Abstract. – OBJECTIVE: Recent studies have revealed the vital role of long non-coding RNAs (lncRNAs) in tumor progression. This study aims to determine whether lncRNA HOXA-AS2 functions in the metastasis of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: Quantitative Real-Time-Polymerase Chain Reaction (QRT-PCR) was conducted to detect HOXA-AS2 expression in NSCLC tissues. Wound healing assay and transwell assay were performed to evaluate the function of HOXA-AS2 in mediating the behaviors of NSCLC cells. Furthermore, the interaction between IGF2 and HOXA-AS2 in mediating the metastasis of NSCLC was analyzed.

RESULTS: By comparing with the expression level in adjacent tissues, HOXA-AS2 expression was higher in NSCLC samples. Moreover, HOXA-AS2 knockdown inhibited invasion and migration of NSCLC cells and, conversely, HOXA-AS2 overexpression obtained the opposite results. In addition, the mRNA and protein expressions of IGF2 were downregulated via HOXA-AS2 knockdown. Besides, the expression of IGF2 was positively correlated to the expression of HOXA-AS2 in NSCLC tissues.

CONCLUSIONS: In this work, HOXA-AS2 could enhance migratory and invasive abilities of NSCLC cells by upregulating IGF2, which might offer a potential therapeutic target for NSCLC.

Key Words: Long non-coding RNA, HOXA-AS2, NSCLC, IGF2.

Introduction

Lung cancer is one of the leading causes of cancer-related death in the world1. Although tremendous advances have been made to reduce the mortality of lung cancer, the prognosis of lung cancer remains dismal with the 5-year survival rate of only 16%2. Non-small cell lung cancer (NSCLC) is the major subtype of lung cancer and accounts for almost 85% of all lung cancer cases3. Surgical resection combined with chemotherapy is the main management for early-stage NSCLC. However, most of NSCLC patients experience disease progression and need further intervention4. Metastasis is the leading cause of mortality in NSCLC. Therefore, it is crucial to clarify the molecular basis underlying the metastasis of NSCLC and to improve the poor prognosis of NSCLC.

With the development of techniques including high-throughput sequencing and microarrays, researchers have discovered that more than 90% of the mammalian genome can be transcribed into noncoding RNAs (ncRNAs). Long non-coding RNAs (lncRNAs), one subgroup of ncRNAs, are important clusters of transcripts without or barely have protein-coding potential. Recently, evidence has proved that lncRNAs are important regulators in many biological behaviors, including carcinogenesis in cancers. For example, lncRNA FTH1P3 enhances the development of oral squamous cell carcinoma by modulating the expression of fizzled 55. LncRNA TP73AS1 dramatically promotes cell apoptosis and depresses cell proliferation in colorectal cancer by functioning as a competing endogenous RNA for sponging miR-103 to modulate the expression of PTEN6. By regulating Sirt7 and PI3K/AKT/mTOR pathway, lncRNA MEG3 depresses cell proliferation and invasion and induces autophagy in glioma7. In addition, lncRNA CDKN2BAS promotes cell growth and migration in hepatocellular carcinoma by mediating miR-153-5p/ARHGAP18 signaling pathway8. However, the function of HOXA-AS2 in NSCLC and the potential molecular mechanism remained unknown so far.
In our research, we found out that the expression of lncRNA HOXA-AS2 was significantly higher in NSCLC tissues. Moreover, HOXA-AS2 promoted the in vitro migration and invasion of NSCLC cells. Furthermore, our study explored the underlying molecular mechanism of HOXA-AS2 in mediating the metastasis of NSCLC.

**Patients and Methods**

**Patients and Sample Collection**

Totally 60 NSCLC cases were enrolled in this research. These NSCLC patients underwent surgery at the Liaocheng People's Hospital. NSCLC tissues and adjacent normal tissues were surgically resected and preserved at -80°C. Informed consent was obtained from every participant before the surgery. The protocol of the study was authorized by the Ethics Committee of the Liaocheng People's Hospital.

**Cell Culture**

Shanghai Model Cell Bank (Shanghai, China) provided us SPCA1, A549, PC-9, H1975 and normal human bronchial epithelial cell (16HBE). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin, and maintained in a 5% CO2 incubator at 37°C.

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

The total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed to complementary deoxyribose nucleic acids (cDNAs) using the Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The following primers were used for qRT-PCR: HOXA-AS2, forward 5'-CCCGTAGGAAGAACCGATGA-3' and reverse 5'-TTTAGGCTTCCAGACAGC-3'; GAPDH, forward 5'-CCAAAATCAGATGGGGCAATGCTGG-3' and reverse 5'-TGAATGGCATGGACTGTGTTCA-3'. Thermal cycle was as follows: 30 sec at 95°C, 5 sec at 95°C and 35 sec at 60°C, for a total of 40 cycles.

**Cell Transfection**

Lentivirus expressing short-hairpin RNA (shRNA) against HOXA-AS2 was provided by GenePharma (Shanghai, China). HOXA-AS2 shRNA (sh-HOXA-AS2) and the empty vector (control) packaged in 293T cells were then used for cell transfection. Meanwhile, the lentiviral virus targeting HOXA-AS2 was cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). HOXA-AS2 lentiviruses (HOXA-AS2) and the empty vector (control) packaged in 293T cells were then used for transfection in A549 cells. 48 h later, detection of the HOXA-AS2 expression level in these cells was conducted using Real Time-quantitative Polymerase Chain Reaction (RT-qPCR).

**Western Blot Analysis**

Reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was utilized to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After loading on the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), membranes were incubated with primary and secondary antibodies provided by Abcam (Cambridge, MA, USA). The chemiluminescent film was applied for the assessment of the protein expression with Image J software (NIH, Bethesda, MD, USA).

**Wound Healing Assay**

Cells seeded in the 6-well plate were transfected with empty vector or HOXA-AS2 shRNA, respectively. Until 90% confluent, cells were scratched by a sterile 10 μL pipette tip and incubated in at 37°C in a humidified incubator containing 5% CO2. The wound closure was determined at 24 h. The experiments were performed three times.

**Transwell Assay**

5×10^4 cells in 200 μL of serum-free RPMI-1640 were applied on the top side of transwell chamber (8 μm in pore size, Corning, Corning, NY, USA) pre-coated with or without 50 μg of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The bottom chamber was applied with RPMI-1640 containing 10% FBS. 48 h later, after being wiped by cotton swab, the top surface of chambers was immersed for 10 min with precooled methanol and stained in crystal violet for 30 min. Invasive cells were counted in three randomly selected fields per well.
LncRNA HOXA-AS2 in non-small cell lung cancer

Statistical Analysis

Statistical analysis was conducted by Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA). Data were presented as mean ± SD (Standard Deviation). Independent t-test was used to compare continuous data. It was considered statistically significant when \( p<0.05 \).

Results

Expression Level of HOXA-AS2 in NSCLC Samples and Cells

First, the expression level of HOXA-AS2 was detected by performing qRT-PCR in NSCLC samples and cell lines. The result revealed that HOXA-AS2 was significantly upregulated in tumor tissue samples (Figure 1A). HOXA-AS2 expression in NSCLC cells was remarkably higher when compared with that in normal human bronchial epithelial cells (16HBE; Figure 1B).

HOXA-AS2 Promoted Migration of NSCLC Cells

SPCA1 and A549 cell lines were used for the overexpression and knockdown of HOXA-AS2 in this study. HOXA-AS2 expression was detected by qRT-PCR for verifying the transfection efficacy (Figure 2A and 2B). Wound healing assay revealed that the knockdown of HOXA-AS2 inhibited migration of SPCA1 cells (Figure 2C). Conversely, the overexpression of HOXA-AS2 promoted the migratory ability of A549 cells (Figure 2D).

HOXA-AS2 Enhanced Invasion of NSCLC Cells

Transwell assay revealed that knockdown of HOXA-AS2 suppressed the invasive ability of SPCA1 cells (Figure 3A). Furthermore, transwell assay revealed that after HOXA-AS2 was overexpressed, the invasive ability of A549 cells was promoted (Figure 3B and 3C).

The Interaction Between IGF2 and HOXA-AS2 in NSCLC Cells

IGF2 expression was significantly lower in HOXA-AS2 shRNA (sh-HOXA-AS2) group when compared with that in empty vector (control) group as qRT-PCR data revealed (Figure 4A). Besides, the expression level of IGF2 was markedly higher in HOXA-AS2 lentiviruses (HOXA-AS2) group compared with that in empty vector (control) group (Figure 4B). Western blot identically revealed that knockdown of HOXA-AS2 inhibited the protein level of IGF2 in NSCLC cells (Figure 4C). Moreover, after HOXA-AS2 was overexpressed, IGF2 was upregulated at the protein level (Figure 4D).

The Interaction Between IGF2 and HOXA-AS2 in NSCLC Tissues

Furthermore, we found out that the expression of IGF2 in NSCLC tissues was remarkably higher when compared with that of adjacent tissues (Figure 5A). Correlation analysis demonstrated that the IGF2 expression level was positively correlated to HOXA-AS2 expression in NSCLC tissues (Figure 5B).
In recent years, emerging researches have revealed that lncRNAs function as crucial regulators in the molecular processes of NSCLC development. For instance, by sponging miR-27b-3p, lncRNA KCNQ1OT1 facilitates cell proliferation and invasion in the progression of NSCLC by upregulating the expression of HSP90AA1. By serving as a sponge of miR-497, lncRNA SNHG1 promotes the development of NSCLC via regulating IGF1-R expression. The overexpression of lncRNA MIN...

**Discussion**

In recent years, emerging researches have revealed that lncRNAs function as crucial regulators in the molecular processes of NSCLC development. For instance, by sponging miR-27b-3p, lncRNA KCNQ1OT1 facilitates cell proliferation and invasion in the progression of NSCLC by upregulating the expression of HSP90AA1. By serving as a sponge of miR-497, lncRNA SNHG1 promotes the development of NSCLC via regulating IGF1-R expression. The overexpression of lncRNA MIN...

**Figure 2.** HOXA-AS2 promoted NSCLC cell migration. **A**, HOXA-AS2 expression in SPCA1 cells transfected with HOXA-AS2 shRNA (sh-HOXA-AS2) or the empty vector (control) was detected by qRT-PCR. GAPDH was used as an internal control. **B**, HOXA-AS2 expression in A549 cells transfected with HOXA-AS2 lentiviruses (HOXA-AS2) or the empty vector (control) was detected by qRT-PCR. GAPDH was used as an internal control. **C**, Wound healing assay showed that wound closure of SPCA1 NSCLC cells significantly decreased via knockdown of HOXA-AS2. **D**, Wound healing assay showed that wound closure of A549 cells markedly increased via overexpression of HOXA-AS2. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05. **p<0.01.

**Figure 3.** HOXA-AS2 promoted NSCLC cell migration and invasion. **A**, Transwell assay showed that number of invasive cells significantly decreased via knockdown of HOXA-AS2 in SPCA1 cells (magnification, 40×). **B**, The transwell assay showed that number of invasive cells remarkably increased via overexpression of HOXA-AS2 in A549 cells (magnification, 40×). The results represent the average of three independent experiments.
CR facilitates cell growth and metastasis in NSCLC by negatively modulating miR-126/SLC7A5 axis. LncRNA CYTOR promotes the abilities of proliferation, migration and invasion in NSCLC cells and induces radiotherapy-resistance by sponging miR-195. Moreover, overexpression of LncRNA-p21 suppresses cell apoptosis in NSCLC by directly downregulating PUMA.

Figure 4. Interaction between HOXA-AS2 and IGF2 in NSCLC cells. A, QRT-PCR results showed that IGF2 expression was inhibited in HOXA-AS2 shRNA (sh-HOXA-AS2) compared with the empty vector (control). B, QRT-PCR results showed that IGF2 expression was upregulated in HOXA-AS2 lentiviruses (HOXA-AS2) compared with the empty vector (control). C, Western blot assay revealed that the protein level of IGF2 decreased in HOXA-AS2 shRNA (sh-HOXA-AS2) compared with the empty vector (control). D, Western blot assay revealed that the protein level of IGF2 increased in HOXA-AS2 lentiviruses (HOXA-AS2) compared with the empty vector (control). The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *p<0.05. **p<0.01.

Figure 5. Interaction between HOXA-AS2 and IGF2 in tissues. A, IGF2 was significantly upregulated in NSCLC tissues compared with adjacent tissues. B, The linear correlation between the expression level of IGF2 and HOXA-AS2 in NSCLC tissues. Data are presented as the mean ± standard error of the mean. *p<0.05. **p<0.01.
HOXA cluster antisense RNA 2 (HOXA-AS2), which is 1048 bp in length, is a lncRNA located in the HOXA cluster between the HOXA3 and HOXA4 genes. Knockdown of HOXA-AS2 inhibits cell proliferation, promotes cell apoptosis in acute myeloid leukemia and also induces chemotherapy-resistance via regulating the expression of miR-520c-3p. HOXA-AS2 promotes the tumor growth, cell migration and cell stenmess in bladder cancer via downregulating miR-125b expression. Serving as a ceRNA of miR-520c-3p, HOXA-AS2 facilitates cell proliferation and invasion in osteosarcoma via promoting epithelial-mesenchymal transition. By acting as a sponge to miR-520c-3p, HOXA-AS2 enhances the progression of papillary thyroid cancer. In this research, we figured out that the HOXA-AS2 was remarkably upregulated in both NSCLC samples. Besides, HOXA-AS2 knockdown suppressed migratory and invasive abilities of NSCLC cells. Conversely, HOXA-AS2 overexpression promoted NSCLC cells to migrate and invade. The above results indicated that HOXA-AS2 might act as an oncogene in NSCLC.

As a member of the IGF/insulin signaling pathway, insulin-like growth factor 2 (IGF2), which possesses the capacities of anti-apoptosis and mitosis, is widely identified to be involved in tumor development and metastasis. For example, overexpression of IGF2 is remarkably correlated with the sensitivity of IGF2-mediated AKT/mTOR pathway, curcumin in functions as a tumor suppressor in the development of urothelial tumor and predicts a poor prognosis. In this research, we figured out that the HOXA-AS2 was remarkably upregulated in both NSCLC samples. Besides, HOXA-AS2 knockdown suppressed migratory and invasive abilities of NSCLC cells. Conversely, HOXA-AS2 overexpression promoted NSCLC cells to migrate and invade. The above results indicated that HOXA-AS2 might act as an oncogene in NSCLC.

Conclusions

We demonstrated that HOXA-AS2 was remarkably upregulated in NSCLC. Besides, HOXA-AS2 could enhance migration and invasion of NSCLC cells by upregulating IGF2. These findings suggested that HOXA-AS2 may contribute to therapy for NSCLC as a candidate target.

Conflict of Interests

The authors declared no conflict of interest.

References


