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Overexpression of HOTTIP promotes proliferation and drug resistance of lung adenocarcinoma by regulating AKT signaling pathway

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Abstract. – OBJECTIVE: Lung adenocarcinoma is an important pathological type of lung cancer. Drug resistance is the main reason for failure of lung adenocarcinoma therapy. The purpose of this study is to explore the role of HOTTIP in the progression of lung adenocarcinoma and in drug resistance.

PATIENTS AND METHODS: Differentially expressed lncRNAs in normal lung tissues and lung adenocarcinoma tissues were analyzed in The Cancer Genome Atlas (TCGA) database, followed by analysis of differential lncRNAs in treated sensitive and insensitive groups. HOTTIP was found to be highly expressed in lung adenocarcinoma tissues and in drug-resistant tissues. Next, the expression of HOTTIP in clinical samples and its relation to clinical data were analyzed. Then, we examined the effect of HOTTIP in lung adenocarcinoma by detecting changes in cell proliferation and drug resistance after overexpression and interference with HOTTIP.

RESULTS: By analyzing the normal and lung adenocarcinoma tissues from TCGA database and the treatment of sensitive and insensitive samples, we found that HOTTIP was overexpressed in lung adenocarcinoma and significantly increased in the treatment-insensitive group. Similar results were obtained in clinical samples. In order to explore the role of HOTTIP in lung adenocarcinoma, the proliferation ability of A549 and the drug resistance of A549/PA were significantly reduced after interfering with HOTTIP. Overexpression of HOTTIP, proliferation ability of A549 and drug resistance of A549/PA was significantly enhanced.

CONCLUSIONS: HOTTIP can promote the progression of lung adenocarcinoma, and the formation of lung adenocarcinoma resistance regulated by the protein kinase B (AKT) signaling pathway.

Key Words:
HOTTIP, Lung adenocarcinoma, AKT, Drug resistance, TCGA.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for 28% of cancer deaths in male and 26% in female[1]. Among them, lung adenocarcinoma accounted for about 40% of the primary lung cancer and is the main form of lung cancer. At present, the main chemotherapy-based comprehensive treatment regimen was adopted; however, the efficacy of advanced non-surgical lung cancer remained at a low level, the efficiency was only 20-40%, 5-year survival rate was only 15%. One of the reasons was multi-drug resistance. Therefore, studying the mechanism of drug resistance is of great significance for the treatment of lung adenocarcinoma and improving the survival of patients[2,3].

More and more researches showed that the human genome encodes a large number of long non-coding RNAs (lncRNAs) and is involved in the regulation of the biological functions of cells. LncRNAs are a class of RNA molecules with over 200 nucleotides in length and are transcribed by RNA polymerase II and do not encode proteins[4-6]. In previous studies, it was considered as "transcriptional noise". However, recent studies have shown that lncRNAs can participate extensively in genomics regulation and are closely related to many diseases including tumors[7].

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Recent studies have confirmed that lncRNA was involved in the chemoresistance of almost all tumors, including Non Small Cell Lung Cancer (NSCLC)[8-11]. Xia et al[12] reported that the down-regulation of MEG3 could promote the resistance of lung cancer cells to drug resistance of DDP through the Wnt/β-catenin signaling pathway. LncRNA GAS5 regulated NSCLC cell resistance to DDP by autophagic cell death[13]. LncRNA NEAT1 can enhance the sensitivity of lung cancer cells to DDP as a molecular sponge combined with miR-98-5p[14].

HOTTIP, as an important lncRNA, plays an important role in many tumors, including non-small cell lung cancer[15], liver cancer[16,17] and pancreatic cancer[18]. It has a clear role in promoting resistance to formation in osteosarcoma[19] and pancreatic cancer[20]. However, its role in lung adenocarcinoma has not been studied. Therefore, the study of the role of HOTTIP in lung adenocarcinoma and its relationship with the formation of resistance could help to understand the mechanism of lung adenocarcinoma in depth and provide a theoretical basis for treatment.

Patients and Methods

Data Acquisition and Collection

Genome-wide expression profiles of lung adenocarcinoma and normal lung tissues, as well as their response to chemotherapy in The Cancer Genoma Atlas (TCGA) database, were downloaded by the GDC (Genoma Data Commons) tool. 30 patients with lung adenocarcinoma treated in our hospital, their adjacent tissues in recent 2 years, and data of chemotherapy response, were collected. This study was approved by the Ethics Committee of Jining No. 1 People's Hospital. Signed written informed consents were obtained from the patients and/or guardians.

Cell Culture

The cell-derived human lung epithelial cell line 16HBE used in this experiment was purchased from Sciencecell Research Laboratories (Carlsbad, CA, USA) and the human lung adenocarcinoma cell line A549 was purchased from the Chinese Academy of Medical Sciences (Beijing, China). Paclitaxel resistant cell line A549/PA was constructed by continuous treatment with 0.1 μmol/L paclitaxel (Sigma-Aldrich, St. Louis, MO, USA) for 1 month followed by 5 μmol/L paclitaxel to maintain paclitaxel resistance (Gibco, Rockville, MD, USA). Cells were cultured in a bottle with Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and cultured in a 5% CO_2, 37°C cell incubator.

Cell Transfection

Cells were placed in a 6-well plate. When the cell density was about 70%, cells were transfected with si-NC and si-LINC01116 sequences according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the medium was changed 6 h after transfection. HOTTIP interference sequences and overexpression plasmids were purchased from RuiBo (Guangzhou, China). The interference sequences were: si-HOTTIP1 #: GCACAGAGAUAAUGGCAAAUU; si-HOTTIP2 # AGCCACATATTCAAGAGATATG.

RNA Extraction and qRT-PCR

24 h after transfection, cells were collected with 1 mL of TRIzol, then the total cellular RNA was extracted, cDNA was reverse transcribed, and all procedures were performed as described. The expression of HOTTIP was detected by qRT-PCR. The reaction system was 5 μL. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control, and each sample was performed in triplicate. The primer sequences were: HOTTIP (Forward) 5'-CCTAAAGCCACGCTTCTTTG-3', HOTTIP (Reverse) 5'-TGCAGGCTGGAGATCCTACT-3'; GAPDH (Forward) AGGAGCGAGATCCCGCCAACA, GAPDH (Reverse) CGGCCGTCACGCCACATCTT.

Cell Proliferation Assay by Cell Counting Kit-8 (CCK8) Method

Two groups of transfected cells were placed into 96-well plates. The serum-free medium was replaced after it was cultured for 6, 24, 48, 72 and 96 h. 10 μL of CCK8 were added to each well. After incubation at 37°C and 5% CO_2 for 1 h, the OD value was measured at 450 nm. Each measurement was performed in quintuplicate.

Drug Resistance Experiment

Cells were cultured in medium containing different concentrations of Paclitaxel (0.01, 0.1, 1, 5 and 10 μmol/L). Cell viability was detected by the CCK8 assay 48 h later. The half maximal inhibitory concentration (IC50) was calculated by the Probit regression model.

Western Blot

Expression alteration of AKT protein in transfected cells were detected, A549/PA cells were transfected with si-HOTTIP, pcDNA-HOTTIP and NC, respectively, and then cells were lysed with cell lysate after 72 h. The total protein was extracted; the protein of bicinchoninic acid (BCA) method was quantified for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, the electrophoretically separated protein was electro transferred to the NC membrane. 10% skim milk was used for blocking at room temperature for 1 h, 1:500 mouse anti-human AKT antibody and 1:1000 mouse anti-human GAPDH antibody were added and incubated at 4°C overnight. The membrane was washed with Tris-buffered saline and Tween 20 (TBST) for 3 times. Goat anti-mouse secondary antibody (diluted 1:2000) was added and incubated at 37°C for 1 h and washed 3 times with Tris-buffered saline and Tween 20 (TBST). Enhanced chemiluminescence (ECL) detection was performed. GAPDH was used as an internal reference for analyzing the results.

Statistical Analysis

Statistic package for social science (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used to analyze the data. The measurement data were expressed as mean ± difference (`x ± s), and the data were compared by *t*-test. Graphpad software was used for drawing. $p<0.05$ indicated that the difference was statistically significant.

Results

HOTTIP is Highly Expressed in the Lung Adenocarcinoma From TCGA Database and is Also Highly Expressed in the Chemotherapy-Insensitive Group

The normal lung and lung adenocarcinoma tissue expression profiles were downloaded from the TCGA website via the GDC tool, as well as the profiling data for results of treatment regimens of these patients. By analyzing the normal and lung adenocarcinoma tissues by the edger R package, we found that HOTTIP was overexpressed in lung adenocarcinoma (Figure 1A and 1C). The same method was used to analyze sensitive and insensitive data from lung cancer and found that HOTTIP was more expressed in the chemotherapy-insensitive treatment group (Figure 1B and 1D).

HOTTIP is Highly Expressed in Lung Adenocarcinoma and in Chemotherapy-Sensitive Groups

The results of qRT-PCR showed that the expression of HOTTIP in 30 cases of lung adenocarcinoma was significantly increased (Figure 2A), and the expression of HOTTIP in chemotherapy-insensitive group was significantly increased (Figure 2B). These data suggested that HOTTIP may be involved in the development of lung adenocarcinoma and the formation of drug resistance mechanisms.

HOTTIP Can Promote the Proliferation of Lung Adenocarcinoma Cells

To explore the role of HOTTIP in lung adenocarcinoma progression, we constructed the HOTTIP small interference sequence and overexpression plasmids. By qRT-PCR experiments, we found that HOTTIP expression in A549 cell line increased significantly, and was more increased in resistant A549 cell line (Figure 2C). Then, we screened out the most efficient siRNAs by PCR. After transfection of siRNA into A549 cell line, we found that proliferation of A549 cell line was weakened (Figure 2D and 2F). At the same time, we found that proliferation of A549 cells was significantly enhanced after transfection with the HOTTIP overexpression plasmid (Figure 2E and 2G). These findings suggested that HOTTIP can significantly promote the proliferation of lung adenocarcinoma cells.

HOTTIP Can Promote Drug Resistance of Lung Adenocarcinoma Cells

Based on the above results, we found that the expression of HOTTIP in drug-insensitive and drug-resistant cell lines was significantly higher than that in chemotherapy-sensitive and non-drug resistant cell lines. These results suggested that HOTTIP may be involved in drug resistance of lung adenocarcinoma. Results showed that the IC50 value of A549 cell line and A549/PA cell line were 2.13 ± 0.15 and 7.03 ± 0.52 μmol/L, respectively (Figure 3A and 3B). These results suggested that A549/PA was more drug resistant.

To further explore the role of HOTTIP in lung adenocarcinoma resistance, we found that after knockdown of HOTTIP, the IC50 value of A549/PA was significantly decreased (Figure 3E). After over-expressing HOTTIP, the value of A549/PA was significantly increased (Figure 3F). These results indicated that HOTTIP promoted A549/PA resistance formation. In addition, the expres-

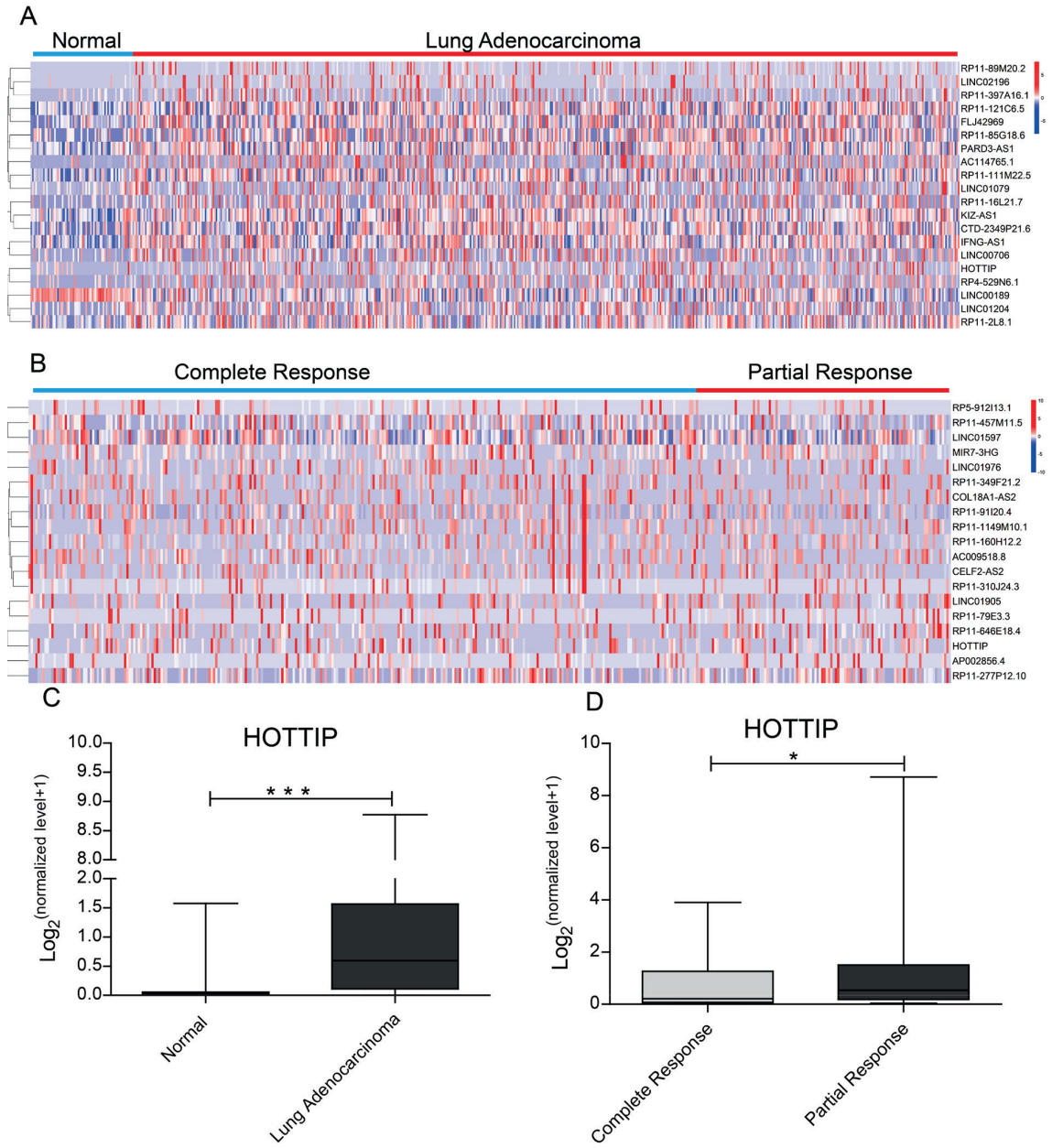

Figure 1. HOTTIP is upregulated in LAD and partial response tissue. ***A,*** Heatmap of dysregulated LncRNA in normal lung tissue and LAD. ***B,*** Heatmap of dysregulated LncRNA incomplete response patients and partial response patients. ***C,*** HOTTIP was up-regulated in LAD. ***D,*** HOTTIP was up-regulated in partial response patients.

sion of AKT was significantly decreased after interfering with HOTTIP, and the expression of AKT was significantly increased after HOTTIP was over-expressed (Figure 3G).

Discussion

To date, except for surgery, chemotherapy is the most important treatment for lung cancer, especially for NSCLC. Chemotherapy can inhibit the recurrence and metastasis of NSCLC to some extent and improve the prognosis of patients. Chemoresistance, however, produced during chemotherapy may reduce the effectiveness of chemotherapy and limit the clinical use of chemotherapy. The HOX gene is a type of organism that specializes in regulating biological forms of genes. According to the sequence similarity and chromosomal location, HOX genes can be

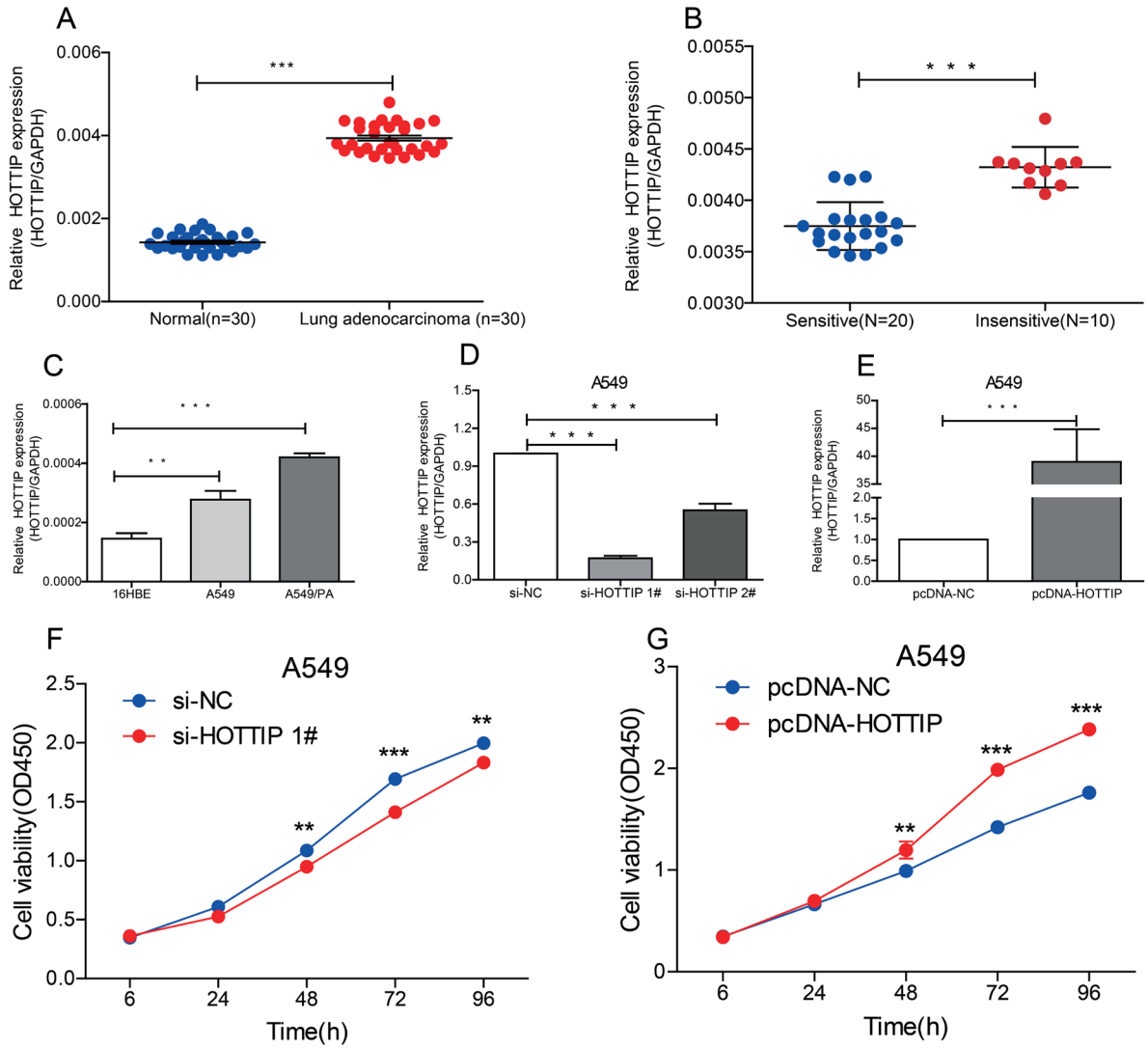

Figure 2. HOTTIP could promote tumor proliferation in LAD. ***A,*** HOTTIP was up-regulated in LAD patients (n = 30). ***B,*** HOTTIP was up-regulated in insensitive patients (n = 10). ***C,*** HOTTIP was up-regulated in A549 cell line and higher in A549/PA cell line. ***D,*** The efficiency of si-HOTTIP in A549 cell line. ***E,*** The overexpression efficiency of pcDNA-HOTTIP in A549 cell line. ***F,*** Knockdown of HOTTIP could depress the proliferative ability in A549 cell line. ***G,*** Overexpression of HOTTIP could promote the proliferative ability in A549 cell line.

divided into 4 clusters, which are HOXA (chromosome 7), HOXB (chromosome 17), HOXC (chromosome 12) and HOXD (chromosome 2). The expressed proteins of HOX gene regulate embryonic development and cell growth and differentiation through DNA sequence-specific binding[21]. Wang et al[22] firstly found a lncRNA localized to the 5' end of the HOXA chromosome in fibroblasts; it was located at the distal end of the body (e.g., hands, feet, and foreskin). This lncRNA was located on chromosome 7p15.2, with full-length of 3764 nt. Therefore, this lncRNA was named as "HOXA transcript at the distal tip (HOTTIP)". Li et al[19] found there was a significant difference of HOTTIP expression in pancreatic ductal and chronic pancreatitis by gene chip comparison. They also found that the expression of HOTTIP in pancreatic cancer tissue was significantly increased compared with the paracancerous tissue. It was further confirmed by qRT-PCR that HOTTIP was overexpressed in human pancreatic ductal carcinoma cell lines. Using siRNA interference technology to down-regulate the expression of HOTTIP in pancreatic ductal carcinoma cells, the proliferation and invasion ability of cancer cells were significantly reduced.

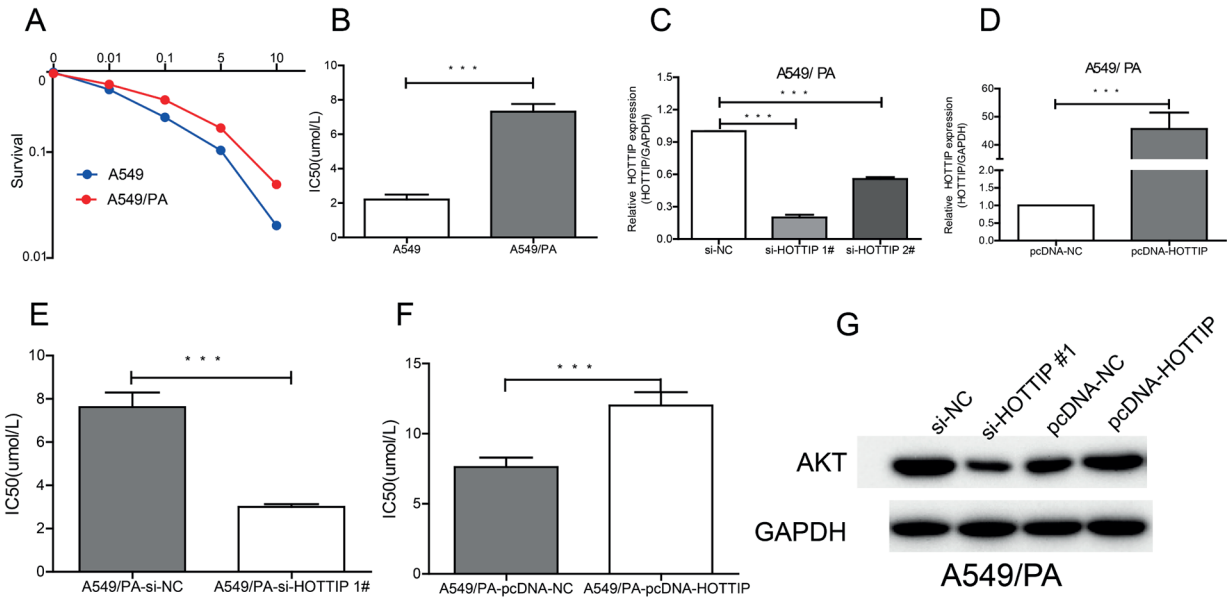

Figure 3. HOTTIP could promote drug resistance in A549. ***A-B,*** The IC50 in A549/PA cells was up-regulated compared with A549 cells. ***C,*** The efficiency of si-HOTTIP in A549/PA cell line. ***D,*** The overexpression efficiency of pcDNA-HOTTIP in A549/PA cell line. ***E,*** The IC50 of A549/PA cells to paclitaxel was depressed by HOTTIP knockdown. ***F,*** The IC50 of A549/PA cells to paclitaxel was increased by HOTTIP overexpression. ***G,*** AKT was decreased by HOTTIP knockdown and increased by HOTTIP overexpression.

Meanwhile, the sensitivity of cancerous cells such as gemcitabine and other chemotherapy drugs after the down-regulation of HOTTIP was also improved[23]. Quagliata et al[17] firstly reported that HOTTIP and HOXA13 were highly expressed in hepatocellular carcinoma samples, and their expression levels were correlated with the malignancy of the tumor. The proliferation and migration of hepatoma cell lines that knocked out the HOTTIP gene were significantly delayed, suggesting that HOTTIP was involved in the proliferation and metastasis of hepatocellular carcinoma[22]. TSANG and other animal experiments found that *in vivo* knockdown of HOTTIP can significantly reduce tumorigenicity and metastasis[24]. A large number of evidence suggested that miRNAs also silenced lncRNA gene expression[25-27]. miR-125b has been shown to inhibit endogenous HOTTIP expression as a negative transcriptional regulator of HOTTIP[24].

In the present study, we first found that HOTTIP was highly expressed in lung adenocarcinoma tissue by analyzing TCGA database, the expression of which was higher in chemotherapy-insensitive group than in sensitive group. In lung adenocarcinoma tissues, we also found that HOTTIP expression was significantly increased in lung adenocarcinoma and was higher in the treatment-insensitive group. In addition, we found that in the group of advanced lung adenocarcinoma, the expression of HOTTIP was higher than that of the early stage, and the larger the tumor volume was, the higher the expression of HOTTIP was. This suggested that HOTTIP played an important role in the progression of lung adenocarcinoma and the resistance formation of lung adenocarcinoma. To explore the role of HOTTIP in lung adenocarcinoma tumor progression, we constructed siRNA and overexpression plasmids to exogenously regulate the expression of HOTTIP. After interfering with HOTTIP, proliferation of A549 decreased significantly, in contrast, after over-expression of HOTTIP, proliferation of s549 was significantly enhanced. To explore the role of HOTTIP in lung adenocarcinoma resistance, we examined the IC50 of paclitaxel in A549 and A549/PA and found that the IC50 of A549/PA was higher. At the same time, the drug resistance index of A549/PA was higher. Next, we found that the IC50 value of HOTTIP in A549/PA was significantly decreased, and the IC50 value was significantly increased after HOTTIP was over-expressed. AKT is a key regulator of AKT pathway, a large number of studies have shown that it had an important relationship with drug resistance[28-30]. Our Western blot results showed

that after knockdown of HOTTIP, the expression of AKT was significantly decreased. After over-expressed HOTTIP, AKT expression was significantly increased. These results suggested that HOTTIP may modulate the formation of lung adenocarcinoma resistance through the AKT pathway.

In this work, HOTTIP was proved to promote the progression of lung adenocarcinoma by analyzing database, clinical samples and basic cell biology experiments, and it can promote the drug resistance formation of lung adenocarcinoma.

Conclusions

We showed that HOTTIP can promote the progress of lung adenocarcinoma, and the resistance formation of lung adenocarcinoma via AKT signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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