LncRNA BX357664 inhibits the proliferation and invasion of non-small cell lung cancer cells

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Abstract. – OBJECTIVE: To explore the level of long non-coding RNA (lncRNA) BX357664 in non-small cell lung cancer (NSCLC) and its role in the development of NSCLC. Meanwhile, the potential regulatory mechanism of BX357664 was also what we were interested in.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to examine the level of BX357664 in 82 pairs of cancer tissues and adjacent normal tissues collected from patients with NSCLC, and the relationship between BX357664 level and pathological parameters or prognosis of NSCLC patients was analyzed. Further verification by RT-qPCR was to examine BX357664 expression in NSCLC cell lines, and BX357664 overexpression model was constructed using lentivirus in NSCLC cell lines including SPCA1 and H1299. In addition, cell counting kit-8 (CCK-8), cell clone formation assay, and transwell assay were performed to analyze the influence of BX357664 on the biological function of NSCLC cells. Western Blot was conducted to explore its underlying mechanisms.

RESULTS: RT-qPCR results indicated that BX357664 in NSCLC was remarkably lower than that in normal tissues. Compared with patients with highly-expressed BX357664, patients with lowly-expressed had worse tumor stage, higher incidence of lymph node metastasis or distant metastasis and lower overall survival rate. In addition, compared with NC group, the proliferation, invasion and migration ability of cells in BX357664 overexpression group was attenuated significantly, and the key proteins in TGF-β1/Smad pathway including transforming growth factor-β1 (TGF-β1), p-Smad2, p-Smad3, N-cad, Vimentin and MMP-9 were also remarkably reduced.

CONCLUSIONS: BX357664 level was significantly reduced in tumor tissues of NSCLC patients, resulting in advanced tumor staging, lymph node metastasis, distant metastasis, and poor prognosis. Additionally, BX357664 may inhibit the proliferation as well as invasion and migration of NSCLC cells by regulating TGF-β1/Smad pathway.

Key Words: BX357664, TGF-β1/Smad signaling pathway, Non-small cell lung cancer, Prognosis, Metastasis.

Introduction

Lung cancer refers to malignant tumors originating from the bronchial or bronchiole mucosal epithelium. The incidence and mortality rate of lung carcinoma are so high that it has become one of the most common malignant tumors in the world, and the number of patients with carcinoma of lung is increasing year by year. Hence, this disease has become a huge threat to human life and the first cause of cancer-related death¹⁻³. Lung carcinoma is mainly divided into two categories, which are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)⁴⁻⁵. Among them, the incidence of the latter accounts for more than 85% of this cancer, and advanced patients account for 40-50%; what’s worse, current radiotherapy and chemotherapy are not sensitive enough to these advanced patients⁵. Non-small cell lung cancer is basically divided into squamous cell carcinoma and non-sinusoidal cell carcinoma (mainly with large cell carcinoma, adenocarcinoma, and other cell types), of which squamous cell carcinoma accounts for about 30-35% of all lung cancers and central type is the most common⁶⁻⁷. Adenocarcinoma accounts for about 35-40% of all lung cancers, which has a higher incidence in non-smokers and the surrounding type is more common⁸. Based on the high recurrence rate in early stage of non-small cell lung cancer and the lack of effective treatment in the advanced stage of the disease, the 5-year survival rate of patients with NSCLC ranges from about 65% of patients in tumor node metastasis (TNM) stage I to less than
% of patients in stage IV, and the overall 5-year survival rate is less than 16%8-10. Patients in stage III or higher without treatment has an average survival of less than 7 months, so the therapeutic method of NSCLC has become a clinical problem9,10. In recent years, as the advancement of science and technology, certain progress has been made in diagnosis and treatment of NSCLC. With the discovery of a series of tumor-targeting genes, a number of drugs targeting NSCLC-involved genes have emerged11. With more and more in-depth research on the pathogenesis of tumor development and development, it is now basically determined that the body’s response to the tumor are caused by changes in certain gene activities and expression in tumor cells. Long non-coding RNA (LncRNA) is one kind of non-coding RNA with the length greater than 200 nucleotides12,13. Although lncRNA does not encode proteins, its involvement can constitute a complicated and very important network of gene expression regulation14,15. Recent studies15 have shown that lncRNAs play a vital role in the development of normal tissues and the regulation of cellular pluripotency as well as cell differentiation. In addition, lncRNAs are involved in the control of multiple molecular pathways, causing changes in gene expression that ultimately regulate cell proliferation, apoptosis, and cell migration15-17. Therefore, the expression disorder of lncRNAs is closely related to various diseases in humans, such as tumor formation15,18. As a new LncRNA, LNCRNA BX357664 can participate in lots of pathophysiological processes such as cell adhesion, migration and proliferation19,20. Since its discovery, many studies have shown that BX357664 is dysregulated in many malignant tumors, such as kidney cancer, colorectal cancer, etc. Meanwhile, its expression level is closely associated with the pathological grade, clinical stage and prognosis of some tumors, but its expression in NSCLC has not been reported19,20.

The transforming growth factor-β1 (TGF-β1) signaling pathway regulates downstream transcription factors-mediated epithelial mesenchymal transition (EMT) to promote tumor invasion and metastasis21. TGF-β1 has been shown to induce EMT in a variety of epithelial cells, including lens, hepatocytes and mammary epithelial cells21-23. Experimental studies21-23 of cell culture in vitro and metastatic tumors in vivo have both confirmed that TGF-β1 can regulate EMT. Therefore, TGF-β1 plays a crucial role in EMT no matter in vitro or in vivo21. The TGF-β1 signaling network is mainly comprised of two transduction pathways including the classical Smad-dependent pathway and the Smad-independent pathway24-26. Therefore, this work aims to explore whether BX357664 can mediate the molecular mechanism of invasion and metastasis of NSCLC through TGF-β1/Smad signaling pathway and thus provide experimental evidence for its clinical application.

In our research, we examined the expression of LncRNA BX357664 in 82 pairs of NSCLC tissues and adjacent tissues and analyzed the relationship between BX357664 level and clinicopathological factors to explore how BX357664 affects the occurrence and progression of NSCLC via TGF-β1/Smad signaling pathway.

**Patients and Methods**

**Patients and NSCLC Samples**

82 cases of primary NSCLC lesions and paracancerous tissues (parallel tissues were more than 5 cm away from cancerous tissues) were resected, and personal information and detailed clinical data of each patient were collected. All patients were diagnosed as NSCLC by postoperative pathological analysis, and no anti-tumor treatment such as radiotherapy or chemotherapy was performed before surgery. The study was approved by the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University and relevant informed consent forms were signed by patients and their families.

**Cell Lines and Reagents**

The human lung cancer cell lines (SKMES1, SPCA1, H358, H1299, A549) and normal lung cell line, 16HBE, were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). High Glucose Dulbecco’s Modified Eagle Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). The cells were cultured with DMEM medium containing 10% fetal calf serum (FCS) in a 37°C, 5% CO₂ incubator.

**Cell Transfection**

Sequences of knockdown or overexpression and the corresponding control were designed by Shanghai Jima Co., Ltd. (Shanghai, China) according to the gene sequence of BX357664 to be loaded in the lentiviral vector. The cells in logarithmic growth phase were seeded into 6-well plates, and the appropriate amount of virus solu-
tion (calculated according to viral MOI) was added and incubated in a cell culture incubator for 48 hours. The fluorescence intensity was evaluated under microscope to evaluate the transfection efficiency for RT-qPCR analysis and cell function experiments. Finally, the stably transfected cell line was screened with puromycin.

**Cell Proliferation Assay**

After 48 h of transfection, cells were digested and seeded into 96-well plates at 2000 cells per well. After 6 h, 24 h, 48 h and 72 h, the cells were added with cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) reagent, respectively. After incubation for 2 hours, the OD value of each well was measured using a microplate reader.

**Cell Scratch**

Marker pen was first used to draw a horizontal line with a ruler on the back of the 6-well plate. About $5 \times 10^5$ cells were added in each well, and the specific amount was different depending on the cells. The next day, tip of gun was used to draw another line perpendicular to the horizontal one, and the tip of the gun should be vertical. The cells were washed 3 times with phosphate-buffered saline (PBS), the scratched cells were removed, and serum-free medium was added. Cell samples were taken out at 0, 12, 24, 48 hours for taking pictures.

**Transwell assay**

After transfection for 48 hours, the cells were trypsinized and resuspended in serum-free medium. After cell counting, cell density was adjusted to 2.0×10⁵/mL, and the transwell chambers with matrigel or without matrigel were placed in a 24-well plate. 200 μL of the cell suspension was added to the upper chamber, and 500 μL medium containing 10% fetal bovine serum (FBS) were added to the lower chamber. After 48 hours of incubation, the chamber was taken out, fixed with 4% paraformaldehyde for 30 minutes, and stained by crystal violet for 15 minutes. After washed with PBS, the inner surface of the chamber was carefully cleaned to remove the cells in inner layer. The stained migrated cells in the outer layer of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

**Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using Quant script RT kit (China Tiangen, Beijing, China). The BX357664 mRNA was quantified by Real-time fluorescent quantitative PCR using the apparatus of ABI’s 7900HT. The experimental procedure was performed according to the Invitrogen specification (Carlsbad, CA, USA), and $2^{-\Delta\Delta C_{t}}$ was used to indicate the relative level of mRNA in tissues. The following primers were used for RT-qPCR reactions: BX357664: forward, 5'-GGCGTTGGTTTTGATGGAGTG-3', and reverse, 5'-AGGCTGCAGAGTTGAGATCG-3'; GAPDH, forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAACGCAACTA -3'.

**Western Blot Assay**

Cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 x g for 15 min at 4°C. Total protein concentration was then calculated using NSCLCA Protein Assay Kit. The extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed according to standard procedures. The primary antibodies were TGF-β1, p-Smad2, p-Smad3, N-cad, Vimentin, MMP-9 and β-actin, and the secondary antibodies were anti-mouse and anti-rabbit, all purchased from Cell Signaling Technology (CST, Danvers, MA, USA).

**Statistical Analysis**

After the data was checked, the database was recorded using Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation (x̄±s). The continuous variables were analyzed by t-test, while the categorical ones were analyzed using χ²-test or Fisher’s exact probability method. Kaplan-Meier method was applied to evaluate the survival time of patients, and the difference between each curve was compared by Log-rank test. p<0.05 was considered to be statistically significant.

**Results**

**BX357664 was Lowly Expressed in NSCLC Tissues and Cell Lines**

RT-qPCR analysis revealed that the mRNA level of BX357664 was markedly decreased in tumor tissues compared with the adjacent normal tissues. The difference was statistically significant (Figure 1A, 1B). At the same time, BX357664 in...
NSCLC cells was also found significantly lower than that in normal lung cells (Figure 1C), especially in SPCA1 and H1299 cells, which were hence chosen for subsequent experiments.

**BX357664 Level was Correlated with Clinical Stage, Lymph Node or Distant Metastasis and Overall Survival of NSCLC Patients**

Based on the RT-qPCR results, tissues were divided into highly-expressed BX357664 group and lowly-expressed group, with the number of each group being counted. Chi-square test was used to analyze the relationship between the lowly-expressed BX357664 and some individual information of patients including age, sex, tumor location, clinical stage, lymph node metastasis and distant metastasis. As shown in Table I, the low level of BX357664 was positively correlated with clinical stage, lymph node metastasis, and distant metastasis of NSCLC, but not with age, gender, and tumor location. In addition, we collected relevant follow-up data in order to figure out the relationship between the level of BX357664 and the prognosis of patients with NSCLC. Kaplan-Meier survival curves indicated that low expression of BX357664 was conspicuously associated with poor prognosis of NSCLC. The lower the level of BX357664, the worse the prognosis (p<0.05; Figure 1D). The above results indicated that BX357664 might be a new biological indicator for predicting the prognosis of NSCLC.

**Over-Expression of BX357664 Inhibited Cell Proliferation**

In order to investigate the effect of BX357664 on the proliferation of NSCLC cells, we first successfully constructed BX357664 overexpression model (Figure 2A, 2B), and tested cell proliferation of the control group and BX357664 overexpression group using CCK8 assay. As shown in Figures 2C and 2D, cells in BX357664 expression group presented a significant decrease in proliferative ability compared with the NC group. And cell clone formation experiments showed the same trend (Figure 2E).

Figure 1. A-B. The expression of BX357664 in 82 pairs of NSCLC tissue was significantly decreased by RT-qPCR. C. Expression levels of BX357664 in 5 NSCLC cell lines (SKMES1, SPCA1, H358, H1299, A549) and normal lung cell line (16HBE) by RT-qPCR. D. Kaplan-Meier survival curves of patients with NSCLC based on BX357664 expression. Patients in the low expression group had a significantly more unfavorable prognosis than those in high expression group. A representative data set is displayed as mean ± SD values. *p<0.05, **p<0.01.
Over-Expression of BX357664 Inhibited Cell Migration and Invasion

Transwell assay was performed to explore the effects of BX357664 on the migration and invasion of NSCLC cells. The results indicated that compared with the NC group, the number of migrated NSCLC cells in BX357664 overexpression group was remarkably reduced, suggesting that their migratory and invasive ability was strikingly enhanced (Figure 3A, 3B), which was consistent with the results of cell scratch test (Figure 3C, 3D).

Over-Expression of BX357664 Suppressed the Activation of TGF-β1/Smad Pathway

To figure out how BX357664 affect cell proliferation or invasive and migratory capacity, we examined the levels of key proteins including TGF-β1, p-Smad2, p-Smad3, N-cad, Vimentin and MMP-9 in the TGF-β1/Smad pathway after knockdown of BX357664. And the results indicated that the above proteins were strikingly decreased (Figure 4).

Discussion

Lung cancer remains the leading cause of cancer-related deaths worldwide, with approximately 80% of cases being non-small cell lung cancer (NSCLC)3-4. Carcinoma of lung is generally diagnosed in advanced stages, which predicts poor prognosis and limitations in treatment options3-4. The progression of lung carcinoma involves the interaction of genetic, epigenetic and environmental factors that can cause the dysregulation of key oncogenes and tumor suppressor genes, ultimately leading to the activation of cancer-related signaling pathways3-4. Over the past decade or so, the discovery of multiple molecular aberrations inducing lung cancer has driven the rapid development of lung cancer research1. There are still about half of NSCLC cases lacking targetable mutation sites, and there exist different degrees of drug resistance in targeted therapy4-7. Therefore, finding alternative treatment strategies for lung carcinoma is of great significance. Nowadays the pharmacological regulation of the epigenome has been used to treat lung cancer, and molecular biology has found that targeted therapy can prevent tumor growth to avoid its heterogeneity3-4. In this study we discuss the current application of molecular targeted therapy in the treatment and discovery of diagnostic markers of lung cancer8,9. Researches in early diagnosis, metastasis, recurrence and adjuvant therapy after advanced NSCLC have become the focus of current research9. Recent studies19,20 have discovered that BX357664 plays a vital role in a variety of diseases, including tumors, but it is unclear whether BX357664 is indispensable in the diagnosis, treatment, and prognosis of NSCLC. Therefore, investigating BX357664 level in NSCLC and analyzing its correlation with clinical prognosis will be beneficial to improve the diagnosis and therapy method of NSCLC as well as improve prognosis of patients.
Long non-coding RNA (LncRNA) is a non-coding RNA of greater than 200 nucleotides in length\textsuperscript{12,13}. It participates in a complex and very important gene expression regulatory network, which can subtly regulate gene expression\textsuperscript{13}. Researches have demonstrated that lncRNAs are essential in normal tissue development and regulation of cell pluripotency and differentiation. In addition, lncRNAs are involved in the control of multiple molecular pathways, causing changes in gene expression, and ultimately regulating cell proliferation, apoptosis and migration\textsuperscript{14,15}. Therefore, the expression disorder of lncRNAs is closely related to many diseases, such as tumor formation\textsuperscript{14}. In addition to being widely expressed in normal tissues, the protein product expressed by BX357664 gene has high levels of expression in various tumor cell tissues: kidney cancer, colorectal cancer, etc.\textsuperscript{19,20}. Studies have observed that BX357664 exerts great influence on migratory and
The invasive capacity of tumor cells. The differential expression of BX357664 in tumor tissues is closely related to tumor metastasis and prognosis. In our work, we first verified that BX357664 level was dramatically down-regulated in NSCLC tissues compared with adjacent normal tissues and positively related to NSCLC staging, lymph node or distant metastasis and poor prognosis. Therefore, we believe that BX357664 may play a role of suppressing cancer. To further understand the impact of BX357664 on the biological function of NSCLC cells, we constructed a

Figure 3. A-B, SPCA1 cells transfected with BX357664 displayed significantly lower migration and invasion capacity. C-D, H1299 cells transfected with BX357664 displayed significantly lower migration and invasion capacity. E, The efficiencies of cell scratch in SPCA1 and H1299 cells with BX357664 over-expression. A representative data set is displayed as mean ± SD values. *p<0.05, **p<0.01.
BX357664 overexpression model using lentivirus. And the results of CCK8 and tranwell assay demonstrated that BX357664 could inhibit the progression of NSCLC; however, the specific molecular mechanism remains elusive. The TGF-β1/Smad signaling pathway can be found in various biological tissues of human. Its main functions include transduction of complex receptor signals on the cell surface through autocrine and paracrine pathways, regulation of cell growth, differentiation, apoptosis, and other functions21-23. It has been found that TGF-β stimulates epithelial cells to first form a ligand receptor complex with TGF-β type II receptor on the cell membrane, and phosphorylates TGF-β type I receptor by TGF-β type II receptor kinase (TβRI) to transmit intracellular signal transduction substances. Smad2/3 is phosphorylated and then binds to Smad4 in the cytosol to form a heterotrimer or tetramer and undergo nuclear translocation, which enters the nucleus and interacts with nuclear transcription factors to promote the expression of the EMT-related markers so as to finally activate EMT. Most of these transcription factors are transcriptional repressors of E-cadherin, which induce the transformation of epithelial phenotype by inhibiting the expression of E-cadherin24-26. It was observed that in keratinized epithelial cells, TGF-β-activated Smad2/3 controls the transdifferentiation of keratinocytes and the expression of the oncogene antagonist MAD125. Mutations in the Smad2 and Smad4 genes were found in some breast cancer patients27. Similarly, Smad3 expression is associated with bladder cancer and colorectal cancer28,29. In the Smad-dependent pathway, Smad7 and Smad6 prevent the development of EMT as an EMT negative factor21-23. Smad6 competitively binds to Smad1 to form an inactive homologous polymer, which negatively regulates the TGF-β/Smad pathway24-26. When the ligand activates the TGF-β type I receptor, Smad7 binds to the membrane receptor, which dephosphorylates Smad2/3 and inhibits the TGF-β transduction pathway24-26. To explore whether BX357664 regulates the development of NSCLC through TGF-β1/Smad pathway, we examined the key proteins including TGF-β1, p-Smad2, p-Smad3, N-cad, Vimentin and MMP-9 by Western Blot. The results revealed that the expression levels of the above proteins were signifi-
cantly decreased, suggesting a negative regulation of BX357664 on the TGF-β1/Smad pathway.

Conclusions

BX357664 was found to have an extremely low expression in NSCLC tissues or cells, which could affect the tumor staging, lymph node or distant metastasis, and poor prognosis. In addition, BX357664 may inhibit cell proliferation or invasive and migratory capacity of NSCLC by regulating TGF-β1/Smad pathway.

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Conflict of Interests
The authors declared no conflict of interest.

References


