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Novel long non-coding RNA LBX2-AS1 indicates poor prognosis and promotes cell proliferation and metastasis through Notch signaling in non-small cell lung cancer

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Abstract. – OBJECTIVE: Recent reports have suggested that long non-coding RNA LBX2 antisense RNA 1 (LBX2-AS1) acts as an important regulator in cancer progression. This study aimed to investigate the clinical significance of LBX2-AS1 in non-small cell lung cancer (NSCLC) patients and its biological functions.

PATIENTS AND METHODS: The expressions of LBX2-AS1 were examined in 165 paired NSCLC tissues and adjacent normal tissues from NSCLC patients by qRT-PCR. The clinical significance of LBX2-AS1 was determined using a series of statistical methods. The effects of LBX2-AS1 knockdown on NSCLC cell proliferation, migration, and invasion were investigated by CCK-8 assays, colony formation assays, EdU proliferation assays, Wound healing assays, and transwell assays. The promotive roles of LBX2-AS1 on Notch1 signal were determined using RT-PCR and Western blot.

RESULTS: We found that LBX2-AS1 was highly expressed in NSCLC tissues and cell lines. The increased levels of LBX2-AS1 were observed to be positively correlated with TNM stage, histological grade, and lymph node metastasis. Furthermore, the Kaplan-Meier survival curves indicated that patients with higher expressions of LBX2-AS1 had unfavorable overall survival. Lost-of-functions assays revealed that the knockdown of LBX2-AS1 in H1299 and A549 cells inhibited cell proliferation, migration, and invasion. Mechanistic studies revealed that the suppression of LBX2-AS1 resulted in the reduced expressions of Notch1, p21, and Hes1, suggesting that LBX2-AS1 might promote the activation of the Notch pathway.

CONCLUSIONS: Our study identified a novel NSCLC-related lncRNA LBX2-AS1, which may represent a novel prognostic biomarker and a potential therapeutic target for NSCLC.

Key Words:
LncRNA, LBX2-AS1, Notch signaling, Biomarker, Non-small cell lung cancer, Metastasis, Proliferation.

Introduction

Lung cancer is the sixth most commonly diagnosed cancer and a major cause of tumor-associated deaths worldwide, accounting for 19% (1.5 million) of tumor deaths in 2015[1,2]. It has been reported that a growing trend of incidence and mortality rate of lung cancer is expected to continue until 2035[3]. Non-small cell lung cancer (NSCLC) accounts for 70-85% of all lung cancers. Although various clinical strategies of treatments such as chemotherapy and operation have been frequently used for the clinical management of NSCLC, the clinical outcome of patients suffering from NSCLC remains unfavorable[4,5]. One of the major reasons why the long-term survivals of patients benefit a few from the traditional treatments is that many patients diagnosed at an advanced stage could develop metastasis and recurrence[6,7]. In addition, because of the limited understanding of the molecular mechanisms underlying NSCLC progression, it is not easy to discover accurate methods against tumor metastasis. Thus, for the improvement of prognosis of NSCLC patients, it is necessary to understand the mechanism of NSCLC progression and discover novel biomarker in molecular level.

In the past 20 years, many researches[8,9] have provided a clue that non-coding RNAs,

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including long non-coding RNAs (lncRNAs) and microRNA, are involved in oncogenesis, including NSCLC. LncRNAs are defined as non-coding transcripts more than 210 nucleotides in length with no capacity in the coding proteins[10]. The potential function of lncRNAs in human beings have attracted growing attention due to their involvement with various biological and physiological processes[11,12]. Many lncRNAs have been reported to display important epigenetic regulations of lncRNAs in tumorigenesis by serving as an oncogene or tumor suppressor[13,14]. Notably, the frequent changes in lncRNAs levels have been demonstrated by the use of high throughput sequencing in various tumor tissues and blood, suggesting their potential as novel prognostic and diagnostic biomarkers[15,16]. However, to date, only a few dysregulated lncRNAs in NSCLC have been functionally characterized.

LBX2 antisense RNA 1(LBX2-AS1), transcribed from the intron of chromosome 2p13.1, was a newly discovered lncRNA which was initially reported to act as a tumor promoter and predict poor prognosis of esophageal squamous cell carcinoma patients[17]. However, we searched for some references online and found that the studies of LBX2-AS1 in other tumors were limited. In this work, we aimed to explore whether LBX2-AS1 was abnormally expressed in NSCLC and its clinical value. Then, the oncogenic activities of LBX2-AS1 were also examined in NSCLC cell lines.

Patients and Methods

Patients and Tissue Samples

A total of 165 paired NSCLC specimens, and the corresponding adjacent normal lung tissues were collected from patients diagnosed NSCLC who received surgical operation in the Chongqing Public Health Medical Center. All collected tissues, including NSCLC samples and non-tumor samples, were reassessed by two pathologists and stored at -80° C for the determination of lncRNAs levels. Patients (76 women and 88 men) whose median age was 63 years (range 25-80) did not receive radiotherapy and chemotherapy before operations. This investigation was performed under the approvals of the Ethics Committee of the Chongqing Public Health Medical Center, and all the samples were collected with the patient's informed consent.

Cell Culture and Transfection

NSCCL cell lines A549, PC9, and H1975 were acquired from the Cell Bank of the CAS (Pudong, Shanghai, China). Other two NSCLC cells (SPC-A1 and H1299) were provided by Ph.D. Wang (Chongqing Public Health Medical Center). Human lung epithelial cells (BEAS-2B) were obtained from the ATCC (Manassas, VA, USA). The above cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Kunming, Yunan, China) which contained 8% fetal bovine serum (FBS, Invitrogen, Haidian, Beijing, China), 100 U/ml penicillin (MCE, Haidian, Beijing, China), and 100 μg/ml streptomycin (Invitrogen, Haidian, Beijing, China), within a humidified atmosphere containing 5% CO_2 at 37° C.

SiRNA targeting LBX2-AS1 (si-LBX2-AS1#1 and si-LBX2-AS1#2) and its negative controls (NC-siRNA) were purchased from GenePharma Company (Shanghai, China). The transfections were performed by the use of the Lipofectamine 2000 kit (Invitrogen, Haidian, Beijing, China) based on the standard progress. Then, qRT-PCR was performed to demonstrate the suppression of LBX2-AS1.

RNA Extraction and QRT-PCR

The total RNAs from tumor and normal specimens were extracted using TRIzol (#15596026, Invitrogen, Haidian, Biejing, China). A novel spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the purity and concentration of all RNAs. For mRNAs detections, the reverse transcription kit (#218061, Qiagen, Hilden, Germany) was used for the reverse transcription of the total RNAs (400 ng). The levels of LBX2-AS1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected using qRT-PCR assays which were performed using a standard SYBR-Green method on ABI 7500/7500 Real Time-PCR System (Biosystems, Foster City, CA, USA). GAPDH was used as an endogenous control. Real time-PCR experiments were performed under the conditions: 95°C, 10 min; 95°C, 15 s; 60°C, 1 min (40 cycles). The relative expressions of LBX2-AS1 were determined by using the ΔCT (lncRNA-GAPDH) method. The primers (Lilai Technology, Pudong, Shanghai, China) were designed as follows: for LBX2-AS1, the forward primer was 5'-ACACAGCTCCATTGGCA-3' and the reverse primer was 5'-ATGACCAGTAGACCAGG-3'. For human GAPDH, the forward prim-

er was 5'-AGGTCGGTGTGAACGGATTTG-3' and the reverse primer was 5'-GGGGTCGTTGATGGCAACA-3'.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assays were applied to analyze the influence of LBX2-AS1 on the proliferation of tumor cells. The parenzyme (Biofine, Tongzhou, Beijing, China) was used to digest the cells which were collected in the logarithmic phase. Then, the treated cells were seeded in 48-well plates and were cultured for 24, 48, 72, and 96 h, respectively after the introduction of si-LBX2-AS1 and NC-siRNA. Then, the CCK8 solution was added to each well. Absorbance (OD value) at a wavelength of 450 nm was determined using a microplate reader (Bio-Rad, Hercules, CA, USA) and used for the calculation of the number of living cells.

Colony Formation Assay

For the clone formation assay, the cells transfected with si-LBX2-AS1 or NC-siRNA were suspended in ATCC 30-2001 medium (Mairuibao, Shijingshan, China) with 10% FBS. Then, we used 24-well plates for the seed of the above cells at a density of 800 cells per well, which were further incubated at 37°C. After two weeks, the cells were washed two times with Phosphate Buffer Saline (PBS), fixed with methanol (#311, Yuli, Chongqing, China) and stained with Sigma-48900 (Giemsa solution, Beinuo, Pudong, Shanghai, China). Finally, the clones in the special plates were stained with crystal violet for half an hour, photographed, and calculated.

EdU Incorporation Assays

For the EdU proliferation assays, the Cell-Light imaging detection kit (RiboBio, Guangdong, Guangzhou, China) was bought from Gema Technology (Pudong, Shanghai, China). The treated cells, including A549 and H1299, were seeded in six cm plates for 1 day. Then, the cells culture was provided with 50 mM EdU and further incubated for 1 day. Subsequently, the cells were fixed using 4% paraformaldehyde. The Apollo reaction cocktails were used to react to the cells permeabilizated using Triton X-100 (Aolaibo, Haidian, Beijing, China) for thirty min. After being stained using Hoechst 33342 (C1029, Beyotime Biotechnology Tongzhou, Beijing, China), the DNA contents of the cells were detected by using a fluorescence microscope. EdU-positive cells were counted by calculating three fields which were imaged at 100× magnification.

Wound Healing Assays

3×10^5 cells were seeded in 6-well plate, cultured in DMEM with 10% FBS at 37°C, and transfected with si-LBX2-AS1 or NC-siRNA. Once cultures reached 85% confluence, the cells layer was scratched using a 200-µl pipette tube and doused with PBS. Subsequently, the cells were incubated with serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) medium for 48 h at 37° C. The images were randomly captured in at least five three fields for each well, and the percentage of migration was calculated.

Cell Invasion Assays

The cells (30000/well) were cultured in DMEM (X77, Gibo, Tongzhou, Beiijing, China) with FBS for 24 h and then were suspended in the top chamber of an insert pre-coated with Matrigel (326535, Corning, Shenfu Technology, Shanghai, China). The lower chamber was filled with 600 µL of DMEM containing 10% FBS. The cells, 24 h later, that stayed at top chamber were removed using a cotton swab. In addition, the cells that had invaded the lower surface of the membranes were fixed and stained using 5% crystal violet. The cells on the lower chamber were calculated by the use of photographic images.

Western Blotting

Twenty-four hours after transfection, the lysates of A549 and H1299 cell lines were collected from the cells cultured on six-well plates at 10^5 cells per well using RIPA buffer (Shishang Technology, Tongzhou, Beijing, China) provided protease suppressors. The proteins were quantified using a BCA Protein Assay kit and separated by using 10% of sodium SDS-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane. Subsequently, five percent of non-fat milk (Gema, Pudong, Shanghai, China) were used for blocking the above membranes in TBS (28358, Thermo Fisher, Waltham, MA, USA) at 4°C overnight and then incubated with the following primary antibodies including Notch 1, p21, Hes1, and GAPDH. The primary antibodies were purchased from Aobosen (Haidian, Biejing, China). Then, the membranes were incubated with secondary antibodies, including anti-mouse or anti-rabbit (Santa Cruz Biotechnology, Haidian, Beijing, China). GAPDH was used as a control. The chemiluminescent detection system was used for the detection of the collected results.

Statistical Analysis

The Statistical Product and Service Solution SPSS software 16.0 (SPSS Inc., Chicago, IL, USA) was applied in the current work. The differences between the two groups (high and low) were examined using the *t*-test. The correlations between the clinical characteristics and the levels of LBX2-AS1 were analyzed using the Chi-square test. The Kaplan-Meier analysis was employed for the survival analysis between the groups. The prognostic values of LBX2-AS1 were analyzed using the Cox proportional hazards model. *p*-values less than 0.05 were considered statistically significant.

Results

Expressions of LBX2-AS1 are Distinctly Downregulated in NSCLC Specimens and Cell

To identify whether LBX2-AS1 acted as a dysregulated lncRNA, we used Cancer RNA-Seq Nexus to analyze the expression pattern of LBX2-AS1 in NSCLC. As shown in Figure 1A, the results suggested LBX2-AS1 as a highly expressed lncRNA in NSCLC tissues, especially in advanced stages tissues (Figure 1A). Then, we validated the above results in 165 paired NSCLC and normal lung specimens using qRT-PCR, finding that the average levels of LBX2-AS1 were distinctly increased in the collected NSCLC specimens in comparison with the matched normal specimens ($p < 0.01$, Figure 1B). Moreover, the levels of LBX2-AS1 in five NSCLC cell lines were examined, and the results indicated that the levels of LBX2-AS1 were higher in five NSCLC cells compared to BEAS-2B cells (Figure 1C).

LBX2-AS1 Overexpression Was a Prognostic Factor in NSCLC Patients

All NSCLC samples which were examined by RT-PCR were classified into the high LBX2-AS1 expressing group (n = 82) and the low LBX2-AS1 expressing group (n = 83) based on the median LBX2-AS1 levels in NSCLC specimens. Then, the Chi-square tests were applied to study the association between LBX2-AS1 and the clinicopathological factors. As shown in Table I, we showed that the expression levels of LBX2-AS1 were correlated with TNM stage ($p = 0.017$), histological grade ($p = 0.022$), and lymph node metastasis ($p = 0.013$). Then, we further explored the clinical significance of LBX2-AS1 by analyzing the survival data from the five-year follow-up period. The results of the Kaplan-Meier analysis indicated that the five-year overall survival of the higher LBX2-AS1 expressions group was distinctly shorter than that of the lower LBX2-AS1 expression group ($p = 0.0018$). Moreover, the univariate and multivariate assays were applied for the further exploration of the prognostic values of LBX2-AS1 in NSCLC patients. As shown in Table II, it was observed that LBX2-AS1 expression was an independent predictor of poor survivals in NSCLC patients (HR = 3.021, 95% CI: 1.118-4.327, $p = 0.016$), as well as TNM stage ($p = 0.019$), histological grade ($p = 0.024$), lymph node metastasis ($p = 0.018$). In brief, our data suggested that LBX2-AS1 acted as a promising prognostic indicator for NSCLC patients.

LBX2-AS1 Accelerated the Proliferation and Metastasis of NSCLC

According to the finding from clinical assays in Table I, higher LBX2-AS1 levels were associated with lymph node metastasis, indicating that this lncRNA might promote the growth and metastasis of NSCLC. For further exploration of the biological effects of LBX2-AS1 in NSCLC, we down-regulated the expressions of LBX2-AS1 in A549 and H1299 by the use of si-LBX2-AS1. The results of RT-PCR showed the transfection efficiency (Figure 2A). The data of CCK-8 assays certified that the knockdown of LBX2-AS1 distinctly reduced the viability of A549 and H1299 cells (Figure 1B). The colony formation assays also revealed that colony formation decreased after silencing LBX2-AS1 (Figures 1C and 1D). In addition, by using the EdU incorporation assays, it was observed that the percentage of EdU-positive cells deceased with the suppression of LBX2-AS1. On the other hand, we also performed Wound healing assays and transwell assays for the examination of the possible influence of LBX2-AS1 on the metastasis abilities of NSCLC cells. As expected, we observed that the significant suppression of LBX2-AS1 distinctly inhibited NSCLC cells migration and invasion (Figure 3A-3C). Taken together, LBX2-AS1 was confirmed to act as a tumor promoter player in the progression of NSCLC.

LBX2-AS1 Functions as an Oncogene by Promoting Notch Signaling Activity

Reicher et al[18] have confirmed the involvement of lncRNAs and Notch signaling, which acts

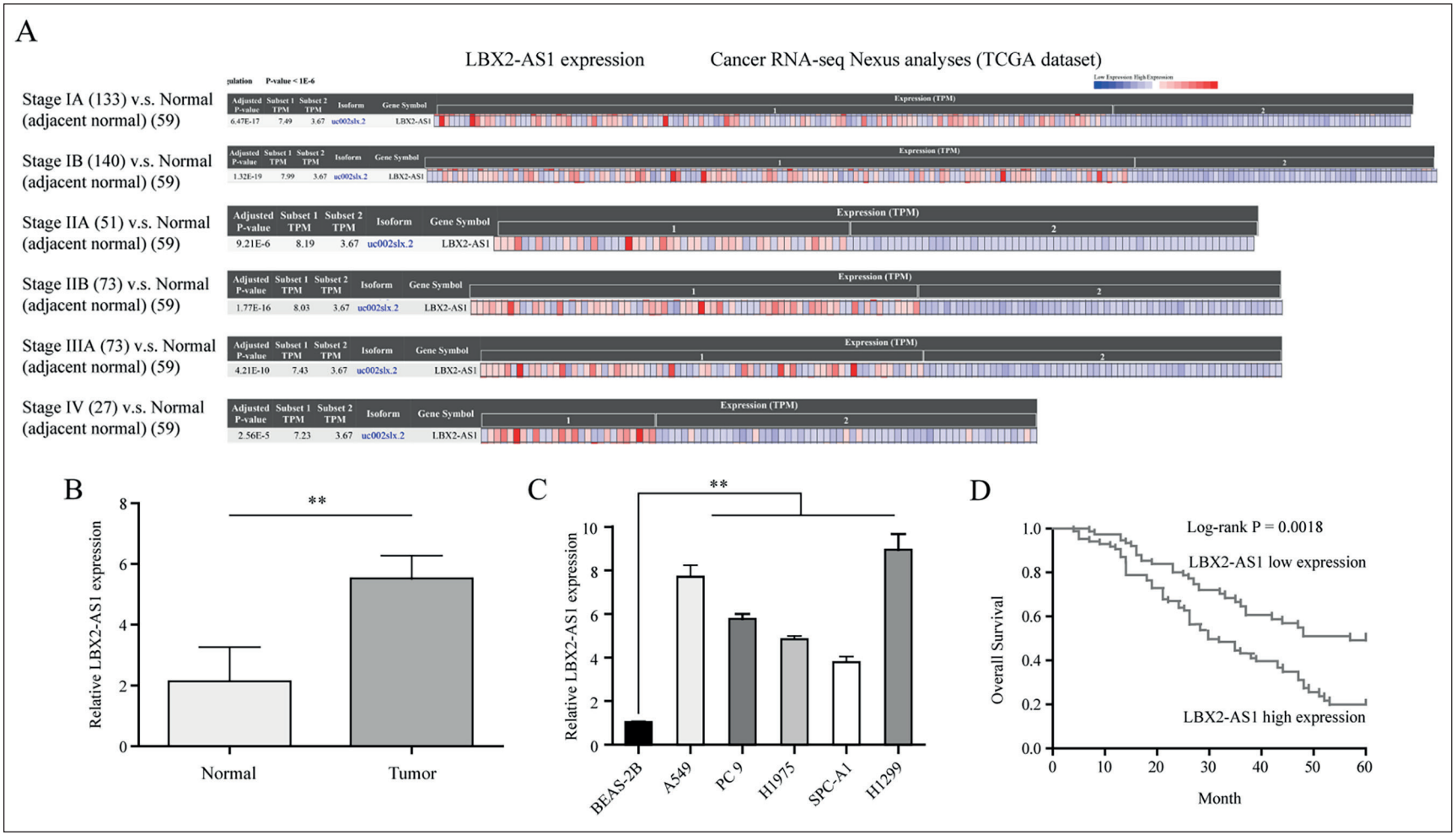

Figure 1. The expressions of LBX2-AS1 in malignant and normal lung tissues and cells. ***A,*** Online tool (Cancer RNA-seq Nexus analyses) was used for the analysis of LBX2-AS1 levels. ***B,*** LBX2-AS1 expression in tumor tissue and adjacent tissue by RT-PCR. ***C,*** Expression levels of LBX2-AS1in five NSCLC cell lines and normal BEAS-2B cells. The cells experiments were carried out in triplicate. ***D,*** The correlation of LBX2-AS1 expressions with the overall survival was determined using the Kaplan-Meier methods. $^{**}p < 0.01$. $^{*}p < 0.05$.

Table I. Clinicopathological features associated with LBX2-AS1 expressions in 165 NSCLC patients.

Variable	N = 165	LINC00675 expression		*p*-value
		High	Low	
Age (years)				0.186
< 55	89	40	49	
≥ 55	76	42	34	
Sex				0.455
Male	104	54	50	
Female	61	28	33	
History of smoking				0.265
Ever	112	59	53	
Never	53	23	30	
Tumor size				0.186
≤ 3 cm	87	39	48	
> 3 cm	78	43	35	
TNM stage				0.017
I/II	111	48	63	
III/IV	54	34	20	
Histological grade				0.022
Well and moderately	101	43	58	
Poorly	64	39	25	
Lymph node metastasis				0.013
Negative	119	52	67	
Positive	46	30	16	

as a common tumor-related signaling pathway. To delve into the potential mechanism involved in the cancer-promotive roles of LBX2-AS1 in NSCLC, the possible associations between LBX2-AS1 and Notch signaling were studied using Western blot and RT-PCR. As shown in Figure 4A and 4B, higher levels of Notch1, p21, and Hes1 were detected in H1299 and A549 cells after the knockdown of LBX2-AS1. In addition, the similar expression trend of the above factors in the protein level was also observed (Figures 4C and 4D). Based on these results, LBX2-AS1 may serve as a tumor promoter *via* modulating Notch signaling.

Discussion

Up to date, China has an increasing incidence of NSCLC patients. The research progress of personalized therapy required sensitive biomarker to guide clinical progress[5]. Although tremendous efforts have been made for the identification of the molecular biomarkers with implemented values, it remains an enormous challenge. Recently, detecting the levels of lncRNAs in tumors was considered a novel predictive method due to their dysregulation related to tumor progression and the advancements of the chip technology[19,20]. Our study identified a tumor-related lncRNA LBX2-

Table II. Multivariate survival analysis of overall survival and disease-free survival in 214 osteosarcoma patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age	1.552	0.653-1.942	0.378	–	–	–
Sex	0.892	0.463-1.775	0.281	–	–	–
History of smoking	0.933	0.582-2.172	0.145	–	–	–
Tumor size	1.544	1.127-2.321	0.118	–	–	–
TNM stage	3.261	1.382-4.774	0.011	2.986	1.174-4.328	0.019
Histological grade	3.452	1.319-4.421	0.018	3.019	1.128-4.129	0.024
Lymph node metastasis	3.217	1.477-5.031	0.007	2.995	1.187-4.348	0.018
LBX2-AS1 expression	3.427	1.329-4.827	0.009	3.021	1.118-4.327	0.016

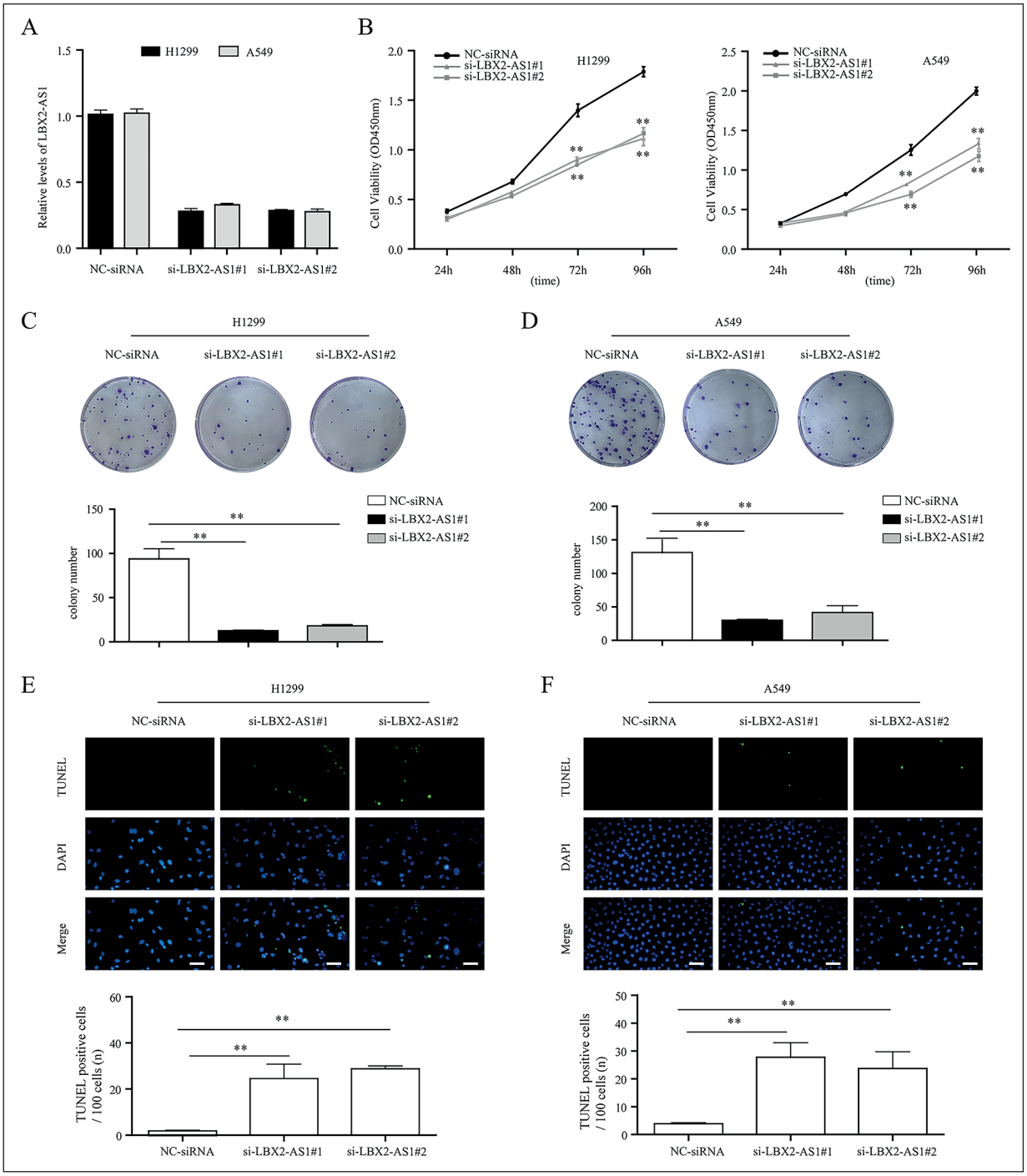

Figure 2. LBX2-AS1 silencing inhibits NSCLC cell proliferation. ***A,*** Expression of LBX2-AS1 in H1299 and A549 cells transfected with NC-siRNA, si-LBX2-AS1#1, or LBX2-AS1#2. ***B,*** Cell proliferation curves in H1299 and A549 by CCK-8 assays. ***C, D,*** Colony formation of H1299 and A549 cells were determined by the colony formation assays (Magnification: 10×). ***E, F,*** The representative images of experiments of TUNEL assay (red) and NSCLC cells (Magnification: 100×). $^{**}p < 0.01$. $^{*}p < 0.05$.

AS1 which was found to be distinctly upregulated in NSCLC. By using the clinical assays, higher levels of LBX2-AS1 were discovered to be correlated with lymph node metastasis and histological grade, as well as advanced TNM stage. Further investigation of clinical data revealed that NSCLC patients with high LBX2-AS1 expressions exhibited poorer overall survival. For the

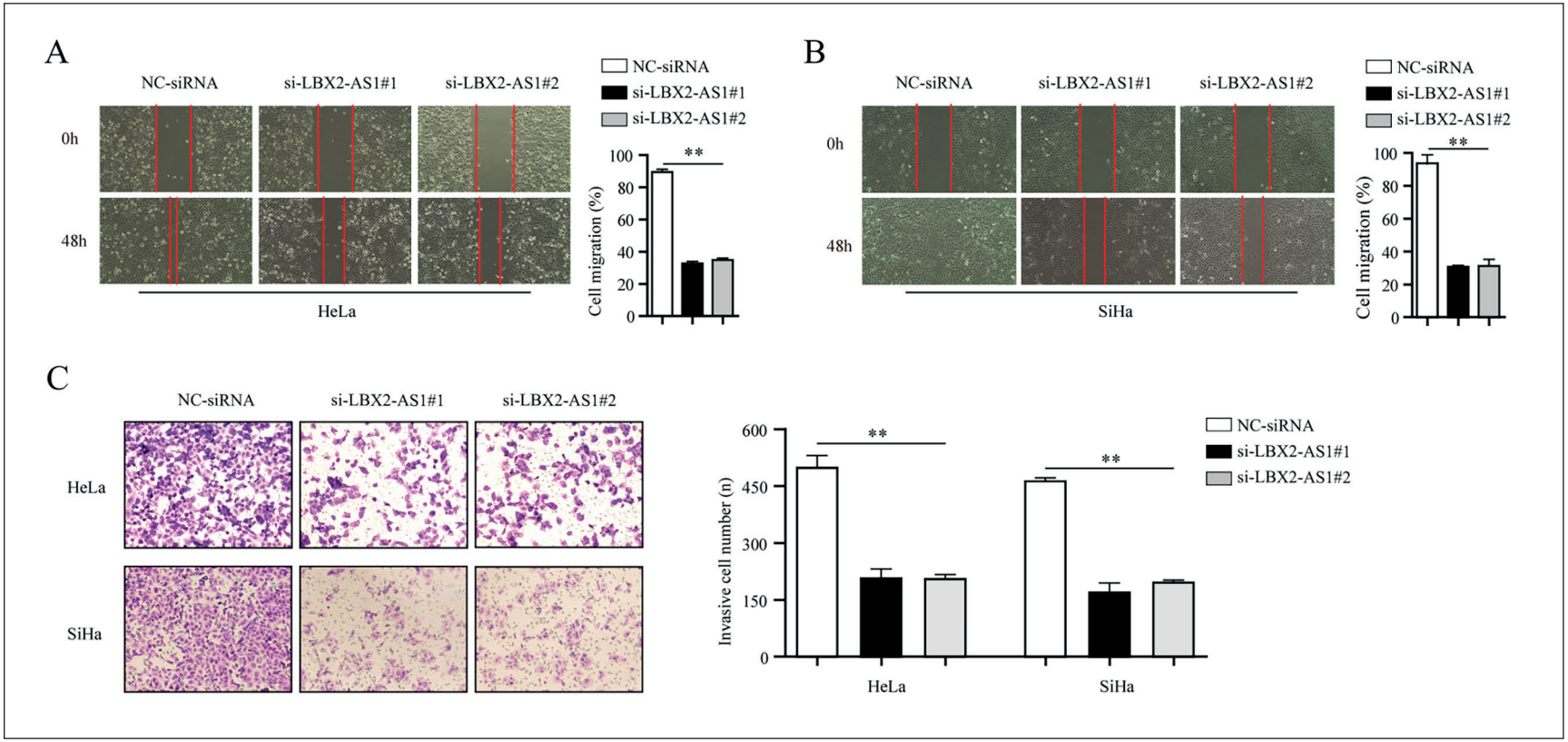

Figure 3. The effects of LBX2-AS1 downregulation on NSCLC cell migration and invasion. ***A, B,*** Scratch assays displayed that LBX2-AS1-silenced H1299 and A549 cells decreased motility compared to the control cells (Magnification: 10×). ***C,*** LBX2-AS1 knockdown suppressed the invasion of H1299 and A549 cells as verified by the transwell assays (Magnification: 40×). $^{**}p < 0.01$. $^{*}p < 0.05$.

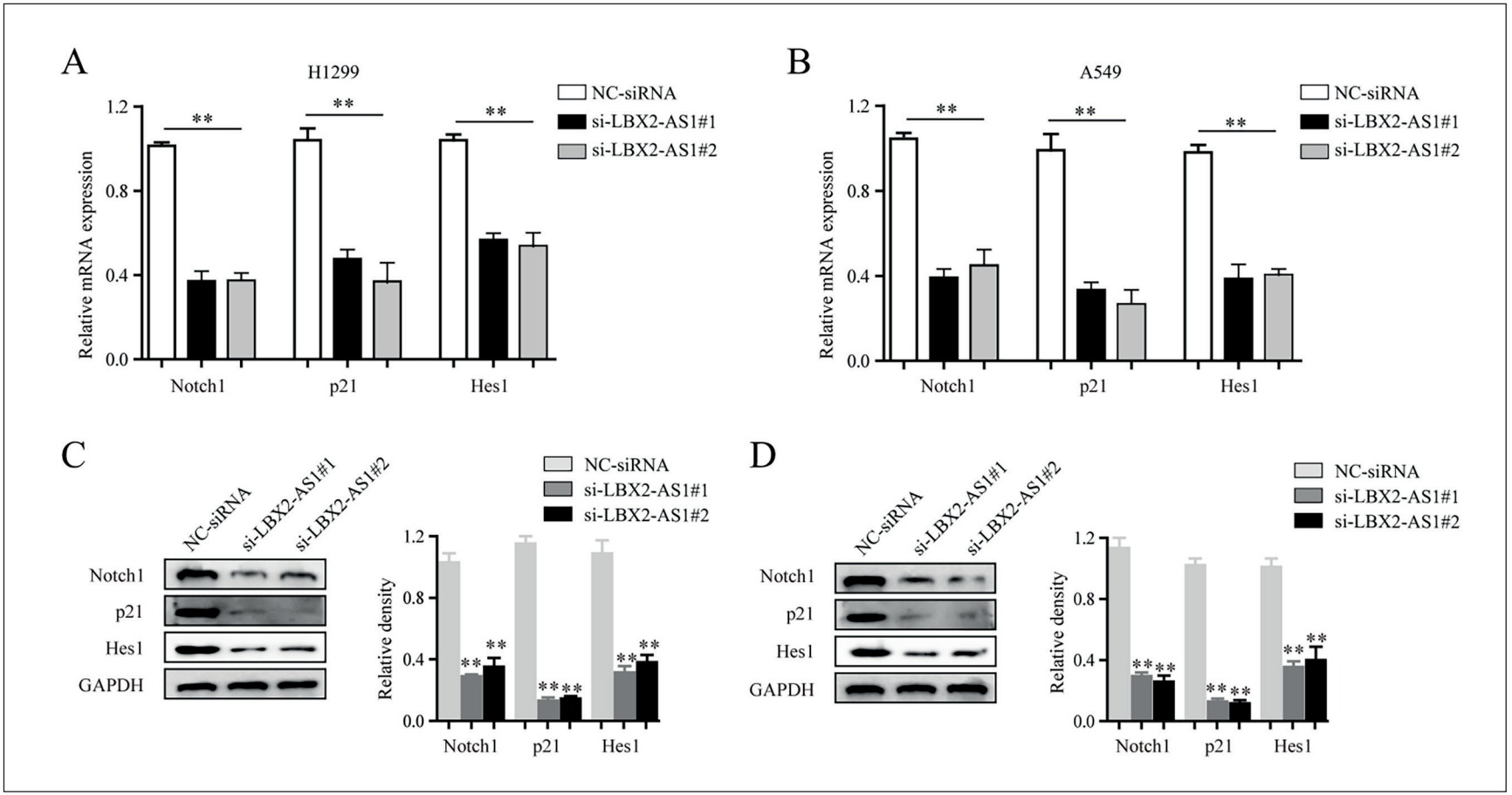

Figure 4. Knockdown of LBX2-AS1 suppressed the Notch signaling. ***A, B,*** RT-PCR assays showed the levels of Notch1, p21, and Hes1 in H1299 and A549 cells treated with si-LBX2-AS1. ***C, D,*** The proteins levels of Notch1, p21, and Hes1 in the above cells were determined by Western blot. $^{**}p < 0.01$. $^{*}p < 0.05$.

exploration of clinical application of LBX2-AS1, the results of the multivariate analyses detected LBX2-AS1 as an independent prognostic biomarker for patients with NSCLC. Overall, our findings provided a novel marker for predicting the survival of NSCLC patients.

Previously, some cancer-associated lncRNAs have been functionally characterized in various tumor, including NSCLC, through various *in vitro* and *in vivo* assays[21]. LncRNAs acted as tumor promoters or oncogenes in the specific type of tumor. For instance, a newly discovered NSCLC-related lncRNA SNHG1 was identified by Lu et al[22], and they performed a series of cells experiments, finding that the overexpression of SNHG1 promoted the proliferation and metastasis of tumor cells by sponging miR-145-5p. LncRNA PCAT6, one of the most upregulated lncRNAs in lung cancer, was found to be related to advanced clinical progress and poor prognosis. Functional assays confirmed its oncogenic ability in NSCLC due to its knockdown and by suppressing the cell growth and invasion *via* binding to EZH2[23]. Wang et al[24] suggested that lncRNA OIP5-AS1 levels were distinctly increased in NSCLC tissues and its silence using si-OIP5-AS1 displayed tumor-suppressive roles by suppressing cell proliferation and metastasis *via* targeting miR-378a-3p. Recently, in two investigations, LBX2-AS1 was firstly reported to be highly expressed in glioma and predict a poor clinical outcome[25,26]. Subsequently, in esophageal squamous cell carcinoma, LBX2-AS1 was firstly confirmed to have oncogenic effects in tumor cells progress *via* regulating ZEB1 mRNAs[17]. Those findings highlighted the potential effects of LBX2-AS1 in tumor progression. Thus, we wondered whether LBX2-AS1 acted as a functional regulator in NSCLC. Using si-LBX2-AS1, our group succeeded in downregulating the levels of LBX2-AS1 in NSCLC cell lines. Then, a series of cell experiments were performed, and the results indicated that the silence of LBX2-AS1 expression distinctly resulted in the inhibition of the proliferation and metastasis of NSCLC cells. Our findings suggested that LBX2-AS1 acted as an oncogene in NSCLC and could be considered as a novel marker for this tumor.

In recent years, several signaling pathways involved in the modulation of oncogenesis have been functionally identified, such as PI3K-AKT, NF-κB1, Notch pathways, and so on[27-29]. Notch signaling, a highly conserved pathway, has been confirmed to play a critical role in modulating various cellular processes, which suggests that this pathway participates in tumor progression[30,31]. As expected, the frequent mutations of the Notch genes are observed in almost various tumors. However, the upstream mechanism involved in the dysregulation of Notch signaling remained largely unclear. Recently, the potential of lncRNAs acting as novel regulators for Notch signaling have been frequently reported[32,33]. Thus, we wondered whether LBX2-AS1-mediated carcinogenic roles in NSCLC were involved in the regulation of Notch signaling. Then, we performed RT-PCR and Western blot for the examination of the related proteins of Notch signaling, finding that the expressions of Notch1, P21, and Hes1 at both protein and mRNA levels were distinctly downregulated in H1299 and A549 cells after the knocking down of LBX2-AS1. Our findings revealed that LBX2-AS1 displayed its oncogenic function by modulating the Notch signaling. However, the additional mechanisms of LBX2-AS1 in the modulation of NSCLC progression require to be further explored.

Conclusions

We provided a molecular basis for the roles of LBX2-AS1 in NSCCL and also suggested a novel prognostic biomarker and therapeutic target for NSCLC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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