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Original Research

Long noncoding RNA HOTTIP contributes to the progression of prostate cancer by regulating HOXA13

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Abstract: Prostate cancer is a leading cause of cancer-related mortality in men worldwide and there is a lack of effective treatment options for advanced (metastatic) prostate cancer. Long non-coding RNAs (lncRNAs) play important roles in diverse biological processes, such as cell growth, apoptosis and migration. However, little is known about the molecular mechanism of lncRNA-HOTTIP-mediated prostate cancer cell proliferation and apoptosis. The aim of this study was to elucidate the involvement of lncRNA HOTTIP in prostate cancer tumorigenesis and further investigate the role of HOXA13 in this process. Here, we showed that HOTTIP silencing inhibited cell survival pathway *in vitro* and *in vivo* by reducing the protein expression of Bcl-2 and enhancing Bax. We further demonstrated that knockdown of HOTTIP inhibited the expression of cell cycle regulatory protein Cyclin D1 and induced cell cycle arrest in G0/G1 phase. Additionally, depletion of HOXA13 by RNA interference (si-HOXA13) revealed that HOTTIP silencing suppressed cell growth at least partly through regulating HOXA13. In conclusion, down-regulation of HOTTIP and HOXA13 was associated with cell growth and cell cycle, and exerts tumor-suppressive functions in the genesis and progression of prostate cancer, providing a potential attractive therapeutic approach for this malignancy.

Key words: Prostate cancer, Long noncoding RNA HOTTIP (lncRNA-HOTTIP), HOXA13, proliferation, apoptosis.

Introduction

Prostate cancer is one of the most commonly diagnosed cancer and the second most common cause of cancer death in men in the US. Thus, understanding carcinogenesis and development mechanism of prostate cancer is urgently needed for developing new therapies. However, the human transcriptome comprises largely of non-coding RNAs (ncRNAs) and there is currently much interest in the roles of such transcripts both in basic biology and in major pathologies such as cancer (1).

Long non-coding RNAs (lncRNAs), which are currently defined as transcripts containing >200 nucleotides without evident protein coding function, were once considered to be transcriptional "noise".[2] But more and more studies have revealed that lncRNAs play significant roles in a large range of biological processes, including cell differentiation, proliferation and apoptosis (2, 3). Moreover, they are also important factors in pathophysiology including cancer. Importantly, a number of cancer-specific lncRNAs have been identified, which may be employed as novel biomarkers for diagnosis and as therapy targets (4, 5).

HOXA transcript at the distal tip (HOTTIP) is HOX-associated lncRNA transcribed from the 5′ tip of the HOXA locus, and HOTTIP is associated with the PRC2 and WDR5/MLL1 chromatin modifying complexes and directly binds WDR5. HOTTIP primarily coordinates expression of genes associated the HOXA locus in fibroblasts (6). Accumulating evidences showed a close association between HOTTIP and HOXA13 (7). Another recent study reported that HOTTIP enhances pancreatic cancer cell proliferation, survival and migration (8). A previous study also showed that HOTTIP is a negative prognostic factor in patients with liver cancer, and increased HOTTIP expression was associated with enhanced liver cancer metastasis (7). However, the underlying role and mechanism of HOTTIP in prostate cancer remain unknown. The focus of this study was to identify the roles that HOTTIP plays in prostate cancer, and to uncover the potential mechanisms by which HOTTIP contributes to disease pathogenesis.

In the present study, we explored the role of HOTTIP in the regulation of proliferation, cell cycle, and tumorigenesis of prostate cancer. We showed that targeted silencing of HOTTIP suppressed cell proliferation, cell cycle, and tumorigenesis. Furthermore, we demonstrated that HOXA13, which is located in physical contiguity with HOTTIP, is a significant target of HOTTIP, and is involved in the progression of prostate cancer.

Materials and Methods

Cell culture

The prostate cancer cell lines (PC3 and DU145) were purchased from Chinese Academy of Sciences (Shanghai, China). Both were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco).

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RNA interference and plasmid transfection

Small interfering RNAs (siRNAs) for HOTTIP (si-HOTTIP), HOXA13, and a non-specific control (si-NC) were purchased from Sigma-Aldrich. The cells were incubated at 37°C in a humid atmosphere with 5% CO_2. Cells were transfected with si-HOTTIP or si-NC using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested after 48 hours of transfection for RNA and protein extraction. The stably transfected cells were prepared for tumor formation assay in nude mouse model.

RNA isolation and quantitative real-time Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed for HOTTIP and HOXA13, with GAPDH as an internal control. Total RNA was then converted to cDNA by reverse transcription using oligodT primers and SuperScript II reverse transcriptase (Invitrogen). qRT-PCR was performed for HOTTIP and HOXA13 mRNA using GAPDH as an internal control. For qRT-PCR, three replicates of each sample were amplified in a 20 µl reaction mixture containing SYBR Green reaction mix (Qiagen, Germany) and 0.5 mM of primer, and analyzed using a Roche Light-Cycler (Roche, Basel, Switzerland). Relative transcriptional folds were calculated as $2^{-\Delta\Delta Ct}$.

Western blot analysis

Cells were washed in PBS and lysed with RIPA buffer (Thermo Scientific, Rockford, IL, USA), and a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) was used to calculate the protein concentration of each sample. Equivalent amounts of proteins were separated by SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride membranes for immunoblotting. The membranes were blocked in 5% fat-free milk for 2 h at room temperature, washed three times, then incubated with the following primary antibodies: anti-HOXA13, anti-Cyclin D1, anti-Bax, anti-Bcl-2, and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA). The membranes were incubated with the specific HRP-conjugated secondary antibodies (Santa Cruz Biotech) at room temperature for 1 h. ECL chemiluminescence kit (Beyotime, China) was used to visualize the bands. GAPDH was used as a loading control.

Cell proliferation assay

Prostate cancer cells were seeded at a density of 2,500 cells per well in 96-well plates and transfected with HOTTIP or HOXA13 siRNA, as well as negative controls. At the indicated time points, 100 µl MTT (0.5 mg/ml, Sigma, Saint Louis, MO, USA) was added to each well and cells were incubated for 4 hours at 37°C in a humidified chamber. Then the solution was discarded and 200 µl DMSO (Sigma) was added into the well to dissolve formazan crystals. Finally, plates were shaken for 15 min for complete dissolution. The optical density (OD) at 570 nm was measured by Windows Revelation QuickLink software, on Opsys MR spectrophotometer (DYNEX Technologies, Denkendorf, Germany). All the experiments were performed in triplicate.

Flow cytometry analysis of cell cycle

Transfected PC3 and DU145 cells were cultured in six-well plates. For cell cycle assay, the cells with HOTTIP knockdown were harvested and fixed in 70% ethanol at -20°C overnight. Fixed cells were washed with PBS and incubated in 400 µl PBS, 50 µl RNase (1 mg/ml) and 10 µl propidium iodide (PI, Sigma, MO, USA) (2 mg/ml, Keygen biotech, Nanjing, China) for 30 min at room temperature, followed BD Biasciences FACS Calibur Flow Cytometry (BD Biasciences, NJ, USA). The experiment was performed independently three times for each cell line.

Tumor formation assay in a nude mouse model

Animal care and protocols were approved by the Institutional Animal Care and Use Committee of Ankang Central Hospital (China). Four-week-old athymic nude mice (BALB/C mice) were randomly divided into two groups (n = 5 in each group): si-NC and si-HOTTIP. 5×10^6 DU145 cells (0.2 ml) that stably transfected with si-NC and si-HOTTIP were injected subcutaneously on the right flanks of the mice. The diameters of the tumors were measured every three days. Twenty-four days after inoculation, the mice were sacrificed by cervical dislocation, and the tumors were resected, measured and weighed. Tumor volume was calculated using the equation: volume = $0.5 \times W^2 \times L$ (W, width; L, length).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 16.0 (IBM Chicago, IL, USA). The chi-square test (X^2 test), Fisher's exact test for non-parametric variables, and Student's t test for parametric variables were used (two tailed). The measurement data were expressed as the mean ± SD. Statistically significant differences were established at $P < 0.05$.

Results

HOTTIP regulated the cell proliferation of and cell cycle

To investigate the role of HOTTIP in prostate cancer progression, we first transfected si-NC and si-HOTTIP into PC3 and DU145 cell lines. qRT-PCR analysis of HOTTIP expression was performed 48 hours after transfection. Compared with control cells, HOTTIP expression was and reduced in both PC3 and DU145 transfected with si-HOTTIP (Figure 1A). MTT assays were performed and OD values were measured at 24, 48 and 72 h after transfection. The results revealed that depletion of HOTTIP reduced cell proliferation compared with si-NC in both cell lines (Figure 1B, C).

To further investigate the growth inhibition observed following HOTTIP knockdown, we compared the cell-cycle profiles of HOTTIP knockdown cells and controls by flow cytometry. Suppression of HOTTIP led to an increase in the percentage of cells in the G0/G1 phase and a decrease in the number of cells in the S-phase compared to the cells transfected with the scrambled siRNA in PC3 (Figure 2A, 2B) and DU145 cell (Figure 2C, 2D). These findings suggested that HOTTIP silencing could inhibit the proliferation of prostate cancer cells *in vitro*.

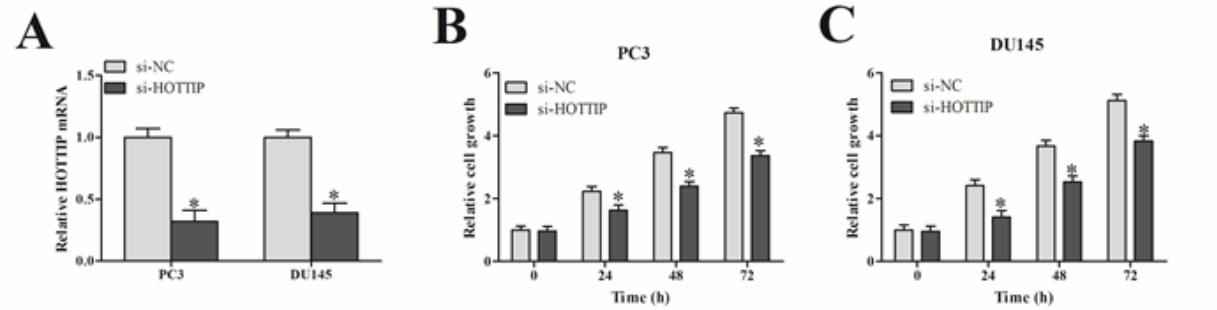

Figure 1. The effects of HOTTIP on cell proliferation. (A) HOTTIP expression levels in PC3 and DU145 cells following the treatment with a non-specific oligonucleotide (si-NC) or a siRNA against HOTTIP (si-HOTTIP). MTT assays were performed to determine the viability of PC3 (B) and DU145 (C) cells at the indicated time points after transfection. Cells transfected with a non-specific oligonucleotide (si-NC) were used as controls and significant. Data represent the mean ± S.D. from three independent experiments. **P*<0.05 vs. si-NC.

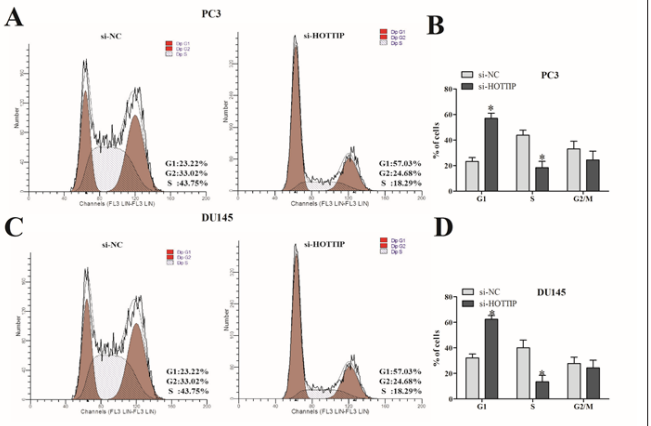

Figure 2. Effects of HOTTIP in cell cycle. The effect of si-HOTTIP (knockdown) on cell cycle distribution in PC3 **(A)** and DU145 **(C)** cells were determined by flow cytometry. **(B, D)** The respective proportion of cells in the G1 phase, S phase, and G2/M phase. Data represent the mean ± S.D. from three independent experiments. **P*<0.05 vs. si-NC.

HOTTIP regulated the expression of Bax, Bcl-2 and Cyclin D1

To explore the mechanism by which HOTTIP induced growth arrest and apoptosis, western blot assay was carried out to examine the expression of cell cycle and apoptosis associated proteins Cyclin D1, Bax and Bcl-2. As shown in Figure 3A and 3B, Bax was upregulated and Cyclin D1 and Bcl-2 were downregulated in si-HOTTIP-transfected cells compared with si-NC-transfected cells in both PC3 (Figure 3A) and DU145 (Figure 3B). Next, we performed qRT-PCR assay to examine the expression of Cyclin D1, Bax and Bcl-2 mRNA, and results showed that Cyclin D1 mRNA was markedly regulated by HOTTIP. However, there was no significant change in Bcl-2 mRNA (Figure 3C and 3D).

Effects of HOTTIP downregulation on cells tumorigenesis *in vivo*

To investigate whether the downregulation of HOTTIP could suppress the tumor formation *in vivo*, si-HOTTIP or si-NC stably-transfected DU145 cells were inoculated into the athymic nude mice. Consistent with the results, the tumors formed in the si-HOTTIP group were obviously smaller than those in the si-NC group (Figure 4A), and the tumor weights derived from cells transfected with si-HOTTIP were also significantly less than those in the si-NC group (Figure 4B). These results suggested that downregulation of HOTTIP could significantly inhibit prostate cancer cell growth *in vivo* and then resulted in delayed tumor progression.

HOXA13 partly mediates the effect of HOTTIP on cell growth

The siRNA-mediated knockdown of HOTTIP resulted in a clear reduction of HOXA13 expression in primary human fibroblast (31). To explore whether the same holds true in prostate cancer cell lines, we first

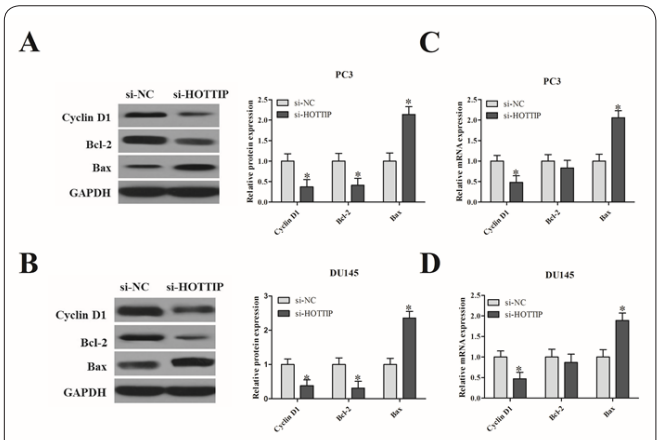

Figure 3. The expression of Cyclin D1, Bcl-2 and Bax. The effect of HOTTIP knockdown on the protein expression of Cyclin D1, Bax and Bcl-2 was evaluated by western blotting in PC3 **(A)** and DU145 **(B)** cells. **(C** and **D)** The effect of HOTTIP-knockdown on mRNA levels of Cyclin D1, Bax and Bcl-2 was confirmed by qRT-PCR. **P*<0.05 vs. si-NC.

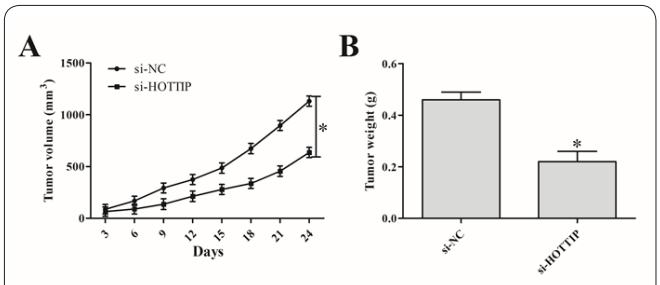

Figure 4. Effects of HOTTIP on tumor growth. HOTTIP was silenced in DU145 cells which were then used in the nude mice as xenografts. **(A)** Tumor volumes were determined for up to 25 days. **(B)** Tumor weights were measured after the animals were sacrificed at Day 25. Cells transfected with si-NC were used as controls, and five mice were used in each treatment group. **P*<0.05 vs. si-NC.

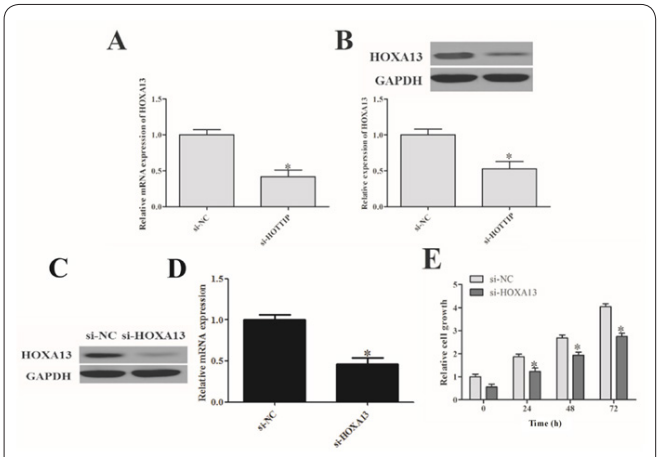

Figure 5. OXA13 partly mediates the effect of HOTTIP on prostate cancer biology. DU145 cells were transfected with HOTTIP or HOXA13 siRNA for 48 h. **(A)** qRT-PCR was performed to evaluate the mRNA level of HOXA13. **(B)** HOXA13 protein expression levels were analyzed by Western blotting. The effect of HOXA13 knockdown on HOXA13 mRNA and protein levels was evaluated by Western blotting **(C)** and qRT-PCR **(D)**. **(E)** Cell viability of DU145 was determined at the indicated time points by MTT assays. Data represent the mean ± S.D. from three independent experiments. **P*<0.05 vs. si-NC.

evaluated the effect of HOTTIP knockdown on the expression of HOXA13 by qRT-PCR in DU145 cells. As shown in Figure 5A, depletion of HOTTIP strongly inhibited the expression of HOXA13. Inhibition of HOXA13 levels was further confirmed by western blotting, which was consistent with its mRNA levels (Figure 5B). To gain further insights into the regulation of the HOTTIP/HOXA13 gene axis in prostate cancer, we knocked down HOXA13 using siRNAs. In DU145 cells 48 h post siRNA delivery, we observed the reduction of HOXA13 levels (Figure 5C), which was consistent with its mRNA expression levels (Figure 5D). Those results indicate that the interdependently regulated expression of HOTTIP and HOXA13 has a synergistic role in the pathogenicity of prostate cancer.

To investigate the functional roles of HOXA13, the cell proliferation was analyzed after transfecting the DU145 cells with siRNA of HOXA13. Strikingly, down-regulation of HOXA13 also inhibited cell growth (Figure 5E). Taken together, these results suggest that the regulatory function of HOTTIP in DU145 biology acts, at least in part, by controlling HOXA13.

Discussion

LncRNAs were once considered to be nucleic acids without any functions. But recent studies have indicated that lncRNAs are important players in the carcinogenesis and aggressive progression of human malignancies. More and more lncRNAs have been identified, and biological characterization of these demonstrates that lncRNAs are master regulators of embryonic pluripotency, differentiation, and body axis patterning (9). Of note, lncRNAs may also play roles as drivers of tumor suppression or exert oncogenic functions in a wide variety of cancer types, by sustaining tumor cell proliferation, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and promoting invasion and metastasis (2, 10, 11).

To date, many transcribed lncRNAs have been implicated in gene silencing, but the potential roles of lncRNAs in gene activation are much less understood. Recently, a lncRNA named HOTTIP, which resides at the 5′ tip of the HOXA locus and coordinates the activation of multiple 5′ HOXA genes *in vivo* (6), has been identified as one of 231 lncRNAs associated with the human HOX loci (12). Furthermore, expression of HOTTIP has been identified as a negative prognostic factor in hepatocellular carcinoma patients (7); however, the functional role of HOTTIP in prostate cancer progression remains unknown. In the current study, we for the first time demonstrate that the lncRNA HOXA transcript at the distal tip HOTTIP, is associated with prostate cancer progression and disease outcome.

The pro-oncogenic functions and negative prognostic significance of HOTAIR have been reported for several cancers including pancreatic cancer (7). In the present study, we further demonstrated that knockdown of HOTTIP in prostate cancer cell lines suppressed cell viability, induced cell apoptosis and G0/G1 phase arrest and inhibited the G1/S cell cycle transition. Similar to these findings, our *in vivo* data revealed that the average tumor weight and volume decreased markedly in mice injected with si-HOTTIP transfected DU145 cells compared to the control. All of these suggested that HOTTIP played an important role in the occurrence and development of prostate cancer.

Failure to induce apoptosis is a crucial factor that leads to the formation of cancer (13). The Bcl-2 protein plays an important role in preventing cancer cell apoptosis and Bax is known for its pro-apoptotic activity (14, 15). In our current work, we found that downregulation of HOTTIP induced apoptosis of PC3 and DU145 cells by the downregulation of the Bcl-2 protein and the upregulation of the Bax protein. However, the expression of Bcl-2 mRNA did not change markedly. Cyclin D1 is a key cell cycle regulator during the G1/S transition and abrogation of its expression leads to G1 cell cycle arrest (16). Our study also confirmed that knockdown of HOTTIP induced G0/G1 arrest possibly by downregulation of Cyclin D1 in PC3 and DU145 cells.

Among the HOXA genes, HOXA13, has been shown to play a crucial role in tumorigenesis of the liver and bladder and in esophageal cancer (17). In order to detect how HOTTIP and HOXA13 are participated in the process of prostate cancer, we conducted experiments *in vitro*. When we transfected HOTTIP siRNA with DU145 cells, and we found the knockdown of HOXA13 expression. In addition, HOTTIP expression levels were also decreased, when siRNA of HOXA13 was transfected. As reported, it seems that a loop regulation may also exist in our cell lines and may be a critical mechanism to maintain HOXA13 expression. Indeed, we admit that the current results can't explain how HOXA13 regulate HOTTIP expression, which needs further experiments in our next study. Of note, knocking down either HOTTIP or HOXA13 resulted in reduced cell proliferation. Taken together, our results support a mechanism whereby the HOTTIP/HOXA13 axis plays a critical role in prostate cancer tumorigenesis.

Collectively, our present study demonstrates that HOTTIP plays a significant role in the genesis and progression of prostate cancer. Our results also indicated

that HOTTIP exerts its function in prostate cancer at least partly by regulating HOXA13. Further studies are required to validate the molecular axis involving HOTTIP and HOXA13 as a predictive biomarker, as well as a therapeutic target in prostate cancer. A deeper understanding of the function and downstream signaling pathways influenced by HOTTIP/HOXA13 deregulation may provide novel insights into the mechanisms underlying prostate cancer tumorigenesis.

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