Long Noncoding RNA HOTTIP Promotes Nasopharyngeal Cancer Cell Proliferation, Migration, and Invasion by Inhibiting miR-4301

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Background: The long noncoding RNA (lncRNA) HOTTIP is involved in gastric cancer tumorigenesis, papillary thyroid carcinoma, colorectal cancer, lung adenocarcinoma, and hepatocellular carcinoma, but it is unclear how HOTTIP exerts roles in nasopharyngeal carcinoma (NPC). The present study investigated HOTTIP function during NPC development.

Material/Methods: HOTTIP levels in cancer specimens and cell lines were analyzed using qRT-PCR. HOTTIP function in NPC was determined by Cell Counting Kit-8 (CCK8) and Transwell assay.

Results: HOTTIP expression was increased in NPC tissues. Higher levels of HOTTIP are correlated with lower survival in NPC patients. HOTTIP silencing suppressed the proliferation, cell cycle, migration, and invasion of NPC cells. HOTTIP served as a sponge for miR-4301. miR-4301 expression was significantly inhibited by HOTTIP in NPC cells. miR-4301 overexpression dramatically inhibited NPC cell proliferation, migration, and invasion.

Conclusions: This study showed that HOTTIP acts as an oncogene in NPC by sponging miR-4301.

MeSH Keywords: Cell Proliferation • Nasopharyngeal Neoplasms • RNA, Long Noncoding

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Background

Nasopharyngeal carcinoma (NPC) is an aggressive head and neck cancer, originating in the nasopharyngeal epithelium [1]. Due to advances in radiotherapy and chemotherapy, the outcomes of NPC patients have recently improved [2]. However, distant metastasis and recurrence still remain major obstacles to patient survival [3]. In order to better heal NPC, it will be critical to elucidate the potential mechanism underlying NPC pathogenesis.

Long noncoding RNAs (lncRNAs) are more than 200 nucleotides in length and have limited coding potential [4]. Accumulating evidence suggests that lncRNA participates in multiple biological processes, including development, immunity, and cancer [5–7]. Importantly, many studies have shown that dysregulated lncRNA expression is correlated with tumorigenesis [8]. lncRNAs are demonstrated to regulate cancer cell behaviors via regulating gene expression by serving as miRNA sponges or interacting with specific proteins [9,10]. For instance: IncRNA AK096174 enhances growth and metastasis of gastric cancer through WDR66 [11], lncRNA-MEG3 suppresses gastric cancer cell growth and invasion via targeting miRNA-21 [12], lncRNA MALAT1 upregulation promotes NPC progression by ERK/MAPK signaling [13], and lncAKHE promotes liver cancer cell proliferation and invasion by activating the NOTCH2 pathway [14].

The function of lncRNA HOTTIP has been investigated in multiple cancers, including thyroid carcinoma [15] and colorectal cancer [16]. Most studies indicate HOTTIP has oncogenic roles [16]. However, the function of HOTTIP in NPC is unclear. We showed HOTTIP was overexpressed in NPC samples and the expression level of HOTTIP is negatively associated with survival rate of NPC patients. We showed that HOTTIP knockdown suppresses NPC cell proliferation, migration, and invasion, and HOTTIP binds to miR-4301 and represses its activity. In summary, this study reveals the important function of HOTTIP in NPC progression and elucidates its molecular mechanism.

Material and Methods

Patient samples

We obtained 47 NPC samples and matched adjacent non-tumor tissues from the First Affiliated Hospital of Soochow University from May 2014 to February 2016. Associations between expression of HOTTIP and clinicopathological characteristics in 47 NPC patients are listed in Table 1. Samples were stored immediately in liquid nitrogen. Our study was approved by the Ethics Committee of our hospital. Informed consent was obtained before experiments.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction and qPCR analysis were carried out as described before [17]. U6 was chosen as the internal control. Relative expression was calculated based on the 2-ΔΔCt method. The primer sequences were as follows: HOTTIP (Forward, 5’-TCCACTACTTGCTTGG-3’; Reverse, 5’-TGAGGCTGCTGAGACTCCT-3’), miR-4301 (Forward, 5’-GGGCGCAACACCAGATGTT-3’; Reverse, 5’-GCGCTCTTGGAGAAGAGAG-3’).
CCK-8 proliferation assays

This assay was performed as previously reported [18].

Transwell assays

The abilities of tumor cell migration and invasion was assessed using Transwell assay according to a previous report [18].

Cell cycle analysis

Cells were fixed with 70% ethanol overnight at 4°C and stained with propidium iodide, followed by FACS analysis using a previously described protocol [19].

Luciferase assay

The putative binding site for miR-4301 in HOTTIP was predicted using a bioinformatics tool (http://mirdb.org/miRDB/index.html). For luciferase reporter assay, the sequence including the putative binding site or the mutant type was constructed into the pGL3-basic vector (Promega Corp., Madison, WI, USA). Then luciferase reporter assay was conducted as previously reported [20].

Statistical analysis

Statistical analyses were carried out using SPSS 22.0 (IBM, SPSS, Chicago, IL, USA). A t test and one-way analysis of variance (ANOVA) followed by a Tukey’s post hoc test was used to compare 2 or more groups for statistical significance. Kaplan-Meier analysis was used to evaluate the correlations between HOTTIP and the survival of NPC patients by Kaplan-Meier curve analysis. *P<0.05.

**Figure 1.** HOTTIP was upregulated in NPC tissues. (A) The expression levels of HOTTIP in 47 pairs of NPC tissues and adjacent normal tissues were measured by qRT-PCR. (B) Relative expression of HOTTIP in metastatic NPC tissues (n=19) and nonmetastatic NPC tissues (n=28). (C) Relative expression of HOTTIP in NPC cell lines. (D) Higher expression of HOTTIP predicted poorer prognosis by Kaplan-Meier curve analysis. * P<0.05.
curves combined with log-rank test were used to analyze overall survival rates. All experimental results were from at least 3 independent assays and data are presented as mean ±SD. P<0.05 was considered as a significant difference.

**Results**

**HOTTIP was upregulated in NPC tissues**

To explore the role of HOTTIP in NPC progression, its expression levels in NPC tissues and normal tissues were determined using qRT-PCR. The results indicated that HOTTIP level was upregulated in cancer tissues compared to non-cancer tissues (Figure 1A). Notably, HOTTIP expression was higher in metastatic NPC tissues than in nonmetastatic tissues (Figure 1B), suggesting that HOTTIP regulates metastasis of NPC. Moreover, HOTTIP expression was higher in NPC cell lines, including SUNE-1 and C666-1 cells, than in NP69 (Figure 1C). To determine whether HOTTIP is an important biomarker, we divided these samples into HOTTIP high expression (n=23) and low expression (n=24) groups according to HOTTIP expression value in tissues (using median value as cutoff). Kaplan-Meier curves were plotted and indicated that high level of HOTTIP was correlated with lower survival rate in NPC patients (Figure 1D).

**Knockdown of HOTTIP inhibited NPC cell proliferation**

We then investigated the function of HOTTIP in NPC cells. We transduced SUNE-1 cells with siRNAs against HOTTIP, and the qRT-PCR analysis indicated that HOTTIP expression was reduced in SUNE-1 cells after transfection with siHOTTIP (Figure 2A). We then performed CCK8 assay and found that HOTTIP knockdown significantly inhibited SUNE-1 cell proliferation (Figure 2B). Cell cycle progression is a major cause of proliferation; therefore, we assessed whether HOTTIP has a similar effect on the cell cycle progression of SUNE-1 cells by FACS. Results illustrated that HOTTIP knockdown resulted in more cells arrested in G0/G1 phase and decreased numbers of cells in S and G2/M phases (Figure 2C).
Knockdown of HOTTIP suppressed NPC cell migration and invasion

Metastasis is a major cause of poor outcomes of NPC patients, so we next assessed the effect of HOTTIP on metastasis using Transwell assay. The results showed that knockdown of HOTTIP markedly reduced the migrated and invaded SUNE-1 cells (Figure 3A, 3B). These results show that HOTTIP exerts an oncogenic role in NPC by facilitating proliferation, migration, and invasion.

HOTTIP serves as a miR-4301 sponge

Increasing evidence indicates that lncRNAs serve as miRNA sponges in cancer [9]. To determine the mechanism of HOTTIP, we performed bioinformatics analysis using an online tool (http://mirdb.org/miRDB/custom.html). The results suggested that HOTTIP might be a sponge for miR-4301, and there was a potential binding site of miR-4301 in HOTTIP (Figure 4A). Through luciferase reporter assay, we found that overexpressing miR-4301 impaired the activity of HOTTIP-WT but not HOTTIP-MUT reporter plasmid (Figure 4B), indicating a direct interaction between HOTTIP and miR-4301. Furthermore, HOTTIP knockdown promoted the expression of miR-4301 in SUNE-1 cells (Figure 4C), whereas ectopic expression of miR-4301 repressed the expression of HOTTIP (Figure 4D). Interestingly, miR-4301 expression was downregulated in NPC tissues (Figure 4E) and further decreased in metastatic NPC tissues (Figure 4F), suggesting that miR-4301 might work as a tumor suppressor and negatively regulate NPC metastasis.

miR-4301 overexpression inhibited NPC cell proliferation, migration, and invasion

The function of miR-4301 in NPC has not been previously elucidated. We sought to determine whether miR-4301 is responsible for the function of HOTTIP in NPC. qRT-PCR analysis showed that miR-4301 expression was significantly upregulated in SUNE-1 cells after transfection with miR-4301 mimics (Figure 5A). According to CCK8 and Transwell assays, miR-4301 upregulation significantly suppressed the proliferation, migration, and invasion of SUNE-1 cells (Figure 5B–5D), suggesting a tumor-suppressive role of miR-4301 in NPC.

Discussion

As one of the most common malignant head and neck cancers, NPC originating from nasopharyngeal epithelium leads to many cancer-related deaths worldwide [1]. However, the molecular mechanism underlying NPC progression remains elusive.
Recently, evidence has indicated that lncRNA expression is associated with tumor progression and can predict disease outcome [21]. In our study, we investigated the role of HOTTIP in NPC progression. HOTTIP expression was elevated in NPC tissues and cell lines. HOTTIP knockdown suppressed the proliferation, migration, and invasion of NPC cells. Mechanistically, we identified HOTTIP as a miRNA sponge for miR-4301. We also demonstrated that HOTTIP promoted NPC progression via inhibiting miR-4301. Our study contributes to understanding of the mechanism of NPC progression.

LncRNAs are a large group of noncoding RNAs with no protein-coding ability. Increasing evidence indicates a close relationship between LncRNA expression and cancer development. For instance, LncRNA NEAT1 regulates ovarian cancer cell resistance to paclitaxel through promoting ZEB1 expression [22], and LncRNA ZFAS1 targets miR-484 to increase proliferation and metastasis of colorectal cancer [23]. Previous studies suggest HOTTIP is a key regulator of cancer development and drug resistance in various cancers, including thyroid carcinoma [15], small cell lung cancer [24], and colorectal cancer [16]. In addition, Fu et al. reported that HOTTIP regulates the stem cell property of pancreatic cancer cells [25]. Zhang et al. showed that HOTTIP accelerates epithelial-mesenchymal transition in glioma through the miR-101/ZEB1 pathway [26]. Lin et al. reported that HOTTIP promotes growth and invasion in esophageal squamous carcinoma [27]. This evidence demonstrates that HOTTIP serves as an oncogene. However, the role of HOTTIP in NPC is unclear. We showed that HOTTIP promoted NPC cell proliferation, migration, and invasion, indicating HOTTIP also acts as an oncogene in NPC. Moreover, our results indicate that HOTTIP might be a potential prognostic marker for NPC patients.

MicroRNAs (miRNAs) are another class of noncoding RNAs, with a length of about 22 nucleotides. miRNAs regulate gene expression via binding to the complementary site of the 3’-UTR of target mRNA. Dysregulation of miRNA expression levels leads to carcinogenesis [28], and the expression of miRNAs is inhibited by LncRNAs [9]. In our study, we found that HOTTIP serves as a sponge of miR-4301 and that HOTTIP knockdown significantly promoted the expression of miR-4301 in NPC cells. A previous study showed that miR-4301 promotes cell death in breast cancer [29], suggesting miR-4301 acts as a tumor suppressor. However, the role of miR-4301 in NPC remains elusive. To determine whether HOTTIP regulates NPC progression via inhibiting miR-4301, we analyzed the effects of miR-4301 on NPC cells. By CCK8 and Transwell assays, we found that miR-4301 overexpression significantly suppressed NPC cell proliferation, migration, and invasion.

Figure 4. HOTTIP served as a miR-4301 sponge. (A) Predicted binding site of miR-4301 in HOTTIP by bioinformatics analysis. (B) Luciferase reporter assay indicated that miR-4301 overexpression repressed the luciferase activity of HOTTIP-WT but not HOTTIP-MUT. (C) HOTTIP knockdown promoted the expression of miR-4301 in SUNE-1 cells. (D) Overexpression of miR-4301 inhibited the expression of HOTTIP in SUNE-1 cells. (E) The expression levels of miR-4301 in 47 pairs of NPC tissues and adjacent normal tissues were measured by qRT-PCR. (F) Relative expression of miR-4301 in metastatic NPC tissues (n=19) and nonmetastatic NPC tissues (n=28). * P<0.05.
miR-4301 overexpression inhibited NPC cell proliferation, migration, and invasion. (A) Relative expression of miR-4301 in SUNE-1 cells transfected with miR-4301 mimics was determined by qRT-PCR. (B) Overexpression of miR-4301 inhibited the proliferation of SUNE-1 cells. (C, D) Overexpression of miR-4301 suppressed the migration and invasion of SUNE-1 cells by Transwell assays. *P<0.05.

Conclusions

In conclusion, our study, for the first time, reveals the function of HOTTIP in NPC. Our results demonstrated that HOTTIP facilitates NPC progression by serving as a miR-4301 sponge, suggesting that the HOTTIP/miR-4301 axis is a potential target for NPC therapy.

Conflict of interests

None.
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