been discussed [15]. For instance, IncRNA MEG3 can repress EC tumorigenesis and progression via regulating PI3K pathway [16]. LncRNA TDRG1 promotes endometrial carcinoma tumorigenicity via targeting VEGF-A protein [17]. In addition, IncRNA HAND2-AS1 represses endometrioid endometrial carcinoma invasion and metastasis via inactivating neuromedin U [18].

The first annotation of LINC00158 also known as C21orf42 has been confirmed in the sequence of human Chromosome 21 [19]. In addition, the interactions between BCL11B-LINC00158 have been speculated to participate in the development of severe asthma [20]. By using bioinformatics tools (LncBase Predicted v.2), some microRNAs are predicted as the downstream target for LINC00158. For instance, miR-181a-5p can be negatively regulated by IncRNA CCAT1 to repress EC cell proliferation and migration [21]. miR-130b-3p has been identified in EC by integrative analysis of TCGA data [22]. Hence, these data indicate that LINC00158 might be involved in EC progression. Molecular function of LINC00158 in endometrial cancer is poorly known and we concentrated on its function on EC.

In this study, we identified a novel highly expressed LINC00158 in EC. We investigated its expression, functional role and underlying mechanisms in EC progression. We found LINC00158 expression was obviously increased in EC while HMGB2 was also elevated in EC tissues. HMGB2 is a crucial protein in multiple cancers. Therefore, we hypothesized that loss of LINC00158 greatly repressed EC progression via inhibiting HMGB2.

**Materials And Methods**

**Clinical samples**

None of EC patients received hormone treatment nor chemoradiation before surgery. The informed consent form all the patients were obtained. 20 normal endometrial tissues from the patients with hysteromyoma in the same period were included as the control group and 40 EC tissues samples were collected. This research was approved by the Ethics Committee Beijing Friendship Hospital Affiliated to Capital Medical University.

**Cell culture**

EC cells (Ishikawa, HEC1-A, HEC1-B and AN3CA cells) and the immortalized endometrial fibroblast cell T-HESCs were obtained from Cell Bank of the Chinese Academy of Science (Shanghai, China). DMEM added with 100 U/mL penicillin/streptomycin and 10% FBS (HyClone, Logan, UT, USA). Cells were incubated in a 5% CO₂ incubator at 37°C.

**Lentivirus transduction**
Short hairpin RNAs (shRNAs) for LINC00158 knockdown were constructed (Invitrogen, Carlsbad, CA, USA) and expressed using pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). EC cells \((1 \times 10^6)\) per well were grown into six-well plates overnight and transfected with vector. The efficacy of transfection was verified by PCR.

**CCK-8 assay**

CCK-8 (Beyotime, Nantong, China) assay was carried out in accordance with manufacturer’s regimen. 3000 cells were seeded into 96-well plates each well. 10 µL CCK-8 reagent (Beyotime, Shanghai, China) was added to the cells and they were maintained at 37°C for 2 hours. OD values were tested at 450 nm by a spectrophotometer (BioTek Instruments, Winooski, VT, USA).

**EdU assay**

Cells were treated using 50 nmol/L EdU (RiboBio, Guangzhou, China) at for 2 hours and fixed using 4% formaldehyde. Then, the cells were treated with 1,000 µL Cell-Light™ EdU Apollo®488 (RiboBio, Guangzhou, China). The nuclear staining was done using DAPI for half an hour. The fluorescence intensity was analyzed using inverted microscope.

**Apoptosis assay**

Cell apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). EC cells were harvested and washed using 1×PBS. Then, cells were added with 5 µl Annexin V-FITC and 5µl PI. Afterwards, cells were incubated for 15 mins in the dark. Apoptosis was assessed using fluorescence activated cell sorting (FACS).

**Cell cycle assay**

Cells were seeded into six-well plates. Cells were trypsinized, centrifuged and then washed twice using PBS. 70% ice-cold ethanol was added to fix the cells and cells were maintained at minus 20°C. Cell cycle detection kit (BD, New Jersey, USA) was used to incubate the cells for 30 min at 4°C. Cell cycle analysis was carried out using flow cytometry.

**Wound-healing assay**

The scratches were created in the monolayers using 200 µL pipette tips. Then, cells were washed three times using PBS and cells were cultured in FBS-free medium. After transfection, we captured the photomicrographs at 0, 24 and 48 hours.

**Cell invasion assay**

Matrigel transwell chambers were used to do cell invasion assays. To be brief, 30 µL the matrix was diluted 1:15, placed into the upper chamber of the Transwell chamber. Cells were suspended in 200 µL FBS-free culture medium and then loaded to the upper chamber. 600 µL complete culture medium with
10% FBS was added to the lower chamber. After incubation for 48 hours, the cells were fixed using 4% paraformaldehyde. The residual cells in the upper chamber were removed by a cotton swab. The cells were stained in the lower chamber using crystal violet. Images were taken using an Olympus fluorescence microscope.

**qRT-PCR**

Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was subjected to reverse transcription using PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). qRT-PCR was carried out using SYBR green (Takara, Tokyo, Japan) using Bio-Rad CFX96 system to test the expression of LINC00158 and HMGB2 mRNA. Gene expression was calculated using $2^{-\Delta\Delta C_t}$ method. Primers were displayed in Table 1.

**Western blot analysis**

Protein samples were extracted and then quantified using 5×loading buffer. Then, the proteins were denatured at 95°C, separated on electrophoresis on 10% SDS polyacrylamide gels. The proteins were transferred to PVDF membranes. Then, the membranes were blocked in 5% skimmed milk at room temperature. Primary antibodies against HMGB2 and GAPDH (1:1000; Proteintech, Proteintech Group, USA) overnight. Then, the membranes were washed three times using TBST, incubated with the corresponding secondary antibodies for 2 hours. Finally, the protein bands were visualized by the enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**RIP assay**

RIP experiment was carried out using Magna RIP Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Briefly, cells were lysed using RIP buffer and incubated with ProteinA/G magnetic beads bounded with primary antibodies against Ago2 or IgG (Abcam, Cambridge, MA, USA). Enrichment of LINC00158 was assessed by qRT-PCR.

**Animal tumorigenesis model**

Animal experiments were conducted under the requirements of the Animal Care and Ethics Committee. Six-week old female BALB/c nude mice from Shanghai Animal Laboratory Center were divided into two groups. HEC-1A cells infected with LV-NC or LV-LINC00158 ($1 \times 10^7$) were resuspended in 200 μL culture medium and subcutaneously injected into the right flanks of the mice. The mice were housed in a specific sterile environment suitable and regularly observed. We removed the xenograft tumors and weighed them. Our work was based on the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Immunohistochemistry**
The sections of tumor tissues were dewaxed, hydrated and washed twice using PBS. After blocking in 5% goat serum, the cells were incubated with anti-Ki-67 antibody at 37 °C for 2 hours. After the section was washed using PBS for 5 minutes, the section was incubated with the secondary antibody at 37 °C for 1 hour. We captured the images using microscope.

Statistical analysis

Data were analyzed by using SPSS 19.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 6.0 Software (GraphPad Inc., San Diego, CA, USA). Correlations were analyzed using Spearman's correlation test. Two-tailed t tests were carried out to compare mean values. P < 0.05 was considered to be significant.

Results

LINC00158 was greatly increased in EC.

Firstly, we detected the expression of LINC00158 in 40 cases of EC and 20 cases of normal endometrium using quantitative PCR. In Figure 1A, higher expression level of LINC00158 was observed in EC than that in normal endometrial tissues. Then, the median expression of LINC00158 in EC was employed as the boundary. EC group was further assigned into high LINC00158 expression group and low LINC00158 expression group. Additionally, LINC00158 expression in positive lymph node metastasis was also higher than that with negative lymph node metastasis as displayed in Figure 1B. For another, the expression level of LINC00158 in EC patients with FIGO stage III was obviously elevated than those in stage I and II in Figure 1C.

LINC00158 promoted EC cell proliferation.

Moreover, in Figure 2A, we found that LINC00158 was up-regulated in EC cells (Ishikawa, HEC1-A, HEC1-B and AN3CA cells) compared to the immortalized endometrial fibroblast cell T-HESCs. Then, LINC00158 was successfully reduced in AN3CA and HEC1-A cells in Figure 2B. Loss of LINC00158 repressed EC cell survival as indicated by CCK-8 assay (Figure 2C and 2D). Consistently, EdU assay proved that AN3CA and HEC1-A cell proliferation was inhibited by the down-regulation of LIN00158 (Figure 2E and 2F).

LINC00158 induced EC cell apoptosis and blocked EC cell cycle progression.

Then, in Figure 3A and 3B, it was shown by flow cytometry assay that AN3CA and HEC1-A cell apoptosis was enhanced by loss of LINC00158. Next, we confirmed that down-regulation of LINC00158 greatly blocked AN3CA and HEC1-A cell cycle progression in G1 phase (Figure 3C and 3D).

Decrease of LINC00158 restrained EC cell migration and invasion.

Next, the migration capability of AN3CA and HEC1-A cells was evaluated using wound-healing assays. As shown in Figure 4A, 4B, 4C and 4D, LINC00158 shRNA significantly suppressed the wound closure in AN3CA and HEC1-A cells. Then, transwell invasion assay was carried out to explore whether LINC00158
affected EC cell invasion capacity. In Figure 4E, 4F, 4G and 4H, decrease of LINC00158 greatly retarded AN3CA and HEC1-A cell invasion.

**HMGB2 was increased in EC.**

Then, the expression of HMGB2 was evaluated in EC tissues. As exhibited in Figure 5A, HMGB2 expression in EC tissues was significant increased than that in normal endometrial tissues. In addition, we found that HMGB2 expression in positive lymph node metastasis was greatly up-regulated than that with negative lymph node metastasis in Figure 5B. HMGB2 expression in EC patients with FIGO stage III was also induced than those in stage I and II as shown in Figure 5C. Subsequently, a positive correlation between HMGB2 and LINC00158 was indicated in Figure 5D.

**LINC00158 could interact with HMGB2.**

In order to investigate whether LINC00158 can regulate HMGB2 expression, EC cells were infected with LINC00158 shRNA. As demonstrated in Figure 6A and 6B, HMGB2 mRNA expression was obviously inhibited by loss of LINC00158 in AN3CA and HEC1-A cells. Additionally, in Figure 6C and 6D, the protein expression of HMGB2 exhibited the same tendency as the mRNA expression of HMGB2 in EC cells. Finally, RIP assay was carried out to confirm the correlation between HMGB2 and LINC00158 as manifested in Figure 6E and 6F.

**LINC00158 silencing inhibited EC progression via repressed HMGB2 in vivo.**

After studying the in vitro function of LINC00158, in vivo assays were then conducted using a xenograft tumor model. HEC1-A cells were infected with LV-NC or LV-shLINC00158 and then injected into nude mice intraperitoneally. In Figure 7A, the tumors were peeled and it was observed that the tumors in LV-shLINC00158 group was much smaller. In addition, the IHC data was revealed and we observed Ki-67 was significantly inhibited by LINC00158 shRNA (Figure 7B). Finally, we proved that LINC00158 inhibition was able to strongly repress HMGB2 mRNA and protein expression (Figure 7C, 7D and 7E).

**Discussion**

As well established, IncRNAs have complex secondary and tertiary structure. They can exhibit their regulatory effects through binding proteins, RNA and DNA [23]. Accumulating studies have reported that IncRNAs are differentially expressed in tumors including in endometrial carcinoma [24-26]. In our present research, we found that LINC00158 was greatly up-regulated in EC. LINC00158 was reduced in AN3CA and HEC1-A cells. Then, we observed that down-regulation of LINC00158 depressed EC cell proliferation, induced cell apoptosis and repressed EC cell cycle. We exhibited that HMGB2 was increased in EC and there was a positive correlation between HMGB2 and LINC00158. Subsequently, we proved that knockdown of LINC00158 repressed EC progression through inhibiting HMGB2.

LINC00158 has been speculated to participate in the development of severe asthma [20]. In our present study, we observed LINC00158 was greatly increased in EC tissues compared with the normal
endometrial tissues. Hence, we hypothesized that LINC00158 might act as an oncogene in EC. Then, we observed that loss of LINC00158 greatly repressed cell viability, migration and invasion. Additionally, EC cell apoptosis was repressed and cell cycle was blocked in G1 phase. Moreover, in nude mice, we confirmed that down-regulation of LINC00158 could restrain EC tumorigenicity and tumor growth. Beside LINC00158, it is still worth to study other potential essential IncRNAs in modulating EC initiation and progression. In addition, the aberrant expression and clinical significance of LINC00158 in other cancers are worth to be explored.

Next, the mechanism by which LINC00158 induced the tumorigenicity of EC cells was focused on. The family of HMGB has four-members (HMGB1, HMGB2, HMGB3 and HMGB4) \[27\]. HMGB is ubiquitous in eukaryotic cells and it can non-specifically binds with DNA in various crucial biological interactions \[28\]. The HMGB member can display different physiological functions in tumor progression. The altered expression of HMGB2 is a crucial prognostic marker in multifarious human cancers \[29-31\]. For example, HMGB2 induces cervical cancer cell proliferation through activating AKT pathway \[32\]. HMGB2 promotes human gastric cancer malignance and can indicate the poor survival outcome \[33\]. In addition, lncRNA CRCMSL can inhibit colorectal carcinoma invasion and metastasis through shuttling HMGB2 \[34\]. LncRNA HOXA11-AS contributes to glioma cell growth and metastasis through targeting miR-130a-5p and regulating HMGB2 \[35\]. This implies that LINC00158 may regulate malignant behaviors of EC cells via HMGB2, which needs further study.

At present, we reported HMGB2 was increased in EC tissues and a negative association between LINC00158 and HMGB2 was indicated. Loss of LINC00158 was able to repress HMGB2 expression in EC cells. The direct correlation between them was confirmed by RIP assay in our study. One of the most well-known functional model for lncRNAs is that lncRNAs can function as ceRNAs to sponge miRNAs via sequence complementarity \[36\]. In our future study, we need to investigate the potential mechanism of LINC00158 in inducing HMGB2 in EC cells.

Conclusion

LINC00158 manifested an oncogenic role in EC progression. Our findings identified a novel IncRNA LINC00158 which was up-regulated in EC. LINC00158 knockdown inhibited the tumor growth of EC cells, and repressed tumor growth of HCC in vivo via interacting with HMGB2. Our data suggested that LINC00158 might provide a novel insight into EC via inducing HMGB2 expression.

Declarations

Ethics approval and consent to participate

This research was approved by the Ethics Committee Beijing Friendship Hospital Affiliated to Capital Medical University. And the informed consent form all the patients were obtained. The animal
experiments were based on the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Yun Liu designed and supervised the study; Yixuan Cai and Min Hao performed the experiments; Yue Chang collected and did the analysis; Yixuan Cai prepared the manuscript; Yun Liu revised the manuscript; All authors read and approved the final manuscript.

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Tables

Table 1. Primers used for real-time PCR

| Genes   | Forward(5’-3’)          | Reverse(5’-3’)          |
|---------|-------------------------|-------------------------|
| GAPDH   | CAAGGT CATCCATGACAACCTTTG | GTCCACCACCCCTGTGCTGTAG  |
| HMGB2   | GGACCCAATGCTCTAAAGGCC   | TGCCCTTGGCACGATATGCAGCA |
| LINC00158 | GGAAATCTTTGGGATGC   | TTCTGGTTCGGGTGAAG      |
LINC00158 was up-regulated in EC tissues. (A) The alternation of LINC00158 expression in EC tissues. (B) LINC00158 expression was positively correlated with lymph node metastasis. (C) LINC00158 expression was positively correlated with histological grade. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. ***P<0.001.
Figure 2

Effects of LINC00158 on endometrial carcinoma cell proliferation. (A) LINC00158 expression in EC cells (Ishikawa, HEC1-A, HEC1-B and AN3CA cells) and Immortalized endometrial fibroblast cell T-HESCs. (B) LINC00158 expression in AN3CA and HEC1-A cells. Cells were infected with LV-shLINC00158 or LV-NC for 48 hours. (C and D) Effects of LINC00158 on AN3CA and HEC1-A cells survival. CCK-8 assay was performed to detect cell viability. (E and F) Effects of LINC00158 on AN3CA and HEC1-A cell proliferation.
EdU assay was carried out to test cell proliferation. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05, ** P<0.01, ***P<0.001.

Figure 3

Effects of LINC00158 on endometrial carcinoma cell apoptosis and cell cycle. (A and B) Effects of LINC00158 on AN3CA and HEC1-A cell survival. Flow cytometry was used to detect cell apoptosis. (C and D) Effects of LINC00158 on AN3CA and HEC1-A cell cycle. Flow cytometry was conducted to detect cell cycle.
cycle. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05, ** P<0.01, ***P<0.001.

Figure 4

Effects of LINC00158 on endometrial carcinoma cell migration and invasion. (A and B) Effects of LINC00158 on AN3CA cell migration. Would healing assay was performed to detect cell migration capacity. (C and D) Effects of LINC00158 on HEC1-A cell migration. (E and F) Effects of LINC00158 on
AN3CA cell invasion. (G and H) Effects of LINC00158 on HEC1-A cell invasion. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05, ** P<0.01, ***P<0.001.

Figure 5

HMGB2 was induced in EC tissues. (A) The alternation of HMGB2 expression in EC tissues. (B) HMGB2 expression was positively correlated with lymph node metastasis. (C) HMGB2 expression was positively correlated with histological grade. (D) Correlation between expression of LINC00158 and HMGB2 in tissue specimens. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05, ** P<0.01, ***P<0.001.
Figure 6
HMGB2 with LINC00158. (A and B) HMGB2 mRNA expression in AN3CA and HEC1-A cells. Cells were infected with shRNA of LINC00158 for 48 hours. (C and D) HMGB2 protein expression in AN3CA and HEC1-A cells. (E and F) The enrichment ratio of LINC00158 with HMGB2 antibody compared with IgG, as determined by RIP assay in AN3CA and HEC1-A cells. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05, ** P<0.01, ***P<0.001.
Figure 7

Loss of LINC00158 repressed EC progression via inducing HMGB2 in vivo. Twelve 8-week old female BALB/c nude mice were injected with AN3CA cells infected with LV-NC (six mice) or LV-shLINC00158 (six mice). (A) Tumors isolated from the two groups. (B) IHC staining of Ki-67 in tumor tissues. (C) Expression of LINC00158 in the tumor tissues. (D and E) Expression of HMGB2 in the tumor tissues. Three independent experiments were carried out. Error bars stand for the mean ± SD of at least triplicate experiments. *P<0.05, ** P<0.01, ***P<0.001.