

Original Article

HOTTIP/HOXA13 axis is positively associated with cell proliferation in glioma

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Abstract: Glioma ranks as the most frequent type of primary brain tumor occurring in the central nervous system of adults. Recent evidence highlights the long non-coding RNA (lncRNA) HOXA transcript at the distal tip (HOTTIP) as a crucial determinant in the progression of human cancers. The present study was to examine the functions and mechanisms of HOTTIP in regulating glioma tumorigenesis. The expression of HOTTIP in 20 glioma tissues and 10 non-cancerous brain tissues was determined by qRT-PCR. Both RNA interference (RNAi) and overexpression approaches were utilized to study the functions of HOTTIP in LN229 cell proliferation, by using CCK-8 and flow cytometry assays. We uncovered that HOTTIP was upregulated in gliomas when compared with non-cancerous brain tissues. Knockdown of HOTTIP significantly inhibited cell proliferation and induced G0/G1 arrest *in vitro*, while overexpression of HOTTIP led to the opposite effects. Furthermore, we observed the marked upregulation of HOXA13 by HOTTIP in LN229 cells, and knockdown of HOXA13 by RNAi revealed that HOTTIP promoted glioma cell proliferation at least partly by regulating HOXA13. Finally, by correlating the expression data and analyzing the prognosis value of HOTTIP and HOXA13 in gliomas, we verified that the levels of HOTTIP and HOXA13 are positively associated with each other. The expression of HOTTIP and HOXA13 were both associated with higher tumor grade. Our study identifies the key role of HOTTIP/HOXA13 axis in cell proliferation of glioma and provides novel insights on the functions of lncRNA in glioma.

Keywords: Glioma, lncRNA, HOTTIP, cell proliferation, HOXA13

Introduction

Gliomas are the most common and malignant primary brain tumor in adults, constituting more than 70% of all primary neoplasms that developed in the central nervous system [1, 2]. Among these, the most frequent (65%) and lethal histological type is the glioblastoma, classified as WHO grade IV. Now surgical cure and adjuvant therapies are main therapies toward glioma and the world has achieved much progress in treating this disease over the last 20 years. However, complete tumor resection is almost impossible, because of the tendency of local invasion, leading to inevitable recurrence after surgery. The prognosis fact of patients with malignant glioma also remains grim. Notably, the median overall survival of glioblastoma patients is merely 10 to 12 months [3]. Therefore, it is essential to reveal the molecular mechanisms of glioma progression for discovering effective therapies.

Recent evidence has identified emerging key players in glioma pathogenesis including long non-coding RNAs (lncRNAs) [4, 5]. lncRNAs represent a novel class of RNAs with no functional protein-coding ability, containing >200 nucleotides and are typically transcribed by RNA polymerase II [6]. They constitute for more than 90% of the mammalian transcriptome and play important roles in regulating gene expression, thus impact on various physiological processes, including development, differentiation, and metabolism [6]. In the past few years, strong evidence indicates that lncRNAs play vital roles in cancer cell proliferation, migration, invasion and survival [7-10].

HOXA transcript at the distal tip (HOTTIP) is a HOX-associated lncRNA transcribed from the 5' tip of the HOXA locus, and is associated with the polycomb repressive complex 2 (PRC2) and WD repeat domain 5 (WDR5) [11]. It has been demonstrated that HOTTIP coordinates expres-

HOTTIP/HOXA13 axis in glioma

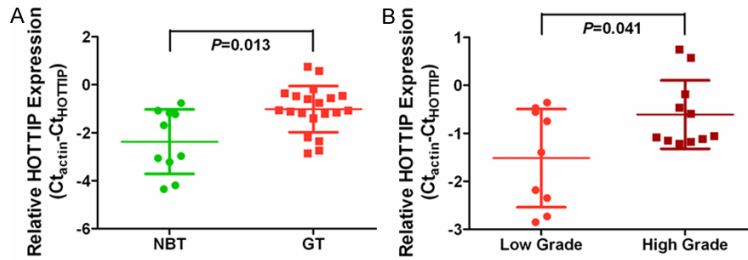


Figure 1. HOTTIP expression in non-cancerous brain tissues and glioma tissues. A. HOTTIP was up-regulated in non-cancerous brain tissues, compared with glioma tissues. B. HOTTIP was up-regulated glioma tissues with high tumor grade. NBT: non-cancerous brain tissues; GT: glioma tissues.

tients gave written informed consent to the study, which was approved by the Ethics Committee of Ruian People's Hospital. Patients' specimens and related clinico-pathological data including complete follow-up were obtained from the pathology department. Tissues were frozen in liquid nitrogen immediately and stored at -80°C before used.

Table 1. Correlation of the expression of HOTTIP with clinicopathologic feature

Clinical Characteristics	HOTTIP Decreased ($\Delta\Delta\text{Ct}\leq-1.2$) n=6	HOTTIP Non-decreased ($\Delta\Delta\text{Ct}>-1.2$) n=14
Age		
<45	3	6
≥ 45	3	8
		$P=1.0000$
Gender		
Male	4	8
Female	2	6
		$P=1.0000$
Tumor Location		
Left	3	6
Right	3	8
		$P=1.0000$
Tumor Grade		
I+II	5	4
III+IV	1	10
		$P=0.0498$

sion of genes associated with the HOXA locus in fibroblasts [11]. Moreover, several recent reports have shown a close association between HOTTIP and HOXA13 in cancers [12-14]. Up to date, the expression, function and mechanism of HOTTIP in gliomas remain largely unclear. The aim of this study was to identify the role of HOTTIP in the regulation of glioma cell proliferation.

Materials and methods

Patients and tissues

All patients were recruited between 2015 and 2016 in the Ruian People's Hospital. All pa-

Cell culture

Human glioma LN229 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were maintained in DMEM medium containing 10% FBS (Gibco, Carlsbad, CA) and cultured at 37°C with 5% CO_2 .

Reagents and transfections

SiRNA mixtures targeting HOTTIP or HOXA13 were from Santa Cruz. Lenti-virus mediate HOTTIP overexpression was from Genepharma Company (Shanghai, China). Plasmids mediate HOXA13 overexpression was generated by molecular cloning methods. Transfections were performed by lipofectamine2000 (Life Technology, Carlsbad, CA, USA) as per the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated with TRIzol reagent (Life Technology, Carlsbad, CA, USA) according to the manufacturer's instructions. Next, 2 μg of total RNA was reverse transcribed in a final volume of 20 μl using Oligo (dt)₁₈ primer (Promega). Quantitative real-time PCR assays were performed to detect HOTTIP expression using the SYBR Premix Ex Taq (Takara, Japan) according to the manufacturer's instructions. The relative levels of HOTTIP were determined by qRT-PCR by gene-specific primers. Actin was used as reference gene because its expression showed minimal variation in different cell lines and cancer specimens.

Western blot

Cultured cells were lysed in RIPA buffer containing protease inhibitor and PMSF (Beyotime,

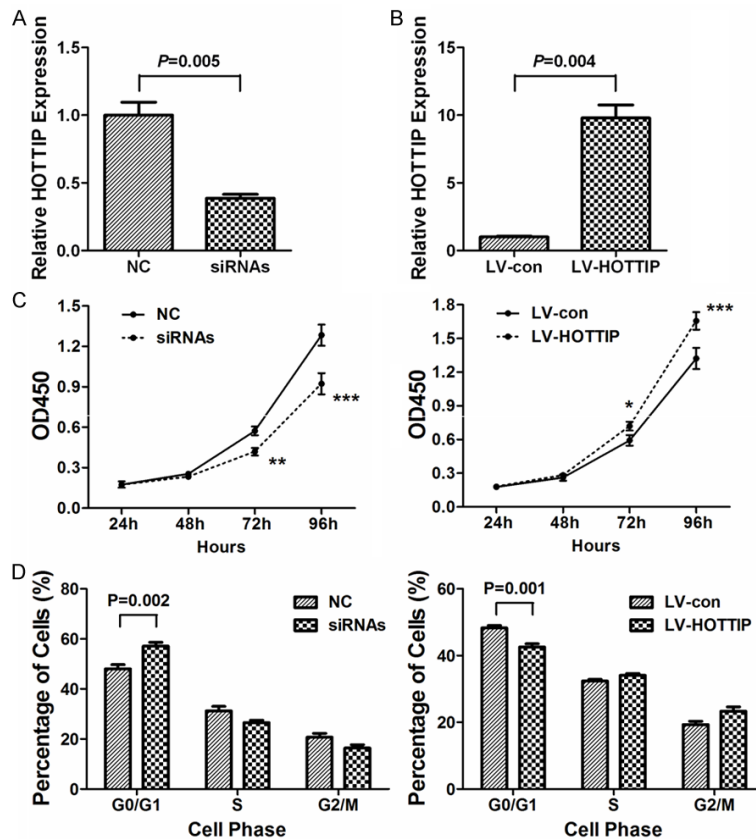


Figure 2. HOTTIP promotes glioma cell proliferation and cell cycle progression. A. LN229 cells were transfected with HOTTIP siRNAs or control siRNAs. 48 h later, cells were harvested for qRT-PCR rest. B. LN229 cells were infected with HOTTIP lenti-virus or control lenti-virus with MOI=20. 72 h later, cells were harvested for qRT-PCR rest. C. CCK-8 assay was performed in LN229 cells by using HOTTIP siRNAs or overexpression lenti-virus. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ versus control group. D. Cell cycle analysis was performed in LN229 cells by using HOTTIP siRNAs or overexpression lenti-virus.

China) using standard procedures. The concentrations of total protein were measured using the BCA Protein Assay Kit (Beyotime, China). Briefly, 40 μ g protein was separated using 10% SDS-PAGE and was transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat dry milk in PBST for 1 h at room temperature and were incubated with HOXA13 (1:500, Abcam) or Actin (1:10000, Sigma) antibodies at 4°C overnight. Then the membrane was incubated with anti-Rabbit IgG or anti-Mouse IgG secondary antibodies (Beyotime, China), which were conjugated to horseradish peroxidase. Western blot bands were visualized using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Shanghai, China). Actin was used as loading controls.

CCK-8 test

Cell proliferation assays were performed using Cell Counting Kit-8 (Dojindo, Japan). Cells were plated in 96-well plates (1×10^4 cells/well). 10 μ l CCK-8 was added to each well at different time periods (24 h, 48 h, 72 h and 96 h) and incubated at 37°C for 2 h. The absorbance of each well at OD450 was measured using a microplate spectrophotometer. The experiments were performed in triplicate.

Cell cycle analysis

LN229 cells for cell cycle analysis were stained with propidium iodide by the CycleTEST PLUS DNA Reagent Kit (BD Biosciences) according to the manufacturer's instructions. Acquisition and analysis were performed by FACS Calibur Flow Cytometer (Beckman Coulter, USA) following the protocol. The percentage of cells in G0/G1, S, and G2/M cell phase were counted and compared. The experiments were performed in triplicate.

Statistical analysis

For statistical analysis, the Grouped Student's t-test for parametric variables was used (two-tailed). The linear correlation analysis was performed between expression of HOTTIP and HOXA13. The κ^2 test were performed between HOTTIP or HOXA13 expression and clinic feature. All tests were two-sided and P -values <0.05 was considered to be statistically significant. Analysis was performed using the SPSS Software v19.0 (IBM, New York, USA).

Results

HOTTIP is up-regulated in glioma tissues

To investigate HOTTIP expression in glioma, we performed qPCR analysis on total RNA ex-

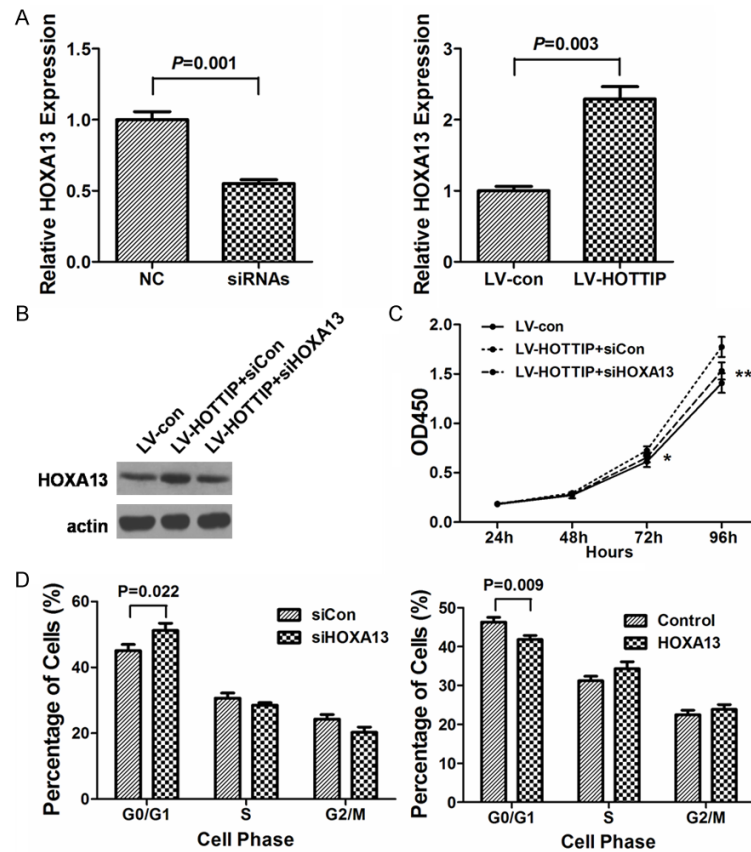


Figure 3. HOXA13 is a downstream target of HOTTIP in glioma cells. (A) LN229 cells were transfected with HOTTIP siRNAs or infected with overexpression lenti-virus. 48 h or 72 h later, cells were harvested for qRT-PCR test to detect the HOXA13 expression. (B) LN229 cells were infected with overexpression lenti-virus. 72 h later cells were transfected with HOTTIP siRNAs or control siRNAs. Another 48 h later, cells were harvested for western blot analysis to detect the HOXA13 protein expression. (C) LN229 cells were treated as described in (B). Sequentially, cells were plated in 96-wells plate and CCK-8 assay was performed as described in Materials and Methods section. (D) Cell cycle analysis was performed in LN229 cells by using HOXA13 siRNAs or overexpression plasmid. *: $P < 0.05$; **: $P < 0.01$; siCon group versus siHOXA13 group.

tracted from 20 glioma tissues and 10 non-cancerous brain tissues. Relative expression of HOTTIP [$\log_2$ (actin/HOTTIP)] was shown in **Figure 1A**. The result revealed that HOTTIP expression was significantly higher in glioma tissues compared with non-cancerous brain tissues ($P < 0.05$). Furthermore, we found that elevated HOTTIP expression was predominantly found in higher grade gliomas ($P < 0.05$), as shown in **Figure 1B**. We next investigated the clinical significance of the correlation between HOTTIP expression and clinicopathologic features. As shown in **Table 1**, expression of HOTTIP was correlated with higher tumor grade ($P = 0.0498$). These re-

sults suggested a potential tumor promoting effect of HOTTIP in glioma.

HOTTIP promotes LN229 cell proliferation and cell cycle progression

To explore whether higher level of HOTTIP determined the glioma progression in vitro, LN229 cells were used to perform functional studies. Knockdown of HOTTIP was accomplished by siRNA transfection, while overexpression of HOTTIP was done by lenti-virus infection (**Figure 2A** and **2B**). Cell proliferation assessed by CCK-8 assay showed that silence of HOTTIP significantly inhibited the proliferation of LN229 cells, while overexpression of HOTTIP accelerated the speed of LN229 cell proliferation (**Figure 2C**). To determine whether the pro-growth effect of HOTTIP on LN229 cells involved its positive impact on cell cycle progression, we performed flow cytometry analysis. The results showed that knockdown of HOTTIP caused an increase in the percentage of G0/G1 phase, while overexpression of HOTTIP led to the opposite (**Figure 2D**).

HOXA13 is a downstream target of HOTTIP in LN229 cells

Having identified the cell proliferation promoting effect of HOTTIP in LN229 cells, we then went further to study the underlying mechanisms. Since previous studies in foreskin fibroblasts show that HOTTIP knockdown decreases expression of 5' HOXA genes, particularly HOXA13 [11], we focused on this clue and confirmed this in our study. The siRNA-mediated knockdown of HOTTIP resulted in a clear reduction of HOXA13 expression, in the contrast, HOTTIP overexpression increased HOXA13 expression in LN229 cells (**Figure 3A**). As is

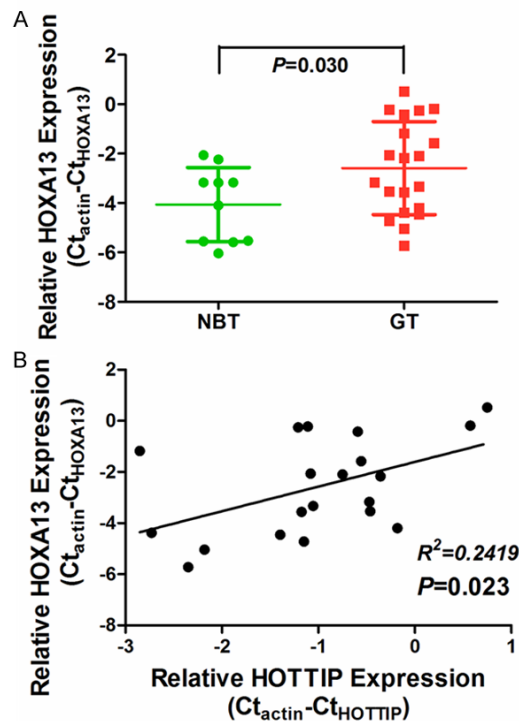


Figure 4. HOTTIP and HOXA13 are positively associated with each other in glioma tissues. A. HOXA13 was up-regulated in non-cancerous brain tissues, compared with glioma tissues. B. Correlation scatter plot of HOTTIP and HOXA13 expression glioma tissues of tested biopsies. Actin was used as reference gene.

Table 2. Correlation of the expression of HOXA13 with clinicopathologic feature

Clinical Characteristics	HOXA13 Decreased ($\Delta\Delta Ct \leq -1$) n=15	HOXA13 Non-decreased ($\Delta\Delta Ct > -1$) n=5
Age		
<45	8	1
≥ 45	7	4
		$P=0.3189$
Gender		
Male	9	3
Female	6	2
		$P=1.0000$
Tumor Location		
Left	7	2
Right	8	3
		$P=1.0000$
Tumor Grade		
I+II	9	0
III+IV	6	5
		$P=0.0379$

known, HOXA13 is a potential GBM diagnostic marker and promotes glioma progression by activating the Wnt and TGF- β pathways [15]. To determine whether HOTTIP promotes glioma cell proliferation by regulating HOXA13, we employed HOXA13 siRNA in HOTTIP overexpressing cells to inhibit the endogenous HOXA13 expression in LN229 cells. Strikingly, knockdown of HOXA13 in HOTTIP overexpressing cells decreased its growth rate (Figure 3B and 3C). Moreover, cell cycle analysis showed that knockdown of HOXA13 caused an increase in the percentage of G0/G1 phase, while overexpression of HOXA13 led to the opposite (Figure 3D). This finding explained the tumor promoting role of HOTTIP in LN229 cells, by acting through HOXA13 signaling.

High HOTTIP/HOXA13 expression is associated with poor prognosis

We next analyzed the expression of HOXA13 in glioma tissues. Figure 4A illustrated that HOXA13 was significantly overexpressed in gliomas in comparison with non-cancerous brain tissues. Spearman analysis using qRT-PCR expression data indicated that the levels of HOTTIP and HOXA13 are positively associated with each other in a total of 20 cases of glioma tissues, Figure 4B). Also, we investigated the clinical significance of the correlation between HOXA13 expression and clinicopathologic features. As shown in Table 2, expression of HOXA13 was also correlated with higher tumor grade ($P=0.0379$). These data outline the positive correlation between HOTTIP and HOXA13, as well as identify both of them as possible markers of clinical outcome in glioma patients.

Discussion

HOTTIP is a recently identified lncRNA shown to be dysregulated in various cancers and served as a potential prognosis biomarker in human cancers [16-18]. An increasing body of literature has reported that dysregulation of HOTTIP is associated with various malignancies including gastric cancer, pancreatic cancer, breast cancer and hepatocellular carcinoma [18]. Among these reports, HOTTIP is regarded as an oncogenic lncRNA, and HOTTIP upregulation correlates with enhanced cell proliferation, migration/invasion and reduced apoptosis [18]. Located in physical con-

tiguity (chr 7p15.2) with the HOXA13 gene, HOTTIP could directly activate the expression of several 5' HOXA genes, through an interaction with the WDR5-mixed lineage leukemia (MLL) complex to increase histone H3 lysine 4 trimethylation [11]. HOXA13 is the most posterior of the HOX clusters in 7p15.2, and is known as a transcriptional regulator of mammalian embryogenic development and is deregulated in tumorigenesis [19, 20].

Another previous study concerning on the role of HOTTIP in glioma concluded an opposite standpoint from ours. They uncovered that HOTTIP was decreased in glioma tissues and cell lines, and overexpression of HOTTIP inhibited the growth of glioma both *in vitro* and *in vivo* [21]. We speculate that differential cell line usage might led to different consequence, but could not explain more about its totally variant expression pattern in two cohorts of glioma tissues. We stated that both HOTTIP and HOXA13 are increased in gliomas, both promote cell proliferation *in vitro*. We also point out that HOTTIP transcriptionally regulates the expression of HOXA13, in accord with the impression that HOTTIP directly modulates expression of 5' HOXA genes. Furthermore, the positive influence of cell cycle progression by HOTTIP might guide the growth promotion effect of this lncRNA, which might be mediated through HOXA13. These findings we obtained from above *in vitro* set the rationale to perform additional work *in vivo* to gather more insights into the role of HOTTIP/HOXA13 in glioma.

Our current study verifies that up-regulation of HOTTIP/HOXA13 is associated with glioma cell proliferation and clinical high grade. Through tissue/cell based experiments, we provide new insights into the role and mechanism of HOTTIP in glioma tumorigenesis, and suggest that HOTTIP represents a potential therapeutic target for glioma.

Disclosure of conflict of interest

None.

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