## ORIGINAL ARTICLE

## Long-chain non-coding RNA HOTTIP enhances oral cancer cell proliferation and migration capacity by down-regulating miR-206

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### Summary

**Purpose:** The purpose of this study was to explore the specific role and potential mechanism of long non-coding RNA HOTTIP in the progression of oral cancer.

**Methods:** HOTTIP in oral cancer tissues and adjacent normal tissues was measured by quantitative real-time polymerase chain reaction (qRT- PCR) technology. After knockdown of HOTTIP expression in oral cancer cell lines, Cell Counting Kit (CCK-8), scratch healing experiment, and Transwell assay were carried out to explore cell proliferation and migration capacity. Furthermore, in order to discover the underlying mechanism, a dual luciferase experiment was designed to verify the binding of HOTTIP to the downstream miR-206 based on the prediction results of the bioinformatics prediction website. Finally, we designed a cell function recovery experiment using co-transfection technology to further confirm the regulation of HOTTIP on miR-206.

**Results:** HOTTIP was abnormally increased in oral cancer. At the same time, the survival analysis showed that the higher

expression of HOTTIP was significantly correlated with a shorter overall survival. The results of cell functional experiments found that HOTTIP played a role in promoting tumor proliferation and migration in oral cancer cells. Besides, when HOTTIP was knocked down in oral cancer cell lines, miR-206-206 was remarkably up-regulated. Dual-luciferase reporter gene experiment confirmed the binding relationship between miR-206 and HOTTIP. In addition, miR-206 was found to be differently expressed in oral cancer tissues from that in normal control tissues. Cellular function experiments verified that HOTTIP could promote tumor proliferation activity and migration by altering miR-206 in oral cancer.

**Conclusions:** HOTTIP promotes the tumor proliferation activity and migration of oral cancer cells through modulating miR-206.

*Key words:* oral cancer, HOTTIP, miR-206, cell proliferation, cell migration

## Introduction

Oral cancer ranks eighth among all malignancies [1], and among patients diagnosed with oral cancer for the first time, less than 60% have a life expectancy of more than 5 years. Oral squamous cell carcinoma (OSCC) is a malignant tumor that invades the mouth, accounting for about 90% of oral cancer [2]. The areas involved in oral cancer include the oral cavity, pharynx and salivary glands, and the malignant transformations are

collectively referred to as oral cancer [3,4]. Despite that great advances have been made in the treatment of oral cancer, mortality and morbidity have not improved significantly over the past 30 years [5]. Therefore, it is urgent to discover and elucidate the specific role of gene mutations in tumorigenesis and continuous progress so as to provide new ideas for the further diagnosis and treatment of OSCC.

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Long non-coding RNAs (lncRNAs) are defined as a class of novel transcripts that cannot encode proteins [6]. They have more than 200 bases in length and are involved in many biological processes, including tumorigenesis and tumor development [7,8]. In addition, lncRNAs could regulate intracellular signaling networks and maintain tumor cell proliferation and anti-apoptotic capabilities, etc, which have important prognostic significance for tumor treatment [9]. For example, lncRNA ZFAS1 was found to be an oncogene in bladder cancer, which participates in regulating the expression levels of key proteins in various tumors [10]. Meanwhile, as a star gene, lncRNA MALAT1 has been shown to be related to the progression of liver cancer [11]. Knockdown of pcat-1 was found to inhibit the cell growth of bone marrow tumors through p38 pathway and MAPK pathway [12].

LncRNA HOTTIP has been shown to function as a potential oncogene in kidney cancer, gastric carcinoma and prostate cancer through many oncogenic signaling pathways [13-16]. Meanwhile, LncRNA HOTTIP could play a role in a wide variety of tumor progress [14-16]. However, the biological function and mechanism of HOTTIP in OSCC are still unknown. Here, through bioinformatics analysis and experimental confirmation, we revealed that HOTTIP could act as an oral cancer susceptibility gene. We further demonstrated that HOTTIP could promote the progression of the oral cancer, providing new targets for the diagnosis and treatment of this cancer.

## Methods

#### Patients and specimens

A total of 23 pairs of oral cancer specimens were collected and classified into cancer tissues and normal adjacent tissues according to pathological results. The ethics of this experiment was reviewed by the Ethics Review Committee of Hospital of Shandong University. All patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki.

#### *Quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNAs were extracted using the TRIzol (Invitrogen, Carlsbad, CA, USA) kit according to the product instructions. cDNAs were synthesized by reverse transcription system (Promega, Madison, WI, USA). QRT-PCR was used to determine the gene expression using the SYBR green reagent and ABI7500 platform. Then the calculated miRNA and mRNA expression values were relatively quantified based on U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used are shown in Table 1.

Table	1. qRT-PCR	primer sequence	s
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Name	Sequence (5'-3')
miR-206-F	GATTCGCCAAAGGAAATAGC
miR-206-R	GTTACAAGGTCATCCAAGAC
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
GAPDH-F	CTGGGCTACACTGAGCACC
GAPDH-R	AAGTGGTCGTTGAGGGCAATG
HOTTIP-F	CCT AAAGCCACGCTTCTTTG
HOTTIP-R	TGCAGGCTG GAGATCCTACT

#### Cell culture

Four oral cancer cell lines (Fadu, CAL-27, SCC-25 and Tca8113) and corresponding normal epithelial cell lines (OMECs) (ATCC; Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in an incubator with 5% CO2.

#### Cell transfection

Oral cancer cell lines CAL-27 and Tca8113 were seeded into 24-well plates, and transfection was performed when the cells reached a confluence of about 80%. The concentration of the interfering reagent for transfection was 50 nM. Finally, the cell transfection efficiency was measured and calculated based on the qRT-PCR results.

#### Cell Counting Kit (CCK-8) test

Cell proliferation experiments were performed using the CCK-8 kit (Beyotime, Shanghai, China). Specifically, the CAL-27 and Tca8113 cell lines were cultured in a 96-well plate at 1000 cells per well for 24, 48, and 72 hours, followed by addition of CCK-8 reagent and incubation at 37°C for 2 hours. A microplate reader (BioRad, Hercules, CA, USA) was used to determine the optical density of each well at 450 nm, and the results were analyzed statistically.

#### Wound healing assay

Transfected cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to  $5 \times 10^5$  cells/mL. The density of the plated cells was determined according to the size of the cells, and the confluence of the cells reached 90% or more the next day. After the stroke, cells were rinsed gently with PBS for 2-3 times and observed again after incubation in lowconcentration serum medium for 24 h.

#### Transwell assay

Transfected cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to  $5 \times 10^5$  cells/mL. A cell suspension of 200 µL ( $1 \times 10^5$  cells) was added to the upper chamber, and 700µL of a medium containing 20% FBS was added to the lower

chamber. After incubated in a 37°C incubator for 48 hours, the chamber was removed, fixed with 4% paraformaldehyde for 30 minutes, and stained with 0.2% crystal violet for 15 minutes. Subsequently, the cells were washed with PBS, and the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner layer cells. The perforated cells stained on the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

#### Statistics

All data analysis was performed using GraphPad 7.0 (La Jolla, CA, USA). HOTTIP expression was compared between groups using a t-test, and the rank sum test

was used to evaluate the survival analysis of HOTTIP and oral cancer patients' overall survival. Pearson correlation analysis was used to calculate the expression of HOTTIP gene and the expression of miR-206 gene. P<0.05 was considered statistically significant.

#### Results

#### HOTTIP is abnormally highly expressed in oral cancer and is associated with poor prognosis

We used qRT- PCR to measure the expression level of HOTTIP gene in oral cancer tissues, which was abnormally increased in tumor tissues (Figure 1A). Then we analyzed the survival prognosis



**Figure 1.** (**A**) qRT-PCR was used to measure the expression level of HOTTIP in 23 pairs of clinical samples; (**B**) qRT-PCR was used to determine the expression level of HOTTIP in different oral cancer cell lines, and it was found that the expression of HOTTIP was higher than that of normal oral epithelial cell lines; (**C**) The overall tumor survival time of oral cancer patients with a high expression of HOTTIP was shorter than that of patients with a low expression of HOTTIP (\*\*p<0.01, \*\*\*p<0.001).



**Figure 2. A:** Transfection of si-HOTTIP in oral cancer cell lines CAL-27 and Tca8113 could target the knockdown of HOTTIP gene expression. **B, C:** CCK8 cell proliferation experiment results showed that knockdown of HOTTIP can inhibit the proliferation of oral cancer cell lines CAL-27 and Tca8113. **D:** The results of Scratch healing experiment showed that knockdown of HOTTIP expression could inhibit the migration ability of oral cancer cell lines CAL-27 and Tca8113. **E:** Transwell cell migration experiment results showed that knockdown of HOTTIP expression could inhibit the cell migration ability of oral cancer cell lines CAL-27 and Tca8113 (\*p<0.05, \*\*p<0.01).

of patients and detected the relationship between patient survival and HOTTIP expression level, and found that the overall survival of patients with high expression of HOTTIP shortened, suggesting that HOTTIP may act as a potential negative prognostic marker. Meanwhile, we also detected the expression of HOTTIP in oral cancer cell lines (Fadu, SCC-25, CAL-27 and Tca8113) and oral normal epithelial cell line (Hs 680.Tg), and found that HOTTIP was highly expressed in oral cancer cell specimens (Figure 1B-1C). These results indicate that HOTTIP was abnormally highly expressed in oral cancer and its expression was associated with poor prognosis.



Figure 3. A: According to the prediction results of bioinformatics, miRNA-211, miRNA-195, miR-206 have a region of binding site to HOTTIP. After HOTTIP knockdown, the respective expression levels of these miRNAs were determined; **B**: qRT-PCR was performed on 23 pairs of clinical samples to determine the expression levels of HOTTIP and miR-206. C: According to the prediction results of the lncRNASNP website, both HOTTIP and miR-206 had a combined 3'UTR region. D: The results of the luciferase reporter gene experiment proved that HOTTIP and miR-206 could combine with each other in oral cancer cells E: qRT-PCR was used to determine the expression level of miR-206 in 23 pairs of clinical samples. It was found that the expression level of miR-206 in the tumor group was decreased significantly (\*p<0.05, \*\*p<0.01).

Knocking down of HOTTIP inhibits proliferation and migration ability of oral cancer cell lines

To further explore the functions of HOTTIP in OSCC, si-HOTTIP and corresponding negative controls were transfected into CAL-27 and Tca8113 cell lines. The transfection efficiency was confirmed using qRT-PCR technology (Figure 2A). Next, we used the CCK-8 assay to measure the proliferation of cells. The results showed that knocking down of HOTTIP in oral cancer cells significantly inhibited the proliferation of CAL-27 and Tca8113 cell lines (Figure 2B, 2C). Additionally, we used a scratch healing experiment to measure the lateral migra-

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**Figure 4. A, B:** Co-transfection of si-HOTTIP + miR-206 Inhibitor in oral cancer cell lines CAL-27 and Tca8113 could reverse the decline in the proliferation of oral cancer cell lines caused by HOTTIP knockdown; **C:** Co-transfection of si-HOTTIP + miR-206 Inhibitor in oral cancer cell lines CAL-27 and Tca8113 could increase the distance of cell scratch healing migration, indicating that the cell's lateral migration ability was partially restored. **D:** Co-transfection of si-HOTTIP + miR-206 Inhibitor in oral cancer cell lines CAL-27 and Tca8113 increased the number of penetrating cells, indicating that the longitudinal migration ability of the cells was partially restored (\*p<0.05 \*\*p<0.01, #p<0.05).

tion ability of oral cancer cells, and found that after transfection with si-HOTTIP, the migration rates of CAL-27 and Tca8113 cells were decreased significantly (Figure 2D). Similarly, Transwell assay showed that the migration capacity of CAL-27 and Tca8113 cell lines was significantly inhibited after knocking down HOTTIP in oral cancer cells (Figure 2E). The above experimental results confirmed that the lncRNA HOTTIP is of great significance to maintain the proliferation and migration abilities of oral cancer cells.

## HOTTIP gene expression level is negatively correlated with miR-206 in oral cancer cells and tissues

Previous studies have found that miRNA-211, miRNA-195, and miR-206 are potential target genes for HOTTIP (template literature). At first, we determined the changes in the expression levels of these miRNAs after knocking down HOTTIP in oral cancer cells, and found that miR-206 was significantly changed (Figure 3A); Then we measured the expression levels of HOTTIP and miR-206 in

23 clinical samples, which showed that the expressions of HOTTIP and miR-206 had a negative correlation in oral cancer tissues (Figure 3B). Subsequently, we predicted the binding sites of the two according to the lncRNA-SNP website and found that the 3'UTR region of miR-206 has a binding site for HOTTIP (Figure 3C). Furthermore, the results of the dual luciferase reporter assay proved that HOT-TIP could bind to miR-206 in oral cancer cells (Figure 3D). Besides, qRT- PCR was used to determine miR-206 expression in clinical samples, which revealed that miR-206 was remarkably decreased in the tumor group (Figure 3E). These results indicated that HOTTIP can modulate the expression level of the downstream miR-206 in OSCC.

# miR-206 can reverse the effects of HOTTIP on oral cancer cell function

In order to further understand the specific regulation of HOTTIP and miR-206 signal axis on oral cancer cell function, CCK-8, scratch healing test and Transwell assay was performed after co-

transfection of mi206 inhibitors and si-HOTTIP, and it was found that after miR-206 knockdown could reverse the inhibitory effect of si-HOTTIP on cell proliferation (Figure 4A, 4B). Similarly, cell scratch healing experiment results showed that the distance of cell scratch migration healing was increased after silencing miR-206, indicating that the cell's lateral migration ability was partially restored (Figure 4C). In addition, Transwell results suggested that silencing miR-206 was able to restore the number of penetrating cells caused by HOTTIP knockdown (Figure 4D). The above experimental results further confirmed that lncRNA HOTTIP could promote tumor progression by inhibiting miR-206 expression in oral cancer.

#### Discussion

The incidence of oral cancer is at the forefront of malignant tumors, and the prognosis of patients is usually poor due to delayed diagnosis and disease metastasis [17]. Highly sensitive and specific indicators are urgently needed to predict the occurrence and progression of tumors. In recent years, research on lncRNAs has received widespread attention. Due to their important roles in regulating gene transcription and translation as well as the activation of intracellular signaling pathways, lncR-NAs are considered to be an important molecular mechanism for tumor progression [18]. With the advent of technologies such as high-throughput sequencing, more and more lncRNAs have been found to be abnormally expressed in tumor specimens, and act as early biomarkers for tumor diagnosis including NALT1 in gastric cancer [19], PVT1 in childhood tumors [20], and PCA3 in prostate tumors [21]. At the same time, the regulatory relationship at the gene level also provides new options for therapeutic targets for tumors [22].

The HOTTIP gene, HOXA distal transcript antisense RNA (NCBI Gene 100316868), is a lncRNA found to promote tumor progression in a variety of tumors and be involved in the process of stem cell self-renewal and repair [23], immune escape [24], and Wnt /  $\beta$ -catenin signaling pathway [25]. In previous studies, HOTTIP was found to be abnormally

expressed in oral cancer, and bioinformatics suggested it to be a potential biological marker [26]. Our research found that the expression of HOTTIP was markedly higher in clinical tumor specimens and cell lines than that of normal control ones. At the same time, patients with a high expression of HOTTIP had shorter survival time. To further clarify the specific role of HOTTIP in oral cancer, we performed cell biology experiments. CCK-8, cell scratch assay, and Transwell experiments revealed that the ability of OSCC cells to proliferate and migrate was markedly reduced after HOTTIP was silenced. These results showed that HOTTIP had a clear carcinogenic effect in oral cancer. Previous studies have suggested that HOTTIP's potential downstream molecules in oral cancer include hsamiRNA-211, hsa-miRNA-195 and hsa-miR-206. Therefore, we verified by cell transfection and qRT-PCR technology that miR-206 expression changed significantly at the cell level and had a negative correlation with HOTTIP expression. miR-206 is a tumor-related small RNA, which is involved in the progression of lung cancer [27], prostate cancer [28] and breast cancer [29]. Hence, we adopted a dual luciferase reporter gene experiment to confirm their binding relationship and verified their expression relationship, which was found to be negatively related. Further cellular experiments showed that miR-206 can reverse the inhibitory effect of HOTTIP knockdown on the proliferation and migration abilities of OSCC cells. Collectively, our experiments explained the that HOTTIP may play a role in OSCC by modulating miR-206. Our research may provide new target option and clear experimental evidence for the clinical diagnosis and treatment of oral cancer.

#### Conclusions

HOTTIP has an abnormally high expression in oral cancer, which plays a role in promoting tumor progression and leads to poor prognosis of tumors.

#### **Conflict of interests**

The authors declare no conflict of interests.

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