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Long noncoding RNA HOXA-AS2 acts as an oncogene by targeting miR-145-3p in human non-small cell lung cancer

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Abstract. – OBJECTIVE: Recent studies have proved that long non-coding RNAs (lncRNAs) play important roles in many diseases, especially malignancies. The aim of this study was to investigate the exact role of lncRNA HOXA-AS2 (Hoxa cluster antisense RNA 2) in the development of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was utilized to detect HOXA-AS2 expression in NSCLC patients. The Wound healing assay and transwell assay were conducted to explore the function of HOXA-AS2 on NSCLC metastasis. Furthermore, the mechanism assays were used to explore the interaction between HOXA-AS2 and microRNA-145-3p (miR-145-3p).

RESULTS: HOXA-AS2 expression level in NSCLC tissues was significantly higher than adjacent tissues. HOXA-AS2 expression was negatively correlated with disease-free survival of NSCLC patients. Moreover, the functional assays showed that the migration and invasion of NSCLC cells were significantly inhibited after HOXA-AS2 *in vitro* silence. Furthermore, the luciferase reporter gene assay also revealed that miR-145-3p was a direct target of HOXA-AS2 in NSCLC.

CONCLUSIONS: Our results indicated that HOXA-AS2 could enhance the migration and invasion abilities of NSCLC by targeting miR-145-3p. Furthermore, these findings suggested that HOXA-AS2 might be a potential therapeutic target for NSCLC.

Key Words:
Long non-coding RNA, HOXA-AS2, Non-small cell lung cancer (NSCLC), MiR-145-3p.

Introduction

Lung cancer is one of the most frequent cancers in the world, both in terms of incidence and mortality. Meanwhile, it remains a public threat to the society[1]. Approximately 234,030 cases are diagnosed with lung cancer worldwide in 2018[2]. Non-small cell lung cancer (NSCLC) accounts for about 85% of lung cancer cases. The surgical resection is the main intervention for NSCLC patients diagnosed in early stages. Currently, tremendous advances have been made in the therapeutic treatment of NSCLC. However, the 5-year survival rate of NSCLC patients is still lower than 15%. Therefore, it is crucial to understand the underlying molecular mechanism of NSCLC and to find out new biomarkers for NSCLC treatment.

90% of the mammalian genome is transcribed to non-coding ribonucleic acids (RNAs). Long non-coding RNAs (lncRNAs) are defined as non-coding RNAs with longer than 200 nucleotides in length. Recent studies have uncovered that lncRNAs are a new frontier field in the research of malignant diseases. For instance, lncRNA UCA1 accelerates the proliferation and cisplatin resistance in oral squamous cell carcinoma by modulating SF1 and suppressing miR-184[3]. In addition, activated by zinc finger E-box binding homeobox 1 (ZEB1), lncRNA HCCL5 accelerates the viability, migration, epithelial-mesenchymal transition (EMT), and malignancy of hepatocellular carcinoma[4]. Furthermore, lncRNA ATB promotes the migration and invasion of glioma cells by activating astrocytes by suppressing the expression of microRNA-204-3p[5].

Scholars have revealed that microRNA (miRNA) plays a crucial role in the regulation of various biological behaviors, including cell proliferation, apoptosis, and metastasis. Likewise, activated by K-Ras carcinogenic signal, miR-155 facilitates the proliferation of pancreatic cancer cells by regulating ROS stress[6]. By targeting FMNL2, miR-613 functions as a tumor suppres-

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sor gene in the progression of colorectal cancer[7]. MiR-126 plays an important role in breast cancer by interacting with a variety of molecules. Meanwhile, it may help to interfere with the inhibition of breast cancer cell metastasis[6]. Through downregulation of transcription factor FOXO1, the over-expression of miRNA-370 promotes the proliferation of human prostate cancer[8].

LncRNA HOXA-AS2 (Hoxa cluster antisense RNA 2) plays an important role in tumor development and metastasis. In this study, we aimed to investigate the function of lncRNA HOXA-AS2 in NSCLC, as well as the interaction between HOXA-AS2 and miR-145-3p.

Patients and Methods

Tissue Samples

Totally, 62 patients who underwent surgical resection at Yantai Mountain Hospital were enrolled in this research. Human NSCLC tissues and adjacent non-tumor tissues were obtained from patients during surgery. After surgical resection, all collected tissue samples were snap-frozen in liquid nitrogen immediately for use. No radiotherapy and chemotherapy treatment were performed in any patient before the surgery. This study was approved by the Research Ethics Committee of Yantai Mountain Hospital. Written informed consents were gathered from all the patients before the study.

Cell Culture

The human NSCLC cell lines (A549, SPCA1, PC-9, and H1299) and immortalized human bronchial epithelial cell line (16HBE) were bought from American Type Culture Collection (ATCC: Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA).

Cell Transfection

Lentivirus expressing short-hairpin RNA (shRNA) targeting HOXA-AS2 (HOXA-AS2/shRNA) synthesized by GenePharma (Shanghai, China) was inserted into shRNA expression vector pGPH1/Neo. Subsequently, HOXA-AS2/shRNA was transfected into A549 NSCLC cells according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in tissues and cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, the extracted total RNA was reverse transcribed to complementary deoxyribonucleic acid (cDNA) through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The primers used in this study were as follows: HOXA-AS2 primers forward: 5′-CCCGTAGGAAGAACCGATGA-3′, reverse: 5′-TTTAGGCCTTCGCAGACAGC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATTCA-3′. The thermal cycle was as follows: 30 s at 95°C, 5 s for 40 cycles at 95°C, and 35 s at 60°C.

Wound Healing Assay

The cells were first transferred into 6-well plates (Corning, Corning, NY, USA) and cultured in RPMI-1640 medium overnight. After scratched with a plastic tip, the cells were cultured in serum-free RPMI-1640. Wound closure was viewed at 48 h. Each assay was independently repeated in triplicate.

Transwell Assay

For detecting the migration ability of transfected cells, 5×10^4 cells in 200 μL serum-free RPMI-1640 were transformed to the upper chamber of an 8 μm pore size insert (Millipore, Billerica, MA, USA). To detect the invasion ability of the transfected cells, 5×10^4 cells in 200 μL serum-free RPMI-1640 were transferred to the upper chamber of an 8 μm pore size insert (Millipore, Billerica, MA, USA) coated with 50 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, the lower chamber was added with RPMI-1640 and FBS. 48 h later, the top surface of the chambers was wiped by a cotton swab. Then, the chambers were immersed for 10 min with pre-cooled methanol and stained with crystal violet for 30 min. Three fields were randomly selected to count the data for invasion membrane.

Luciferase Reporter Gene Assay

The 3′-Untranslated Region (3′-UTR) of HOXA-AS2 was cloned into the pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3′-UTR. Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used for site-di-

rected mutagenesis of the miR-145-3p binding site in HOXA-AS2 3′-UTR as mutant (MUT) 3′-UTR. Subsequently, they were transfected into NSCLC cells. The luciferase assay was detected by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Xenograft Model

After HOXA-AS2 was silenced in A549 cells, the cells were replanted into NOD/SCID mice (6 weeks old). The tumor volume was calculated every 5 days as the formula (volume = length × width2 × 1/2). After 4 weeks, the tumors were extracted and calculated. This experiment was approved by the Animal Ethics Committee of Yantai Mountain Hospital.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. The data were expressed as mean ± SD (Standard Deviation). The Student's *t*-test method and the Kaplan-Meier method were utilized when appropriate. $p<0.05$ was considered statistically significant.

Results

HOXA-AS2 Expression Level in NSCLC Tissues and Cells

We first detected HOXA-AS2 expression in 62 NSCLC patients' tissues and 4 NSCLC cell lines via qRT-PCR. The results showed that the HOXA-AS2 expression in NSCLC tissue samples was remarkably higher than adjunct non-tumor tissues (Figure 1A). Similarly, the HOXA-AS2 expression in NSCLC cells was significantly higher than that of 16HBE cells as well (Figure 1B). Subsequently, all patients were divided into two groups according to the median expression of HOXA-AS2, including: high HOXA-AS2 expression group and low HOXA-AS2 expression group. The Kaplan-Meier analysis showed that the disease-free survival of NSCLC patients in high HOXA-AS2 expression group was significantly worse than that of those in low HOXA-AS2 expression group (Figure 2).

Silence of HOXA-AS2 Suppressed Migration and Invasion of NSCLC Cells

In this study, the A549 NSCLC cell line was chosen for the silence of HOXA-AS2 *in vitro*. The transfection efficiency of HOXA-AS2 was verified by qRT-PCR (Figure 3A). The wound healing assay showed that the silence of HOXA-AS2 significantly inhibited the migrated ability of A549 cells (Figure 3B). The transwell assay revealed that the number of migrated cells remarkably decreased after the HOXA-AS2 silence in A549 cells (Figure 3C). Moreover, the transwell assay revealed that the number of invaded cells was significantly reduced after HOXA-AS2 was silenced in the A549 cells as well (Figure 3D).

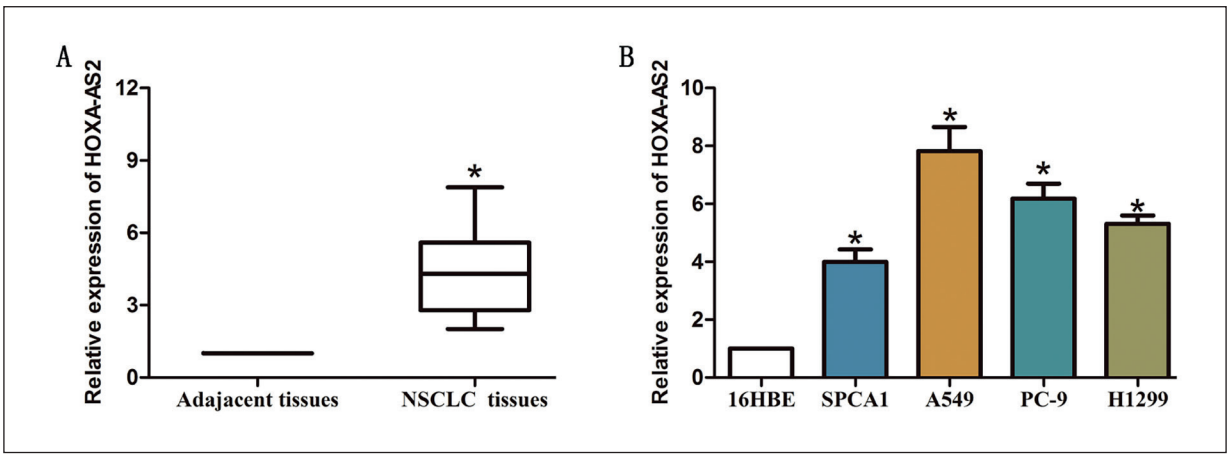

Figure 1. HOXA-AS2 was highly expressed in NSCLC tissues and cell lines. ***A,*** HOXA-AS2 expression significantly increased in NSCLC tissues compared with adjacent tissues. ***B,*** The expression levels of HOXA-AS2 relative to GAPDH were determined in human NSCLC cell lines and normal human bronchial epithelial cell 16HBE by qRT-PCR. The data were presented as mean ± standard error of the mean. *$p<0.05$.

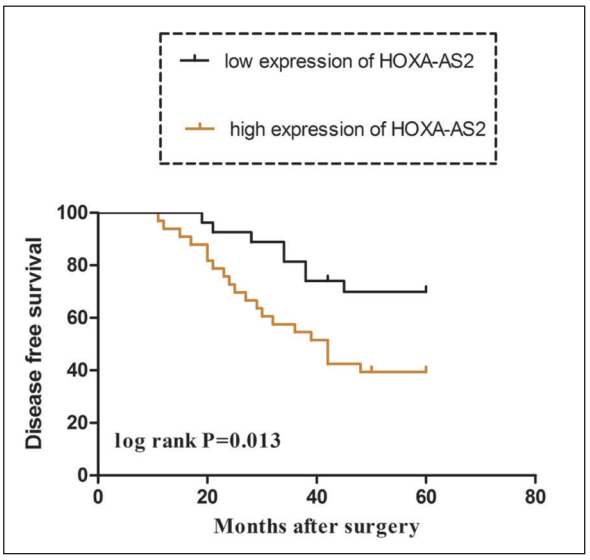

Figure 2. The association between HOXA-AS2 expression and NSCLC patients' prognosis. Higher expression of HOXA-AS2 was associated with worse disease-free survival of NSCLC patients.

Silence of HOXA-AS2 Suppressed Tumor Formation In Vivo

To detect the ability of HOXA-AS2 *in vivo*, the tumor formation assay was conducted in NOD/SCID mice. The results showed that the tumor size in HOXA-AS2/shRNA group was significantly less than that of the control group (Figure 4A). Four weeks later, the tumors were extracted from treated mice, and HOXA-AS2 expression in tumor tissues was detected by qRT-PCR. As a result, HOXA-AS2 was lowly expressed in HOXA-AS2/shRNA group when compared with the control group (Figure 4B).

The Interaction Between MiR-145-3p and HOXA-AS2 in NSCLC

The bioinformation software (DIANA LncBASE Predicted v.2) was used to predict the miRNAs containing a complementary base with HOXA-AS2. MiR-145-3p has been identified as a tumor suppressor in tumor metastasis. Therefore, miR-145-3p was selected from those predicted miRNAs. The predicted binding area of HOXA-AS2 was shown in Figure 5A. The subsequent results demonstrated that miR-145-3p was highly expressed in HOXA-AS2/shRNA cells than that of control cells (Figure 5B). The Luciferase Reporter Gene Assay revealed that the co-transfection of HOXA-AS2-WT and miR-145-3p significantly decreased the luciferase activity (Figure 5C). Furthermore, the miR-145-3p expression level was negatively correlated with HOXA-AS2 expression in NSCLC tissues (Figure 5D).

Discussion

In the past several decades, the morbidity of lung cancer has increased worldwide, especially in industrially advanced countries. However, the main characteristics of NSCLC are migration and invasion of neoplasms, which are responsible for the high mortality rate. Previous studies have demonstrated that lncRNAs are important regulators in NSCLC progression. Up-regulated FGF1 by overexpression of lncRNA RAB1A-2 promotes NSCLC development, eventually leading to poor prognosis[9]. LncRNA PRNCR1 promotes the progression of NSCLC by up-regulating HEY2 through PRNCR1-miR-488-HEY2 network[10].

HOXA-AS2 (Hoxa cluster antisense RNA 2), located on chromosome 6, plays vital roles in various diseases. Authors[11,12] have found that HOXA-AS2 participates in the development of cancers. Likewise, HOXA-AS2 facilitates breast cancer cell metastasis by targeting miR-520c-3p[13]. HOXA-AS2 enhances cell growth ability by targeting P21 and KLF2 in colorectal cancer[14]. In this study, we found that HOXA-AS2 was significantly up-regulated in NSCLC tissues. Besides, the silence of HOXA-AS2 remarkably repressed the migration and invasion of NSCLC cells. The tumor formation assay also revealed that the silence of HOXA-AS2 could remarkably suppress tumor formation *in vivo*. All these results suggested that HOXA-AS2 acted as an oncogene and promoted tumor metastasis of NSCLC.

In this study, we further explored the possible mechanism of HOXA-AS2 function in NSCLC metastasis. Recent studies have shown that lncRNAs interact with microRNAs in malignant tumors. LncRNAs participate in the regulation of tumorigenesis by binding to the related regions of microRNAs as well. Indeed, the knockdown of lncRNA TUG1 depresses cell proliferation and invasion in osteosarcoma *via* sponging miR-153[15]. By interacting with miR-21-5p, lncRNA XIST represses the proliferation and metastasis of the osteosarcoma cells through regulation of PDCD4 expression[16]. LncRNA PVT1 promotes glucose metabolism, cell motility, cell proliferation, and tumor progression in osteosarcoma by modulating miR-497/HK2

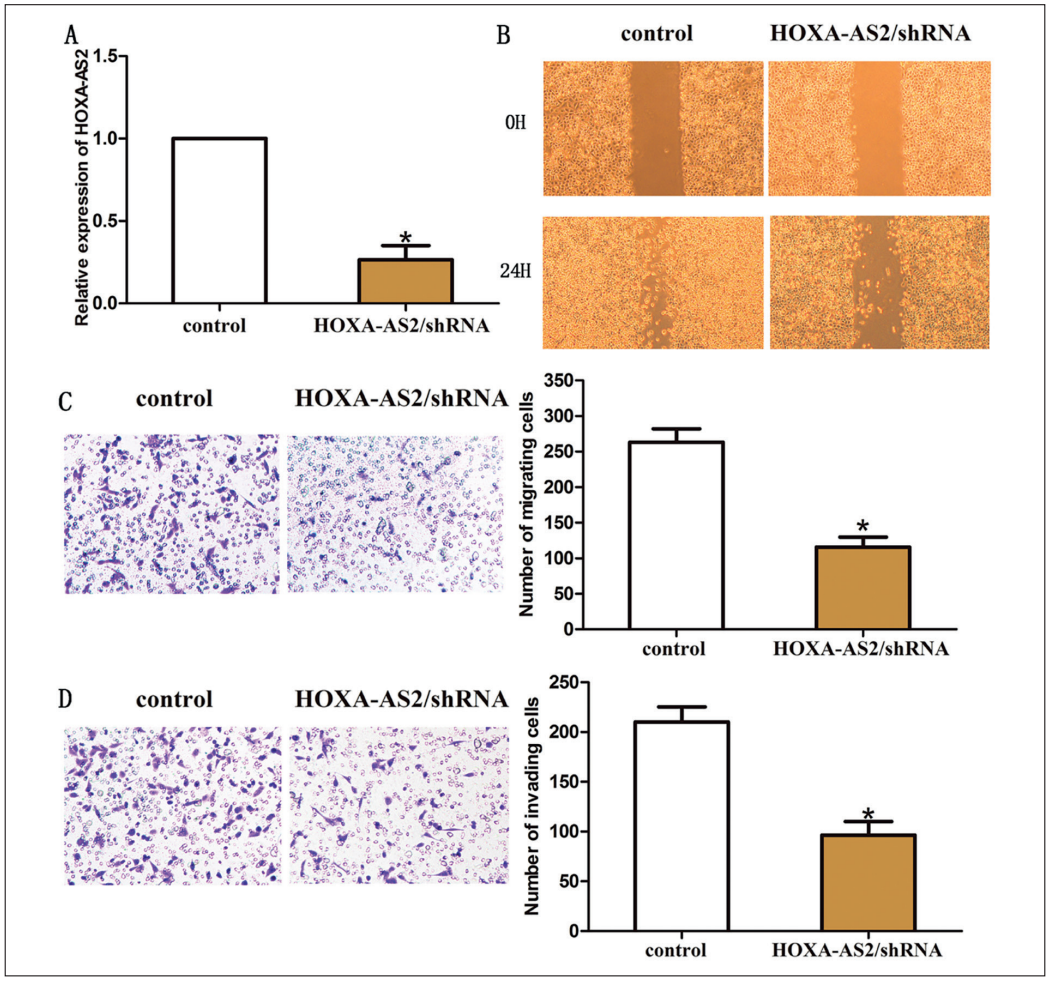

Figure 3. The silence of HOXA-AS2 inhibited the NSCLC cell migration and invasion. ***A,*** HOXA-AS2 expression in A549 NSCLC cells transfected with HOXA-AS2/shRNA and control vector were detected by qRT-PCR. GAPDH was used as an internal control. ***B,*** The wound healing assay showed that the silence of HOXA-AS2 significantly repressed the migration of A549 NSCLC cells (magnification: 10×). ***C,*** The transwell assay showed that the number of migrated cells significantly decreased after the silence of HOXA-AS2 in A549 NSCLC cells (magnification: 40×). ***D,*** The transwell assay showed that the number of invaded cells significantly decreased after the silence of HOXA-AS2 in A549 NSCLC cells (magnification: 40×). The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with control cells.

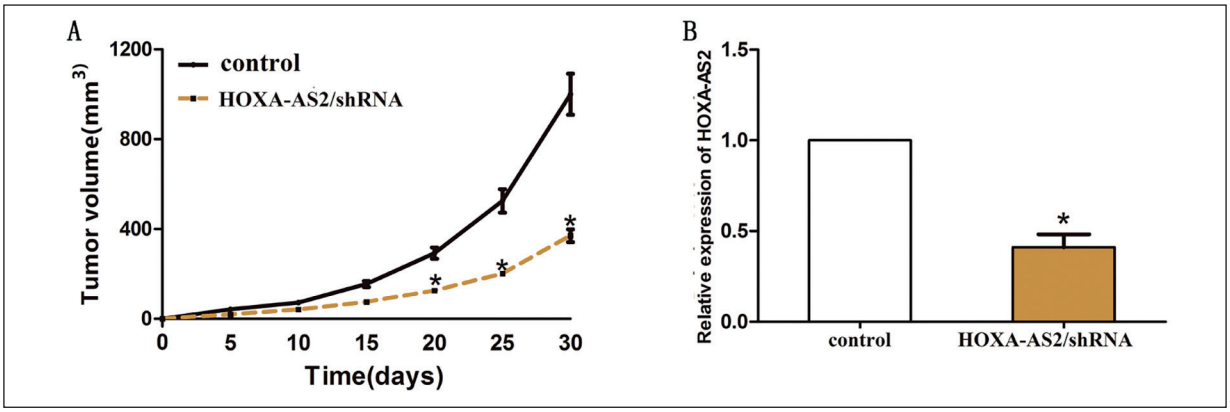

Figure 4. Silence of HOXA-AS2 inhibited tumor formation *in vivo*. ***A,*** After tumor extraction, tumor volume was calculated respectively in control or HOXA-AS2/shRNA group and made into a graph. ***B,*** The relative expression of HOXA-AS2 in tumors was examined by qRT-PCR. Data were presented as mean ± SD of three independent experiments. *p< 0.05.

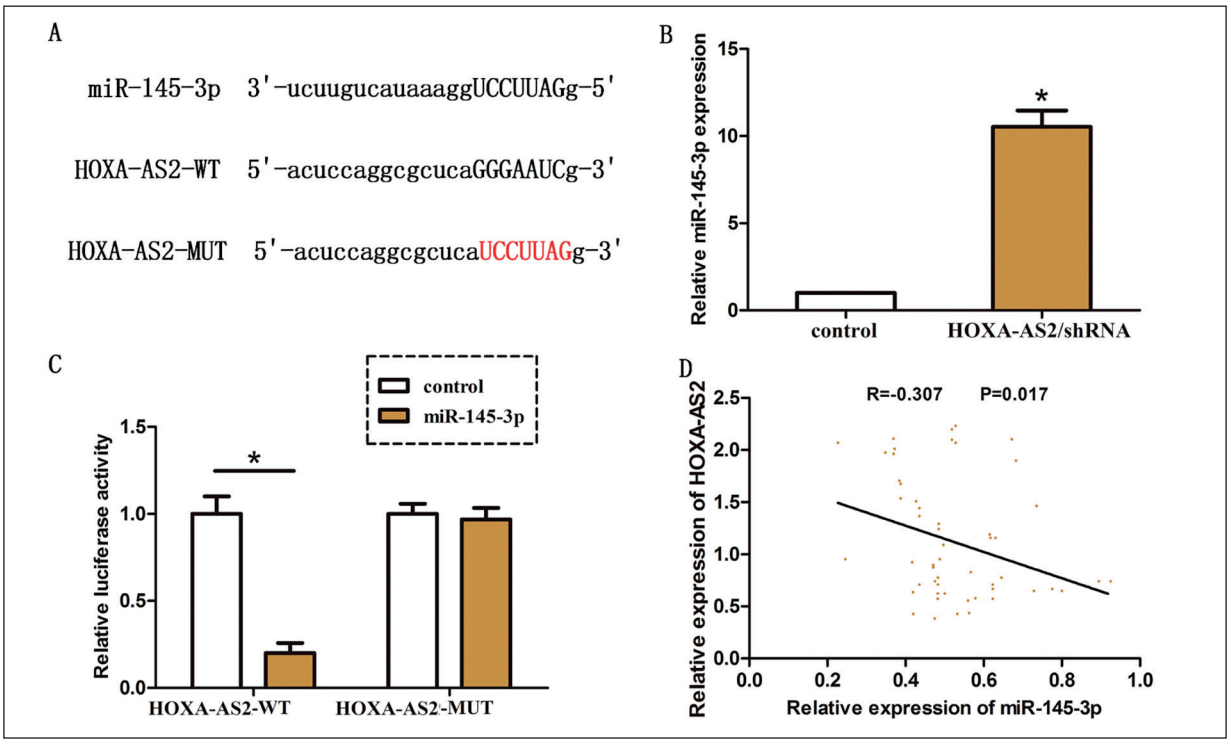

Figure 5. Reciprocal repression between HOXA-AS2 and miR-145-3p. ***A,*** The binding sites of miR-145-3p on HOXA-AS2. ***B,*** MiR-145-3p expression was significantly up-regulated in HOXA-AS2/shRNA group when compared with control group. ***C,*** Co-transfection of miR-145-3p and HOXA-AS2-WT obviously decreased luciferase activity. Co-transfection of miR-control and HOXA-AS2-WT did not change luciferase activity. Meanwhile, co-transfection of miR-145-3p and HOXA-AS2-MUT did not change luciferase activity either. ***D,*** The linear correlation between the expression level of miR-145-3p and HOXA-AS2 in NSCLC tissues. The results represented the average of three independent experiments Data were presented as mean ± standard error of the mean. *$p<0.05$.

axis[17]. By sponging miR-326, lncRNA SNHG1 facilitates tumorigenesis in osteosarcoma through the regulation of NOB1[18].

The bioinformation software was utilized to predict the potential target microRNAs of HOXA-AS2. MiR-145-3p was found abnormally expressed in multiple malignant tumors, including NSCLC. MiR-145-3p inhibits cell proliferation and induces cell apoptosis in osteosarcoma by regulating HDAC4[19]. UHRF1 regulated by miR-145-3p is associated with the repression of cell metastasis in bladder cancer[20]. Moreover, miR-145-3p is identified as a tumor suppressor gene in NSCLC[21,22]. In the present report, we firstly investigated the interaction between miR-145-3p and HOXA-AS2. The results demonstrated that miR-145-3p could directly bind to HOXA-AS2 *via* luciferase reporter gene assay. In addition, miR-145-3p expression was significantly suppressed by the upregulated HOXA-AS2. Furthermore, the expression of miR-145-3p was negatively correlated with HOXA-AS2 in NSCLC tissues. The above results indicated that HOXA-AS2 promoted tumor metastasis of NSCLC by targeting miR-145-3p.

Conclusions

These results showed that HOXA-AS2 was remarkably upregulated in NSCLC tissues and cells. HOXA-AS2 could facilitate the migration and invasion of NSCLC cells by targeting miR-145-3p. Meanwhile, our study first discovered the HOXA-AS2/miR-145-3p axis in NSCLC. Our findings might provide a candidate target for NSCLC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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