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

J. YANG[1], Y.-M. DU[2], B. LI[1]

[1]Department of Pathology, Jinzhou Medical University, Jinzhou, China
[2]Department of Vascular Surgery, the First Affiliated Hospital of Jinzhou Medic iversit Jinzhou, China

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 prog is of NSCLC patients was analyzed. Furth fication by RT-qPCR was to examine BX 64 expression in NSCLC cell lines, and BX35 4 overexpression model was constructed us lentivirus in NSCLC cell lines including SPC and H1299. In addition, cell co t-8 (CCK 8), cell clone formation ass d t well assay were performed to an e the i ence of BX357664 on the biolog unctio cells. Western Blot its underlying mec sms.

RESULTS: RT R results ated that BX357664 in N s remarkab er than that in norm sue mpared wi patients with highly xpressed 7664, patients with lowly-ex sed had wors or stage, higher incide of lymph node me stasis or distant met sis an wer overall survival rate. In ad co ed wi NC group, the proliferation, and n tion ability of cells in 576 rexpr on group was attenuated ican key proteins in TGF-β1/Sm pathwa luding transforming growth fac 1 (TGF-β1), p-Smad2, p-Smad3, N-cad, V MMP-9 were also remarkably reced.

CONCLUSIONS: BX357664 level was signifi y reduced in tumor tissues of NSCLC pa tie 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 NS cells gulati GF-β1/Smad pathwa

Key Words:
BX35766 , TGF-β1 signaling pathway, Non-small c g cancer, H sis, Metastasis,

roduction

ancer refers to malignant tumors originating bronchial or bronchiole mucosal epithelium. The incidence and mortality rate of carcinoma are so high that it has become one t st 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 death[1-3]. 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 patients[5]. 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 common[6,7]. Adenocarcinoma accounts for about 35-40% of all lung cancers, which has a higher incidence in non-smokers and the surrounding type is more common[8]. 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

Corresponding Author: Bo Li, MM; e-mail: libojinzhou@outlook.com

1% 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 problem[9,10]. 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 emerged[11]. 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 nucleotides[12,13]. Although lncRNA does not encode proteins, its involvement can constitute a complicated and very important network of gene expression regulation[14,15]. Recent studies[15] 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, changes in gene expression that ultimately regulate cell proliferation, apoptosis, and cell migration. Therefore, the expression disorder of lncRNA is closely related to various diseases in human such as tumor formation[15,18]. As an lncRNA, LNCRNA BX357664 can participate in lots of pathophysiological processes such as cell invasion, migration and proliferation. Since its discovery, many studies have shown that BX357664 is dysregulated in many malignant tumors, such as kidney cancer, liver 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 reported[19,20].

Transforming growth factor-β1 (TGF-β1) signaling pathway regulates downstream transcription factors-mediated epithelial mesenchymal transition (EMT) to promote tumor invasion and metastasis[21]. TGF-β1 has been shown to induce EMT in a variety of epithelial cells, including lung, hepatocytes and mammary epithelial cells[21-23]. Experimental studies[21-23] 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 vivo*[21]. The TGF-β1 signaling network is mainly comprised of two transduction pathways including the classical Smad-dependent pathway and the Smad-independent pathway[24-26]. 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 pairs of NSCLC tissues and adjacent tissues and analyzed the relationship between BX357664 level and clinicopathological factors. Finally, how BX357664 affects the occurrence and progression of NSCLC *via* TGF-β1/Smad signaling pathway.

Patients and Methods

Patients and NSCLC Samples

62 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% CO^2 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 × 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 2.0×10^5/mL, and 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% formaldehyde for 30 minutes, and stained by crystal violet for 5 minutes. After washed by PBS, the inner surface of the chamber was gently 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 $2^{-\Delta\Delta Ct}$ was used to indicate the relative level of mRNA in tissues. The following primers were used for RT-qPCR reactions: BX357664: forward, 5'-GGCGTGGTTTTGATGGAGTC-3', and reverse, 5'-AGGCTGCAGAGTTGATTCG-3'; GAPDH: forward, 5'-GTGGACATTGCAAAGAC-3', reverse, 5'-AAAGGGTAACGTACTA-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 BCA Protein Assay Kit. 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 ($\bar{x}\pm s$). The continuous variables were analyzed by *t*-test, while the categorical ones were analyzed using χ^2-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

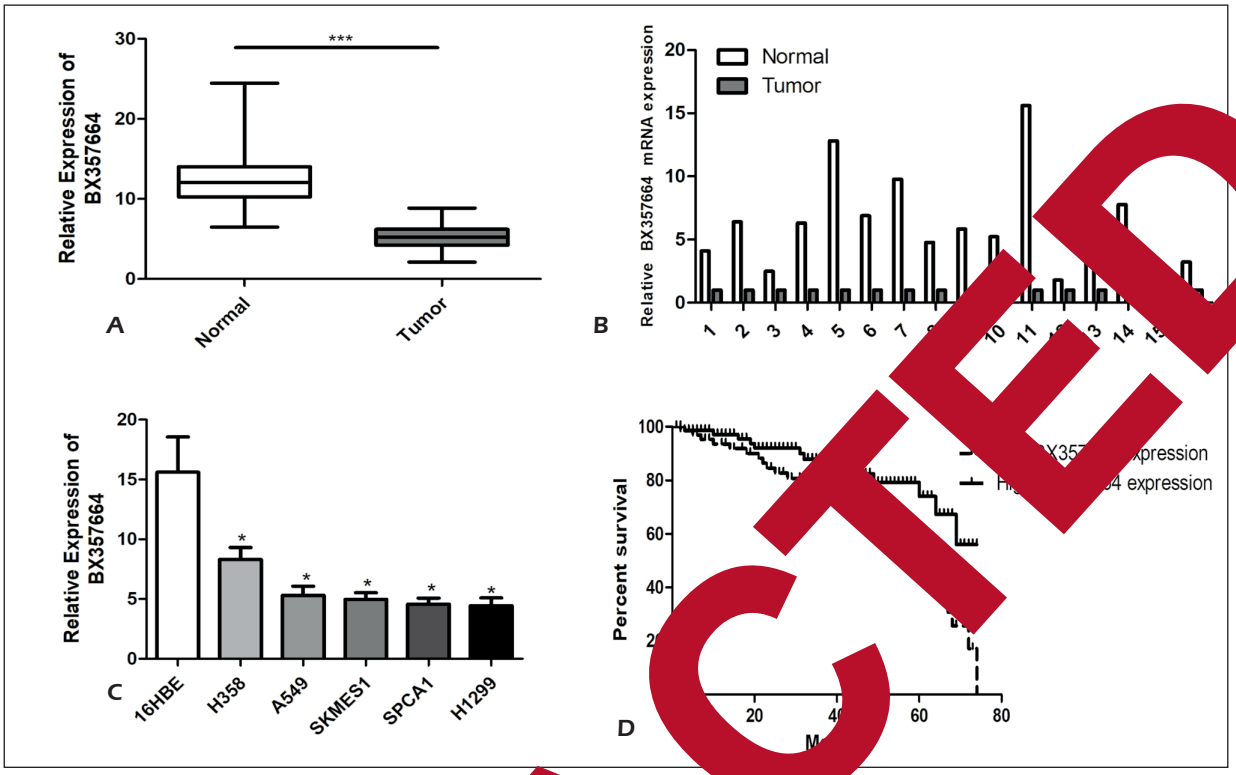


Figure 1. A-B, The expression of BX357664 in 82 NSCLC tissue was significantly decreased by RT-qPCR. **C**, Expression levels of BX357664 in 5 NSCLC cell lines (H358, H1299, A549) and normal lung cell line (16HBE) by RT-qPCR. **D**, Kaplan-Meier survival curves of pa s with sed on BX357664 expression.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

NSCLC cells was also fou ignific y lower than that in normal lun s (Fi pecially in SPCA1 an H12 which e hence chosen for su quent e ments.

BX357664 l Correlate with Clinica tage, ph Node or Dista etastasis Overall Surviv f NSCLC Patie

B on the qPCR results, tissues were divi to hi expressed BX357664 group and lowly group th the number of each p be ount hi-square test was used t lyze t ship between the lowly-expre BX35 and some individual informatio ients including age, sex, tumor location, l lymph node metastasis and distant etastasis. As shown in Table I, the low level of 57664 was positively correlated with clinical st ymph 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 overexpression group presented a significant decrease in proliferative ability compared with the NC group. And cell clone formation experiments showed the same trend (Figure 2E).

Table I. Association of BX357664 expression with clinicopathological characteristics of NSCLC.

Parameters features	No. of cases	BX357664 expression		*p*-value
		High (%)	Low (%)	
Age (years)				
< 60	35	21	14	
≥ 60	47	25	22	
Gender				
Male	40	25	15	
Female	42	22	20	
T stage				0.045
T1-T2	46	31	15	
T3-T4	36	16	20	
Lymph node metastasis				0.0
No	48	33		
Yes	34	15	19	
Distance metastasis				0.034
No	63	38		
Yes	19	8	11	

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 indi[illegible]d that compared with the NC group, the nu[illegible] migrated NSCLC cells in BX357664 overe[illegible]s-sion group was remarkably reduced, sugge[illegible] that their migratory and invasive ability w[illegible] strikingly enhanced (Figure 3A[illegible] which wa[illegible] consistent with the results [illegible]ch test (Figure 3C, 3D).

Over-Expression [illegible]BX357[illegible] Suppressed th[illegible]tivation of TGF-β1/S[illegible]way

To figure [illegible] how [illegible]7664 affect cell proliferation[illegible]asive and [illegible]tory capacity, we examin[illegible]he levels of key [illegible]teins including TGF[illegible] p-Sma[illegible] p-Smad3, N-cad, Vimentin an[illegible]P-9 [illegible]he TGF-β1/Smad pathway after knock[illegible] BX35[illegible]. And the results in-[illegible]ted [illegible]he ab[illegible] proteins were strikingly [illegible]sed ([illegible]

Discussion

[illegible]ung cancer remains the leading cause of can-ce[illegible]ated deaths worldwide, with approximately 80% of cases being non-small cell lung cancer (NSCLC)[1-3]. Carcinoma of lung is generally diagnosed in advanced stages, which predicts poor [illegible]nosis and lim[illegible]ations in treatment options[3,4]. [illegible] progression [illegible] lung carcinoma involves the i[illegible]tion of g[illegible]tic, epigenetic and environmen[illegible]ator[illegible]t can cause the dysregulation of key [illegible]es and tumor suppressor genes, [illegible]ltimately leading to the activation of cancer-re-[illegible]ignaling pathways[3-5]. Over the past decade [illegible]e discovery of multiple molecular aberrations inducing lung cancer has driven the rapid development of lung cancer research[5]. There are still about half of NSCLC cases lacking targetable mutation sites, and there exist different degrees of drug resistance in targeted therapy[4-7]. 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 heterogeneity[3,4]. In this study we discuss the current application of molecular targeted therapy in the treatment and discovery of diagnostic markers of lung cancer[8,9]. Researches in early diagnosis, metastasis, recurrence and adjuvant therapy after advanced NSCLC have become the focus of current research[9]. Recent studies[19,20] 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.

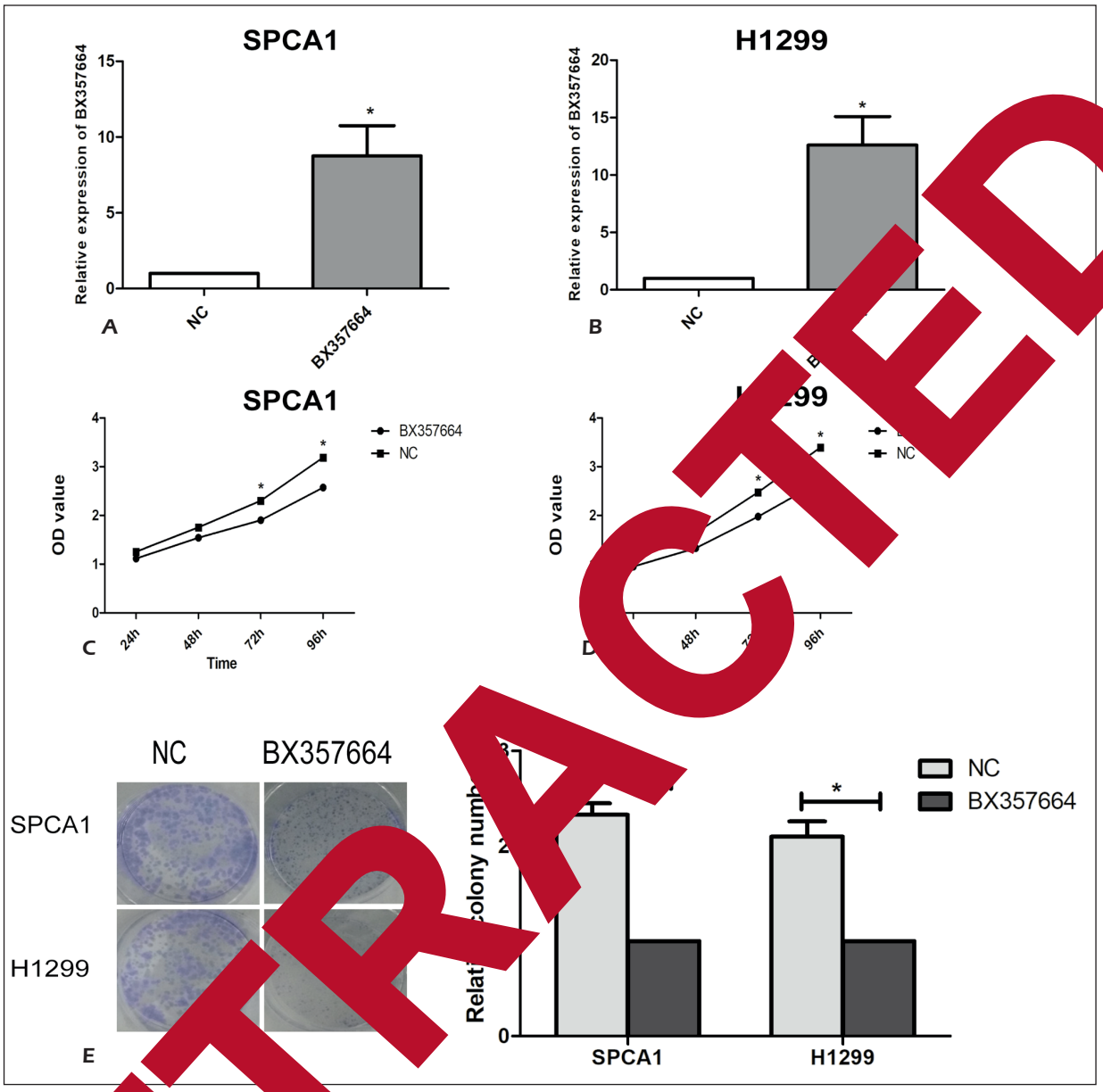
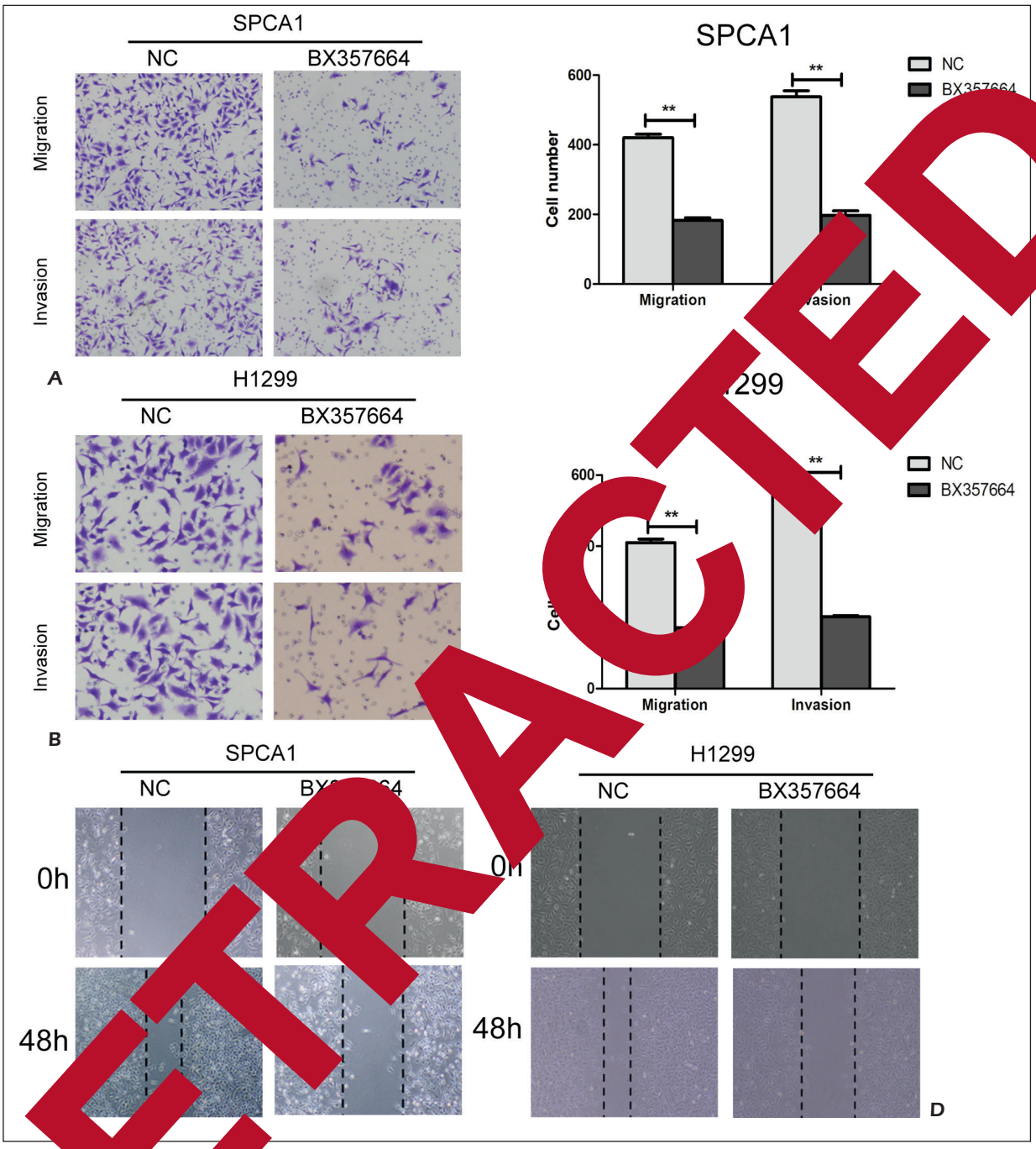


Figure 2 ..., Western Blot an... PCR were used to verify the efficiency of BX357664 over-expression in SPCA1 and H1299 c...es. **C-D**. Growth curve ...alysis showing the cell growth of SPCA1 and H1299 cells with BX357664 over-expression. ...e efficie... of cell colony formation in SPCA1 and H1299 cells with BX357664 over-expression. A representative dat... displa...s mean ± SD values. *p<0.05, **p<0.01.

...ng n... ...g RNA (LncRNA) is a nor...ding R... ...f greater than 200 nucleotides in... [12,13]. It participates in a complex and ...t gene expression regulatory network, which can subtly regulate gene expression[13]. ...arches have demonstrated that lncRNAs are e...al 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[14,15]. Therefore, the expression disorder of lncRNAs is closely related to many diseases, such as tumor formation[15]. 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.[19,20]. Studies have observed that BX357664 exerts great influence on migratory and



re 3. SPCA1 s transfected with BX357664 displayed significantly lower migration and invasion capacity. **C-D**, cells t th BX357664 displayed significantly lower migration and invasion capacity. **E**, The efficiencies of cel tch in S nd H1299 cells with BX357664 over-expression. A representative data set is displayed as mean ± SD val $p<0.05$, * 0.01.

vasive capacity of tumor cells. The differential ession of BX357664 in tumor tissues is closely ted to tumor metastasis and prognosis[19,20]. 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

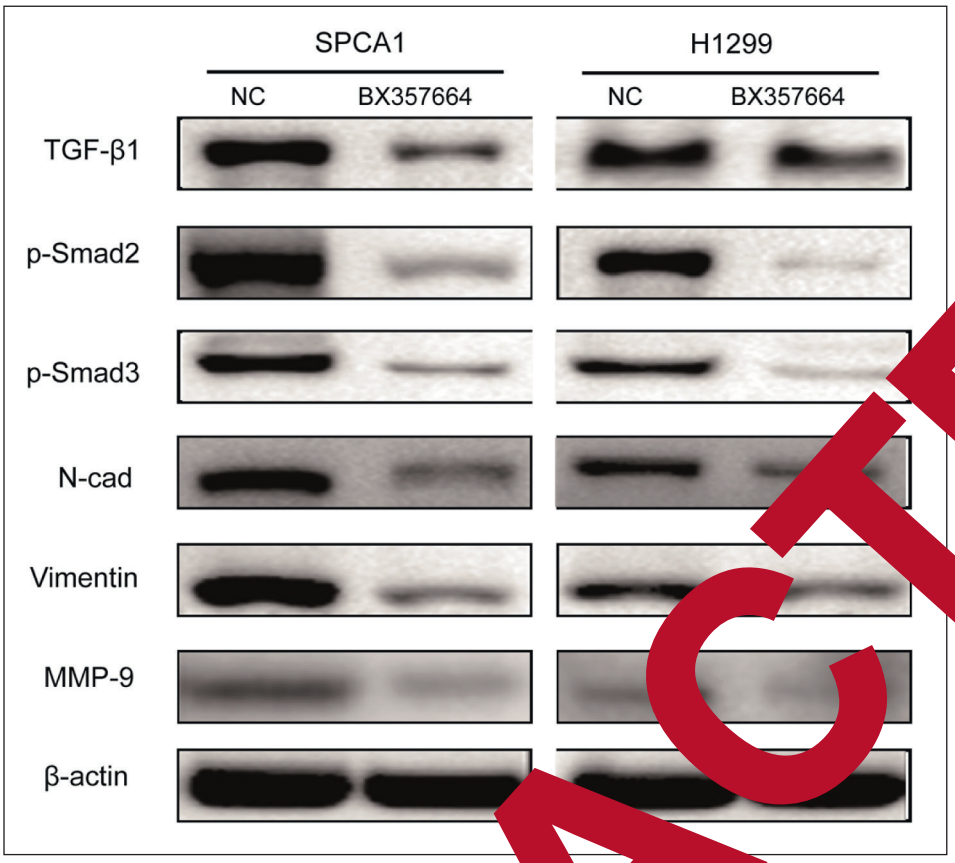


Figure 4. Over-expression of BX357664 expression significantly decreased the expression of TGF-β1/Smad signal pa... including TGF..., p-Smad3, ..., Vimentin and MM...

BX357664 overexpression mo... lentiv... rus. And the results of CCK8 ... ll assay ... demonstrated that BX357... could ...ibit the progression of NSCLC; h... er, the ... lecular mechanism re... ins... TGF-β/Smad signaling pat... y can b... d in various biological tissue... human. Its ... functions include trans... complex ... ptor signals on the c... surfa... rough autocrine and paracrine ...ways, regu... of cell growth, differe...on, apoptosis, an... er functions[21-23]. It ha... n foun... at TGF-β stimulates epithelial cel... first t... a ligand receptor complex with TGF-... ecepto... the cell membrane, and ...phor... TG... ype I receptor by TGF-β ... rece... se (TβRI) to transmit intracel... signal... sduction substances. Smad2/3 is ...rylated and then binds to Smad4 in the ...so... m a heterotrimer or tetramer and ...dergo nuclear translocation, which enters the ...eus and interacts with nuclear transcription fa... 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-cadherin[24-26]. 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 MAD1[25]. Mutations in the Smad2 and Smad4 genes were found in some breast cancer patients[27]. Similarly, Smad3 expression is associated with bladder cancer and colorectal cancer[28,29]. In the Smad-dependent pathway, Smad7 and Smad6 prevent the development of EMT as an EMT negative factor[21-23]. Smad6 competitively binds to Smad1 to form an inactive homologous polymer, which negatively regulates the TGF-β/Smad pathway[24-26]. 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 pathway[24,26]. 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 in the TGF-β1/Smad pathway after overexpression of BX357664 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.

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