Hsa_circ_0075960 Serves as a Sponge for miR-361-3p/SH2B1 in Endometrial Carcinoma

Bo Wu¹, Ailing Ren², Ying Tian³, and Ruizhen Huang⁴

Abstract

Although the cases of endometrial carcinoma (EC) is gradually increasing across the world, its etiology and pathogenesis remain unknown. The present study is the first to define the role and biological function of circRNA hsa_circ_0075960 in the development and progression of EC. We first determined that hsa_circ_0075960 is aberrantly expressed in EC cells. Then, we uncovered that the downregulation of hsa_circ_0075960 suppressed cell proliferation and promoted cell apoptosis of EC cells, suggesting that hsa_circ_0075960 could inhibit the progression of EC in vitro. In addition, we identified that miR-361-3p was the direct target of hsa_circ_0075960. Further analysis revealed that hsa_circ_0075960 affected the development of EC via sponging miR-361-3p. Interestingly, we verified that the level of SH2B1 was controlled by the downregulation of hsa_circ_0075960 and that the negative effect caused by hsa_circ_0075960 could be reversed via miR-361-3p inhibition. Our cumulative results revealed that the novel tumor regulator hsa_circ_0075960 functioned as a sponge for miR-361-3p/SH2B1 in EC cells and regulated the progression of EC through the modulation of miR-361-3p.

Keywords

circRNA, miRNA, endometrial cancer, SH2B1, hsa_circ_0075960

Abbreviations

EC, endometrial cancer; circRNA, circular RNA; miRNA, microRNA; CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; RT-qPCR, real-time quantitative polymerase chain reaction.

Introduction

Endometrial carcinoma (EC), which is one of the most common gynecological malignancies occurring globally, has the fourth highest cancer incidence in females, causing 12160 deaths in the United States in 2019 alone.¹,² Based on its clinical, pathological, and molecular features, EC is often categorized into 2 types. The type I cancer is generally related to estrogen and progesterone stimulation, accounting for 80% occurrence in uterine cancers.³ On the other hand, type II cancer possibly arises from atrophic endometrium and occurs relatively rarely when compared with type I cancer. Although most ECs are diagnosed at an early stage and the 5-year survival rate of stage I EC is 96%, patients with advanced EC continue to have a poor prognosis with the 5-year survival rate of only 17%. Thus, it is imperative to search for new diagnostic alternatives and treatment strategies for EC.

Accumulating evidence have indicated that tumorigenesis is induced by epigenetic and genetic changes, and the emerging role of non-coding RNAs (ncRNAs) has been recently suggested in cancerogenesis. NeRNAs can be categorized into linear non-coding RNAs and circular non-coding RNAs (circRNA). Unlike the linear RNAs, the circRNA has a closed-loop structure and lacks the 5’caps or 3’poly-A tails.⁴

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CircRNAs were once regarded as the outgrowth of aberrant splicing despite their wide presence in eukaryotic cells. In addition, the involvement of circRNAs has recently been demonstrated in various cellular aspects such as cellular differentiation, tissue homeostasis, and disease development. Adding to its role in tumorigenesis, circPVT1 has been identified as a proliferative factor and a prognostic marker in gastric cancer. Chen et al. reported that circular RNA hsa-circ-0072309 inhibited the progress of renal carcinoma by affecting the phosphoinositide 3-kinase (PI3 K) and mTOR pathways. circRNAs have also been demonstrated to play roles in the development of EC. Zong et al. also identified that circ_PUM1 promoted the progress of EC by targeting the miR-136/NOTCH3 pathway. In addition, Shen indicated that hsa_circ_0002577 could regulate miR-197/CTNNB1 axis and activate the Wnt pathways to promote the EC progression. In addition, Chen et al. performed transcriptome sequencing to identify numerous circRNAs that were differentially expressed between malignant and normal endometrial tissues. Despite all the efforts, the underlying role of circRNAs in EC remains unclear.

In our study, we identified the novel molecular mechanisms involved in the tumorigenesis of EC. We performed RT-qPCR assay to identify circRNA hsa_circ_0075960, which was differentially presented in the EC cell lines in comparison with the normal endometrium. Then, we found that hsa_circ_0075960 affected the progress of EC by affecting cell apoptosis, cell proliferation, and cell migration. Furthermore, we demonstrated that miRNA miR-361-3p was a potential target of hsa_circ_0075960 by using a luciferase assay. Moreover, we found that miR-361-3p could control the level of SH2B1. In short, we identified that circRNA hsa_circ_0075960 could affect the level of SH2B1 by sponging miRNA miR-361-3p in EC cells.

Materials and Methods

Cell Culture and Transfection

The EC cell lines Ishikawa, RL-952, HEK-1-A, and JEC as well as normal endometrial cells hESC were purchased from the Shanghai Institute of Cell Biology of Chinese Academy of Sciences (Shanghai, China). The cell medium 1640 and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from the Thermo Company (Gibco, USA) and supplemented with 10% fetal bovine serum and 1% P/S (Gibco). All cell lines were cultured in a humidified incubator at 37°C under 5% CO2. Cell transfection was conducted with the lipofection reagent lipo3000 as per the manufacturer’s instructions (Life Science, USA).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Assay

Trizol reagent was used to extract total RNA from cells as per the kit manual (Life, USA). The concentration and purity of RNA were measured with the NanoDrop ND-1000 (Thermo Fisher Scientific, Inc., USA). cDNA was reverse transcribed using the PrimeScript™ RT Reagent Kit (Takara, Japan). Real-time quantitative PCR was performed by using the SYBR Premix Ex Taq™ II Kit (Takara). GAPDH and U6 were used as internal control. The relative expression levels of the genes involved in this study were calculated using the comparative threshold cycle (Ct) (2-ΔΔCt) method. When necessary, the fold-change was calculated through the normalized levels of genes in the experiment group to the mean of those in the control group.

Cell Proliferation Assay

Cell growth was detected using the Cell Counting Kit-8 (Dojindo, Japan). The cells (2 × 10^3) were fed into each well of a 96-well plate. After culturing for 24 h, 10 μL of the CCK-8 medium was added into each well and incubated for 4 h at 37°C. The OD value was collected at different time-points at 450-nm wavelength using the Infinite 200 PRO (Tecan, Switzerland).

Cell Apoptosis Assay

After being transfected for 12 h, the cells were harvested, washed, and re-suspended with PBS. The Annexin V-FITC and PI were added to the cell suspension and incubated for 15 min in the dark. Then, flow cytometer was performed to detect FITC-positive and PI-positive cells. The FlowJo software was used to analyze the data.

Wound Healing Assay

The cells (1 × 10^6) were fed into each well of a 6-well plate with 2-mL cell media. A scratch was then created using a 200-L pipette tip and the widths of the scratch was measured under a microscope at 0 and 48 h. The wound healing rate was calculated using the following equation:

\[
\text{Scratch width at } 48 \text{ h/Scratch width at } 0 \text{ h} \times 100\%
\]

Dual Luciferase Reporter Assay

The luciferase reporter vectors with wild-type or mutant 3' UTR of circ_0075960 or SH2B1 were constructed. The cells were co-transfected with miR-361-3p mimics or miR-NC using the Lipo3000. After 48 h, the luciferase activity was determined by using a dual-luciferase reporter assay kit (Promega, USA).

Statistical Analysis

The GraphPad Prism 6.0 software was used to analyze the data (La Jolla, CA). Student’s t-test or one-way analysis of variance (ANOVA) was applied to evaluate the difference between the different groups. Data was presented as mean ± standard deviation (SD). P < 0.05 was considered to be statistically significant.
Results

Hsa_circ_0075960 Was Excessively Presented in EC Cells

A previous study demonstrated that a novel circRNA hsa_circ_0075960 was upregulated in extracellular vesicles (EVs) isolated from the serum of patients with advanced EC, although the role of hsa_circ_0075960 remains unclear. To identify whether hsa_circ_0075960 was a circular RNA, we performed a head-to-tail splicing assay and Sanger sequencing of the junctional sequences (Figure 1A). Then, we designed the convergent and divergent primers. The results revealed that the divergent amplified circRNAs in cDNA, but not genomic DNA (gDNA), indicating that hsa_circ_0075960 has a circular structure (Figure 1B). We also performed an RNase digesting assay to confirm that hsa_circ_0075960 was more stable (data not shown).

To explore whether hsa_circ_0075960 is an appropriate biomarker of EC, we performed the qRT-PCR assay and determined the level of hsa_circ_0075960 in the EC cell lines and normal endometrial cells. Our results indicated that the expression of hsa_circ_0075960 was upregulated in EC cells than in normal endometrial cells (Figure 1C; ***P < 0.001), which suggested that the dysregulation of hsa_circ_0075960 may play a role in EC. To further identify the effect induced by hsa_circ_0075960 on EC, we employed RNA interference to downregulate hsa_circ_0075960 in EC cells. As shown in Figure 1D, the level of hsa_circ_0075960 was validated by qRT-PCR, and the U6 was regarded as an internal control. Our cumulative results indicated that the RNA interference could efficiently inhibit the level of endogenous hsa_circ_0075960 (Figure 1D, ***P < 0.001).
The Downregulation of Hsa_circ_0075960 Suppressed the Progress of EC Cells

To further identify the effect induced by hsa_circ_0075960 on EC, we employed RNA interference to downregulate hsa_circ_0075960 in EC cells, followed by performing CCK-8 assay to detect cell proliferation of EC cells. As shown in Figure 2A, downregulation of hsa_circ_0075960 could attenuate cell proliferation when compared with the control group. In addition, we utilized Annexin-FITC and PI staining to label the apoptotic cells and performed flow cytometry analysis to evaluate the apoptotic cell ratio in sh-NC and sh-circ_0075960 groups. The apoptotic cell ratio was significantly lower in the sh-circ_0075960 group compared to the sh-NC group. Furthermore, wound healing assay was performed to assess the migration ability of EC cells. As shown in Figure 2D-E, the wound healing rate in the sh-circ_0075960 group was significantly lower than that in the sh-NC group, indicating that hsa_circ_0075960 downregulation suppressed the migration ability of EC cells.

Figure 2. Hsa_circ_0075960 inhibition suppressed the progress of endometrial cancer cells. A, CCK-8 assay was performed to identify cell proliferation in EC cells transfected with sh-NC and sh-circ_0075960. B, Flow cytometry analysis by Flowjo software exhibited the apoptosis cell ratio in sh-NC and sh-circ_0075960 group. C, Statistic data of apoptosis cell. D-E, Wound healing assay indicated hsa_circ_0075960 inhibition suppressed the migration ability of EC cells. Data was presented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.
apoptotic cells. The results of flow cytometry demonstrated that the downregulation of hsa_circ_0075960 promoted cell apoptosis of EC cells (Figure 2B-C). Subsequently, we also performed wound healing assay and found that hsa_circ_0075960 inhibition could suppress the migration ability of EC cells (Figure 2D-E). Taken together, these data indicate that hsa_circ_0075960 inhibition attenuated the EC progression.

Hsa_circ_0075960 Bond Negatively Regulated miR-361-3p

The hypothesis of competing endogenous RNAs (ceRNA) suggest a novel mechanism of interactions among different RNAs. Indeed, circRNAs often act as ceRNA via competitive binding with miRNAs in order to modulate the expressions of the downstream genes. To investigate the underlying mechanisms of the inhibitory effect induced by hsa_circ_0075960, the potential substrates of hsa_circ_0075960 were predicted by bioinformatics software. Then, we screened out potential substrates based on their expression profiles in EC. Among a series of potential targets, we identified a potential binding site of miR-361-3p with hsa_circ_0075960 (Figure 3A). In addition, miR-361-3p was found to contribute to the progression of various tumors.16-20 In order to identify the interaction between hsa_circ_0075960 and miR-361-3p, we mutated the possible binding sites of hsa_circ_0075960 and miR-361-3p. The luciferase assay exhibited that the miR-361-3p mimics reduced the luciferase activity of the hsa_circ_0075960 WT reporter group rather than that of the hsa_circ_0075960 MUT group, indicating that miR-361-3p bond to hsa_circ_0075960 directly (Figure 3B). Although miR-361 had been reported to function in EC,21,22 we confirmed the level of miR-361-3p in the EC cell lines. Our results thus suggested that the level of miR-361-3p in EC was lower compared with that in the normal endometrial cells (control; Figure 3C).

Hsa_circ_0075960 Attenuated the Process of EC via miR-361-3p Sponging

In order to examine whether hsa_circ_0075960 contributed to the process of EC via miR-361-3p regulation, we employed the CCK-8 Kit to confirm the proliferation of EC cells. Our results proved that miR-361-3p could eliminate the effect induced by hsa_circ_0075960 (Figure 4A). Then, we performed flow cytometry to detect the ratio of apoptosis cells. The results demonstrated that the downregulation of miR-361-3p suppressed the effect caused by hsa_circ_0075960 inhibitions on the development of EC cells (Figure 4B-C). In addition, a wound healing assay was performed to detect whether miR-361-3p inhibition could eliminate the inhibitory influence of migration ability caused by the downregulation of hsa_circ_0075960 (Figure 4D-E). Our cumulative results proved that hsa_circ_0075960 functioned as a sponger of miR-361-3p in the EC cells in this study.
SH2B1 Could Be Modulated by Hsa_circ_0075960/ miR-361-3p Axis

Based on the bioinformatics’ prediction, the cytoplasmic adapter protein Src homology 2 B adapter protein 1 (SH2B1) was identified as a possible substrate of miR-361-3p (Figure 5A). SH2B1 have been demonstrated to modulate the signaling pathway by combining different components, including a variety of ligands and their tyrosine kinase receptors. Accumulative evidence have proven that SH2B1 is not only associated...
with metabolic diseases, such as diabetes, but also with cancer progression. From the cancer cell line encyclopedia and human protein atlas, SH2B1 was demonstrated to be expressed in the endometrium with higher expression levels in EC; this report is consistent with those of our unpublished study. From the TCGA dataset, the 5-year survival rate of the patients with high SH2B1 expression was 68%, while that of patients with low SH2B1 expression was 80%, suggesting that SH2B1 may have unfavorable influences on the prognosis of patients with EC. Therefore, we focused our attention to SH2B1 in our study.

To confirm our hypothesis, we conducted the luciferase assay and confirmed the interaction between miR-361-3p and SH2B (Figure 5B). In addition, we detected the protein level of SH2B1 by western blotting; our results showed that hsa_circ_0075960 and miR-361-3p could regulate the expressions of SH2B1 (Figure 5C-D). Taken together, we could conclude that hsa_circ_0075960 sponges miR-361-3p to modulate SH2B1 in the EC cells.

**Discussion**

EC is a life-threatening disease affecting thousands of women across the world. The 5-year survival rate of patients with stage I EC is 96%, while that of stage IV patients is only 17%. Therefore, exploration of new treatment strategies for patients with advanced EC is urgently needed. To search for the underlying modulator of advanced EC, Xu et al. reported the expression profile of circRNA in EVs from EC patients. Based on their RNA-seq analysis, we found that circRNA hsa_circ_0075960 may be upregulated in the serum of patients with EC. However, the molecular mechanism underlying this event remained unclear. We therefore next identified the level of hsa_circ_0075960 upregulated in EC cell lines in comparison with that in normal endometrial cells. Interestingly, we found that hsa_circ_0075960 inhibition could suppress the progress of EC via alleviation of cell proliferation and promotion of cell apoptosis. Furthermore, we found that the possible target of hsa_circ_0075960 was miR-361-3p. Accumulating evidence emphasized the role of miR-361-3p in various tumors. Hu et al. reported that miR-361-3p regulated the EMT induced by ERK1/2 in pancreatic ductal adenocarcinoma and that its control was dependent on DUSP2 mRNA degradation. Chen et al. demonstrated that miR-361-3p and miR-615-5p could be controlled by circular RNA 100146 in non-small cell lung cancers. In addition, miR-361-3p was also reported to function in the gynecological oncology. In cervical cancers, the miR-361-3p expression was regarded as an independent prognostic indicator of favorable survival. In EC, Dong et al. demonstrated that miR-361 regulated the networks involving STAT3 and promoted the progression of EC. Ihira et al. reported that miR-361 was controlled by EZH2 for the suppression of EC development. Considered as an important role of miR-361-3p, we proposed the hypothesis that hsa_circ_0075960 can regulate the development of EC by controlling the miR-361-3p regulation. In addition, we utilized a bioinformatics tool to predict whether the possible substrate of
miR-361-3p is SH2B adapter protein 1 (SH2B1), which has been shown to interact with Grb2, TrkA, and Janus kinase 2. A series of papers have been published that uncovered the role of SH2B1 in the occurrence, progression, and worsening of colorectal cancer, gastric cancer, and NSCLC.\(^{19,27,29}\) In conclusion, we identified a novel biomarker of the EC progression circRNA hsa_circ_0075960, which served as a sponger of miR-361-3p to regulate the expression of SH2B1.

**Authors’ Note**

Bo Wu, Ailing Ren, and Ying Tian made equal contribution to this study, so they were co-first authors. There were no animal experiment and clinical sample from patients involved in this work.

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**Declaration of Conflicting Interests**

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