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Long noncoding RNA HOXD-AS1 promotes non-small cell lung cancer migration and invasion through regulating miR-133b/MMP9 axis

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ABSTRACT

HOXD antisense growth associated long noncoding RNA (HOXD-AS1) was reported to be dysregulated and exert crucial roles in tumorigenesis and progression of multiple malignancies. However, the role and mechanism of action of HOXD-AS1 in the carcinogenesis and progression of non-small lung cell cancers (NSCLC) remains largely unknown. HOXD-AS1, miR-133a and Matrix metallopeptidase 9 (*MMP-9*) mRNA expression were detected by quantitative real-time polymerase chain reaction assays in NSCLC tissues and cell lines. Cell counting kit-8, wound healing and transwell invasion assays were performed to evaluate cell proliferation, migration and invasion abilities, respectively. Luciferase assays were used to investigate binding seeds between miR-133b and HOXD-AS1. Western blot assay were performed to detect protein expression. Here higher expression of HOXD-AS1 was found in NSCLC tumor tissues compared with normal lung tissues, and was associated with lymph node metastasis, high tumor node metastasis (TNM) stage, and poor overall survival rate of patients with NSCLC. Knockdown of HOXD-AS1 significantly inhibited proliferation, migration and invasion of NSCLC cells. Additionally, we found that miR-133b was a direct downstream target of HOXD-AS1 in NSCLC. miR-133b inhibition reverse the inhibitory effect of HOXD-AS1 knockdown on the proliferation, migration, and invasion of NSCLC cells. Furthermore, HOXD-AS1 positively regulated the expression of MMP-9 (a target of miR-133b) in NSCLC cells. These results suggest that HOXD-AS1 might be a potential prognostic biomarker and a novel therapeutic target for treating NSCLC.

1. Introduction

Lung cancer is the most common incident cancer and the leading cause of cancer-related death worldwide, which is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [1]. Among lung cancer, more than 85% of those cases are currently classified as NSCLC [2]. Despite great advances achieved in prognosis and treatment, the overall five-year survival rate of NSCLC is still as low as 15% [3]. Although accumulating evidence have documented that alterations in many oncogene and tumor suppressor are associated with NSCLC progression, the underlying mechanism of NSCLC carcinogenesis remain largely unknown [4]. Therefore, elucidating the molecular mechanisms responsible for NSCLC tumorigenesis is urgently needed to search for high specificity and sensitivity target of this disease.

Long non-coding RNAs (lncRNAs) are generally a class of noncoding RNAs that are over 200 nucleotides in length without evident protein coding functions [5]. Emerging evidence has revealed that lncRNAs are involved in a wide range of biological processes by regulating gene activation and inactivation [6,7]. LncRNAs frequently was dysregulated in various cancers and contributed to tumorigenesis and tumor progression by regulating cell growth, apoptosis, migration, and invasion [8,9]. Numbers of lncRNAs had been reported to be associated with initiation and progression of NSCLC, functioned as oncogene or tumor suppressor [10,11]. The characterization of LncRNAs functions and their clinical applicability in NSCLC is therefore crucial need to search diagnosis marker and therapy agent for NSCLC.

HOXD antisense growth associated long noncoding RNA (HOXD-AS1), an important lncRNA, was reportedly to modulate cell proliferation, apoptosis, migration, invasion, and tumor growth in multiple types of cancers [12–21]. For instance, HOXD-AS1 could promote ovarian cancer cell proliferation and colony formation, and enhanced the migration and invasion capabilities of ovarian cancer cells through miR-608/frizzled family receptor 4 (FZD4) axis in ovarian cancer [12]. Upregulated HOXD-AS1 expression dramatically promoted cell proliferation and invasion of melanoma by regulating RUNX3 [14]. In hepatocellular carcinoma (HCC), knockdown of HOXD-AS1 significantly inhibited migration and invasion of HCC cells *in vitro* and distant lung metastasis *in vivo* by regulating SOX4 [17]. Although a

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study showed that HOXD-AS1 was frequently up-regulated in NSCLC tissues compared to the adjacent normal tissues, and the HOXD-AS1 knockdown reduced proliferation and promoted apoptosis of NSCLC cells by regulating miR-147 [22]. However, the precise role of HOXD-AS1, especially in migration and invasion of NSCLC remains largely unknown. In this study, we investigated the expression and clinical significances of HOXD-AS1 in NSCLC. Using a series of *in vitro* assays we explored the effects of HOXD-AS1 on NSCLC cell proliferation, migration and invasion. Furthermore, the mechanisms underlying the effects of HOXD-AS1 on NSCLC cell migration and invasion are also investigated.

2. Materials and method

2.1. Human tissue specimens and cell culture

Surgically resected NSCLC tissues and corresponding normal lung tissue were obtained from 54 patients at the Department of Department of Respiratory Medicine, The First Hospital of Jilin University (Changchun, China). None of patients received preoperative chemotherapy or radiotherapy before surgical resection. The samples were histologically confirmed to be tumor or non-tumor tissues, and then were quickly frozen in liquid nitrogen until use. Clinical parameters, including pathological features and clinical stage, were retrospectively collected and listed in Table1. Written informed consent was obtained from all of the patients who participated in this study. The project protocol was approved by the Institutional Ethics Committee of the First Hospital of Jilin University.

Normal lung 16HBE epithelial cells and four NSCLC cell lines (A549, H1299, SPCA1, and H358) were bought from Shanghai Institutes for Biological Science, China. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) in a humidified atmosphere containing 5% CO2 at 37 °C.

2.2. RNA isolation and qRT-PCR

Total RNA from cultured cells or tissues was extracted using Trizol reagent (Life Technologies, Carlsbad, CA, USA) using a standard procedure. One microgram of RNA was reversely transcribed into complementary DNA (cDNA) using PrimeScript™ RT Master Mix (TAKARA, Dalian. China) or TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, USA). Quantitative PCR was performed on a 7900HTfast Real-time PCR system (Applied Biosystems, Foster City, CA), using FastStart Universal SYBR-Green Master Mix (Roche, Basel, Switzerland), or with TaqMan microRNA assays. The primers used in this study were listed Table2. The relative expression levels of LncRNA, mRNA, and miRNA were normalized against GAPDH and U6 small nuclear RNA using the comparative cycle threshold (CT) method ($2^{-\Delta\Delta Ct}$).

Table 1
Correlation between clinicopathological features and HOXD-AS1 expression in 52 NSCLC tissues.

Variables	No. of cases	HOXD-AS1 expression		P value
		Low (n %)	High (n %)	
Age (years)				$P > 0.05$
< 60	20	9 (45.0)	11 (55.0)	
≥60	32	15 (46.9)	17 (53.1)	
Gender				$P > 0.05$
Man	28	14 (50.0)	14 (50.0)	
Woman	24	10 (41.7)	14 (58.3)	
TNM stage				$P < 0.05$
I–II	38	22 (57.9)	16 (42.1)	
III–IV	14	2 (14.3)	12 (85.7)	
Tumor size				$P > 0.05$
< 3 cm	34	17 (50.0)	17 (50.0)	
> 3 cm	18	7 (41.2)	11 (58.8)	
Lymph node metastasis				$P < 0.05$
No	40	23 (57.5)	17 (42.5)	
No	12	1 (8.3)	11 (91.7)	

Table 2
Real time PCR primers used for mRNA expression analysis.

Target gene	Prime(5'–3')
U6	F- TCCGATCGTGAAGCGTTC
	R- GTGCAGGGTCCGAGGT
miR-133b	F- AAAGGACCCCAACAACCAGCAA
	R- TTGCTGGTTGTTGGGGTCCTTT
HOXD-AS1	F- CCTTGAAAGTGGGTAAAATGTGC
	R- TAGTTTCCTTGTTCCTTTGTGCTGT
MMP-9	F- GCCTTCGCACTGTGGAGC
	R- GGATACCCGTCTCCGTGCTC
GAPDH	F- AAGGTGAAGGTCGGAGTCAA
	R- AATGAAGGGGTCATTGATGG

Abbreviations: Fforward; mRNAmessenger RNA; PCRpolymerase chain reaction; Rreverse.

2.3. siRNA, miRNAs and transfection

Non-target siRNA control (si-NC) and two siRNAs against HOXD-AS1 (si-HOXD-AS1#1, si-HOXD-AS1#2 were designed and synthesized from GenePharma (Shanghai, China). The miR-133b mimics, negative control mimic (miR-NC), miR-133b inhibitors and corresponding negative mimic(anti-miR-NC) were synthesized by Ribobio (Guangzhou, China). The above-mentioned molecular production were transfected with A549 cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

2.4. Cell proliferation

Transfected cells (2×10^3 cells/well) were plated in 96-well plates in regular growth medium. Cell proliferation was measured using the cell counting kit-8 (CCK-8;Dojindo, Japan) according to the manufacturer's protocol. Optical density (OD) at the wavelength of 450 nm was measured using a Benchmark PlusTM microplate spectrometer (BioRad, Hercules, CA, USA).

2.5. Wound healing assay

A total of 1×10^6 transfected cells were seeded in 6-well plates and grown overnight until reached 100% confluences. Then, a linear wound in the cellular monolayer was created by scraping the confluent cell monolayer with a 200-μl sterile pipette tip. The suspension cells were washed gently with PBS twice and grown in DMEM with free FBS for 24 h. The migration of cells was observed at 0 and 24 h after wounding and then imaged. Wound closure was analyzed in five random fields by ImageJ software (National Institutes of Health, Bethesda, MD, USA) to calculate the migrating distance of cells.

2.6. Cell invasion assay

Cell invasion was evaluated by using transwell cell culture chambers of 8 μm pore size according to the manufacturer's instruction. Briefly, 1.0×10^5 transfected cells in serum-free medium were plated in the upper chamber pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). 600 μl DMEM containing with 10% FBS were added to the lower chamber as a chemoattractant. After 48 h of incubation at 37 °C, cells remaining in the upper chambers were removed using cotton swabs, while cells attached to the lower surfacewere fixed with 4% paraformaldehyde, and stained with 1% crystal violet solution. The