Long non-coding RNA *HOTTIP* promotes tumor growth and inhibits cell apoptosis in lung cancer

H-P. Deng, L. Chen, T. Fan, B. Zhang, Y. Xu and Q. Geng*

Department of Cardiothoracic Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei, China

**Corresponding author:** Qing Geng, Department of Cardiothoracic Surgery, Renmin Hospital of Wuhan University, 238 Jie Fang Road, Wu han, 430060, PR China. E-mail: szgqing@126.com

**Abstract**

Lung cancer is one of the leading causes of cancer-related deaths worldwide. Early diagnosis is the best defense against this threat and is therefore of vital importance. In this study, we investigated the role of long non-coding RNA *HOTTIP* in the tumor growth of lung cancer. Initially, we found that expression of *HOTTIP* was significantly elevated in 20 cases of lung cancer. *HOTTIP* was also differentially expressed in a consecutive of lung cancer cell lines. Furthermore, specific siRNA against *HOTTIP* was employed to deplete expression of *HOTTIP* in A549 cells and NCI-H446 cells. After successfully depletion of *HOTTIP*, cell proliferation and colony formation were significantly inhibited in vitro. Tumor growth in vivo was also suppressed after depletion of *HOTTIP* in a mouse model of lung cancer. Moreover, depletion of *HOTTIP* caused cell cycle arrest in G0/G1 phase and induced significant cell apoptosis. Cell cycle regulators Cdc25C, Cyclin B1 and Cyclin D1 were downregulated, whereas anti-apoptotic factors Bcl-2 and Bcl-xL were up-regulated after *HOTTIP* ablation. These data suggest that lncRNA *HOTTIP* contributes to tumor growth in vivo and in vitro and inhibits cell apoptosis in lung cancer.

**Key words:** lncRNA *HOTTIP*, tumor growth; apoptosis, cell cycle, lung cancer.

**Introduction**

Lung cancer is one of the leading causes of cancer-related deaths around the world (1). Among the lung cancers, non-small cell lung cancer (NSCLC) currently accounts for approximately 80% of all cases (2, 3), of which more than 65% present with locally advanced or metastatic disease. Lack of effective biomarkers and imageology for early detection are the main contributors to the poor prognosis of lung cancer. Newly emerged molecular targeted agents have undoubtedly achieved longer survival in some subtypes of this disease. However, conventional cytotoxic chemotherapy has limited the application of these agents in the treatment of advanced stage of lung cancer (4). Development of chemotherapy resistance even frustrates physicians which accounts for high recurrence rates. Similarly, while transcriptional factor Specificity protein 1 (Sp1) was reported to be required for lung tumor growth and suppress metastasis, it was only accumulated strongly in early stage and declined in late stage (5), with that in breast cancer (6), indicating that inhibition of Sp1 was only seemingly effective in early stage of these malignancies. These findings greatly necessitate our looking for novel developments of effective biomarkers for lung cancer progression.

Recently, the emergence of long non-coding RNAs (lncRNAs) has greatly advanced our understanding of human tumorigenesis. lncRNAs are defined as transcripts with over 200 nucleotides and are typically transcribed by RNA polymerase II (7). Functionally, lncRNAs work as molecular scaffolds for promoting protein-DNA and protein-protein interactions; they also serve as decoys that stabilize interactions between distal and proximal macromolecules (8-11). Mounting reports have documented that aberrant expression of lncRNAs is widely involved in human tumorigenesis and controls the oncogenic activities of cancer cell, such as proliferation, survival and migration/invasion (10, 12). Among the discovered lncRNAs, HOX-associated lncRNAs are of great biological importance. For instance, HOX transcript antisense RNA (*HOTAIR*) and HOXA trans-cript at the distal tip (*HOTTIP*) are two mostly studied lncRNAs in this subgroup, of which *HOTAIR* is the most documented one. *HOTAIR* is a 2.2 kb lncRNA located in the mammalian HOXC locus which serves as sequence-specific scaffold for at least two complexes related to histone modifications, namely polycomb repressive complex (PRC2) and the LSD1/CoREST/REST complex (10-12). Current studies have widely reported that *HOTAIR* is a crucial pro-oncogenic factor that predicts tumor recurrence in hepatocellular carcinoma (HCC) (13). *HOTAIR* is also a negative prognostic factor in various types of cancer, including colorectal cancers (14, 15), pancreatic cancer (16), and oesophageal squamous cell carcinoma (17).

As another HOX-associated lncRNA, *HOTTIP* is also associated with the PRC2 and WDR5/MLL1 chromatin modifying complexes and directly binds WDR5 (18). *HOTTIP* primarily coordinates the activation of 5’HOXA genes in fibroblasts (18), and recent studies showed a close association between *HOTTIP* and HOXA13 in HCCs (19) and pancreatic cancer (20). *HOTTIP* promoted cell proliferation, invasion, and chemoresistance by regulating HOXA13 in pancreatic cancer (20). However, beadchip array argued that *HOTTIP* did not regulate HOXA13 in pancreatic cancer cells, but played a role in regulating several other HOX genes including HOX1, HOXA9, HOXA10, HOXA11 and HOXB2 (21). These findings indicated that *HOTTIP* might have the similar pro-oncogenic functions as the HOX-associated lncRNA *HOTAIR*, but each lncRNA...
might have distinct sets of target genes in human tumors.

Despite the reports in HCC and pancreatic cancer, the role of lncRNA HOTTIP in human lung cancer remains unclear. Herein, the present study aims to determine whether HOTTIP plays critical roles in lung cancer progression in vitro and in vivo. Expression of HOTTIP in lung cancer tissues would be initially assessed, and effects of HOTTIP knockdown in lung cancer cell survival will also be explored.

**Materials and methods**

**Cells and reagents**

Human lung cancer cell lines SK-LU-1, NCI-H1975, NCI-H157, A549, NCI-H446 and Calu-3 were all purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in Dulbecco’s modified eagle medium (DMEM) media (Gibco, Los Angeles, CA, USA) supplied with 10% fetal bovine serum (FBS, Gibco) in a humidified incubator containing 5% CO₂ at 37°C. Cell culture media was refreshed every two days. For cell proliferation detection, CCK-8 kit was purchased from Dojindo (Japan). All the primary and secondary antibodies were purchased from Santa Cruz Biotechnology (CA, USA).

**Human tissues**

A total of twenty cases of lung cancer patients who were admitted to Remin Hospital of Wuhan University were collected. These cases were diagnosed with lung cancer in different stage. Their adjacent non-cancerous tissues were also obtained. All patients were informed of our study protocol and showed their full consent to participate into the present study. Permission to use human tissue sections for research purposes was obtained and approved by an institutional review board at Remin Hospital of Wuhan University.

**Construction of HOTTIP-knockdown lentivirus**

For knockdown of HOTTIP, specific HOTTIP shRNA (shHOTTIP) and scramble control shRNA (shCtr) were inserted into the pLVX-tdTomato-Puro lentiviral vector (Open Biosystems, Rockford, IL). shHOTTIP and shCtr sequences were described previously (20) with shHOTTIP target sequence as 5’-GAUAAUGGCAAAUU-3’.

shRNA lentivirus was used to generate stable HOTTIP-knockdown cells. Our procedure was in accordance with a previous report (20). After construction of HOTTIP-knockdown cells, lentivirus-delivered shRNAs were subject to infect lung cancer cell lines. Briefly, cells were seeded into a six-well plate at a concentration of 8×10⁴ cells/well and infected with the lentivirus at the multiplicity of infection (MOI) of 50. After 96h of co-incubation, infection efficiency was determined by quantitative real-time PCR (qRT-PCR) and under fluorescence microscopy by counting the percentage of GFP positive cells.

**qRT-PCR**

Total RNAs were extracted using TRizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer’s instructions. Extracted total RNAs were then immediately converted into cDNA by inverse transcription using oligoT primers and SuperScript II reverse transcriptase (Invitrogen). For qRT-PCR, each sample was amplified in triplicate in a 20-μL reaction system containing SYBR Green reaction mix (Qiagen,Germany) and 0.5 mM of primers. Samples were analyzed using a Roche Light-Cycler (Roche, Basel, Switzerland). GAPDH was used as an internal control. Primers for HOTTIP were as follows:

- Forward, 5’-CCTAAGCACGCTTCTTTG-3’
- Reverse, 5’-TGCAAGCTGGAGATCTAGT-3’

Sequences for GAPDH primers were as follows:

- Forward, 5’-GTGGACATCCGGAAAGAC-3’
- Reverse, 5’-AAAGGTGTAAACGCAAATCT-3’

Relative gene expression was calculated using the formula 2⁻△CT (CT, cycle threshold) where△CT=CT value of target gene–CT value of GAPDH.

**Western blot analysis**

Total proteins from cells were extracted and subjected to one-dimensional SDS-PAGE electrophoresis. After that, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by using a transfer apparatus. Membranes were later blocked by 5% skim milk in TBS buffer containing 0.05% Tween-20 (TBST) for 1h at room temperature. Subsequently, membranes were washed with TBST three times, and probed with corresponding primary antibodies overnight at 4°C. The secondary antibody was incubated with the membrane at the presence of blocking agent for 1h at room temperature. After three washes with TBST, immune complexes were developed using enhanced chemoluminescent autoradiography (ECL) kit (Amersham, Pittsburgh, PA, USA). GAPDH was synchronously developed as loading control.

**Cell viability assay**

For detection of cell viability, control and HOTTIP-depleted A549 cells and NCI-H446 cells were incubated into 96-well plates (5000 cells/well). Cell viability was monitored for a consecutive of 6 days. On each monitored day, 10μl of CCK-8 solution was added to each well. After incubated for another 2h at 37°C, the absorbance value for each well was determined using a synergy 2 multi-mode microplate reader (Bio Tek Instruments, Winooski, VT, USA) at 570 nm.

**Colonoy formation assay**

Control and HOTTIP-depleted lung cancer cells in the logarithmic growth phase were initially digested with a trypsin solution (Gibco, USA), and then 2ml of the single-cell suspension was seeded onto 10cm plates (800 cells/well) and allowed to form natural colonies. After 12 days of incubation, both groups of colony were stained with crystals violet, followed by washing twice with PBS. Subsequently, cells were fixed by paraformaldehyde and washed again with PBS solution, which was later photographed with an inverted microscope. Colonies with over 50 cells were manually counted.

**Flow cytometry**

A549 cells and NCI-H446 cells which were depleted of HOTTIP (shHOTTIP group) or not (shCtr) were harvested for cell cycle analysis with flow cytometry. Cells were initially fixed in 70% pre-cold ethanol and
Xenograft model of lung cancer

Twelve male BALB/c-nude mice were purchased from SLRC Laboratory Animal Co. (Shanghai, China). Mice were all six-week old and randomly divided into two groups (n=6 per group), namely shHOTTIP group and shCtr group. Mice from each group were housed with ad libitum access to water and food in temperature and light-controlled environment. An equal amount of A549 cells, either depleted of HOTTIP or not, were injected into the right flank of the corresponding group of mice. Tumor dimensions were measured once a week and tumor volume was calculated as described previously (22). Five weeks after the injection, all mice were sacrificed and tumors were dissected for weighing. The protocol of animal experiment was approved by the Institutional Animal Care of Remin Hospital of Wuhan University. All efforts were made to minimize sufferings.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Differences between groups were analyzed with the Student’s t-test using SPSS version 16.0 software. A p-value of less than 0.05 was considered significant.

Results

IncRNA HOTTIP is highly expressed in human lung cancer tissues

Initially, expression of HOTTIP in clinical lung cancer was determined. A total of 20 cases of lung cancer were collected. qRT-PCR showed that expression of HOTTIP was significantly higher in the tumor tissues than the adjacent ones, with the fold changes up to 10 in the No.9 case.
Successful construction of lentivirus-delivered shRNA against HOTTIP in lung cancer cell lines

In view of the high expression of HOTTIP in lung cancer tissues, we further determined the expression of HOTTIP in a set of lung cancer cell lines, including SK-LU-1, NCI-H1975, NCI-H157, A549, NCI-H446 and Calu-3. Our data showed that HOTTIP was differentially expressed in these cell lines. A549 cells and NCI-H446 cells exhibited the highest expression of HOTTIP (Figure 2A), making these two cell lines optimal for subsequent analyses. We used A549 and NCI-H446 cell lines as models of lung cancer for construction of lentivirus-delivered shRNA against HOTTIP (shHOTTIP). After construction, fluorescent microscope showed that both cell lines were highly infected with lentivirus (Figure 2B). Furthermore, qRT-PCR showed that infection with shHOTTIP instead of shCtrl significantly decreased expression of HOTTIP in A549 cells and NCI-H446 cells (Figure 2C). These data suggest the successful construction of lentivirus-delivered shRNA against HOTTIP in lung cancer A549 cells and NCI-H446 cells.

Depletion of HOTTIP slowed down tumor growth in vitro

To investigate effects of HOTTIP knockdown on lung cancer progression, we assessed cell viability and colony formation abilities. Cell viability was monitored in a consecutive of 6 days. We found that cell proliferation rates between shHOTTIP and shCtrl groups began to differ since day 3 in both cell lines. By day 6, cell proliferation rate in shHOTTIP group was significantly lower, accounting for only approximately 50% of the control A549 cells, and about 60% of control NCI-H446 cells (Figure 3A). Consistently, colony formation was also inhibited in both cell lines. Remarkable colonies were observed in the control cells, whereas colonies were rarely present in the shHOTTIP groups. Quantification of the colonies revealed that the number of colony was decreased by up to 57.1% in shHOTTIP-infected A549 cells and even by 66.7% in shHOTTIP-infected NCI-H446 cells (Figure 3C). These observations suggest that depletion of HOTTIP significantly inhibited tumor growth in lung cancer in vitro.

Depletion of HOTTIP arrested cell cycle and induced cell apoptosis

Furthermore, we examined the effects of HOTTIP knockdown on cell cycle progression and cell fate. Strikingly, flow cytometry showed that cell cycle was disturbed in both cell lines after depletion of HOTTIP (Figure 4A). Cell proportions in G0/G1 phase were significantly higher than control cells when HOTTIP was depleted. On the contrary, cell proportions in S phase and G2/M phase were consistently decreased in HOTTIP-depleted A549 cells and NCI-H446 cells (Figure 4B). Morphological changes were also revealed by the Hoechst 33342 staining. In the control cells, the nuclei were stained as homogeneously blue, whereas in the shHOTTIP groups, nuclear fragmentation and bright chromatin condensation were observed (Figure 4C). Apoptotic cells were counted. It was shown that numbers of apoptotic cells were significantly increased by depletion of HOTTIP in both A549 cells and NCI-H446 cells (Figure 4D). These findings suggest that depletion of HOTTIP arrested cell cycle and induced cell apoptosis.

Depletion of HOTTIP inhibited tumor growth in vivo

Moreover, we established a xenograft model of lung cancer. Tumor dimensions were monitored periodically. Tumor volume was calculated accordingly. On week 2, we found that HOTTIP-depleted group exhibited smaller size of tumor than the control mice. By the fifth week, average tumor size in shHOTTIP group was approximately 200mm³, whilst it was over 600 mm³ in the control mice (Figure 5A). All mice were sacrificed on the fifth week. Tumors were dissected and weighed. It was measured that the average tumor weight in shHOTTIP-infected group was significantly lower than the control group.
HOTTIP was significantly decreased as compared with control group (Figure 5B). Consistently, tumor sizes in macroscopic view were generally smaller in shHOTTIP group relative to control group (Figure 5C). Moreover, immunohistochemistry staining showed that PCNA, a nuclear marker indicating cell proliferation, was accordingly less stained in HOTTIP-depleted group. These data strongly suggest that depletion of HOTTIP significantly slowed down tumor growth in the mouse model. In addition, cleaved-caspase-3 which initiates the apoptosis process was strongly stained and up-regulated after depletion of HOTTIP, reinforcing the notion that depletion of HOTTIP induced cell apoptosis.

Knockdown of HOTTIP interrupted expression of cell cycle regulators and apoptosis-related factors

Furthermore, we analyzed expression of cell cycle regulators and apoptosis-related factors in response to knockdown of HOTTIP in A549 cells and NCI-H446 cells. as shown in Figure 6, knockdown of HOTTIP decreased the key cell cycle regulators Cyclin B1, Cyclin D1 and Cdc25C. Bcl-2 and Bcl-xL which are representatives of anti-apoptosis were also decreased in response to HOTTIP knockdown. However, Bad which represents the pro-apoptotic factor was increased after knockdown of HOTTIP. These data were in consistence with above observations that depletion of HOTTIP arrested cell cycle and induced apoptotic death in lung cancer cells.

Discussion

Lung cancer is one of the most prevalent malignancies worldwide, ranking as the first and second leading causes of cancer mortality in males and females, respectively. Lung adenocarcinoma (LAD) is histologically the most common subtype of NSCLC (23). Unfortunately, affected patients have only limited access to early diagnosis and timely interference. Hence, early detection is the best defense against lung cancer and is therefore of vital importance (24).

Currently, lncRNAs have emerged as key regulators of tumor development and progression. The present study proposed that lncRNA HOTTIP played critical roles in lung cancer growth in vitro and in vivo. In the collected 20 cases of lung cancer, expression of HOTTIP in cancer tissues was consistently higher than that in the adjacent non-cancerous tissues. Knockdown of HOTTIP by specific shRNA caused significant inhibition of cell proliferation and colony formation, as well as the tumor growth in a mouse model. While knockdown of HOTTIP slowed down tumor growth, it arrested cell cycle at G0/G1 phase, and induced significant cell apoptosis. This was also supported by our immunoblot analysis. Knockdown of HOTTIP consistently decreased the expression of cell cycle regulators Cyclin B1, Cyclin D1 and Cdc25C and also suppressed expression of Bcl-2 and Bcl-xL, whereas it increased expression of Bad. Cyclin B1, Cyclin D1 and Cdc25C are critical cell cycle regulators and apoptosis-related factors.
cycle regulators which promote cell cycle progression (25). Decreased expression of these proteins supported the notion that depletion of HOTTIP induced cell cycle arrest. Furthermore, Bad is a pro-apoptotic factor, while Bel-2 and Bel-xL are classic anti-apoptotic factors (26). Inhibition of anti-apoptotic factors with the promotion of pro-apoptotic factor Bad strongly suggested that depletion of HOTTIP led to the eventual cell apoptosis after inducing the cell cycle arrest. Taken together, our conclusive data suggest that HOTTIP is a critical mediator of lung cancer growth in vivo and in vitro. HOTTIP could promote cell cycle progression and inhibit the apoptotic death in lung cancer cells.

This is the first report to our knowledge to investigate the role of HOTTIP in human lung cancer. Previous studies have widely reported that HOTAIR, a HOX-associated lncRNA, serves as a critical regulator which is involved in multiple cancers, including HCC, colorectal cancer and pancreatic cancer (10, 13, 14, 17). Similarly, HOTTIP was also reported to be a critical mediator of cancer progression in HCC and pancreatic cancer (19, 20). The significance of our findings would be two-folds: first, our data suggest that HOTTIP also plays critical roles in lung cancer growth. These findings not only extend the list of cancers that HOTTIP is involved in, but also suggest the oncogenic activities of HOTTIP in various human tumors; Second, HOTTIP is also a HOX-associated lncRNA as HOTAIR. Our findings may suggest that HOTTIP and HOTAIR have the similar oncogenic functions in human tumors since they derive from the same subtype of lncRNAs.

One interesting question would be the detailed mechanisms of how HOTTIP regulates lung cancer progression. Current studies have showed that HOTTIP is closely associated with HOXA13. The levels of HOTTIP and HOXA13 were both up-regulated and associated with HCC patients’ clinical progression and predicated disease outcome in HCC. However, a recent study by beadchip array analysis argued that HOTTIP did not regulate HOXA13 in pancreatic cancer cells, but played a role in regulating several other HOX genes including HOXA1, HOXA9, HOXA10, HOXA11 and HOXB2 (21). Hence, current evidences are still controversial. One hypothesis would be that HOTTIP may exhibit distinct mechanisms in pancreatic cancer and liver cancer. However, another report on pancreatic ductal adenocarcinoma showed that knockdown of HOTTIP decreased expression of HOXA13, and HOXA13 partially mediated the effect of HOTTIP (20), leading us to speculate that HOTTIP may regulate strikingly different sets of genes in distinct types of cancers and even at different stages. However, more work needs to be done.

In all, we studied the roles of lncRNA HOTTIP in lung cancer growth in vitro and in vivo. Depletion of HOTTIP significantly slowed down tumor growth and induced cell apoptosis in lung cancer. HOTTIP promotes lung cancer growth possibly by inhibition of cell apoptotic death. The detailed mechanisms remain to be elucidated. Our findings may serve as strong evidence for development of novel therapeutic strategies against lung cancer.

Acknowledgments
This project was supported by the National Natural Science Foundation of China (No. 81170076).

References
16. Kim, K., Jutooru, I., Chadalapaka, G., Johnson, G., Frank, J., Burghardt, R. et al. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene, 2013,
32:1616-1625, doi:10.1038/onc.2012.193