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HOTTIP participates in mammary cancer by promoting cell proliferation via PI3K/AKT pathway

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Abstract. – OBJECTIVE: To investigate the role of HOTTIP in the development of mammary cancer and its underlying mechanism.

PATIENTS AND METHODS: 70 mammary cancer tissues and paracancerous tissues surgically resected from mammary cancer patients were enrolled in this study. HOTTIP expressions in these mammary cancer tissues and paracancerous tissues were detected by qRT-PCR (quantitative real-time polymerase chain reaction). The relationship between HOTTIP expression, prognosis, tumor size, and stage of mammary cancer patients was analyzed. Subsequently, we constructed lentivirus of HOTTIP. Proliferation, apoptosis, cell cycle, and invasion of mammary cancer cells transfected with HOTTIP lentivirus were detected by CCK-8 (cell counting kit-8), colony formation, flow cytometry, and transwell assay, respectively. The effect of overexpressed HOTTIP on PI3K/AKT pathway was detected by Western blot.

RESULTS: HOTTIP was overexpressed in mammary cancer tissues than that of paracancerous tissues. HOTTIP expression was negatively correlated with the prognosis of mammary cancer. Overexpressed HOTTIP remarkably promoted cell cycle, and increased expressions of CyclineD1 and PCNA. Meanwhile, overexpressed HOTTIP inhibited cell apoptosis, whereas promoted proliferation and colony formation abilities. Western blot results demonstrated that overexpressed HOTTIP promotes proliferation of mammary cancer cells *via* PI3K/AKT pathway.

CONCLUSIONS: HOTTIP remarkably promotes proliferative and invasive abilities, but inhibits cell apoptosis of mammary cancer cells via PI3K/AKT pathway.

Key Words
HOTTIP, PI3K/AKT pathway, Proliferation, Mammary cancer.

Introduction

Mammary cancer is one of the most common malignancies in females. The incidence of mammary cancer has been increased in western developed countries. In recent years, the incidence of mammary cancer is also astonishingly increased in China due to the improved life pressure and living standards. More notably, the disease onset has been significantly advanced[1,2]. Current treatment methods of mammary cancer include surgery and chemotherapy. Although the survival rate of mammary cancer patients has been increased, mammary cancer cells could not be completely eliminated. Mammary cancer poses great physical and economic burden on affected patients[3]. So far, great advances have been made to promote the treatment efficacy of mammary cancer. The effective biomarkers in predicting mammary cancer are still lacked, which still needs to be further explored.

Long non-coding RNAs (lncRNAs) are a kind of functional RNAs with approximately 200-100,000 nt in length. LncRNAs were first discovered in mouse DNA transcripts and are mainly located in the nucleus or cytoplasm[4,5]. Functionally, lncRNAs do not encode proteins. They regulate the expression levels of genes at transcriptional and post-transcriptional levels. Besides, lncRNAs are capable of regulating various diseases. For example, lncRNAs are closely related to the occurrence and development of metabolic diseases, neurodegenerative diseases, malignant tumors, and autoimmune diseases[6-8].

Among numerous lncRNAs, HOTTIP (HOXA transcript at the distal tip) has been well studied in recent years. HOTTIP is located on chromo-

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some 7p15.2, the transcript of which contains 4665 nucleotides. It promotes the methylation of H3K4 and activation of the adjacent HOXA by interacting with the WDR5/MLL complex during embryonic development[9]. Other studies pointed out that overexpressed HOTTIP can promote the occurrence of hepatocarcinoma (HCC) and increase the invasion of liver cancer cells *via* negative regulation of microRNA-125b[10]. HOTTIP expression is associated with invasion and prognosis of HCC, which may be served as a biomarker for prognosis of HCC[11]. HOTTIP is also overexpressed in digestive system tumors, such as esophageal cancer, gastric cancer, pancreatic cancer, and colorectal cancer. HOTTIP is closely related to the occurrence and development of tumors, thereby affecting the survival and prognosis of tumor patients[12]. The specific role of HOTTIP in mammary cancer, however, still needs to be further investigated.

Patients and Methods

Sample Collection

Mammary cancer tissues and paracancerous tissues were collected from 70 mammary cancer patients admitted to our hospital from 2015.10-2017.10. Tissue samples were surgically resected and immediately preserved in liquid nitrogen for the following experiments. All patients were pathologically diagnosed as mammary cancer, and they did not receive preoperative anti-tumor therapies. Basic characteristics of age, expressions of ER, PR, and HER2 were all recorded in Table I. This study was approved by the Yantaishan Hospital Hospital Ethics Committee, and all patients were informed consent.

Cell Culture and Transfection

MCF-10A, SKBR3, MDA-MB-231, and MCF-7 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and maintained in a 5% CO_2 incubator at 37°C. Cell transfection was performed when the confluence was up to 80%. LV-Vector or LV-HOTTIP was mixed in serum-free DMEM, respectively, and incubated at room temperature for 5 min. Subsequently, the culture medium containing LV-Vector or LV-HOTTIP was mixed and placed at room temperature for 20 min. The mixture was added in each well and transfected cells were collected 48 h later for the following experiments. All reagents were purchased from GenePharma (Shanghai, China).

Table I. HOTTIP expression is closely related to tumor size, expressions of ER, HER2 and PR.

Clinicopathologic features	Number of cases	LncRNA HOTTIP expression		*p*-value
		Low (n=35)	High (n=35)	
Age (years)		35	35	
≤45	30	16	14	0.6291
>45	40	19	21	
Tumor size				
>4 cm	37	12	25	0.0019*
≤4 cm	33	23	10	
ER status				
Negative	34	24	10	0.0008*
Positive	36	11	25	
PR status				
Negative	28	20	8	0.0034*
Positive	42	15	27	
HER2 status				
Negative	43	30	13	0.00003*
Positive	27	5	22	
Metastasis				
YES	30	13	20	0.1052
NO	40	25	18	

Western Blot

The radioimmunoprecipitation assay (RIPA) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein in each group of cells. The BCA (bicinchoninic acid) method was performed to quantitate 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 5% 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 the Tris-buffered saline and Tween 20 (TBS-T). Chemiluminescence was used to expose 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 4×10^3/well and incubated in a constant temperature incubator. After the cells adhered for 6 h, fresh medium containing 10% FBS was added to the culture plate. After cell were cultured for 0 h, 24 h, 48 h, and 72 h, respectively, 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) solution was added to each well and incubated for another 2 h. The absorbance at a wavelength of 450 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Cells at logarithmic growth were seeded into a 6-cm culture dish. After cell culture for 2-3 weeks, 500 μL of 1% crystal violet was utilized to stain cells at room temperature for 20 min. 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 numbers (>50 cells/clone).

RNA Extraction and Quantitative Real-Time 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 measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The cDNA was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 95°C for 5s, 55°C for 30 s, and 72°C for 30s, 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: CCTAAAGCCACGCTTCTTTG, R: TGCAGGCTGGAGATCCTACT; Cyclin D1, F: AATGAAATTCAGGTTGTTGCAGGAG, R: CATGGCAGTGACACCAACCAG; PCNA, F: GGCCGAAGATAACGCGGATAC, R: GGCATATACGTGCAAATTCACCA.

Cell Apoptosis Detection

For cell apoptosis, cells were digested, washed twice 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 of Annexin V-APC and 5 μL of PI (propidium iodide), and incubated at 4°C for 20 min in the dark before the flow cytometric analysis.

Cell Cycle Detection

For cell cycle, cells were digested, washed and fixed overnight with 70% pre-cooled ethanol. After being washed with PBS, 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 10 000 cells.

Transwell Assay

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

Statistical Analysis

SPSS17.0 (Statistical Product and Service Solutions) statistical software package (SPSS Inc., Chicago, IL, USA) was used for data analysis. Kaplan-Meier was introduced for analyzing the prognosis of mammary cancer patients. The *t*-test was used to analyze the difference between two groups and the chi-square test was used to analyze the classification data. $p<0.05$ was considered statistically significant.

Results

HOTTIP Was Overexpressed in Mammary Cancer

HOTTIP was overexpressed in mammary cancer tissues than that of paracancerous tissues (Figure 1A). Furthermore, mammary cancer patients were assigned to high and low expression group based on their HOTTIP expression levels. Kaplan-Meier data demonstrated that mammary cancer patients with higher expression of HOTTIP presented worse prognosis than those with lower expression level (Figure 1B and 1C). Meanwhile, we found that HOTTIP expression is closely related to tumor size, expressions of ER, HER2, and PR of mammary cancer. However, HOTTIP expression did not correlate with metastasis of mammary cancer (Table I). We next detected HOTTIP expression in mammary cancer cell lines and normal breast cell line. Compared with the normal breast cell line MCF-10A, HOTTIP was overexpressed in mammary cancer cell lines, including SKBR3, MDA-MB-231, and MCF-7 (Figure 1D). Among them, MDA-MB-231 presented a lower expression of HOTTIP. We, therefore, selected SKBR3 and MCF-7 cells for the following experiments.

HOTTIP Promoted Cell Cycle of Mammary Cancer Cells

HOTTIP was remarkably overexpressed after lentivirus transfection (Figure 2A). For cell cycle detection, overexpressed HOTTIP remarkably shortened G0/G1 phase, but prolonged S phase of mammary cancer cells (Figure 2B and 2C). Furthermore, cell cycle-related genes (Cyclin D1 and PCNA) were upregulated after HOTTIP overexpression (Figure 2D and 2E), indicating that HOTTIP could regulate cell cycle of mammary cancer cells.

HOTTIP Promoted Proliferation of Mammary Cancer Cells

The data showed that overexpressed HOTTIP remarkably promoted cell proliferation in a time-dependent manner, which achieved the peak at 72 h (Figure 3A and 3B). Since PI3K/AKT pathway has been reported to be greatly involved in cell proliferation, our results demonstrated that overexpressed HOTTIP remarkably activated PI3K/AKT pathway (Figure 3C and 3D). Meanwhile, overexpressed HOTTIP also increased colony formation ability of mammary cancer cells (Figure 3E). The above data elucidated that HOTTIP could regulate proliferation of mammary cancer *via* PI3K/AKT pathway.

HOTTIP Promoted Invasion of Mammary Cancer Cells

Transwell assay demonstrated that overexpressed HOTTIP remarkably increased the invasive ability of mammary cancer cells (Figure 4A). Meanwhile, flow cytometry results indicated that

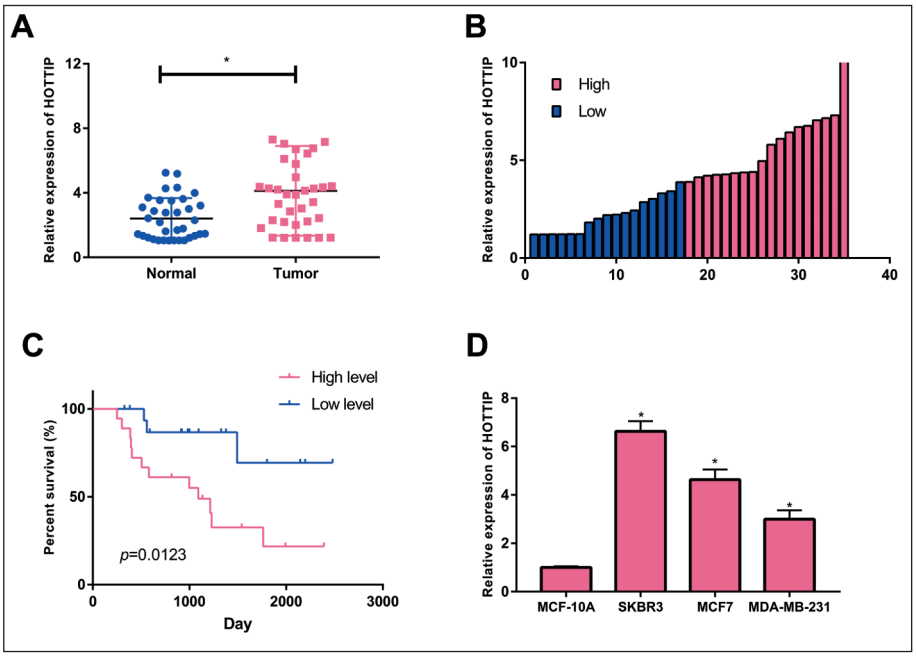

Figure 1. HOTTIP was overexpressed in mammary cancer. **A**, HOTTIP was overexpressed in mammary cancer tissues than that of paracancerous tissues. **B-C**, Kaplan-Meier data demonstrated that mammary cancer patients with higher expression of HOTTIP presented worse prognosis than those with lower expression level. **D**, Compared with the normal breast cell line MCF-10A, HOTTIP was overexpressed in mammary cancer cell lines, including SKBR3, MDA-MB-231, and MCF-7.

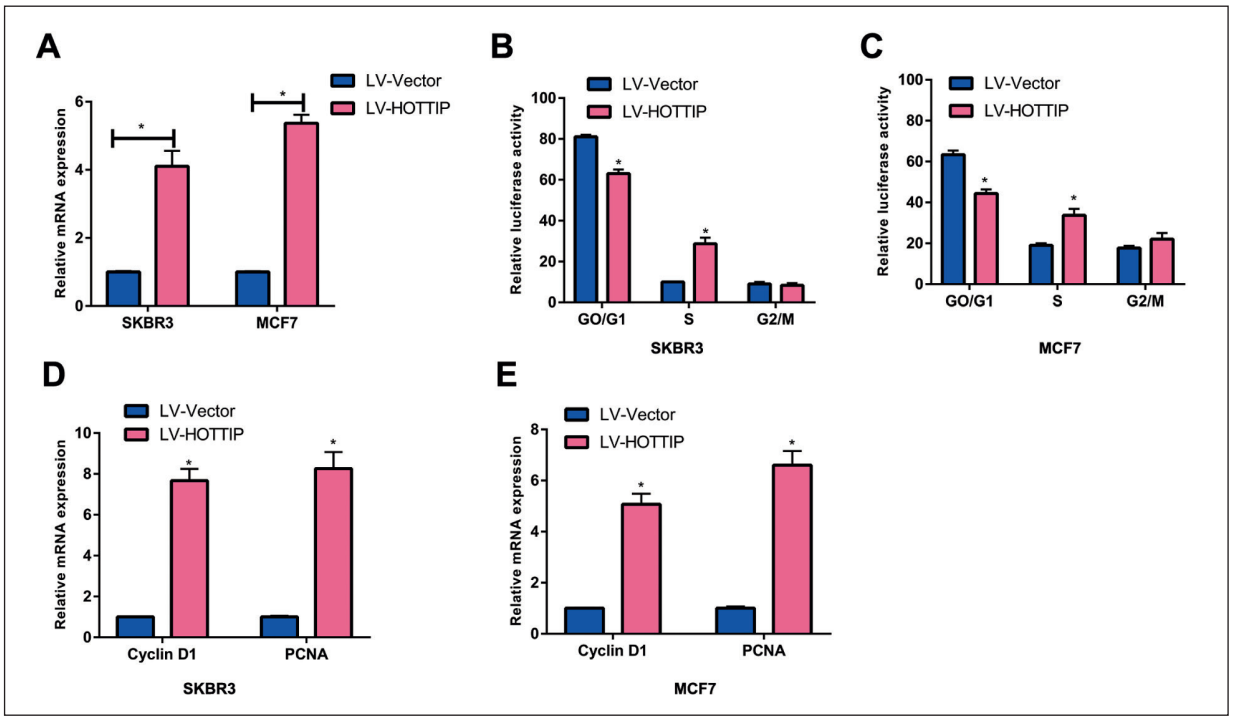

Figure 2. HOTTIP promoted cell cycle of mammary cancer cells. **A**, HOTTIP was remarkably overexpressed after lentivirus transfection. **B-C**, Overexpressed HOTTIP remarkably shortened G0/G1 phase, but prolonged S phase of mammary cancer cells. **D-E**, Cell cycle-related genes (Cyclin D1 and PCNA) were upregulated after HOTTIP overexpression.

apoptosis of SKBR3 and MCF-7 cells were inhibited by HOTTIP overexpression (Figure 4B). The present study suggested that HOTTIP participates in the development of mammary cancer through influencing invasive and apoptotic abilities of mammary cancer cells.

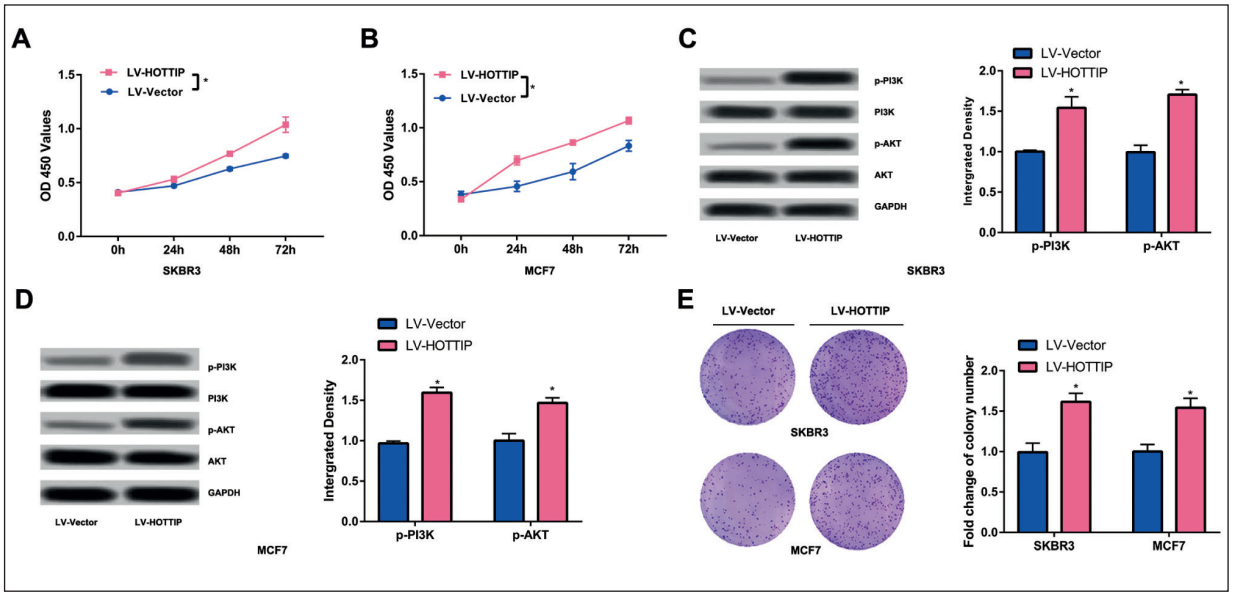

Figure 3. HOTTIP promoted proliferation of mammary cancer cells. **A-B**, Overexpressed HOTTIP remarkably promoted cell proliferation of SKBR3 and MCF-7 cells. **C-D**, Overexpressed HOTTIP remarkably activated PI3K/AKT pathway in SKBR3 and MCF-7 cells. **E-F**, Overexpressed HOTTIP remarkably promoted colony formation ability of SKBR3 and MCF-7 cells.

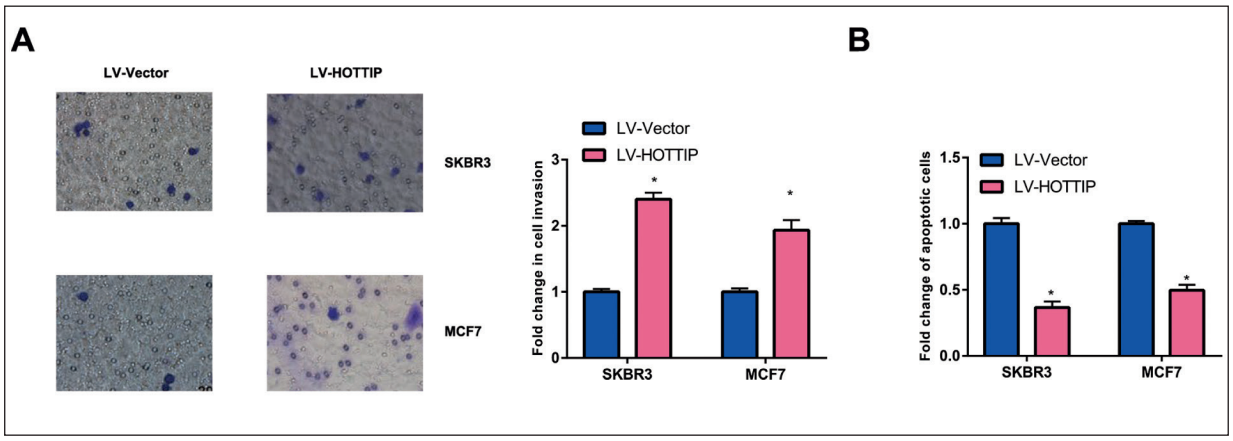

Figure 4. HOTTIP promoted invasion of mammary cancer cells. **A**, Overexpressed HOTTIP promoted invasion of SKBR3and MCF-7cells. **B**, Overexpressed HOTTIP inhibited apoptosis of SKBR3 and MCF-7 cells.

Discussion

Mammary cancer is one of the most common malignancies in females, the mortality of which ranks the first in the world. It is reported[13] that there are about 6.02 million of female malignancies worldwide, including 1.38 million of mammary cancer patients. Therefore, it is urgent to explore the pathogenesis of mammary cancer, so as to provide new ideas for clinical treatment.

LncRNAs have been confirmed to be associated with various cancers. Genomic studies have shown that there are differentially expressed lncRNAs in mammary cancer tissues. For example, lncRNALSINCT5 is overexpressed in mammary and ovarian cancer. Knockdown of LSINCT5 could remarkably reduce the proliferation of tumor cells[14]. It is also found that HOTAIR is overexpressed in primary breast cancer, which is significantly higher in metastatic breast cancer. *In vivo* experiment indicated that HOTAIR could remarkably promote the metastasis of mammary cancer cells, which could be served as an indicator for predicting metastasis and prognosis of mammary cancer patients[15]. Downregulated lncRNANKILA is closely related to metastasis and poor prognosis of mammary cancer. Functionally, NKILA inhibits IκB phosphorylation and NF-κB pathway through complex formation with NF-κB/IκB, so as to participate in metastasis of mammary cancer[16]. Therefore, lncRNAs can participate in the development and drug resistance of mammary cancer[17]. In this study, we found that HOTTIP is overexpressed in mammary cancer tissues, which is a risk factor for poor prognosis. *In vitro* studies have shown that overexpression of HOTTIP can promote cell proliferation, invasion, and cell cycle whereas inhibit apoptosis of mammary cancer cells. Overexpression of HOTTIP significantly activated the PI3K/AKT pathway.

Current studies have found that P13K/AKT pathway is dysregulated in different tumors. PI3K/AKT pathway plays a crucial role in regulating cell proliferation and survival[18]. AKT is an important intersection in the PI3K pathway, which is located at the intersection of various upstream signaling pathways. AKT binds to phosphatidylinositol 3-phosphate (PIP3) through the PH domain, which in turn changes conformation by transferring from the cytoplasm to the cell membrane[19,20]. Meanwhile, cytoplasmic phosphoinositide-dependent protein kinase I (PDK1) binds to PIP3, which leads to the interaction between AKT and PDKI. Subsequently, AKT is phosphorylated by PDK[21]. Phosphorylation of the FOXO family members by AKT leads to the inactivation of FOXO, further reducing the mRNA transcription of apoptosis-related genes (e.g., BH protein and Fas-ligand)[22,23]. BAD was the first discovered target gene of AKT. BAD is a member of the Bcl-2 pro-apoptotic family, which inhibits cell proliferation induced by AKT[24].

Conclusions

We showed that overexpressed HOTTIP remarkably promotes proliferative and invasive abilities, but inhibits cell apoptosis of mammary cancer cells *via* PI3K/AKT pathway.

Conflict of Interest:

The authors declared no conflict of interest.

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