Long Noncoding RNA (lncRNA) HOTAIR Affects Tumorigenesis and Metastasis of Non-Small Cell Lung Cancer by Upregulating miR-613

Caiyu Jiang, Yan Yang, Yang Yang, Lu Guo, Jiang Huang, Xingren Liu, Chi Wu, and Jun Zou

Department of Respiratory and Critical Care Medicine, Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital, Sichuan, P.R. China

Non-small cell lung cancer (NSCLC) is one of the most deadly cancers with poor prognosis. Recent findings suggested that the lncRNA HOTAIR played an important role in tumorigenesis and metastasis. In the present study, HOTAIR was highly expressed in NSCLC tumor tissues and cell lines (H1299, H23, H292, and A549). Downregulation of HOTAIR suppressed cell proliferation and invasion, while it promoted apoptosis of NSCLC cells. The targeting relationship between HOTAIR and miR-613 was first revealed by bioinformatics prediction. miR-613 was found to be lowly expressed in NSCLC tumor tissues and cell lines. Knockdown of HOTAIR increased the expression of miR-613 significantly, and cotransfection with miR-613 inhibitor reversed this increase, indicating that the expression of miR-613 was negatively regulated by HOTAIR. The targeting relationship between HOTAIR and miR-613 was further confirmed through the luciferase report assay. Moreover, the cotransfection of HOTAIR shRNA and miR-613 inhibitor counteracted the tumor inhibition effects of HOTAIR shRNA through promoting cell proliferation and invasion while suppressing apoptosis in NSCLC cells, suggesting that the HOTAIR/miR-613 axis was involved in tumorigenesis and metastasis of NSCLC. In vivo experiments revealed that knockdown of HOTAIR decreased tumor growth and invasion and increased apoptosis and miR-613 expression. In conclusion, our study indicated that downregulation of HOTAIR suppressed tumorigenesis and metastasis of NSCLC via upregulating the expression of miR-613. The HOTAIR/miR-613 axis might provide a new potential therapeutic strategy for NSCLC treatment.

Key words: Hox transcript antisense intergenic RNA (HOTAIR); Drug resistance; Non-small cell lung cancer (NSCLC); Autophagy

INTRODUCTION

Non-small cell lung cancer (NSCLC), which includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and several other types, comprises more than 80% of all lung cancer and accounts for the majority of cancer deaths worldwide. Despite the great advances that have been achieved in early diagnosis and the treatment of NSCLC, the prognosis of NSCLC is still very low, with a 5-year overall survival rate less than 20%. Tumor recurrence and metastasis are the major causes of cancer-related death in human NSCLC. Thus, understanding the mechanism of NSCLC tumorigenesis would help to improve the treatment of this disease. The tumorigenesis of NSCLC is a multistage process, which involves the deregulation of various genes that are crucial for cell proliferation, apoptosis, migration, and other cellular processes. Recently, accumulating evidence had indicated that noncoding RNAs (ncRNAs) play vital roles in the tumorigenesis of NSCLC, providing a new insight into the biology of this disease.

In the human genome, less than 2% of genes are protein-coding genes, and 98% of genes can be transcribed into ncRNAs with no protein-coding capacity. Long noncoding RNA (lncRNA) is a type of RNA molecule that consists of more than 200 nucleotides without protein-coding capacity. Hox transcript antisense intergenic RNA (HOTAIR), identified as an lncRNA, is located within the homeobox C (HOXC) gene cluster on chromosome 12. HOTAIR is reported to be highly expressed in a number of cancers, including cervical cancer, pancreatic cancer, and NSCLC. Research by Gupta and colleagues suggested that HOTAIR had active roles in modulating the cancer epigenome and promoting cancer metastasis. HOTAIR is also reported to
Enhance cell proliferation, migration, and invasion in NSCLC. However, the understanding of the molecular mechanism of HOTAIR in tumorigenesis of NSCLC is still limited.

MicroRNAs (miRNAs) are a group of small ncRNAs that bind to the 3′-untranslated regions (3′-UTRs) of their target mRNAs to regulate the expression of specific genes. Accumulated evidence indicates that miRNAs function as oncogenes or tumor suppressors in different kinds of tumors. miR-613 is found to be involved in many types of tumors. miR-613 is reported to inhibit proliferation and migration in bladder cancer and also inhibit progression of gastric cancer. In NSCLC, it has been found that low miR-613 expression contributed to cell viability and cell cycle progression, and miR-613 may act as a tumor suppressor in NSCLC. Previous studies suggested that lncRNAs could regulate expression of miRNAs, which in turn regulate the expression of specific genes targeted by miRNAs.

In the present study, we found that miR-613 was a potent target of HOTAIR in NSCLC by using bioinformatics. We explored the mechanism of the HOTAIR/miR-613 axis in the tumorigenesis and metastasis of NSCLC. We found that HOTAIR was highly expressed while miR-613 was lowly expressed in NSCLC tumor tissues and cell lines. There exists a negative relationship between the expression of HOTAIR and miR-613, and silencing of HOTAIR decreased tumorigenesis and metastasis of NSCLC by upregulating miR-613. Our findings suggest a new direction for the treatment of NSCLC.

**MATERIALS AND METHODS**

**Clinical Specimens**

A total of 30 NSCLC tumor tissues and 30 adjacent histological normal tissues were collected from patients who underwent surgical treatment at Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital (P.R. China). All cases were reviewed by a pathologist and histologically confirmed as NSCLC based on histopathological evaluation. The specimens were immediately snap frozen in liquid nitrogen and stored at −80°C until further experiments. The study was approved by the Research Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital.

**Cell Culture**

The NSCLC cell lines H1299, H23, H292, and A549 and human bronchial epithelial cell line (HBE) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare Life Science, Logan, UT, USA) at 37°C with 5% CO2.

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

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The expression of HOTAIR was quantified by qRT-PCR using a SYBR Premix ExTaq Reverse Transcription PCR Kit (Takara, Dalian, P.R. China), and GAPDH was used as an internal control for normalization. The primers were HOTAIR, 5′-GGCAAATGTCAAGGGTT-3′ (forward) and 5′-GTTAACACCGACTGGGA-3′ (reverse), and GAPDH, 5′-CGCTGAGTGTCGTAATGGAGT-3′ (forward) and 5′-CGTCAAAGGTTAGGATGGG-3′ (reverse). For the detection of the miR-613 level, TaqMan Assay Kit (Applied Biosystems, Foster City, CA, USA) was used for qRT-PCR. U6 was used as an internal control for normalization. The primers were miR-613, 5′-GTCGATTCAGTGCAGGCTCACAGGACA-3′ (forward) and 5′-CAGCCGATCGTCCCTGGAATGTA-3′ (reverse), and U6, 5′-CAGCCGATCGTCCCTGGAATGTA-3′ (forward) and 5′-AAGCTTTAGCAGGATGGG-3′ (reverse). The relative level was calculated by relative quantification (2^{-ΔΔCt}) method.

**Transfection**

Cells were seeded into 96-well plates at 5×10^3 cells per well and incubated in complete medium for transfection. Short-hairpin RNA (shRNA) directed against the human IncRNA HOTAIR (shRNA-HOTAIR) and negative control HOTAIR scramble were purchased from GenePharma (Shanghai, P.R. China). HOTAIR shRNA and scramble were transfected into H1299 cells using Lipofectamine 2000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer’s protocol. For miR-613 transfection, miR-613 mimic or miR-613 inhibitor and negative control miR-613 mock (GenePharma) were transfected into H1299 cells using Lipofectamine 2000 reagent (Life Technologies Corporation) according to the manufacturer’s protocol.

**Cell Counting Kit-8 (CCK-8) Assay**

Cell proliferation assay was performed with CCK-8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol. The cells were seeded into 96-well plates with 1×10^5 cells per well in triplicate. CCK-8 (10 μl) was added to each well at different time points (0, 1, 2, 3, 4, and 5 days) and then incubated in the dark for 2 h at room temperature. The absorbance was measured at 450 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).
**Cell Apoptosis Analysis**

For cell apoptosis analysis, cells at $2 \times 10^5$ were washed in ice-cold PBS. Then cells were stained with FITC-annexin V and propidium iodide (Beyotime, Beijing, P.R. China) following the manufacturer’s instructions. Cell apoptosis was analyzed in a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**Transwell Invasion Assay**

For Transwell invasion assay, a chamber 6.5 mm in diameter with an 8-mm pore size (Corning, Corning, NY, USA) was used. Cells ($5 \times 10^5$) in medium without FBS were seeded onto the Matrigel-coated membrane matrix of the upper chamber, and the lower chamber was filled with medium supplemented with 10% FBS. After 24 h of incubation, cells on the top of the filter were removed, and cells on the bottom of the filter were fixed in 4% paraformaldehyde and stained with crystal violet. The number of invaded cells was counted under light microscopy (Olympus, Tokyo, Japan).

**Western Blot**

Proteins were extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, P.R. China) containing protease inhibitors. Proteins were then separated by 10% SDS-PAGE gel and transferred into polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA) after electrophoresis. The membranes were incubated with primary antibodies overnight at 4°C after blocking with 5% skim milk for 1 h at room temperature. The primary antibodies (Cell Signaling Technology, Beverly, MA, USA) used in this study include Ki-67, proliferating cell nuclear antigen (PCNA), Bax, Bcl-2, matrix metalloproteinase-3 (MMP-3), vascular endothelial growth factor (VEGF), and GAPDH. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at room temperature. The antibody-bound proteins were detected using the ECL system (Bio-Rad Laboratories, Hercules, CA, USA).

**Bioinformatics Method**

In order to evaluate HOTAIR’s potential target genes, the following online miRNA target prediction algorithms were used: PicTar (http://www.picтар.org/), miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org/vert_71/), and Microcosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/).

**Luciferase Reporter Assay**

The fragment of HOTAIR containing the target sequence of miR-613 was inserted into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to form the reporter vector HOTAIR wild type (HOTAIR-WT), while HOTAIR mutated type (HOTAIR-MUT) contained a mutated binding site. Cells ($5 \times 10^5$) were seeded into a 96-well plate to reach 60% confluence. The cells were then cotransfected with HOTAIR-WT or HOTAIR-MUT and miR-613 mimics using Lipofectamine 2000. The Dual-Luciferase Reporter Assay System (Promega) was used for testing the luciferase activity.

**In Vivo Animal Study**

The 4-week-old BALB/c nude mice were supplied by the Experimental Animal Center of Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital. The animal experiments in this study were approved by the Medical Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital. All animal experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Mice were divided randomly into two groups: control group and HOTAIR shRNA group, with five mice in each group. H1299 cells (1 $\times 10^7$/mice) transfected with or without HOTAIR shRNA were subcutaneously injected into the flank area of mice in different groups. Tumor volumes ($\text{mm}^3$) were measured every 5 days and calculated according to the following formula: tumor volumes ($\text{mm}^3$) = length $\times$ width$^2$/2. The mice were sacrificed 25 days postinjection, and tumor tissues were harvested for further analysis.

**TUNEL Assay**

Tumor tissues were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. The TUNEL assay was performed using Colorimetric TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, P.R. China) according to the manufacturer’s protocol. The numbers of TUNEL$^+$ cells of six random fields were counted under light microscopy. The cells displaying brown staining in the nucleus were counted as positive apoptotic cells. The cell apoptosis rate was calculated as the percent of TUNEL$^+$ cells relative to the total cells.

**Immunohistochemistry**

The 4-μm-thick sections of tumor tissues were used to detect the expression of VEGF by immunohistochemistry. The sections were submerged in hydrogen peroxide to block the endogenous peroxidase after being deparaffinized and rehydrated. Citrate buffer was used to perform antigen retrieval in a water bath at 95°C for 35 min. The sections were blocked with 5% bovine serum...
albumin and incubated overnight at 4°C with the primary antibody anti-VEGF (1:1,000; Abcam, Cambridge, UK). After incubation with peroxidase-conjugated polymer for 30 min, diaminobenzidine (DAB; Beyotime Institute of Biotechnology) was used for detection.

**Statistical Analysis**

All results are presented as mean±standard deviation (SD). Student’s t-test was performed to analyze the difference between groups by SPSS 19.0 software. Values of p<0.05 were considered statistically significant.

**RESULTS**

**HOTAIR Is Upregulated and miR-613 Is Downregulated in NSCLC Tumor Tissues and Cell Lines**

The expression of HOTAIR and miR-613 was detected in NSCLC tumor tissues and cell lines by qRT-PCR. Our data showed that the expression of HOTAIR was upregulated while the expression of miR-613 was downregulated in NSCLC tumor tissues compared with normal tissues. In addition, the expression of HOTAIR was also upregulated and the expression of miR-613 was downregulated in NSCLC cancer cell lines (H1299, H23, H292 and A549).

**Figure 1.** Hox transcript antisense intergenic RNA (HOTAIR) is upregulated while miR-613 is downregulated in non-small cell lung cancer (NSCLC) tumor tissues and cell lines. (A, B) Relative expression of HOTAIR in normal tissues, tumor tissues, tumor cell lines, and normal cell line was detected by quantitative real-time polymerase chain reaction (qRT-PCR). (C, D) Relative expression of miR-613 in normal tissues, tumor tissues, tumor cell lines, and normal cell line was detected by qRT-PCR. The bars show means±standard deviation (SD) of three independent experiments. **p<0.01, ***p<0.001 compared with normal tissues in (A) and (C); *p<0.05, **p<0.01, compared with HBE cells in (B) and (D).

**Figure 2.** Downregulation of HOTAIR suppressed cell proliferation and invasion, while it promoted apoptosis of NSCLC cells. H1299 cells were transfected with HOTAIR scramble or HOTAIR short-hairpin RNA (shRNA). Untreated H1299 cells were used as the control group. (A) Relative expression of HOTAIR in different groups was detected by qRT-PCR. (B) Cell proliferation was determined by the cell counting kit-8 (CCK-8) assay. (C, D) Cell apoptosis was detected by flow cytometric analysis. (E, F) Invasion ability of cells was compared using Transwell model. (G–L) Relative protein levels of proliferation-related proteins [Ki-67 and proliferating cell nuclear antigen (PCNA)], apoptosis-related proteins (Bax and Bcl-2), and invasion-related proteins [matrix metalloproteinase-3 (MMP-3) and vascular endothelial growth factor (VEGF)] were detected through Western blot. The bars show means±SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, compared with HOTAIR scramble group.
HOTAIR shRNA in H1299 cells was detected by qRT-PCR. The knockdown efficiency of HOTAIR was increased and the expression of miR-613 was decreased in NSCLC tumor tissues and cell lines.

**Downregulation of HOTAIR Suppressed Cell Proliferation and Invasion and Promoted Apoptosis of NSCLC Cells**

To investigate the effects of HOTAIR in NSCLC, the shRNA was used to specifically downregulate the expression of HOTAIR. The knockdown efficiency of HOTAIR shRNA in H1299 cells was detected by qRT-PCR (Fig. 2A). Downregulation of HOTAIR suppressed cell proliferation and promoted cell apoptosis compared with the HOTAIR scramble group (Fig. 2B–D). The invasion ability of H1299 cells was also suppressed by HOTAIR downregulation (Fig. 2E and F). Besides that, the expression of proliferation-related proteins (Ki-67 and PCNA), antiapoptosis protein Bcl-2, and invasion-related proteins (MMP-3 and VEGF) was decreased, whereas the expression of proapoptotic protein Bax was increased significantly by HOTAIR shRNA (Fig. 2G–L). Taken together, our results suggested that downregulation of HOTAIR inhibited cell proliferation and invasion while it promoted apoptosis of NSCLC cells.

**miR-613 Is Negatively Regulated by HOTAIR and Inversely Correlated With HOTAIR to Regulate Tumorigenesis and Metastasis in NSCLC**

In order to explore the molecular mechanisms of HOTAIR in NSCLC, putative HOTAIR targets were predicted through bioinformatic analysis. The predicted results showed that miR-613 was one of the potential targets of HOTAIR (Fig. 3A). To explain the relationship between HOTAIR and miR-613, miR-613 mimic and inhibitor were transfected into H1299 cells, and the transfection efficiency is shown in Figure 3B. Remarkably, an increased expression level of miR-613 was observed in the HOTAIR shRNA group, and the cotransfection of HOTAIR shRNA and miR-613 inhibitor significantly counteracted this increase (Fig. 3C). The luciferase reporter assay showed that cotransfection with the miR-613 mimic and HOTAIR-WT in H1299 cells led to a significant decrease in luciferase activity. However, cotransfection with HOTAIR-MUT and the miR-613 mimic did not show a significant difference compared with cells transfected with HOTAIR-MUT (Fig. 3D), confirming the targeting relationship between HOTAIR and miR-613. As indicated above, silencing of HOTAIR suppressed cell proliferation and invasion, and it promoted apoptosis of NSCLC cells. However, cotransfection with miR-613 and HOTAIR shRNA reversed these effects of HOTAIR shRNA significantly through promoting cell proliferation and invasion while inhibiting apoptosis. Moreover, the expression of PCNA and VEGF was increased while the expression of Bax was decreased in the shRNA+miR-613 inhibitor group compared with the shRNA+miR-613 mock group (Fig. 3E–I). In conclusion, our data indicated that HOTAIR negatively regulated the expression of miR-613 in tumorigenesis and metastasis of NSCLC cells.

**Downregulation of HOTAIR Suppresses Tumor Growth and Metastasis and Promotes Apoptosis In Vivo**

Having determined the regulating role of HOTAIR in NSCLC cell lines, we then explored the effects of HOTAIR in tumor progression of NSCLC in vivo. A xenograft mouse model was created by subcutaneous injection of H1299 cells transfected with or without HOTAIR shRNA to BALB/c nude mice. We observed that the tumor volume in the HOTAIR shRNA group grew slower and was also smaller than that in the control group (Fig. 4A). The relative expression of miR-613 was increased in tumor tissues of the HOTAIR shRNA group compared with the control group (Fig. 4B). Silencing of HOTAIR also increased the apoptosis rate and decreased the expression of VEGF significantly compared with the control group (Fig. 4C and D). These results revealed that silencing of HOTAIR suppressed tumor growth and metastasis while promoting apoptosis by upregulation of miR-613 in mice with NSCLC.

**DISCUSSION**

NSCLC is a common malignant tumor with poor prognosis. Although the level of clinical treatment of NSCLC has been improved, the 5-year survival rate is still low. Tumor recurrence and metastasis are two main causes of death in patients with NSCLC. However, the mechanism involved in the tumorigenesis and metastasis of NSCLC is still not well understood and requires further investigations. Previous studies have explained the roles of lncRNAs in tumorigenesis and metastasis of NSCLC. In the present study, we investigated the effects of lncRNA HOTAIR in the tumorigenesis and metastasis of NSCLC in vitro and in vivo. Our results suggested that downregulation of HOTAIR suppressed the tumorigenesis and metastasis of NSCLC through upregulation of miR-613.

Evidence had indicated that HOTAIR was abnormally expressed in various types of tumors including cervical cancer, colorectal cancer, and gastric cancer. Elevated HOTAIR expression has also been found to be correlated with tumor progression of a number of tumors. The study by Li and colleagues revealed that HOTAIR was upregulated in esophageal squamous cell carcinoma (ESCC) cell lines and promoted ESCC cell proliferation and tumor...
HOTAIR shRNA DECREASES DRUG RESISTANCE IN NSCLC

Figure 3. miR-613 is negatively regulated by HOTAIR and inversely correlated with HOTAIR to regulate tumorigenesis and metastasis in NSCLC. H1299 cells were transfected with HOTAIR shRNA, shRNA+miR-613 mock, and shRNA+miR-613 inhibitor, respectively. (A) Bioinformatics analysis of HOTAIR and miR-613. (B) Relative expression of miR-613 in H1299 transfected with miR-613 mock, miR-613 inhibitor, or miR-613 mimic, and control group was detected by qRT-PCR. (C) Relative expression of miR-613 in different groups was detected by qRT-PCR. (D) The interaction between HOTAIR and miR-613 was confirmed by luciferase reporter assay. (E) Cell proliferation was determined by CCK-8 assay. (F) Cell apoptosis was detected by flow cytometric analysis. (G) Invasion ability of cells was compared using the Transwell model. (H, I) Relative protein level of PCNA, Bax, and VEGF was detected by Western blot. The bars show means±SD of three independent experiments. *p<0.05, **p<0.01, compared with the control group; #p<0.05, compared with the shRNA+mock group.
metastasis in mice\textsuperscript{23}. A study by Kim et al. also indicated that knockdown of HOTAIR in pancreatic cancer cells decreased cell proliferation, altered cell cycle progression, induced apoptosis, and inhibited tumor growth in mouse xenograft model\textsuperscript{8}. Consistent with these previous studies, we also observed that the relative expression of HOTAIR was highly expressed in NSCLC tumor tissues and cell lines. Moreover, downregulation of HOTAIR decreased cell proliferation and metastasis, whereas it promoted apoptosis of NSCLC cells, indicating that silencing of HOTAIR suppressed tumorigenesis and metastasis of NSCLC cells. However, the mechanism of how HOTAIR regulated tumor progression in NSCLC was not clearly elucidated. Previous studies revealed that overexpression of HOTAIR targeted polycomb repressive complex 2 (PRC2), a complex composed of EZH2, SUZ12, and EED, and altered H3K27 methylation and gene expression, which finally resulted in tumor invasion and metastasis\textsuperscript{10,24}. HOTAIR was also reported to regulate the expression of miRNA, which in turn regulated the expression of specific genes targeted by miRNA. Cai et al. showed that HOTAIR suppressed the expression of miR-663b via histone modification in pancreatic cancer\textsuperscript{25}. Another study by Wang et al. also indicated that HOTAIR negatively regulated the expression of miR-326 to regulate cell proliferation and migration in lung cancer\textsuperscript{26}.

Figure 4. Downregulation of HOTAIR suppressed tumor growth and metastasis while it promoted apoptosis in vivo. Mice were subcutaneously injected with H1299 transfected with HOTAIR shRNA or untreated H1299 cells. (A) Calculation of tumor volume at 5, 10, 15, 20, and 25 days after injection. (B) Relative expression of miR-613 in tumor tissues was detected by qRT-PCR. (C) Apoptosis index was measured by TUNEL assay. (D) The expression of VEGF in tumor tissues was assayed by immunohistochemistry. The bars show means ± SD of three independent experiments. *p < 0.05, **p < 0.01, compared with control group.
HOTAIR shRNA DECREASES DRUG RESISTANCE IN NSCLC

In the present study, we found that miR-613 might be a potential target of HOTAIR using bioinformatics analysis. Therefore, we focused on the relationship between HOTAIR and miR-613 in tumorigenesis of NSCLC.

miR-613, as a tumor suppressor, was reported to be involved in cell proliferation, invasion, migration, and tumor growth in various kinds of tumors. For example, miR-613 was reported to be downregulated in Wilms’ tumor tissues, and overexpression of miR-613 attenuated Wilms’ tumor cell viability, proliferation, invasion, and migration capacity. miR-613 was also downregulated in gastric cancer cells and suppressed cell proliferation and migration in gastric cancer. Research by Li et al. elucidated that low miR-613 expression contributed to cell viability and cell cycle progression in NSCLC via direct binding to the CDK4 3’-UTR. In agreement with previous studies, our data showed that the expression of miR-613 was markedly decreased in NSCLC tumor tissues and cell lines. In addition, we found that relative expression of miR-613 was elevated by HOTAIR shRNA. Results from dual-luciferase reporter assay further demonstrated the complementary pair binding between HOTAIR and miR-613, suggesting that miR-613 was negatively regulated by HOTAIR.

In pancreatic cancer, it was reported that HOTAIR positively regulated notch3 expression via acting as a competing endogenous RNA for miR-613 binding, and the HOTAIR/miR-613/notch3 axis might be a promising target for therapeutic treatment of pancreatic cancer.

Similarly, in our study, we observed that cotransfection with the miR-613 inhibitor and HOTAIR shRNA promoted cell proliferation and invasion, while it inhibited apoptosis rate compared with the shRNA+miR-613 mock group. Our results showed that inhibition of miR-613 counteracted the tumor inhibition effects of HOTAIR shRNA, indicating that HOTAIR shRNA suppressed tumorigenesis and metastasis through upregulating the expression of miR-613 in NSCLC cells.

Having determined the mechanism of the HOTAIR/miR-613 axis in NSCLC in vitro, in vivo experiments were also carried out for further investigation. Previous research demonstrated that knockdown of HOTAIR suppressed tumor growth in renal cell carcinoma. Downregulation of HOTAIR expression also attenuated tumor growth in thyroid cancer and colorectal cancer. In accordance with these studies, our data showed that knockdown of HOTAIR increased the expression of miR-613, suppressed tumor growth, and promoted cell apoptosis significantly. The expression of invasion-related protein VEGF was also decreased by HOTAIR shRNA. Our results supported the conclusion that HOTAIR shRNA acted as a tumor suppressor of NSCLC through regulating miR-613 in vivo.

Taken together, this present study identified the interaction between HOTAIR and miR-613. Silencing of HOTAIR suppressed tumorigenesis and metastasis of NSCLC through the upregulation of miR-613. The HOTAIR/miR-613 axis might provide a new potential therapeutic strategy for NSCLC treatment.

ACKNOWLEDGMENTS: All the authors conducted the conception and design of the study, the acquisition and interpretation of the data, the drafting of the article, and the final approval of the version to be published equally. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors declare no conflicts of interest.

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics for Hispanics/Latinos, 2012. CA Cancer J Clin. 2012;62(5):283–98.
2. Zarougoudis K, Zarougoudis P, Darwiche K, Boutsikou E, Machairiotis N, Tsakiriadis K, Katsikogiannis N, Kougionoumi I, Karapantzos I, Huang H, Spyropatos D. Treatment of non-small cell lung cancer (NSCLC). J Thorac Dis. 2013;5(Suppl 4):S389–96.
3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin. 2016;66(2):115–32.
4. Bertone P, Stolc V, Royce TE, Rozowsky JS, Urban AE, Zhu X, Rinn JL, Tongprasit W, Samanta M, Weissman S, Gerstein M, Snyder M. Global identification of human transcribed sequences with genome tiling arrays. Science 2004;306(5705):2242–7.
5. Gutmann M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature 2012;482(7385):339–46.
6. Liu XH, Liu ZL, Sun M, Liu J, Wang ZX, De W. The long non-coding RNA HOTAIR indicates a poor prognosis and promotes metastasis in non-small cell lung cancer. BMC Cancer 2013;13:464.
7. Lee M, Kim HJ, Kim SW, Park SA, Chun KH, Cho NH, Song YS, Kim YT. The long non-coding RNA HOTAIR increases tumour growth and invasion in cervical cancer by targeting the Notch pathway. Oncotarget 2016;7(28):44558–71.
8. Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, Burghardt R, Kim S, Safe S. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene 2013;32(13):1616–25.
9. Li N, Wang Y, Liu X, Luo P, Jing W, Zhu M, Tu J. Identification of circulating long noncoding RNA HOTAIR as a novel biomarker for diagnosis and monitoring of non-small cell lung cancer. Technol Cancer Res Treat. 2017;16(6):1060–6.
10. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010;464(7291):1071–6.
11. Zhou C, Ye L, Jiang C, Bai J, Chi Y, Zhang H. Long non-coding RNA HOTAIR, a hypoxia-inducible factor-1alpha activated driver of malignancy, enhances hypoxic cancer cell proliferation, migration, and invasion in non-small cell lung cancer. Tumour Biol. 2015;36(12):9179–88.
12. Wang KC, Chang HY. Molecular mechanisms of long non-coding RNAs. Mol Cell 2011;43(6):904–14.
13. Zeng K, Zheng W, Mo X, Liu F, Li M, Liu Z, Zhang W, Hu X. Dysregulated microRNAs involved in the progression.
of cervical neoplasm. Arch Gynecol Obstet. 2015;292(4):905–13.

14. Wang L, Wang B, Fang M, Guo F, Cui M. Identification of microRNAs and target genes involved in serous ovarian carcinoma and their influence on survival. Eur J Gynaecol Oncol. 2014;35(6):655–61.

15. Yu H, Duan P, Zha H, Rao D. miR-613 inhibits bladder cancer proliferation and migration through targeting SphK1. Am J Transl Res. 2017;9(3):1213–21.

16. Lu Y, Tang L, Zhang Q, Zhang Z, Wei W. MicroRNA-613 inhibits the progression of gastric cancer by targeting CDK9. Artif Cells Nanomed Biotechnol. 2017.

17. Li D, Li DQ, Liu D, Tang XJ. MiR-613 induces cell cycle arrest by targeting CDK4 in non-small cell lung cancer. Cell Oncol. (Dordr) 2016;39(2):139–47.

18. Cai H, Yao J, An Y, Chen X, Chen W, Wu D, Luo B, Yang Y, Jiang Y, Sun D, He X. LncRNA HOTAIR acts a competing endogenous RNA to control the expression of notch3 via sponging miR-613 in pancreatic cancer. Oncotarget 2017;8(20):32905–17.

19. Economopoulou P, Mountzios G. Non-small cell lung cancer (NSCLC) and central nervous system (CNS) metastases: Role of tyrosine kinase inhibitors (TKIs) and evidence in favor or against their use with concurrent cranial radiotherapy. Transl Lung Cancer Res. 2016;5(6):588–98.

20. Schmidt LH, Speiker T, Koschmieder S, Schaffers S, Humberg J, Jungen D, Bulk E, Hascher A, Wittner D, Marra A, Hillejan L, Wiebe K, Wiewrodt R, Muller-Tidow C. The long noncoding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth. J Thorac Oncol. 2011;6(12):1984–92.

21. Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S, Miyano S, Mori M. Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. Cancer Res. 2011;71(20):6320–6.

22. Xu ZY, Yu QM, Du YA, Yang LT, Dong RZ, Huang L, Yu PF, Cheng XD. Knockdown of long non-coding RNA HOTAIR suppresses tumor invasion and reverses epithelial-mesenchymal transition in gastric cancer. Int J Biol Sci. 2013;9(6):587–97.

23. Li X, Wu Z, Mei Q, Li X, Guo M, Fu X, Han W. Long non-coding RNA HOTAIR, a driver of malignancy, predicts negative prognosis and exhibits oncogenic activity in oesophageal squamous cell carcinoma. Br J Cancer 2013;109(8):2266–78.

24. Yao Y, Li J, Wang L. Large intervening non-coding RNA HOTAIR is an indicator of poor prognosis and a therapeutic target in human cancers. Int J Mol Sci. 2014;15(10):18985–99.

25. Cai H, An Y, Chen X, Sun D, Chen T, Peng Y, Zhu F, Jiang Y, He X. Epigenetic inhibition of miR-663b by long non-coding RNA HOTAIR promotes pancreatic cancer cell proliferation via up-regulation of insulin-like growth factor 2. Oncotarget 2016;7(52):86857–70.

26. Wang R, Chen X, Xu T, Xia R, Han L, Chen W, De W, Shu Y. MiR-326 regulates cell proliferation and migration in lung cancer by targeting phox2a and is regulated by HOTAIR. Am J Cancer Res. 2016;6(2):173–86.

27. Wang HF, Zhang YY, Zhuang HW, Xu M. MicroRNA-613 attenuates the proliferation, migration and invasion of Wilms' tumor via targeting FRS2. Eur Rev Med Pharmacol Sci. 2017;21(15):3360–9.

28. Hong Q, Li O, Zheng W, Xiao WZ, Zhang L, Wu D, Cai GY, He JC, Chen XM. LncRNA HOTAIR regulates HIF-1alpha/AXL signaling through inhibition of miR-217 in renal cell carcinoma. Cell Death Dis. 2017;8(5):e2772.

29. Di W, Li Q, Shen W, Guo H, Zhao S. The long non-coding RNA HOTAIR promotes thyroid cancer cell growth, invasion and migration through the miR-1-CCND2 axis. Am J Cancer Res. 2017;7(6):1298–309.

30. Dou J, Ni Y, He X, Wu D, Li M, Wu S, Zhang R, Guo M, Zhao F. Decreasing IncRNA HOTAIR expression inhibits human colorectal cancer stem cells. Am J Transl Res. 2016;8(1):98–108.