

HOTTIP regulates progression of endometrial cancer via activating PI3K/AKT pathway

Q. GUAN, Q. ZHANG, C. ZHANG, Q. LIU, Q.-L. REN

Department of Gynecology, Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing, China

Abstract. – OBJECTIVE: To investigate the possible role of HOTTIP in the pathogenesis of endometrial cancer (EC) and its underlying mechanism.

PATIENTS AND METHODS: 76 EC tissues and 76 adjacent normal tissues were collected in this study. HOTTIP expression was detected by qRT-PCR (quantitative Real-Time Polymerase Chain Reaction), and its relationship with clinical prognosis of EC patients was then analyzed. The effect of in vitro HOTTIP on proliferation, cell cycle, apoptosis, colony formation, and migration was examined, respectively. Furthermore, the impact of HOTTIP on PI3K/AKT pathway was explored.

RESULTS: HOTTIP was remarkably expressed in EC patients. The survival rate of EC patients with high expression of HOTTIP was lower than that of patients with low expression, whereas the pathological grade and tumor size in high expression group were significantly higher than those of low expression group. After up-regulation of HOTTIP by lentivirus transfection, the proliferation, colony formation and migration of EC cells showed a significant increase, whereas cell apoptosis was remarkably inhibited. In addition, high expression of HOTTIP promoted the EC development by activating PI3K/AKT pathway.

CONCLUSIONS: Over-expressed HOTTIP promotes the development of endometrial cancer via activating PI3K/AKT pathway.

Key Words:

HOTTIP, Proliferation, Apoptosis, PI3K/AKT.

Introduction

Endometrial carcinoma (EC) is an epithelial malignancy that originates in the endometrium. It is one of the most serious malignant tumors of the female reproductive system. Recently, the incidence of EC has been rising annually and the disease onset has become younger.

EC accounts for about 7% of the total number of female cancer and 20%-30% of female reproductive tract malignancies. It is reported that EC has become the second tumor with the highest incidence of female reproductive tract malignancy in the United States. Also in China, with the development of social economy, living conditions and hormone replacement therapy, EC incidence has also gradually increasing^{1,2}. A large number of studies have identified several molecules that are closely related to EC development, such as PTEN and CA125³. The molecular pathogenesis of EC, however, has not yet been fully elucidated. As a result, it is urgent to explore the underlying pathogenesis of EC, so as to provide new directions for improving clinical treatment.

Long non-coding RNAs (lncRNAs) are a type of RNA molecules with a transcript length of more than 200 nt. Functionally, lncRNAs do not encode proteins, but regulate gene expressions at epigenetic, transcriptional and post-transcriptional levels^{4,5}. lncRNAs were originally thought to be the noise of genomic transcription and by-products of RNA polymerase II transcription, which did not possess biological function. However, accumulated evidence has shown that lncRNAs are involved in almost all physiological and pathological processes of the body and are particularly related to the occurrence and development of multiple tumors⁷. For example, GAS5 was identified as a tumor suppressor lncRNA, which was capable of inducing cell growth arrest and apoptosis in mammalian cell lines. GAS5 deficiency was associated with tumor formation⁸. HOTTIP was found in human peripheral tissue fibroblasts and was located on 7p15.2. Studies have confirmed that HOTTIP is transcribed from upstream of HOXA13 and then delivered into the cytoplasm to form a non-coding RNA with a length of 3764 nucleotides⁹. It has been reported to regulate cell metabolism as an important

molecular signal, which involves Bax/Bcl-2 and other related cell signaling pathways. Besides, HOTTIP was abundantly expressed in hepatocellular carcinoma, which was positively correlated with the malignancy degree of liver cancer^{10,11}. The role of HOTTIP in endometrial cancer, however, still needs further investigation.

Patients and Methods

Clinical Samples

All samples were collected from EC patients who underwent surgical resection in resection in Affiliated Hospital of Nanjing University of Traditional Chinese Medicine from January 2001 to December 2016. A total of 76 patients were included in our experiment and all specimens resected during the surgery were immediately preserved in liquid nitrogen. Epidemiological information about the patients was shown in Table I. All specimens were confirmed by H&E (hematoxylin and eosin) staining by at least 2 independent pathologists. Patients did not receive any preoperative radiotherapy, chemotherapy or hormone therapy. This study was approved by the Ethics Committee of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. Signed written informed consent was provided for each patient.

Cell Culture

HEC-1A, HEC-1B, Ishikawa, KLE and AN-3CA cells were purchased from the American

Culture Collection (ATCC, Manassas, VA, USA). HEC-1A cells were cultured in Mc505 medium containing 10% FBS (fetal bovine serum), 0.1 mg/mL streptomycin and 100 U/mL penicillin. Ishikawa cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 10% FBS, 0.1 mg/mL streptomycin and 100 U/mL penicillin. Other cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) medium with 10% FBS, 0.1 mg/mL streptomycin and 100 U/mL penicillin. All cells were maintained in a 37°C temperature incubator at 5% CO₂ and 95% humidity. Reagents used in cell culture were purchased from Gibco (Grand Island, NY, USA).

Transfection

LV-Vector or LV-HOTTIP were transfected with Lipofectamine at a final concentration of 4 µg/mL when cell confluence was up to 60%, respectively. Culture medium was changed after transfection for 4-6 h. Cells were transfected again by the same method on the next day. The stably transfected cells were used for subsequent experiments. Lentiviruses required for the experiments were all purchased from GenePharma (Shanghai, China).

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

Total RNA was extracted from the tissues and cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measure-

Table I. Epidemiological information about EC patients.

Clinicopathologic features	Number of cases	LncRNA HOTTIP expression		p-value
		Low (n = 38)	High (n = 38)	
Age (years)	76	38	38	0.3527
Pathologic type	44	18	14	0.5894
Adenocarcinoma	58	20	24	
Others	18	30	28	< 0.0001*
Size	40	8	10	
T4	36	30	10	0.0012*
Pathologic staging	34	8	28	
G1-G2-N3	42	24	10	0.0211*
Metastasis	42	14	28	
None	15	3	12	
Total	61	35	26	

ment of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). The reaction conditions were as follows: 40°C for 6 min and 65°C for 25 min. QRT-PCR reaction conditions were as follows: 95°C for 1 min, 95°C for 30 s, and 60°C for 40 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: HOTTIP, F: CCTA-AAGCCACGCTTCTTTG; R: TGCAGGCTG-GAGATCCTACT.

Western Blot

The radioimmunoprecipitation assay (RIPA) protein lysate was used to extract the total protein in each group of cells. The BCA (bicinchoninic acid) method was performed to quantify the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with Tris-buffered Saline and Tween (TBS-T) enhanced chemiluminescence (ECL) was used to show the protein bands on the membrane.

Cell Counting Kit-8 (CCK-8) Assay

The cells were seeded in 96-well plates at a density of 1×10^4 cells and incubated in constant temperature incubator. After the cells adhered for 6 h, fresh medium containing 10% FBS (fetal bovine serum) was added to each culture plate. After cell culture for 0 h, 24 h, 48 h, and 72 h, respectively, 10 μL of CCK-8 (cell counting kit-8, Dojindo, Kumamoto, Japan) solution was added to each well and incubated for another 2 h. The absorbance at wavelength of 450 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Cells at logarithmic growth were routinely trypsinized, centrifuged and adjusted to a density of 1×10^5 cells/mL. The single cell suspension was added to 6-well plates. After 7 d-incubation,

500 μL of 1% crystal violet was utilized to stain cells at room temperature for 1 h. Under an inverted microscope (Nikon, Tokyo, Japan), 5 randomly selected fields in each well were captured. Image-pro plus software was used to count the colony number (≥ 50 cells/clone).

Transwell Assay

Totally 500 μL of complete medium was added to the lower chamber and placed in a 37°C incubator for 24 h. Cells were seeded in the upper-well at a density of 1×10^5 cells/per well. 24 h later, the upper chamber was removed from the incubator and placed in a 24-well plate. 500 μL of methanol for fixation overnight at 4°C. Cells were then stained with crystal violet and photographed under an inverted microscope. 5 randomly selected fields in each well were captured. Image-pro plus software was used to count the colony numbers (≥ 50 cells/clone).

Flow Cytometry

For cell apoptosis, cells were digested, washed and fixed with 4°C pre-cooled phosphate-buffered saline (PBS) and centrifuged at 1000 rpm for 5 min. Totally 500 μL of PBS was added to resuspend the collected cells. The cells were then resuspended in 100 μL of Annexin V Binding Buffer, mixed with 5 μL Annexin V-APC and 5 μL PI (propidium iodide), and incubated at 4°C for 20 min in the dark before the flow cytometric analysis.

For cell cycle, cells were digested, washed and fixed overnight with 70% pre-cooled ethanol. After being washed with PBS again, about 150 μL of PI (Invitrogen, Carlsbad, CA, USA) was added to stain the cells at 40°C for 30 min in the dark. Flow cytometry was used to examine the distribution of 10^4 cells.

Statistical Analysis

SPSS17.0 (Statistical Product and Service Solutions) statistical software package (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism 5.0 software system (La Jolla, CA, USA) were introduced for data analysis and image editing, respectively. The *t*-test was used to analyze the difference between the two groups and the chi-square test was used to analyze the classification data. $p < 0.05$ was considered statistically significant.

Results

HOTTIP Was Upregulated in EC Tissues

By analyzing the 76 pairs of collected specimen of EC and adjacent normal tissues, we found that HOTTIP was overexpressed in EC tissues than that of adjacent normal tissues (Figure 1A). Based on the expression level of HOTTIP in tumor tissues, subjects were further assigned to high expression and low expression groups (Figure 1B). Overexpressed HOTTIP was closely related to poor prognosis of EC (Figure 1C). More seriously, EC patients with high expression of HOTTIP were often accompanied by lymph node metastasis. The pathological grade and tumor size in high expression group were also higher than those of the low expression group. No significant difference in pathological types was found between the two groups (Table I). Above data indicated that HOTTIP might be involved in the occurrence of endometrial cancer.

HOTTIP Regulated Proliferation, Apoptosis, and Migration of EC Cells

We examined the HOTTIP level in five endometrial cancer cell lines. The results showed that the expression level of HOTTIP was the highest in HEC-1B and lowest in KLE cells (Figure 2A). Therefore, we performed subsequent studies using these two cell lines. Lentivirus was exogenously transfected to increase HOTTIP expression in both cell lines (Figure 2B). *In vitro* results demonstrated that overexpressed HOTTIP did not affect the cell cycle, but markedly promoted the proliferation of EC cells in a time-dependent manner, which achieved the peak at 72 h (Figure 2C-2D). Colony formation assay also demonstrated the contribution of HOTTIP to cell proliferation (Figure 2E). Besides,

HOTTIP exhibited a remarkable inhibitory effect on the apoptosis of tumor cells. Expressions of apoptosis-related genes, including cleaved Caspase-3, were found to be downregulated in the HOTTIP high expression group (Figure 2G). Transwell experiment showed that overexpressed HOTTIP increased migration ability of EC cells (Figure 2H). The above results suggested that HOTTIP could participate in the occurrence of endometrial cancer *via* promoting cell proliferation and migration and inhibiting apoptosis.

HOTTIP Regulated PI3K/AKT Pathway

Given the important role of PI3K/AKT pathway in cell proliferation, we hypothesized that HOTTIP might regulate cell proliferation through PI3K/AKT pathway. Our data demonstrated that overexpressed HOTTIP markedly increased phosphorylation level of PI3K/AKT pathway (Figure 3), indicating that HOTTIP might regulate the proliferation and apoptosis of tumor cells by PI3K/AKT pathway.

Discussion

EC is one of the most common malignant tumors of the female reproductive system. Although most EC patients can be diagnosed and treated in an early stage, about 15% of EC patients are diagnosed with advanced or occult metastases, resulting in poor outcome after surgery, radiotherapy, and chemotherapy, and even high mortality¹²⁻¹⁴. Abnormally expressed long non-coding RNAs (lncRNAs) and their important functions have been reported in various tumors. In this study, we found that HOTTIP expression was upregulated in EC tissues, which was closely

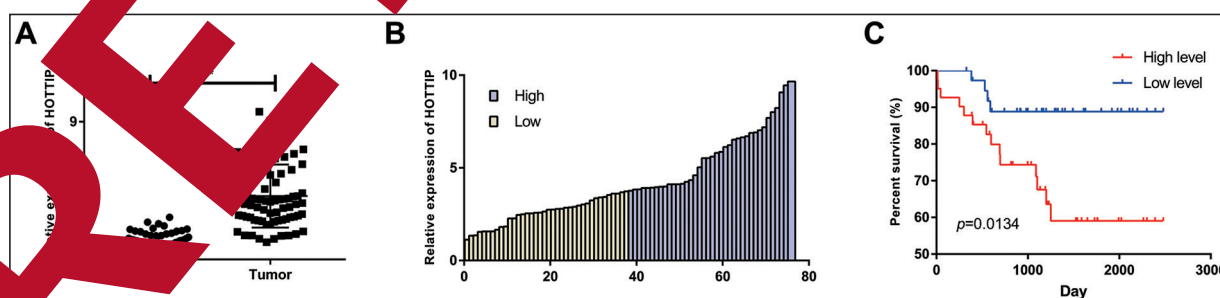


Figure 1. HOTTIP was upregulated in endometrial carcinoma. **A**, HOTTIP was significantly upregulated in EC patients. **B**, Expression of HOTTIP in EC patients. **C**, The survival rate of EC patients with high expression of HOTTIP was lower than that of patients with low expression.

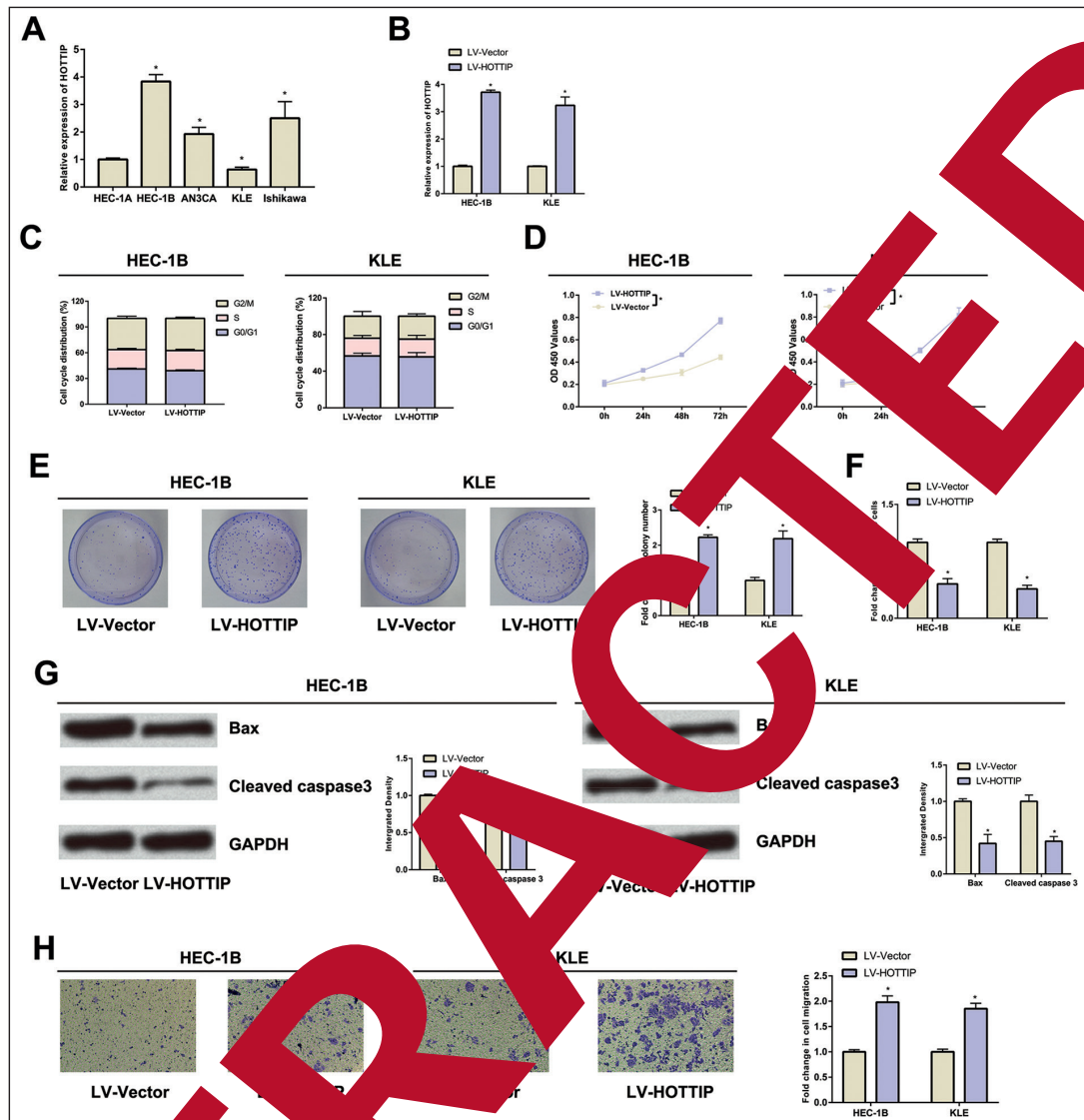


Figure 2. HOTTIP regulated cell proliferation, apoptosis and migration. **A**, HOTTIP expressions in HEC-1A, HEC-1B, Ishikawa, KLE and AN3CA cells. **B**, Detection of HOTTIP expression after transfection of lentivirus. **C-H**, Effect of overexpressed HOTTIP on cell cycle (**C**), cell proliferation (**D**), colony formation (**E**), apoptosis (**F**), apoptotic key proteins (**G**) and cell migration ability (**H**) in HEC-1B and KLE cells.

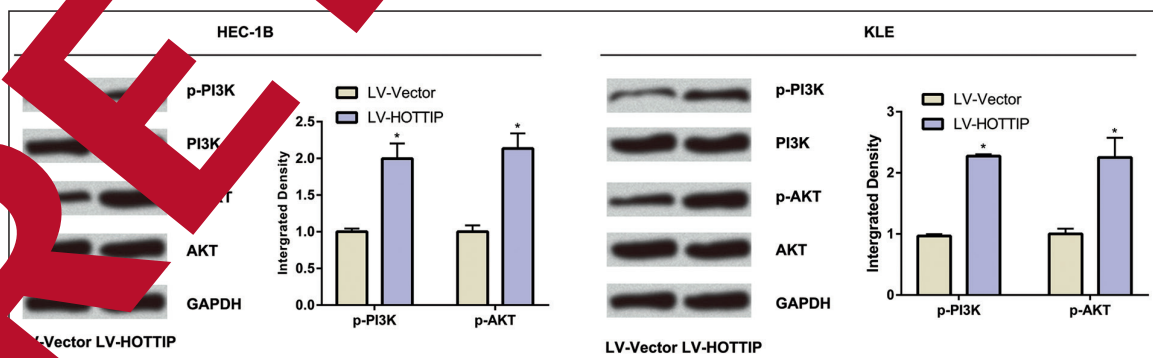


Figure 3. Overexpressed HOTTIP activates PI3K/AKT pathway. The effect of overexpressed HOTTIP on phosphorylation of PI3K/AKT pathway was examined.

related to poor prognosis of EC patients. Our data showed that upregulated HOTTIP could promote cell proliferation and migration, and inhibit cell apoptosis. In addition, HOTTIP might regulate cellular functions by activating PI3K/AKT pathway.

PI3K/AKT pathway is extensively presented in different types of cells, which is capable of regulating cell growth, proliferation, and differentiation¹⁵. Abnormal activation of PI3K/AKT can lead to infinite cell proliferation and resistance to apoptosis, which is associated with the occurrence of many tumors. AKT is proved to be responsible for cell proliferation and apoptosis^{16,17}. Overactivated PI3K/AKT pathway can also directly enhance the aggressiveness and metastasis of tumors through multiple mechanisms. PI(4,5)P₂ and PI(4)P, as the active products of PI3K, stimulate actin-enhanced cell metastasis or promote adhesion of tumor cells and extracellular matrix through integrin and calcium linker. Meanwhile, activated AKT promotes the degradation of extracellular matrix by upregulating the expression of MMP-9, which contributes to the invasion and metastasis of cancer cells^{18,19}. In this study, we found that the activation of AKT was accompanied by the increased cell proliferation and migration, which is consistent with previous studies on other tumors.

Apoptosis is the programmed death of cells controlled by multiple genes. Abnormal regulation of apoptosis and anti-apoptosis is often related to the occurrence and development of tumors. PI3K/AKT pathway plays an important role in the apoptosis resistance of tumor cells through various mechanisms, such as activating activities of Bcl-2 family members, inhibiting caspase family members, and activating apoptotic inhibitor protein family members²⁰. Pro-apoptotic and anti-apoptotic factors either maintain the tissue homeostasis. Bcl-2 family is an important family of apoptosis regulators, which includes anti-apoptosis genes such as Bcl-2 and Bcl-x_l, and pro-apoptotic genes such as Bad and Bax. These gene products interact with each other and affect the survival of tumor cells to a variety of apoptotic signals. For example, non-phosphorylated Bad can heterodimerize with Bcl-2 or Bcl-x_l, resulting in the loss of anti-apoptotic activity. Survival signals stimulated by multiple factors can inhibit apoptosis and promote tumorigenesis by regulating the activity of Bcl-2 family members through PI3K/AKT pathway. It has been reported that activation of PI3K/

AKT phosphorylates Ser136 of Bad. Phosphorylated Bad in turn dissociates from Bcl-2 on the mitochondrial membrane, allowing Bcl-2 to regain its anti-apoptotic effect²³. In our study, overexpressed HOTTIP remarkably inhibited expressions of Bax and cleaved Caspase-3, thereby exerting an anti-apoptotic effect.

Conclusions

We showed that the overexpression of HOTTIP participates in the development of endometrial cancer through promoting cell proliferation and migration, and inhibiting apoptosis via PI3K/AKT pathway.

Conflict of Interest

Authors declare that they have no conflict of interests.

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