

Long Noncoding RNA HOTAIR: An Oncogene in Human Cervical Cancer Interacting With MicroRNA-17-5p

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Increasing evidence has indicated that long noncoding RNAs (lncRNAs) are a class of significant regulators in various tumorigenesis processes. The lncRNA homeobox transcript antisense RNA (HOTAIR) has been reported to act as a functional lncRNA in cervical cancer development. The present study investigated the underlying mechanism of HOTAIR and miR-17-5p in cervical cancer tumorigenesis. The results showed that HOTAIR expression was significantly upregulated in both cervical cancer tissues and cell lines. Loss-of-function experiments showed that HOTAIR knockdown inhibited the proliferation, migration, and invasion of cervical cells. In addition, miR-17-5p expression was downregulated in cervical cancer tissues and cell lines. Pearson's correlation analysis indicated that miR-17-5p expression was negatively correlated to that of HOTAIR. Luciferase reporter assay revealed that miR-17-5p directly targeted HOTAIR 3'-UTR. Rescue experiments showed that miR-17-5p knockdown could reverse the tumor-suppressing effect caused by si-HOTAIR transfection. In summary, our results reveal the tumor-promoting role of HOTAIR in cervical cancer via sponging miR-17-5p, providing a novel therapeutic target for future treatment of cervical cancer.

Key words: Cervical cancer; HOTAIR; Tumorigenesis; miR-7-5p

INTRODUCTION

Cervical cancer is one of the top four malignant cancers in women worldwide, with morbidity and mortality increasing annually. Moreover, most of these cases (approximately 85%) occur in developing countries. China has a rather high morbidity of 7.5/100,000 and mortality of 3.4/100,000 for cervical cancer. In recent decades, the development of cervical cancer screening approaches and HPV vaccine have achieved significant improvement in decreasing cervical cancer morbidity. However, in many developing countries, cervical cancer is still a severe threat to women's health¹⁻³. Therefore, further studies are urgently needed to investigate the tumorigenesis process and discover potential therapeutic targets for cervical cancer.

In recent decades, mounting evidence has indicated that noncoding RNAs (ncRNAs) play an important role in tumorigenesis of various cancers, including microRNAs and long noncoding RNAs (lncRNAs)^{4,5}. The biological functions of miRNAs in malignancy have been widely explored, acting as oncogenes or antitumor genes⁶. However, the roles of lncRNAs have not received much

attention until recently. Emerging evidence indicated that lncRNAs are related with DNA-binding proteins that regulate gene expression, suggesting their multiple regulation in various essential processes, such as cell cycle control, transcription, and translation⁷. Presently, an increasing number of studies suggest that lncRNAs exert biological functions via targeting microRNAs, acting as an miRNA "sponge"⁸.

Homeobox transcript antisense RNA (HOTAIR) is a 2,158-bp lncRNA from the HOXC gene cluster⁹. From previous results, we find that HOTAIR functions as an oncogene in a number of tumors. For instance, HOTAIR is reported to be associated with poor prognosis for breast, liver, colon, pancreatic, and cervical cancers^{10,11}. HOTAIR is also reported to be overexpressed in cervical cancer tissues and cell lines, enhancing cervical cancer growth by regulating protein expression through cellular signal pathways¹²⁻¹⁵. miR-17-5p has been reported as a tumor suppressor in cervical cancer by targeting TP53INP1^{16,17}. HOTAIR has been reported to regulate osteogenic differentiation and proliferation by inhibiting miR-17-5p¹⁸.

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In our study, we detected the expression of HOTAIR in cervical cancer tissues and cells. Moreover, the physiological function of HOTAIR was tested using loss-of-function experiments *in vitro*. The interaction of HOTAIR and miR-17-5p, the downstream target, was also investigated.

MATERIALS AND METHODS

Clinical Sample

Clinical samples of cervical cancer patients and non-cancer patients were derived from 50 female patients (30 cervical cancer patients, 20 noncancer patients) from The First Affiliated Hospital of Xinjiang Medical University between May 2012 and March 2016. The cervical cancer patients were diagnosed according to the International Federation of Gynecology and Obstetrics (FIGO) staging standard (stages IA–IVB), and none of them received adjuvant treatment before surgery. Twenty control samples were obtained from hysteromyoma patients who needed a hysterectomy during the same period. Additionally, patients who suffered from more than two types of gynecological cancers were excluded from this research. Specimens were dipped in liquid nitrogen and then stored at -80°C for further experiments. This research was conducted following the principles in the Declaration of Helsinki, which was approved by The First Affiliated Hospital of Xinjiang Medical University. Written informed consent was obtained from all patients before surgery.

Cell Lines and Cell Culture

Cervical cancer cell lines squamous cell cervical carcinoma (SiHa) and epidermoid cell cervical carcinoma cells, originally derived from a small bowel mesentery metastasis (Caski), were obtained from the American Type Culture Collection (Rockville, MD, USA). SiHa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA), and Caski cells were grown in RPMI-1640 medium (Gibco BRL). Human keratinocyte cell line was also purchased from ATCC and cultured in RPMI-1640 medium. All culture medium was supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 95%

air and 5% CO_2 . Cells used in all the experiments were passaged less than 20 times.

Cell Transfection

Different kinds of transfection mimics were purchased and used to increase or decrease expression of lncRNA HOTAIR or miR-17-5p (GenePharma, Shanghai, P.R. China). According to their usages, transfection mimics were named as miR-17-5p mimic, miR-inhibit mimic, control mimic A, HOTAIR mimic, HOTAIR siRNA mimic, and negative mimic B. Lipofectamine 2000 transfection reagent was used to transfect mimics into cervical cells or control cells following the manufacturer's instructions (GenePharma).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to determine the relative expression levels of lncRNA HOTAIR and miR-17-5p. In brief, the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to attain RNAs from tissues or cells, and then first-strand cDNAs were synthesized from RNAs using a reverse transcription reagent kit (Invitrogen) following the manufacturer's protocols. Primers were all obtained from Applied Biosystems (Foster City, CA, USA). qRT-PCR was performed using the SYBR Green PCR Kit (Toyobo, Co, Ltd, Osaka, Japan). Specifically, each reaction volume contained 2 μl of extracted RNAs, 10 μl of SYBR Green Master PCR Mix, 5 pmol of forward primers and 5 pmol of reverse primers, 1 μl of diluted cDNA template, and certain amounts of sterile distilled water to a final volume of 20 μl . Every procedure was performed under certain conditions: initial denaturation (95°C for 3.5 min), denaturation (95°C for 15 s), annealing (60°C for 50 s), elongation (72°C for 55 s), and the final elongation (72°C for 3 min). U6 was tested as the internal standard. All primers used in this experiment are listed in Table 1. The relative gene expression levels were calculated by comparing to the expression level internal standard using the $2^{-\Delta\Delta\text{CT}}$ method.

Luciferase Assay

The hypothetical connection between the 3'-UTR of HOTAIR and miR-17-5p was verified by luciferase assay. In the preparatory stage, wild-type (WT) or mutant-type

Table 1. Primers for qRT-PCR Analyses

HOTAIR forward	5'-CAGTGGGGAACTCTGACTCG-3'
HOTAIR reverse	5'-GTGCCTGGTGCTCTCTTACC-3'
miR-17-5p forward	5'-TGCGCCAAAGTGCTTACAGTGCA-3'
miR-17-5p reverse	5'-CCAGTGCAGGGTCCGAGGTA TT-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'

(MUT) 3'-UTR sequences of HOTAIR were inserted at the firefly luciferase gene in pRL-CMV vectors (Promega, Madison, WI, USA), purified, and named as reporter mimics. Then cervical cancer Caski cells were seeded in 12-well plates (about 3,500 cells/well) and cotransfected with reporter mimics and miR mimics (miR-17-5p, miR-inhibit, and NC-B mimics) using Lipofectamine 2000. In addition, pRL-CMV vectors carrying the *Renilla* luciferase gene were applied in every sample as internal control for transfection efficiency (Promega). Finally, firefly luciferase activity was determined in 48 h using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocols, which was corrected by *Renilla* luciferase activity before statistical analysis.

Cell Viability Assay

Cells were planted into a 96-well plate (about 3,500 cells/well) and transfected with corresponding vectors. Forty-eight hours after transfection, cell viability was detected using a CCK-8 kit (Beyotime Institute of Biotechnology, Jiangsu, P.R. China) following the manufacturer's instructions.

Cell Migration and Invasion Assay

After cells were transfected with corresponding vectors, cells were harvested during log phase and seeded in Transwell chambers (BD Biosciences, San Jose, CA, USA) filled with RPMI-1640 medium. For the invasion assay, the Transwell chamber was precoated with Matrigel (BD Biosciences). Cells were incubated in the chambers for 16 or 24 h before the migration or invasion assay. In the end, cells in the lower chambers were fixed, stained, and counted in a microscopic field (200×).

Statistical Analysis

All results are presented as mean \pm standard deviation (SD), which were all repeated by at least three independent experiments. Statistical differences were measured by the Student's *t*-test or one-way or two-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Enhanced Expression of HOTAIR in Cervical Cancer Tissues and Cell Lines

qRT-PCR was used to measure the expression level of lncRNA HOTAIR in cervical cancer tissues and cell lines. The results showed that HOTAIR expression was significantly increased in cervical cancer tissues and cell lines compared with normal control samples (Fig. 1A and B). These results provided an indication that lncRNA HOTAIR might function as an oncogene in cervical cancer.

HOTAIR Silencing Suppressed Cell Viability of Cervical Cancer Cells

To clarify the biological function of lncRNA HOTAIR in cervical cancer, HOTAIR siRNA was transfected into two cervical cancer cell lines (SiHa and Caski cells). We then confirmed that HOTAIR expression level was successfully downregulated by the transfection of siRNA using qRT-PCR (Fig. 2A). The CCK-8 assay showed that HOTAIR silencing significantly decreased cell viability of cervical cancer cells in a time-dependent manner (Fig. 2B and C). Moreover, the Transwell assay showed that the migration and invasion abilities of cervical cancer cells were also significantly suppressed after HOTAIR

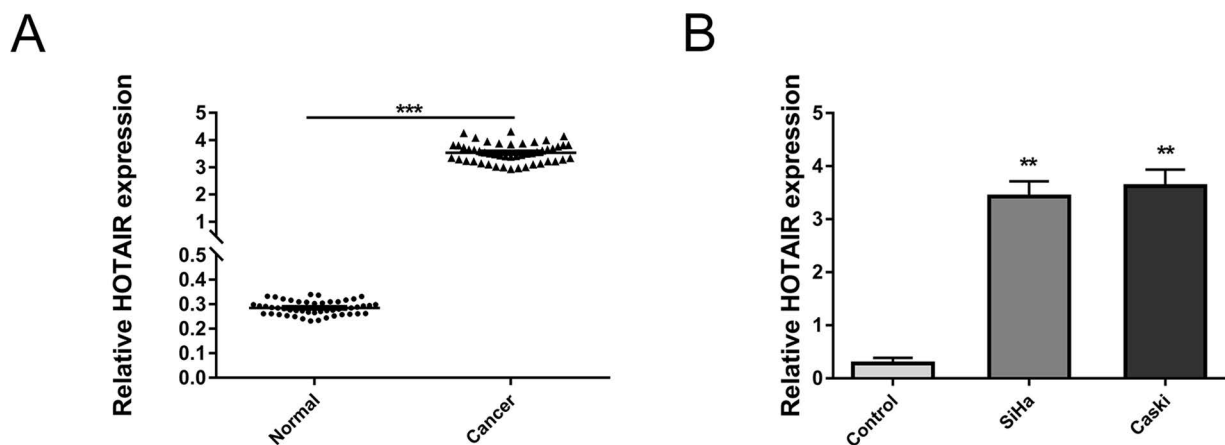


Figure 1. Homeobox transcript antisense RNA (HOTAIR) was overexpressed in cervical cancer tissues and cell lines. (A) The expression level of HOTAIR was significantly higher in cervical cancer tissues than in normal tissues. (B) The expression level of HOTAIR was significantly higher in cervical cancer cell lines than in human keratinocyte cells. Data are presented as mean \pm standard deviation (SD). ** $p < 0.01$, *** $p < 0.001$, calculated with Student's *t*-test.

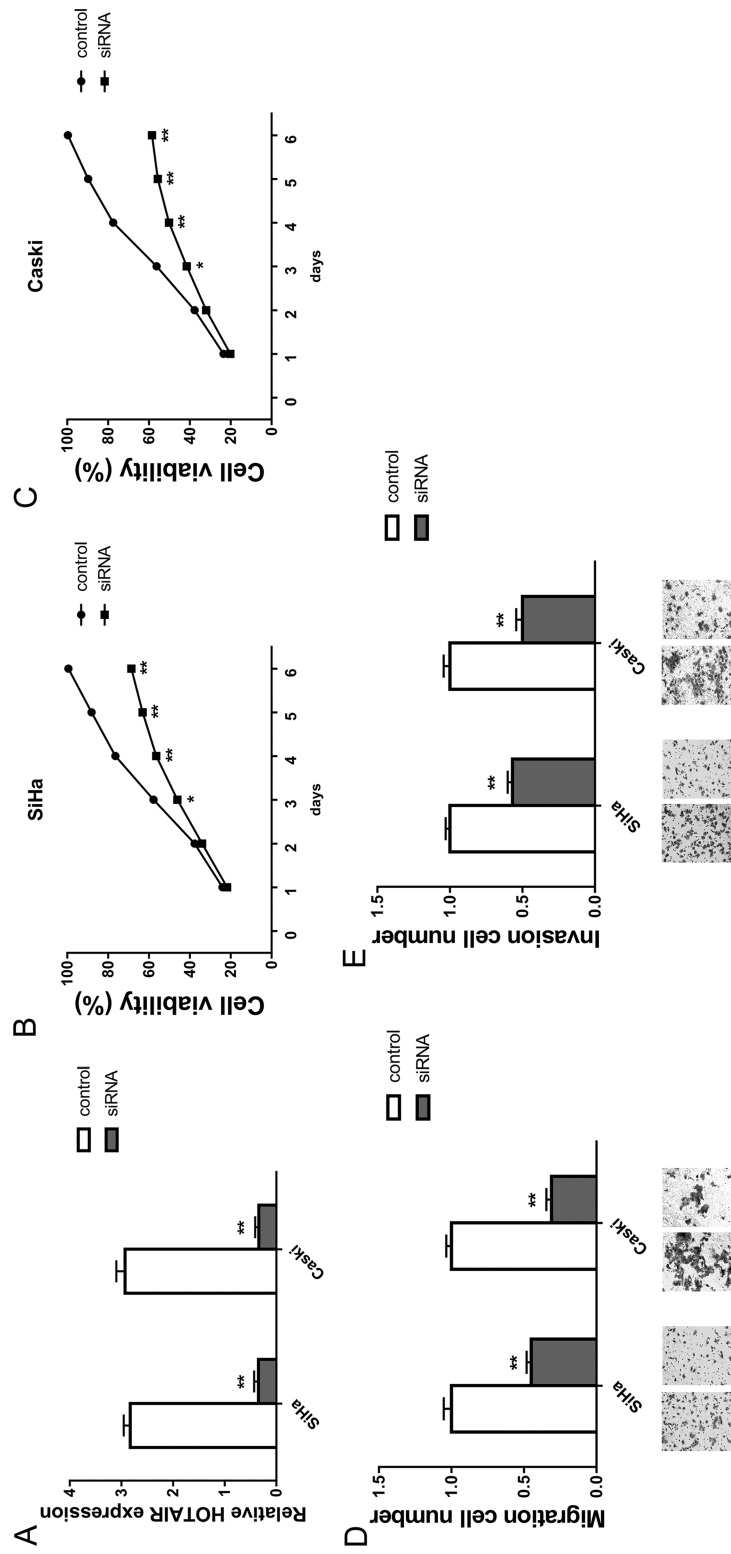


Figure 2. Low expression of HOTAIR inhibited proliferation, migration, and invasion of cervical cancer cells. (A) The expression level of HOTAIR is significantly lower in HOTAIR siRNA-transfected cells than in controls. (B, C) CCK-8 assay results indicated that proliferation of cervical cancer cells was significantly decreased in a time-dependent manner after downregulation of HOTAIR. (D) The migration ability of cervical cancer cells was significantly suppressed after downregulation of HOTAIR. Random fields are shown. (E) The invasion ability of cervical cancer cells was significantly suppressed after downregulation of HOTAIR. Random fields are shown. Data are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, calculated with Student's *t*-test.

silencing (Fig. 2D and E). These results suggested that HOTAIR silencing could suppress proliferation, migration, and invasion of cervical cancer cells.

miR-17-5p Expression Was Downregulated in Cervical Cancer Tissues and Cell Lines

Our research group had obtained miRNA expression profile in cervical cancer tissues compared to normal tissues in previous studies (Fig. 3A). In this profile, we found that the expression level of miR-17-5p was lower in cervical cancer tissues, which suggested that miR-17-5p might be a potential tumor suppressor. Furthermore, the expression of miR-17-5p in cervical cancer tissues and cell lines was determined using qRT-PCR, showing that the miR-17-5p expression level was significantly decreased in cervical cancer tissues and cell lines (Fig. 3B and C). In summary, these results showed that miR-17-5p expression was significantly low in cervical cancer tissues and cells, which suggested its potency as a tumor suppressor in cervical cancer.

Complementary Binding Between HOTAIR and miR-17-5p

Pearson’s correlation analysis was performed, and the results revealed that miR-17-5p expression was negatively correlated with that of HOTAIR (Fig. 4A). Bioinformatics analysis showed the complementary sequences between HOTAIR 3’-UTR and miR-17-5p (Fig. 4B). The dual-luciferase reporter assay further confirmed the binding alignment between HOTAIR and miR-17-5p (Fig. 4C). Taken together, the results indicated that HOTAIR could regulate the expression of miR-17-5p in cervical cancer, and this relation might be the key to understanding mechanisms of their biological functions in cervical cancer.

Biological Function of miR-17-5p and HOTAIR

To investigate if HOTAIR plays a role in the biological function of miR-17-5p in cervical cancer, we conducted experiments on monitoring the proliferation, migration, and invasion abilities of cervical cancer Caski cells with different expression levels of miR-17-5p in a low HOTAIR environment (Fig. 5). Cervical cancer Caski cells were cotransfected with HOTAIR siRNA mimics or NC mimics in combination with miR-17-5p, miR inhibitor, or miR NC mimics, and CCK-8, migration, and invasion assays were performed in each combination group (low miR group, high miR group, and control group). The overall results indicated that downregulation of miR-17-5p could only partially rescue the tumor-suppressive effects caused by low HOTAIR expression, whereas overexpressed miR-17-5p could more intensively inhibit the proliferation, migration, and invasion abilities of cervical cancer Caski cells. In conclusion, miR-17-5p exerted tumor-suppressive effects on cervical cancer, which is highly likely to be connected with the potential oncogene HOTAIR.

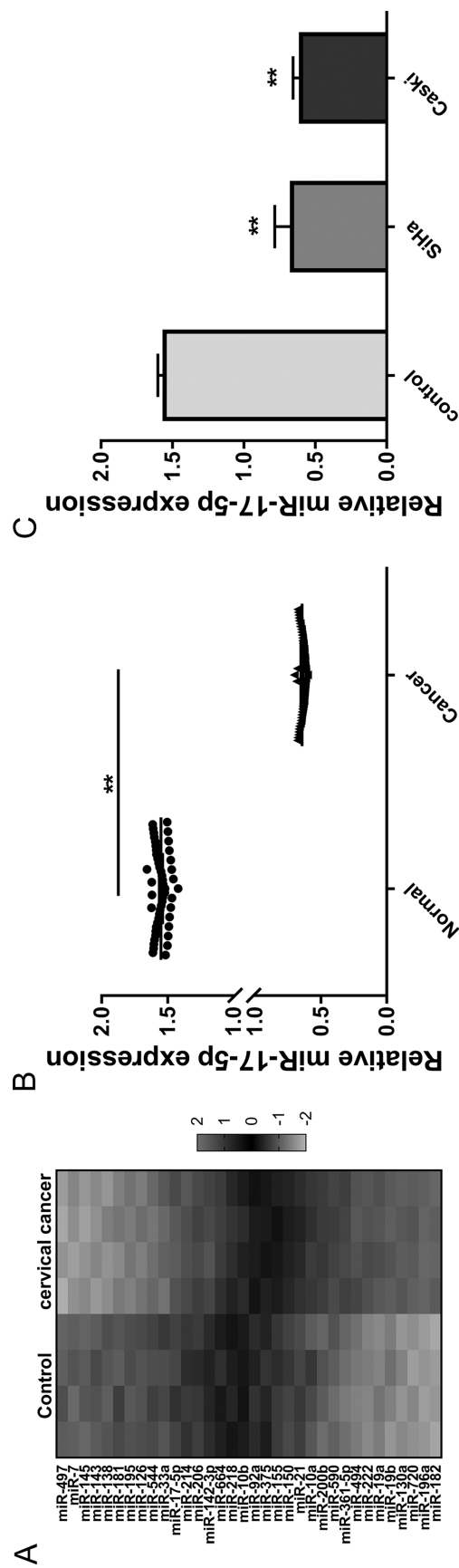


Figure 3. miR-17-5p expression is downregulated in cervical cancer tissues and cell lines. (A) miRNA expression profiles in cervical cancer tissue were determined by miRNA microarray. (B) The expression of miR-17-5p was significantly lower in cervical cancer tissues than in normal tissues. (C) The expression level of miR-17-5p was significantly lower in cervical cancer cell lines than in noncancer cells. Data are presented as mean ± SD. ***p* < 0.01, calculated with Student’s *t*-test.

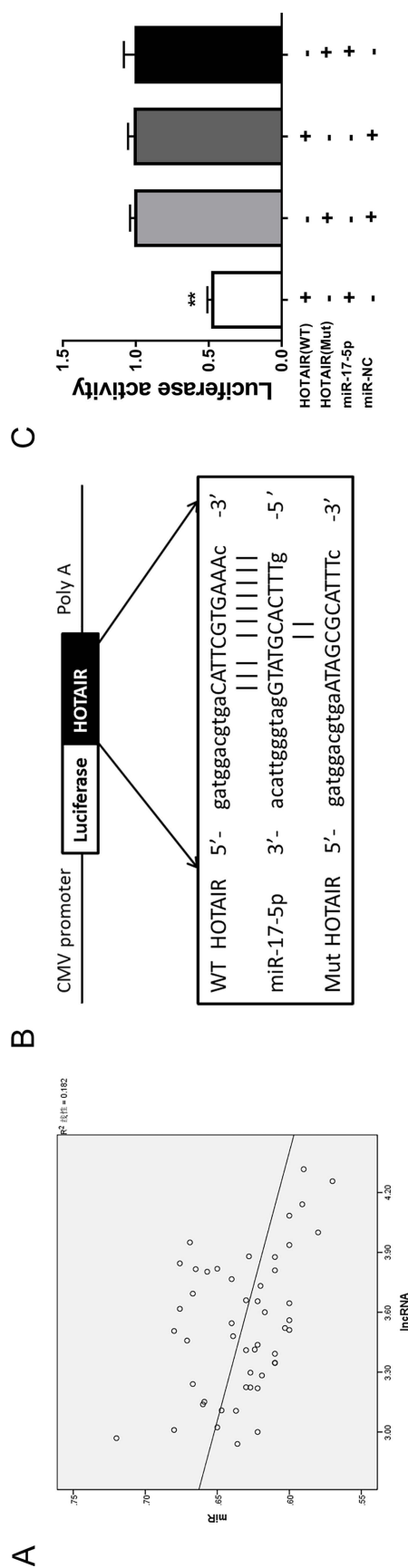


Figure 4. HOTAIR regulated miR-17-5p expression via direct binding. (A) Pearson's correlation analysis showed that the expression of miR-17-5p is negatively related to the expression of HOTAIR ($R^2 = 0.182$). (B) A presumptive complementary region between HOTAIR and miR-17-5p. (C) The dual-luciferase reporter assay indicated that there was a certain possibility of 3'-UTR of HOTAIR (WT) and miR-17-5p sharing a complementary binding sequence (firefly luciferase activity in overexpressed miR-17-5p Caski cells was decreased by 63% compared to NC-transfected Caski cells). Data are presented as mean \pm SD. $**p < 0.01$, calculated with Student's *t*-test.

DISCUSSION

Mounting evidence has directly or indirectly indicated that noncoding RNAs play an essential part in the tumorigenesis of various malignant tumors¹⁹⁻²¹. Among them, lncRNA HOTAIR and miR-17-5p were both reported as important regulatory factors in the development of cervical cancer. In previous reports, we found that lncRNA HOTAIR was reported as an oncogene, which is overexpressed in cervical cancer and associated with tumor progression and poor prognosis¹¹⁻¹³. Although the biological function of miR-17-5p is different in various types of cancer, miR-17-5p was mostly considered as a tumor suppressor in cervical cancer^{16,17}. In addition, a case control study reported that 11 types of miR-17-5p SNPs are related to different pathological types of cervical cancer risks in a Chinese population²². Functional mechanisms of HOTAIR and miR-17-5p have been examined in the past¹⁴⁻¹⁶. It is noteworthy that none of the previous studies connected their functions in cervical cancer. The interaction between lncRNAs and microRNAs has raised more and more attention in recent years, with the growing interest in lncRNA biological functions. Forms of those interactions have been reported to be miRNAs triggering lncRNAs to decay, lncRNAs binding miRNAs to depress mRNAs, and lncRNAs generating miRNAs²³.

Before formally conducting the current research, we detected a prospective complementary region of HOTAIR and miR-17-5p in a pretest using a bioinformatics software. Based on that, we started our experiment with a qRT-PCR assay to determine HOTAIR expression levels in cervical cancer tissues and cell lines. As presented in Figure 1, HOTAIR was upregulated in both cancer tissues and cell lines. We then investigated the biological functions of HOTAIR in cervical cancer. Using siRNA of HOTAIR to suppress the HOTAIR expression in cervical cancer cells, we detected that cell viability and migration and invasion abilities were significantly reduced in the environment of low HOTAIR expression (Fig. 2). Increasing research has indicated that lncRNAs such as HOTAIR can participate in the tumorigenesis of cancers. HOTAIR could function by interacting with microRNAs, which is likely to be a complicated gene network²⁴. Guan et al. found that miR-148b-3p suppressed malignant biological behaviors of glioma cells, such as cell cycle progression and invasion, by directly targeting HOTAIR²⁵. Xu et al. reported that HOTAIR acted as a miR-148a sponge in esophageal cancer to increase Snail2 expression, enhance cell invasion and metastasis abilities, and promote the epithelial-to-mesenchymal transition²⁶. Prior to the Xu et al. study, Sun and his team reported similar results in cervical cancer, which indicated that HOTAIR regulated the expression of HLA-G via absorbing miR-148a²⁷.

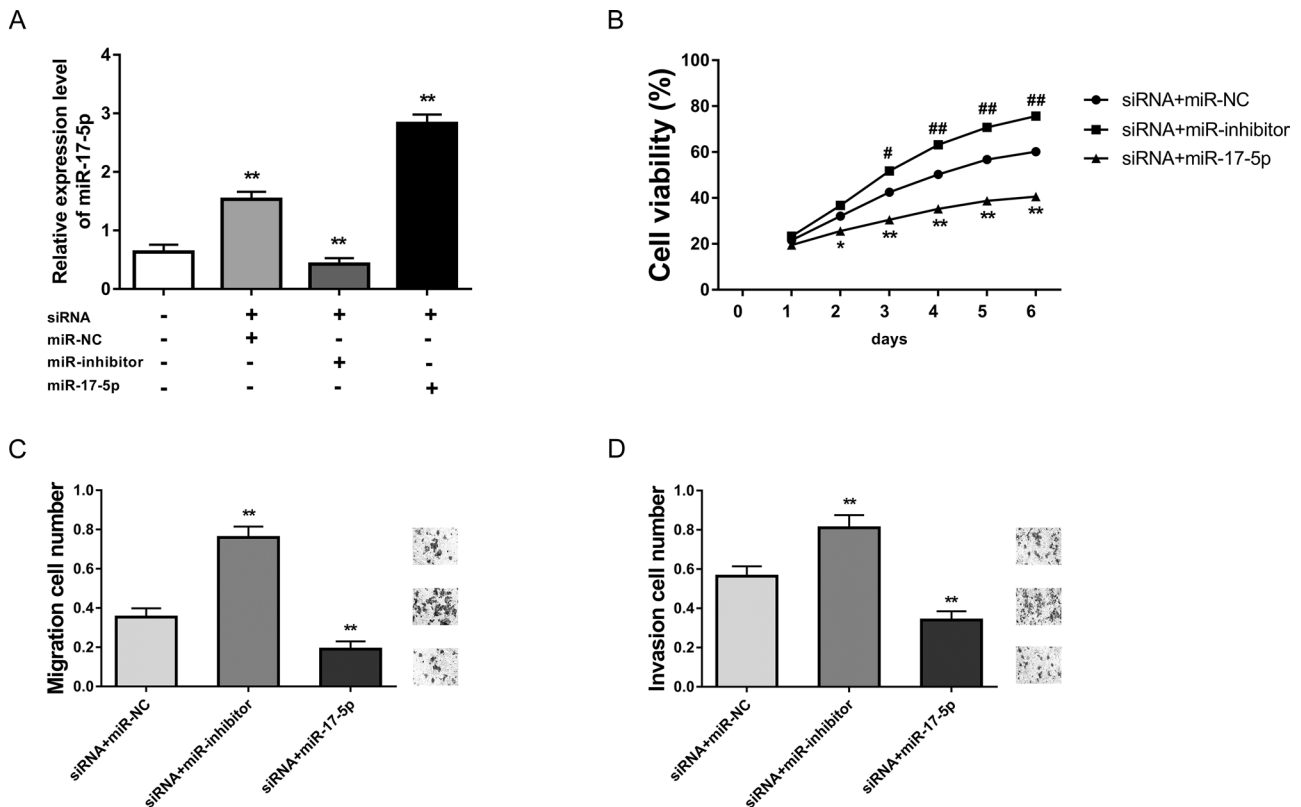


Figure 5. Functions of HOTAIR and miR-17-5p on cervical cancer Caski cells. (A) Expression of miR-17-5p in cervical cancer Caski cells after transfections. (B) Cell viability of cervical cancer Caski cells determined by the CCK-8 assay. (C, D) The cell migration and invasion abilities of cervical cancer Caski cells were determined by Transwell assays. Data are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and # $p < 0.05$, ## $p < 0.01$, calculated with Student's *t*-test.

We continue our study on the basis of our previous miRNA expression profiles of cervical cancer tissue versus normal tissue. We observed that miR-17-5p expression is significantly lower in cervical cancer tissues than normal tissues. miR-17-5p has also been reported to be downregulated in cervical cancer by a previous study¹⁶. We confirmed that using qRT-PCR detected the expression of miR-17-5p in cervical cancer patients' tissues and in cell lines SiHa and Caski. We also found that miR-17-5p expression is negatively correlated with HOTAIR expression, calculated with Pearson's correlation analysis (Fig. 3). This finding provided a hint on the interaction of HOTAIR and miR-17-5p. To our knowledge, the relationship between HOTAIR and miR-17-5p was first proposed by a research team from Augusta University¹⁸. Furthermore, we verified the presumptive connection of HOTAIR and miR-17-5p by detecting luciferase activity after transfecting HOTAIR mimics (mutant or wild type) and miR-17-5p vectors to Caski cells (Fig. 4). The results indicated that the expression of HOTAIR and miR-17-5p in cervical cancer was related by a structural complementarity, and we believe this relation could contribute to a synergistic effect on their biological function in cervical cancer.

Therefore, we investigated the cell viability and migration and invasion abilities of Caski cells after cotransfecting with miR vectors (miR-17-5p, miR inhibitor, and miR NC vectors) and siRNA HOTAIR mimics. The overall results revealed that cotransfection of the miR inhibitor could partially rescue the tumor-suppressive effects caused by siRNA HOTAIR, whereas cotransfection of miR-17-5p could enhance the inhibiting impacts of siRNA (Fig. 4). In general, our results suggested that HOTAIR and miR-17-5p might share a relationship in moderating the development of cervical cancer. Previous research has raised similar opinions. Wei et al. reported that HOTAIR exhibits a role in moderating the osteogenic differentiation by enhancing the DNA methylation level of the miR-17-5p promotor to suppress miR-17-5p expression in nontraumatic osteonecrosis of the femoral head¹⁸. Liu et al. published that HOTAIR could regulate tumorigenesis of gastric cancer in vivo and in vitro through acting as a competing endogenous RNA to sequester miR-331-3p²⁸. Ke et al.'s study showed that knock-down of HOTAIR suppressed the malignant behaviors of glioma cells via increasing miR-326 expression, which further inhibited FGF1 expression to target and block the

PI3K/AKT and MEK1/2 pathways²⁹. However, it is worth noting that our research is the first to establish the synergistic correlation of HOTAIR and miR-17-5p via direct binding, which enhances the cell viability and migration and invasion abilities of cervical cancer.

In conclusion, lncRNA HOTAIR and miR-17-5p both play important roles in the development of cervical cancer, and HOTAIR exerts its tumor-promoting effect by sponging miR-17-5p. Therefore, the HOTAIR/miR-17-5p axis could be a promising therapeutic target for future treatment of cervical cancer.

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