

Original Research Article

LncRNA-ATB inhibits the proliferation and invasion of NSCLC cells by regulating MiR-200s expression

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Abstract

Purpose: To investigate the effect of LncRNA-ATB on the proliferation and invasion of non-small cell lung cancer (NSCLC) cells, and its mechanism of action.

Methods: LncRNA-ATB mRNA levels in carcinoma tissues and normal adjacent tissues of 38 NSCLC patients in Peking University Shougang Hospital were determined by reverse transcription-polymerase chain reaction (RT-PCR). Human NSCLC A549 cell line was divided into control and LncRNA-ATB inhibition (si-ATB) groups, respectively. The proliferation and invasion of cells in each group were assessed. Subsequently, the effect of LncRNA-ATB inhibition on the growth of NSCLC cells was evaluated by subcutaneous tumor formation assay.

Results: The expression of LncRNA-ATB was significantly higher in carcinoma tissues than in normal adjacent tissues in NSCLC patients. Cell counting kit-8 (CCK-8) assay results showed that si-ATB group displayed a weakened ability of the cells to proliferate ($p < 0.05$). Furthermore, deoxyribonucleic acid (DNA) replication ability was weaker in si-ATB group than in the control group. Wound healing assay results showed that the migration ability of cells in the si-ATB group was lower than that in the control group. Also, LncRNA-ATB knockdown inhibited the invasion ability of human NSCLC cells ($p < 0.05$). Tumor formation assay data indicate that LncRNA-ATB knockdown significantly repressed the subcutaneous tumor formation ability of NSCLC cells. Furthermore, LncRNA-ATB knockdown in NSCLC cells up-regulated miR-200a, miR-200b and miR-200c.

Conclusion: The expression level of LncRNA-ATB is elevated in carcinoma tissues of NSCLC patients, and its knockdown suppresses the proliferation and invasion of NSCLC cells by up-regulating miR-200s. This finding suggests that it is a potential strategy for the management of NSCLC patients.

Keywords: LncRNA-ATB, Non-small cell lung cancer (NSCLC), Cell proliferation, Cell invasion, miR-200s

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INTRODUCTION

Lung cancer, especially non-small cell lung cancer (NSCLC), is the most common cancer type globally, and is also top among all types of

cancers in terms of incidence and mortality [1]. Although NSCLC patients account for 85 % of lung cancer patients with various types, only a few patients' overall survival has been improved owing to the large heterogeneity of histology and

the genetics of NSCLC, and most of them are placed on palliative chemotherapy [2,3]. Targeted therapy, the most prospective treatment for NSCLC, identifies genetic differences between patients in a fast and accurate way and provides personalized precision therapy [4]. Therefore, it is of great significance to determine the molecular mechanisms of the occurrence and development of NSCLC in order to facilitate its prevention and treatment.

Human transcriptome includes lots of messenger RNAs (mRNAs) encoding proteins and non-protein-coding transcripts comprising long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) with regulatory and unknown functions [5]. In recent years, the effect of lncRNAs on human diseases has gradually attracted attention. Increasing evidence indicate that lncRNAs, as star molecules that can interact with RNAs and deoxyribonucleic acids (DNAs) and promote or suppress expressions of protein-coding genes, as well as regulate proliferation, differentiation, apoptosis, development and metabolism of mammalian cells [6,7]. For example, lncRNA-UCA1 may promote the development of NSCLC through overexpression, and also act as a biomarker for diagnosis and prognosis in the blood plasma of NSCLC patients [8]. It has been reported that expression of lncRNA-activated by TGF- β (lncRNA-ATB) is increased in multiple tumor tissues (liver cancer, bladder cancer and colon cancer) and has a positive correlation with a patient's poor prognosis [9]. However, the expression and effect of lncRNA-ATB in NSCLC have not been reported yet.

By determining the expression of lncRNA-ATB in carcinoma as well as adjacent tissues of NSCLC patients, and suppressing its expression in human NSCLC A549 cell line, the influence of lncRNA-ATB on the proliferation and invasion of human lncRNA-ATB was examined in this study, as well as its mechanism of action. The aim was to provide references for optimization of the treatment of NSCLC patients.

METHODS

Tissue samples

Carcinoma tissues and adjacent tissues were sampled from 38 NSCLC patients receiving surgical treatment in Peking University Shougang Hospital, rinsed with normal saline to wash away blood, cut into pieces, put into EP tubes, and stored in a refrigerator at -80 °C. This study was

approved by the Ethics Committee of Peking University Shougang Hospital (no. SG-18-021). Signed written informed consent was obtained from all participants before the study. The study followed the guidelines of the Declaration of Helsinki [10].

Human NSCLC A549 cell line

Human NSCLC A549 cell line purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China) was cultured with Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) in a constant temperature incubator at 37 °C and sub-cultured. The NSCLC cells were seeded in a 6-well plate (1.0×10^6 cells per well) with medium and cultured in the constant temperature incubator for 36 h. When cells were grown to 70 - 80 % confluence, the cells were transfected with nonsense sequences and small interfering RNAs (siRNAs) of lncRNA-ATB as per the instructions of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA), followed by culturing in the constant temperature incubator at 37 °C.

Determination of mRNA expression of related miRNAs

Total RNAs were extracted from carcinoma tissues and adjacent tissues of NSCLC patients and human NSCLC A549 cell line using TRIzol method (Invitrogen, Carlsbad, CA, USA), followed by determination of the concentration and purity of the extracted RNAs using an ultraviolet spectrophotometer. Next, mRNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) and kept in the refrigerator at -80 °C. Subsequently, RT-PCR was carried out using a system consisting of 2.5 μ L of 10 \times buffer, 1 μ L of cDNAs, 0.5 μ L of forward primer (20 μ mol/L), 0.5 μ L of reverse primer (20 μ mol/L), 10 μ L of LightCycler® 480 SYBR Green I Master ($\times 2$), and 5.5 μ L of ddH₂O (Table I).

Assessment of cell proliferation

Cells in the logarithmic growth phase in each group were inoculated into a 96-well plate and cultured in a 5 % CO₂ constant temperature incubator at 37°C for 12, 24, 36, 48 and 72 h. Then, the medium was removed, and a color development solution was prepared in the dark.

Table 1: Primer sequences of used in RT-PCR

Target gene		Primer sequence
GAPDH	Forward	5' -GACATGCCGCCTGGAGAAAC- 3'
	Reverse	5' -AGCCCAGGATGCCCTTTAGT- 3'
LcRNA-ATB	Forward	5' -ACGTTGTGCTGATGCTGAA- 3'
	Reverse	5' -CGTGTAGTCGTAGTCGTAGTC- 3'
MiR-200a	Forward	5' -CCGTGTAGTCGTAGTCGTAA- 3'
	Reverse	5' -CTGATGTGCATGCTGATGCTA- 3'
MiR-200b	Forward	5' -CGTAGTCGTAGTCGTAGTCGAT- 3'
	Reverse	5' -ACGTAGTCGTAGTCCAGTCGA- 3'
MiR-200c	Forward	5' -CGATGCGATTGTGACTAACGTA- 3'
	Reverse	5' -CTAGTCGTAGTCCCAACATGTC- 3'

Next, the color development solution (110 μ L/well) was added to the plate, and the plate was incubated in the constant temperature incubator at 37°C for 2 h. Thereafter, the absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 540 nm.

Wound healing assay

Cells in logarithmic growth phase were inoculated into a 96-well plate at a density of about 5×10^4 cells/well. Then, a pipette tip was used to scratch the cells in the middle of the plate, the cells dropped due to scratching were washed away with phosphate-buffered saline (PBS), and the medium was replaced using serum-free medium. Next, the migration of cells was photographed at 0 and 24 h under a high-power microscope (Nikon, Tokyo, Japan).

Transwell assay

Transwell chambers (8 μ m) were coated with matrigel diluted at 1: 8 with culture medium (BD Biosciences, Franklin Lakes, NJ, USA), and incubated in an incubator at 37 °C for 2 h for gelatinization. Then, the two cell lines were severally diluted into single-cell suspensions with serum-free medium, and then seeded in the upper chamber at 5×10^4 cells/100 μ L, with medium containing 10 % FBS added to the lower chamber, followed by 48 h of culture. Then, the transmembrane cells were fixed with 5 % glutaraldehyde, stained with 0.1 % crystal violet and photographed.

5-Ethynyl-2'-deoxyuridine (EdU) staining assay

The LncRNA-ATB gene in human NSCLC cells was knocked down with siRNAs for 24 h. Next, the human NSCLC cells were stained using a Click-iT EdU staining kit (Invitrogen, Carlsbad, CA, USA) in accordance with the instructions and the staining results were photographed using a fluorescence microscope, and 3 fields of view

were randomly selected from each slide for counting and quantification of EdU-positive cells.

Assay of subcutaneous tumor formation in nude mice

A total of 20 nude mice were randomly divided into two groups corresponding to cell grouping. The two groups of human NSCLC cells were re-suspended with a mixture of matrigel and PBS prepared at a ratio of 1:1 to make single cell suspensions.

Next, the two groups of nude mice were anesthetized with diethyl ether, the two groups of cell suspensions were subcutaneously injected into the abdomen and lower limbs of the nude mice (107 cells per mouse). Six weeks later, the nude mice were sacrificed using cervical dislocation, and the tumors were separated to measure their sizes.

Statistical analysis

SPSS statistical analysis software (version 26.0) was utilized for analysis of all data. Measurement data are expressed as mean \pm standard deviation. Differences between two groups were analyzed using the Student's t-test. $P < 0.05$ was taken as statistically significant.

RESULTS

Expression of lncRNA-ATB in carcinoma and normal tissues

The RT-PCR test was conducted in this study to determine the expression level of lncRNA-ATB in carcinoma tissues and normal adjacent tissues of 38 NSCLC patients. The results (Figure 1) showed that the expression level of lncRNA-ATB was significantly (about 6.06 times) higher in carcinoma tissues than in adjacent tissues in NSCLC patients ($p < 0.05$).

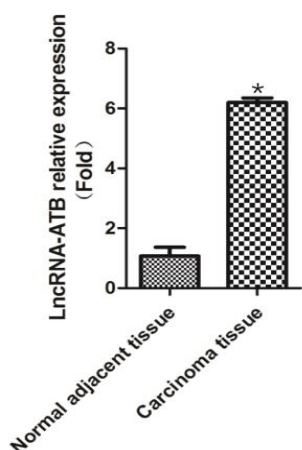


Figure 1: Expression level of lncRNA-ATB in carcinoma tissues and normal adjacent tissues of NSCLC patients. * $P < 0.05$, vs. normal adjacent tissues

Human NSCLC cell line with lncRNA-ATB knockdown

To further explore the effects of lncRNA-ATB on the proliferation and invasion of human NSCLC cells, the human NSCLC A549 cell line with lncRNA-ATB targeted knockdown was constructed. It was discovered through RT-PCR that the mRNA expression of lncRNA-ATB in NSCLC cells was significantly reduced in si-ATB group ($p < 0.05$, Figure 2), indicating successful establishment of the human NSCLC cell line with the specific knockdown of lncRNA-ATB.

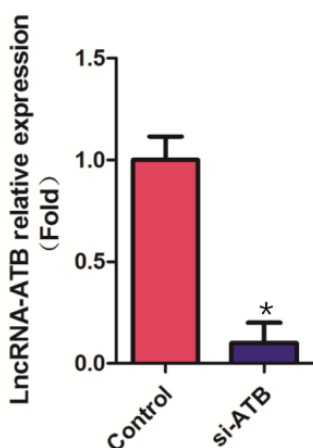


Figure 2: Human NSCLC cell line with lncRNA-ATB knockdown. * $p < 0.05$, vs. control group

Effect of lncRNA-ATB knockdown on proliferation of human NSCLC cells

The results of CCK-8 assay revealed that inhibiting lncRNA-ATB significantly reduced the proliferation level of human NSCLC cells at 12, 24, 36, 48 and 72 h ($p < 0.05$, Figure 3 A).

Results of EdU staining (Figure 3 B) showed that at 24 h after lncRNA-ATB knockdown, the number of EdU-positive cells was significantly reduced in human NSCLC cells ($p < 0.05$), further validating the inhibitory effect of lncRNA-ATB knockdown on the proliferation of human NSCLC cells.

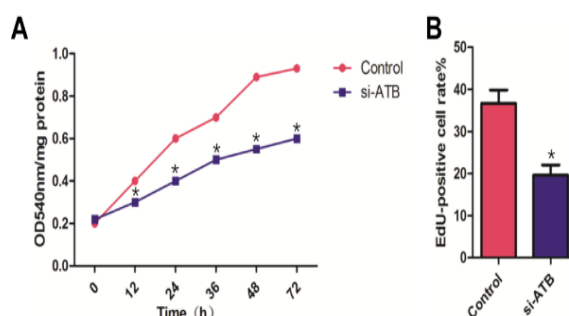


Figure 3: Effect of lncRNA-ATB knockdown on proliferation of human NSCLC cells (x400). * $P < 0.05$, vs. control group

Effect of lncRNA-ATB knockdown on migration of human NSCLC cells

It was discovered through wound healing assay that lncRNA-ATB knockdown decreased the migration level of human NSCLC cells at 24 h ($p < 0.05$, Figure 4), suggesting that lncRNA-ATB has the potential to promote the migration of human NSCLC cells.

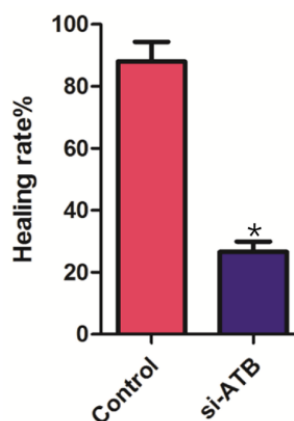


Figure 4: Influence of lncRNA-ATB knockdown on migration of human NSCLC cells. (x20) Control: Control group, si-ATB: si-ATB group. * $P < 0.05$, vs. control group

Effect of lncRNA-ATB knockdown on invasion of human NSCLC cells

The results of Transwell assay showed that the invasion of NSCLC cells was significantly inhibited at 48 h after lncRNA-ATB knockdown ($p < 0.05$; Figure 5).

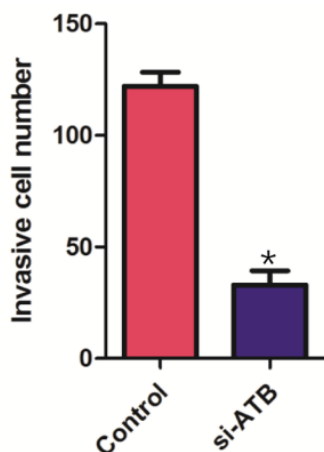


Figure 5: Effect of lncRNA-ATB knockdown on invasion of human NSCLC cells ($\times 200$). * $P < 0.05$, vs. control group

Effect of lncRNA-ATB knockdown in human NSCLC cells on subcutaneous tumor formation in nude mice

The results of subcutaneous tumor formation assay revealed that lncRNA-ATB knockdown evidently repressed the ability of human NSCLC cells to form tumors *in vitro* ($p < 0.05$; Figure 6), a further confirmation that lncRNA-ATB knockdown impeded the growth of human NSCLC cells to some extent.

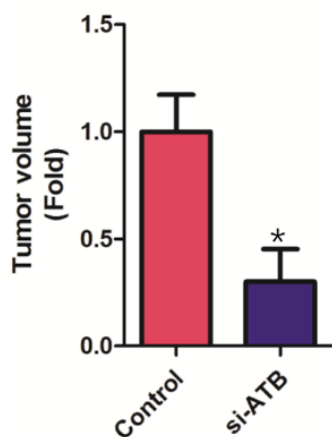


Figure 6: Effect of lncRNA-ATB knockdown in human NSCLC cells on subcutaneous tumor formation in nude mice. * $P < 0.05$, vs. control group

Influence of lncRNA-ATB knockdown on mRNA expression of miR-200s in human NSCLC cells

The results (Figure 7) revealed that lncRNA-ATB knockdown significantly up-regulated the expression levels of miR-200a, miR-200b and miR-200c in human NSCLC cells ($p < 0.05$).

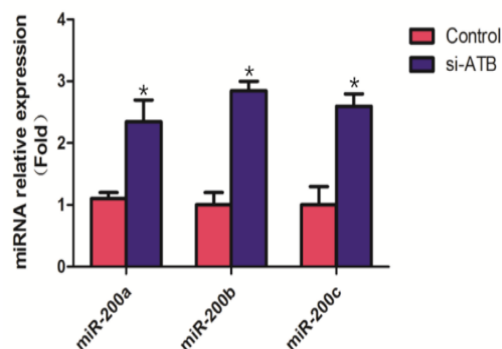


Figure 7: Influence of lncRNA-ATB knockdown on mRNA expression of miR-200s in human NSCLC cells. * $P < 0.05$, vs. control group

DISCUSSION

NSCLC accounts for 85 % of all lung cancer cases, and is a crucial cause of cancer-related death around the world [11]. Although increasing number of scholars have conducted more in-depth studies on the pathogenesis and treatment of NSCLC, prognosis of patients is still poor, with a 5-year survival rate of $< 15\%$ [12]. The major causes of treatment failure and death of NSCLC patients include the following. First, compared with large individual differences among patients, the treatment is relatively single. Second, NSCLC cells have strong proliferative capacity, and are prone to metastasis [13,14]. Therefore, to improve the prognosis and survival rate of NSCLC patients, it is crucial to further study molecular mechanisms of the occurrence and development of NSCLC, and determine key targets of molecular therapy.

lncRNAs is a type of non-coding RNA with a length of over 200 bp [15]. Initially, lncRNAs were regarded as "transcriptional noises", but subsequent research showed that lncRNAs participate in all kinds of important life activities such as epigenetic control, regulation of gene expression, maturation (including splicing and editing) of RNA, and maintenance of chromatin structure [16]. The effect of lncRNAs has been gradually revealed. For instance, lncRNA-NEAT1 can not only act as a biomarker of prognosis but also influence the development of clear cell renal cell carcinoma (CCRCC) by modulating epithelial-mesenchymal transition [17]. Long non-coding RNA-AGER suppresses the proliferation of NSCLC cells by increasing AGER, a related gene of innate immunity [18].

MiRNAs, the small RNAs consisting of about 19-22 bp, negatively regulate the expression of target genes by clipping mRNA and suppressing protein translation. It is estimated that miRNAs

regulate over 30 % of human proteins, so they play an important role in different types of tumors [19].

Five members in the miR-200s family are miR-200a, miR-200b, miR-200c, miR-141 and miR-429 [20]. According to the recent research, miR-200s exhibit totally different biological characteristics in various types of tumors. For example, tumor suppressor gene p53 suppresses the expression of the oncogene CRKL through miR-200s [21]. In osteosarcoma, the overexpression of miR-200s promotes the proliferation, migration and invasion of tumor cells [21]. In contrast, the overexpression of miR-200s in stromata reinforce the invasion of breast cancer cells through the activation of CAF and endothelial-mesenchymal transition [22]. In this research, it was found that the high expression of miR-200s significantly suppressed the proliferation and migration of NSCLC cells, which shows that miR-200s has anti-tumor effect in NSCLC.

Numerous studies have proven that there is cross regulation between lncRNAs and miRNAs. For example, miRNAs cause the decay of lncRNAs, while lncRNAs act as sponges or decoys of miRNAs, can compete with miRNAs in interacting with mRNAs, and are degraded to produce miRNAs [23,24]. lncRNA-CCAT2 negatively regulates the production of miR-145 so as to impede the maturation of colon cancer cells [25]. lncRNA-MALAT1, an endogenous RNA, reduces the miR-200s level in cells through sponge adsorption, therefore inhibiting the expression of ZEB2 and finally repressing CCRCC [26]. In the case of osteosarcoma, by suppressing the expressions of miR-200a, miR-200b, miR-200c, miR-141 and miR-429, lncRNA-ATB can promote the proliferation and invasion of cancer cells, suggesting that lncRNA-ATB has the effect of tumor promotion in osteosarcoma [27]. In the present study, it was found that lncRNA-ATB knockdown suppressed the proliferation, migration and invasion of NSCLC cells, indicating that it plays an anti-cancer role by increasing the expressions of miR-200a, miR-200b and miR-200c in NSCLC cells. Thus, these findings suggest that lncRNA-ATB may suppress the level of miRNA-200s through genes of miRNA sponges or decoys. However, the specific mechanism involved needs to be studied further.

CONCLUSION

The results of this research have revealed, for the first time, that the expression of lncRNA-ATB increases in carcinoma tissues of NSCLC

patients, and that lncRNA-ATB knockdown suppresses the progression of NSCLC by down-regulating miR-200s expression. The results of the current study may provide new strategies for the diagnosis and treatment of NSCLC.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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