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ELK1-induced upregulation of lncRNA HOXA10-AS promotes lung adenocarcinoma progression by increasing Wnt/β-catenin signaling

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ABSTRACT

In recent years, increasing number of lncRNAs have been studied in lung adenocarcinoma (LAD). LncRNA HOXA10-AS was found to be upregulated in LAD tissues. Based on the data of TCGA database, HOXA10-AS was a prognostic factor for lung adenocarcinoma. This study aims to reveal the biological function of HOXA10-AS in LAD. qRT-PCR was applied to test expression levels of HOXA10-AS in both LAD tissues and cell lines. Next, transcription factor ELK1 was demonstrated to upregulate HOXA10-AS in LAD cells through performing bioinformatics analysis and dual luciferase activity. Loss of function assays were performed in two different LAD cell lines. Silenced HOXA10-AS was proved to inhibit LAD progression by affecting cell proliferation, cell apoptosis and cell metastasis and EMT progress. Western blot analysis revealed that HOXA10-AS increased Wnt/β-catenin signaling in LAD cell lines. Finally, rescue assays were carried out to identify the biological function of HOXA10-AS-Wnt/β-catenin signaling in LAD progression. In conclusion, ELK1-induced upregulation of HOXA10-AS improved LAD progression through increasing Wnt/β-catenin signaling.

1. Introduction

Among all cases of lung cancer, 80% are non-small cell lung cancer (NSCLC) [1]. With the increasing incidence, NSCLC has gradually become the important main cause of cancer-related death all over the world. Lung adenocarcinoma (LAD) tends to form haematogenous metastasis. LAD was acknowledged to be the commonest histological type of NSCLC. Moreover, the majority of LAD patients die of relapse and metastasis [2]. Although a lot of efforts have been made in surgery, chemotherapy, radiotherapy, and targeted therapy, the recovery rate and survival rate of LAD patients are still quite low [3,4]. Hence, deep research of the molecular mechanisms involved in LAD progression is crucial for treatment of LAD patient [5].

The human genome projects revealed that only 2%–3% of the human genomes can code proteins. Among these 2%–3% of the human genomes, more than 75% can be transcribed into non-coding RNAs (including miRNAs and lncRNAs). According to previous studies, we knew that miRNAs can act as tumor suppressors or tumor facilitators in various cancers through modulating their target genes [6,7]. In recent years, lncRNAs have attracted more and more attentions due to their regulatory function in cancers [8–11]. LncRNAs can act as ceRNAs by interacting with miRNAs and mRNA in human cancers [12–14]. Upregulation of lncRNAs induced by transcription factors can promote progression and development of human cancers [15–17]. According to the data of TCGA database, upregulation of lncRNA HOXA10-AS was closely correlated with the unfavorable prognosis of LAD patients. Therefore, we chose HOXA10-AS to do further research. In this study, ELK1 was found to be the upstream transcription factor of HOXA10-AS1. Mechanism assays were carried out in two LAD cell lines. The results manifested that ELK1 actually upregulated HOXA10-AS in LAD cells. Moreover, loss-of function assays was designed and applied to prove the effects of silenced HOXA10-AS on LAD progression. It has been widely reported that lncRNAs can modulate carcinogenesis through modulating its downstream signaling pathway [18–22]. In this study, we uncovered that HOXA10-AS could activate wnt/β-catenin signaling. The results of rescue assays certified that HOXA10-AS1 improved LAD progression via wnt/β-catenin signaling. Collectively, we concluded that ELK1-induced HOXA10-AS upregulation improved LAD progression via increasing wnt/β-catenin signaling.

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2. Materials and methods

2.1. Tissue specimens

86 pairs of LAD tissues and the adjacent normal tissues used in this study were collected and obtained from LAD patients who were diagnosed with LAD in the Tongren Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent had been acquired from all patients. This study received approval of the research ethics committee of Tongren Hospital, Shanghai Jiao Tong University School of Medicine.

2.2. Cell culture and transfection

All cell lines used in this study (one bronchial epithelial cell line: 16HBE; five LAD cell lines: A549, H1299, H1975, PC9 and SPC-A1) were commercially obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured or grown in RPMI-1640 or DMEM medium (GIBCO-BRL) in a humidified air at 37 °C with 5% CO_2. Both media were supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) (Invitrogen, Carlsbad, CA). To knock down HOXA10-AS, specific shRNAs and control shRNAs were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Whereas, ELK1 was silenced with specific siRNAs and si-NC (RiboBio, Guangzhou, Guangdong, China).

2.3. RNA isolation and qRT-PCR

TRIzol reagent (Invitrogen) was utilized to isolate or extract total RNA from tissues or cultured cells. Total RNA (1 μg) was reversely transcribed to cDNA in a final volume of 20 μl under standard conditions with the PrimeScript RT Reagent Kit (Takara, Dalian, China). According to the user guide, SYBR Premix Ex Taq (Takara) was used to perform Real-time PCR analyses. The expression of genes was normalized to the expression of GAPDH. Data were collected by means of $2^{-\Delta\Delta CT}$ method.

2.4. Cell proliferation assays

MTT assay was performed to measure cell viability. Cells were seeded in a 96-well plate for about 24 h. At 48 h' post-transfection, MTT solution (5 mg/ml, 20 μl, Invitrogen) was added into each well for cell incubation. Four hours later, the media was removed and 100 μl DMSO was added into each well. The relative viability of indicated cells was evaluated by measuring the optical density (OD) of cell lysates at 560 nm. For colony formation assay, transfected cells were placed in six-well plates at 1000 cells per well and incubated in DMEM medium (Invitrogen) containing 10% FBS for two weeks under a condition of 37 °C. PBS (Invitrogen) was then used to wash cells. The cells were fixed with ten percent formalin. The number of visible colonies was calculated after stained with 0.1% crystal violet (Sigma, USA).

2.5. Transwell assay

Migration ability of indicated LAD cells were assessed by using transwell chambers (8 μm pore size, Corning). To measure cell migration, transfected LAD cells were placed and cultured in the upper well. One day later, the cells migrated through the transwell plate. Next, the cells were fixed with 3% paraformaldehyde and stained with 1% crystal violet. Finally, a light microscope was utilized to calculate the cell number of migration and invasion.

2.6. Western blot analysis

Total proteins of LAD cell lines were lysed with RIPA buffer (Thermo Fisher Scientific, CA, USA). The protein concentrations were measured with the BCA Protein Assay Kit (Beyotime, Shanghai, China). Protein samples were equally separated with 10% SDS-PAGE and then transferred into PVDF membranes. Subsequently, the membranes were incubated with primary antibodies (anti-E-cadherin: ab15148; anti-N-cadherin: ab98952; anti-*c*-myc: ab32072; anti-β-catenin: ab32572; anti-GAPDH: ab9485) at 4 °C overnight and with HRP-conjugated secondary antibody. The protein blots were visualized by using enhanced hemiluminescence reagents and autoradiography. GAPDH was used as the loading control. All antibodies were purchased from Abcam (Cambridge, UK). An enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Rockford, USA) was used to analyze the protein bands in an imaging system (ZG11SCIBRIGHTCL, Bio-Rad, CA, USA).

2.7. Luciferase reporter assay

After the binding sites between ELK1 and the promoter region of HOXA10-AS was predicted. The different fragment sequences were synthesized and cloned into the pGL4 reporter vector. The transfection efficiency was normalized to the Renilla. The luciferase activity was assessed by utilizing the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

2.8. Statistical analysis

Statistical analyses were conducted with SPSS 17.0 software (SPSS Incorporation, Chicago, IL). Difference between two groups was analyzed with Student's t-test, while differences among multiple groups were analyzed with one-way ANOVA. To analyze the correlation between HOXA10-AS expression and overall survival of LAD patients, survival curve was downloaded from TCGA database. Spearman's correlation analysis was applied to analyze the correlation between HOXA10-AS and ELK1. Error bars in figures represent mean ± SD. $^{*}P < 0.05$ was considered statistically significant.

3. Results

3.1. Upregulation of HOXA10-AS1 is correlated with poor prognosis of LAD patients

To find a lncRNA which is closely correlated with lung adenocarcinoma, we searched and downloaded relative data from TCGA database (https://cancergenome.nih.gov/). According to the data of TCGA database, we knew that HOXA10-AS was upregulated in LAD tissue samples. In addition, upregulation of HOXA10-AS was positively correlated with low overall survival rate of LAD patients (Fig. 1A). So we confirmed that high expression of HOXA10-AS1 was a prognostic factor for LAD patients. To obtain further evidence, we examined the levels of HOXA10-AS in 86 pairs of normal tissues and LAD tissues with qRT-PCR. As expected, HOXA10-AS was upregulated in LAD tissues (Fig. 1B). Likewise, the levels of HOXA10-AS were detected in one normal cell line (16HBE) and five LAD cell lines (A549, H1299, H1975, PC9 and SPC-A1). HOXA10-AS was observably overexpressed in LAD cell lines, especially in A549 and SPC-A1 (Fig. 1C). Therefore, A549 and SPC-A1 cell lines were chosen for next experiments.

3.2. HOXA10-AS is upregulated by the transcription factor ELK1

To explore the upstream molecular mechanism by which HOXA10-AS was upregulated, we applied online software JASPER