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LncRNA NBR2 inhibits EMT progression by regulating Notch1 pathway in NSCLC

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Abstract. – OBJECTIVE: The aim of this study was to identify the role of long non-coding RNA (lncRNA) NBR2 in non-small-cell lung cancer (NSCLC) and its possible molecular mechanisms.

PATIENTS AND METHODS: The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to explore lncRNA NBR2 expression in NSCLC cells and tissues. The chi-square test was used to analyze the relationship between lncRNA NBR2 expression and the clinical features of NSCLC patients. The pcDNA3.1 and pcDNA3.1-NBR2 vectors were transfected into NSCLC cells, and the proliferation and migration ability of NSCLC cells were detected using cell counting kit-8 (CCK-8) and transwell assay. The epithelial-mesenchymal transition (EMT)-related genes expression was detected by an EMT RT2 PCR array. QRT-PCR and Western blot was used to analyze the mRNA and protein levels of Notch1, Vimentin, N-cadherin, E-cadherin, HEY1, HEY2, and HEYL.

RESULTS: The expression of lncRNA NBR2 was decreased in NSCLC patients tissues, and the NSCLC patients in the NBR2 low expression group showed a poor prognosis. Meanwhile, the expression of NBR2 in patients with NSCLC was correlated with tumor size. Overexpression of NBR2 suppressed the viability and migration of NSCLC cells and the expression of Notch1 and EMT-related genes in AsPC-1 cells. Simultaneous overexpression of NBR2 and Notch1 could reverse the inhibitory effect of NBR2 on proliferation and migration of NSCLC cells.

CONCLUSIONS: LncRNA NBR2 inhibited the progression of EMT in NSCLC by regulating the Notch1 pathway.

Key Words
LncRNA NBR2, NSCLC, Notch1, EMT.

Introduction

Lung cancer is one of malignant tumor which origins from the bronchi or lung. Lung cancer can be usually divided into two types including non-small-cell lung cancer (NSCLC) and small cell lung cancer (SCLC)[1]. NSCLC is a heterogeneous malignant tumor composed of three subtypes, which is the main pathological type of lung cancer, reaching to 80%-85%[2]. With the continuous medical treatment development, NSCLC patients treated with chemotherapy drugs can prolong their survival time. In recent years, many molecular targeted drugs, such as gefitinib and erlotinib, have been widely used in clinical treatment. However, the five-year survival rate of NSCLC patients is still less than 15%, with localized advanced tumor occurred in approximately 25-30% of cases and metastasis in approximately 40-50% of cases, while the main cause of death is distant metastasis[3,4]. At present, the molecular mechanism of metastasis of NSCLC has not been fully expounded. In-depth exploration of the pathogenesis of NSCLC metastasis is of great significance for finding effective therapeutic targets and improving the survival rate of advanced NSCLC patients.

Long non-coding RNA (lncRNA) is a newly discovered member of the non-coding RNA family, which is over 200 nt in length and has spatial, temporal and tissue specificity[5-8]. A large number of evidence[9-11] has shown that lncRNA was involved in many life processes, including x-chromosome silencing, stem cell pluripotency rearrangement, myocyte differentiation, and regulation of apoptosis and invasion. Studies[12,13] have found that abnormal expression of lncRNA can be involved in the development of many human diseases including tumors through DNA or histone modification, chromatin remodeling, and as an adsorption sponge for microRNAs. Others studies have illustrated that abnormal expression of lncRNA is associated with the development of NSCLC and the metastasis and invasion of tumor cells. For example[14], a highly conserved nuclear lncRNA named MALAT1 is increased in lung cancer tissues and serves as a predicted marker for metastasis. HNF1A-AS1, which is highly expressed in lung adenocarcinoma, can promote

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cell proliferation and metastasis by interacting with DNMT1 and inhibiting E-cadherin expression[15]. These data suggest that lncRNA can play a significant role in the pathogenesis of NSCLC, and can open up new horizons for studying the biological behavior of this disease.

NBR2 is a long non-coding RNA (lncRNA) that has recently been shown to regulate AMP-activated protein kinase (Amp) under energy stress, suggesting that NBR2 also can play a role in inhibiting tumor progression[16-18]. However, its expression pattern, biological function and potential mechanism in NSCLC are still unknown.

Patients and Methods

Tissue Samples and Clinical Data

Cancer tissues and paracancerous tissues of 50 patients with NSCLC were collected, and all fresh tissues were quickly frozen and collected at -80°C for further experiment. All specimens were collected on the morning of the surgery, and all patients signed the informed consent. This investigation was approved by the Ethics Committee of Shanxian Central Hospital.

Cell Culture and Transfection

Human normal lung cell line (BEAS2B) and lung cancer cell lines (A549, AsPC-1, and H460) were selected. The cells were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a constant temperature incubator at 37°C with 5% CO_2. When the cell density reached about 80%, they were digested and passaged. pcDNA3.1 or pcDNA3.1-NBR2 was transfected into AsPC-1 cell line, and the culture medium was changed after 6 hours of transfection.

Quantitative Real -Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from NSCLC cell lines by pre-chilled TRIzol (Invitrogen, Carlsbad, CA, USA) following the instructions. The reverse transcription reaction was performed according to the PrimeScript RT reagent Kit with gDNA Eraser TaKaRa Code: DRR047A Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions. The primer sequences were shown in Table I.

Western Blot

After transfection for 24-48 h, the radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) was used to lyse the cells to extract total protein, and the concentration of protein samples was determined. RND3 and cdk2 primary antibodies (purchased from Cell Signal Technology, Danvers, MA, USA, 1:1000) were added after routine electrophoresis and membrane transferring and incubated at 4°C overnight. After washing the membrane, the Horseradish peroxidase (HRP)-labeled secondary antibody (Cell Signal Technology, Danvers, MA, USA, goat anti-rabbit IgG 1:5000) was used to incubate the membrane for 2 h at room

Table I. Sequences of primers used in quantitative RT-PCR.

Target gene	Primer	Nucleotide sequence
NBR2	F	5'-TGGCACCCACCTTCCCTG-3'
	R	5'-CGGCTTCGCTTCCACTTTT-3'
Notch 1	F	5'-GAGGCGTGGCAGACTATGC-3'
	R	5'-CTTGTACTCCGTCAGCGTGA-3'
E-cadherin	F	5'-CGAGAGCTACACGTTCACGG-3'
	R	5'-GGGTGTCGAGGGAAAAATAGG -3'
N-cadherin	F	5'-TCAGGCGTCTGTAGAGGCTT-3'
	R	5'-ATGCACATCCTTCGATAAGACTG-3'
Vimentin	F	5'-GACGCCATCAACACCGAGTT-3'
	R	5'-CTTTGTCGTTGGTTAGCTGGT-3'
HEY1	F	5'-ATCTCAACAACTACGCATCCCAGC-3'
	R	5'-GTGTGGGTGATGTCCGAAGG-3'
HEY2	F	5'-AGCGAGAACAATTACCCTGGGCAC-3'
	R	5'-GGTAGTTGTCGGTGAATTGGACCT-3'
HEYL	F	5'-CAGTAGCCTTTCTGAATTGCGAC-3'
	R	5'-CCCAGCACAACTCCTCCCTA-3'

temperature. Afterwards, enhanced chemical fluorescence luminescence (ECL) was added (Shanghai Biyuntian Biotechnology, Ltd., Shanghai, China), and the gel imaging analysis system was used to detect the integrated optical density (IOD) values of each strip, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference. The ratio of IOD of each band IOD to that of the internal reference was the relative expression of the protein of interest.

Cell Counting Kit-8 (CCK-8) Assay

After 24 to 48 hours of transfection, the cells were seeded into 96-well plates with 5000 cells per well. The cells were cultured for 6 h, 24 h, 48 h, 72 h, and 96 h, and then, 10 μL of CCK8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to the wells and incubated for 2 h. A microplate reader was used to measure the absorbance of each well at 450 nm. The cell growth curve was then plotted.

Transwell Assay

The ability of cell migration was detected using a transwell chamber. 250,000 AsPC-1 cells were digested and collected; then, the cells were resuspended in 200 μL of serum-free medium and added to a chamber. 500 μL of medium containing 10% FBS was added to the lower compartment. The chamber was taken out after 12 hours, and the chamber was removed with tweezers and cells on the upper surface of the chamber were wiped with a cotton swab.

Epithelial-Mesenchymal Transition (EMT) RT2 Analyzer Polymerase Chain Reaction (PCR) Array

Following the instructions, PCR was performed using an EMT RT2 Profiler PCR Array (Qiagen, Hilden, Germany). 3 glyceraldehyde-phosphate dehydrogenase was used to normalize by comparison of the Ct method. A fold change of more than 5.0 and p less than 0.05 was considered to be significant.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA, USA). The t-test was used to analyze continuous variables between the two groups. The chi-square test was used to analyze categorical variables. The log-rank statistical method was performed for evaluating the overall survival rate between the two groups. $p \leq 0.05$ was considered statistically significant.

Results

LncRNA NBR2 Was Lowly Expressed in NSCLC

By detecting the expression of NBR2 in 50 NSCLC patients' cancer and adjacent tissues, we showed that the expression of NBR2 was significantly decreased in cancer tissues comparing to paracancerous ones. We also divided the tissue samples into high and low expression group according to the median NBR2 expression in NSCLC patients (Figure 1A and 1B). According to the clinical characteristics of patients with NSCLC, we demonstrated that the expression of NBR2 in tissues of NSCLC patients whose tumor size less than 3 cm was higher than those with tumor size over 3 cm (Figure 1C). Further, we found that the overall survival rate of NBR2 low expression group was markedly lower than that of NBR2 high expression group (Figure 1D). The above results indicated that NBR2 expression was decreased in NSCLC and associated with patients' poor prognosis.

Overexpression of LncRNA NBR2 Suppressed Cell Proliferation, Invasion, and Migration of NSCLC Cells

To elucidate the role of NBR2 in the progression of NSCLC, we examined NBR2 expression in NSCLC cell lines by qRT-PCR, and the results demonstrated that, compared to normal lung cell line (BEAS2B), the expression of NBR2 was decreased in A549, AsPC-1, and H460 cells, and the lowest NBR2 level was showed in AsPC-1 cell line, which was selected for subsequent experiments (Figure 2A). We further transfected pcDNA3.1-NBR2 into AsPC-1 cells and detected the expression of NBR2 by qRT-PCR. The results illustrated that the expression of NBR2 was increased in AsPC-1 cells transfected with pcDNA3.1-NBR2 comparing to cells treated with an empty vector (Figure 2B). The CCK8 assay was used to detect cell viability, and the activity of overexpressing NBR2 cells was found decreased (Figure 2C). In addition, the transwell assay revealed that overexpression of NBR2 suppressed cell migration in the AsPC-1 cell line (Figure 2D). Thus, we speculated that NBR2 could suppress the proliferation and migration of NSCLC cells.

Effects of LncRNA NBR2 on the Notch1 Signaling Pathway and EMT in NSCLC Cells

To further explore the mechanism by which NBR2 mediated NSCLC cancer, we transfected pcDNA3.1 or pcDNA3.1-NBR2 into AsPC-1 cells

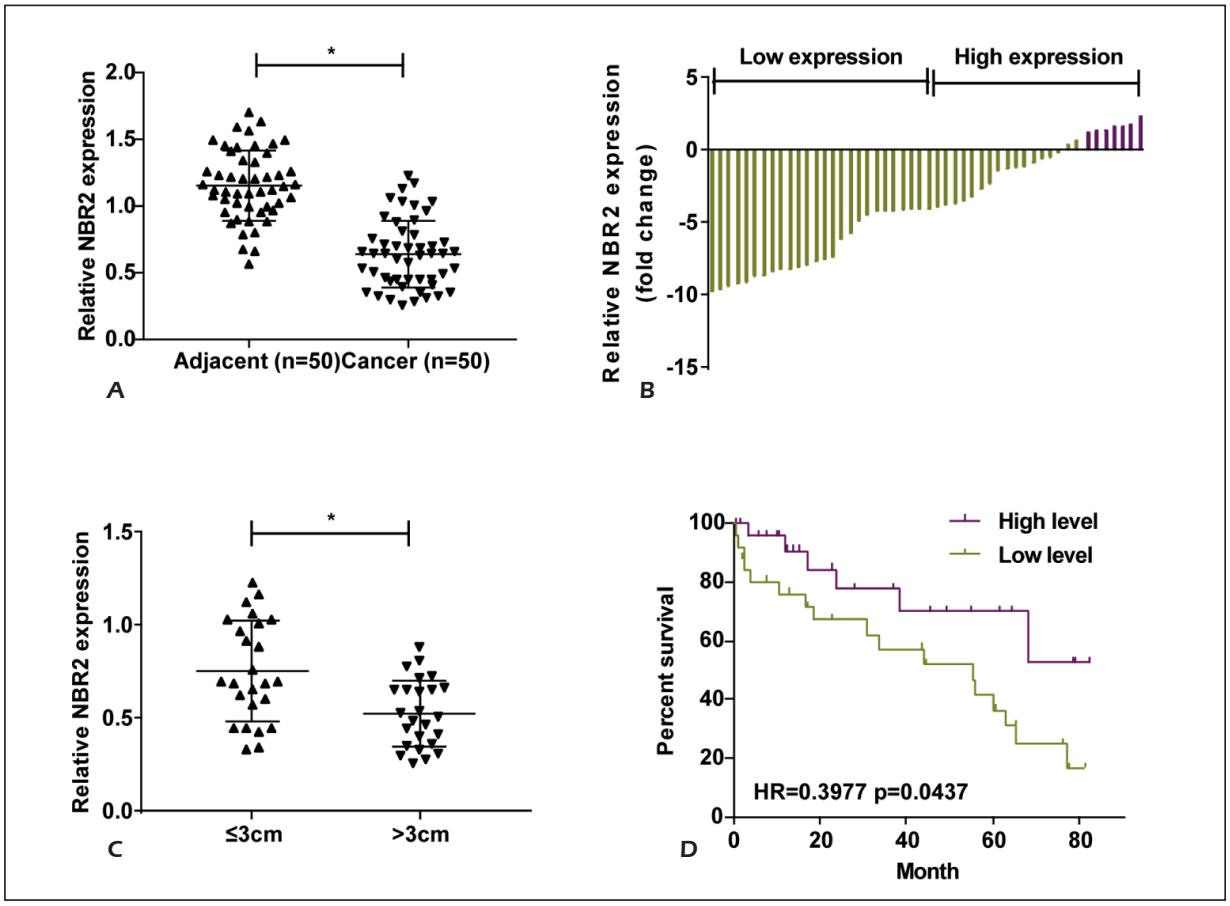

Figure 1. LncRNA NBR2 is lowly expressed in non-small cell lung cancer. **A-B**, qRT-PCR detection of lncRNA NBR2 expression in non-small cell lung cancer tissues was significantly lower than that in adjacent tissues. **C**, qRT-PCR detection of tumor size ≤ 3 cm in patients with non-small cell lung cancer tissue expression of NBR2 is lower than tumor size > 3 cm patients. **D**, Non-small cell lung cancer patients with lower NBR2 expression showed poorer survival than those with high NBR2 expression.

and analyzed the EMT-related genes expression. The results demonstrated that NBR2, Notch1, N-cadherin, Vimentin, JAG1, and EZH2 genes expression was down-regulated, while the expression of E-cadherin, KRT19, and CAV2 genes was up-regulated (Figure 3A). Further, it was found that after overexpression of NBR2, the level of mRNA of Notch1, Vimentin, and N-cadherin were decreased in AsPC-1 cells, while the E-cadherin mRNA expression was oppositely increased (Figure 3B). Western blot analysis also confirmed the same result on the protein levels of the above genes (Figure 3C). In addition, qRT-PCR and Western blot demonstrated that the overexpression of NBR2 suppressed the expression of HEY1, HEY2, and HEYL (Figure 3D and 3E). The above results indicated that the overexpression of NBR2 could inhibit the expression of EMT-related genes in AsPC-1 cells.

Notch1 Overexpression Reversed the Inhibitory Effect of NBR2 on Proliferation and Migration of NSCLC Cells

To determine the role of Notch1 in the development of EMT in NSCLC cells, NBR2 and Notch1 were simultaneously overexpressed in AsPC-1 cells. CCK8 assay revealed that simultaneous overexpression of NBR2 and Notch1 reversed the decrease in cell viability caused by the overexpression of NBR2 alone (Figure 4A). QRT-PCR revealed that simultaneous overexpression of NBR2 and Notch1 reversed the effect of NBR2 alone on the expression of Notch and EMT-related genes (Figure 4B and 4C). Transwell assays detected that overexpression of Notch1 increased cell migration ability in AsPC-1 cells with overexpressed NBR2 (Figure 4D). The above results indicated that Notch1 overexpression reversed the inhibitory effect of NBR2 on proliferation and migration of NSCLC cells.

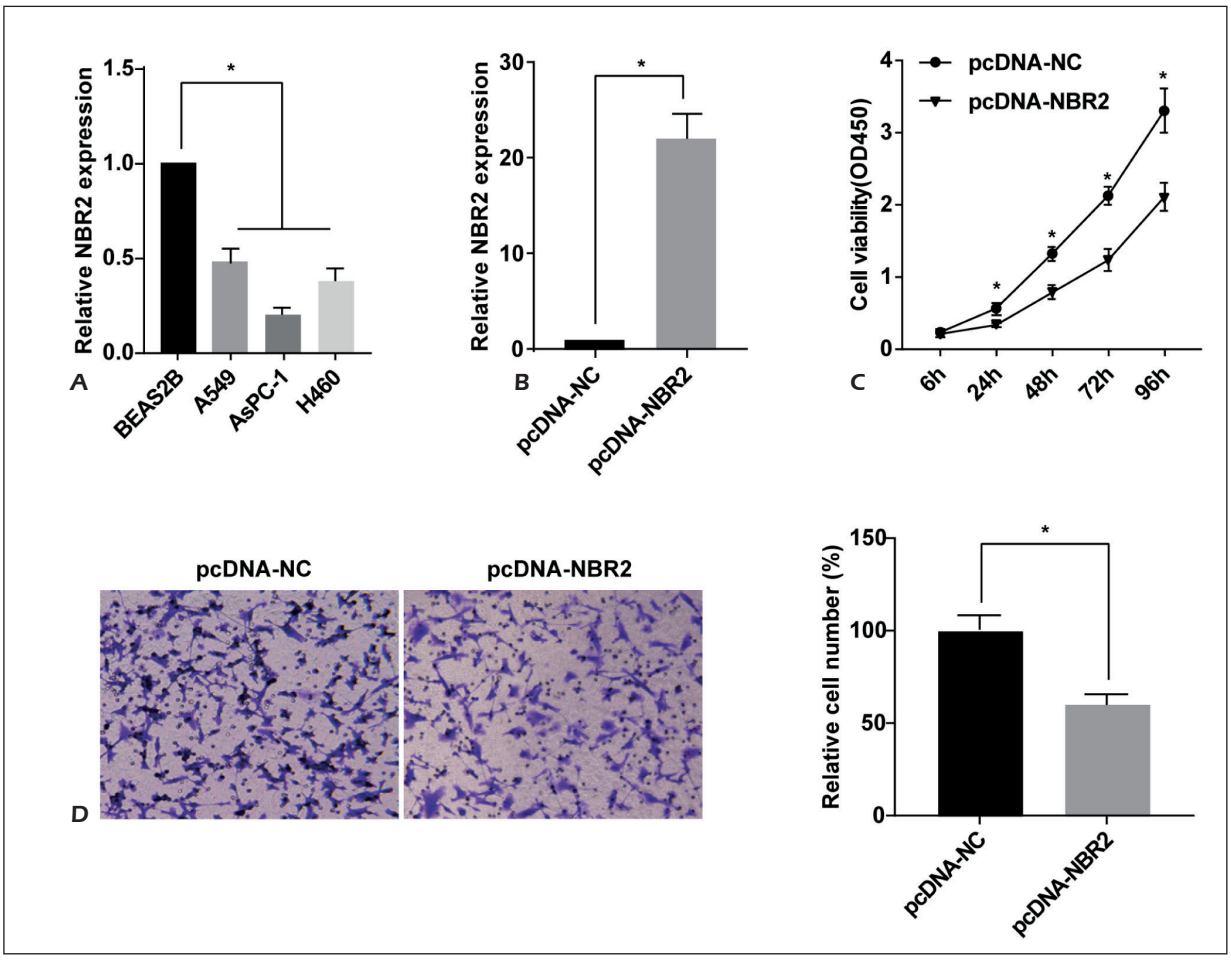

Figure 2. Overexpression of NBR2 inhibits cell proliferation, invasion, and migration of non-small cell lung cancer cells. **A**, qRT-PCR detection of NBR2 down-regulation in non-small cell lung cancer cell lines (A549, AsPC-1, and H460) compared to normal lung cell line (BEAS2B). **B**, After overexpression of NBR2 in AsPC-1 cells, the expression level of NBR2 was measured by qRT-PCR, and the expression of NBR2 was significantly up-regulated in AsPC-1 cells treated with pcDNA3.1-NBR2 compared to cells treated with empty vector. **C**, CCK8 assay showed that the cell viability of pcDNA-NBR2 was decreased in the AsPC-1 non-small cell lung cancer cell line compared with the pcDNA-NC group. **D**, Transwell assay detects that in the AsPC-1 cell line, after overexpression of NBR2, cell migration is weakened (magnification: 40×).

Discussion

Non-small cell lung cancer (NSCLC) accounted for 80% among new cases of lung cancer[19]. Due to early diagnosis lack of biomarkers, tumor cell metastasis remains one of reasons for the challenge of NSCLC treatment[20]. Nowadays, a large number of researches[21-23] have demonstrated that lncRNA plays a significant role in the development of various tumors including NSCLC. For example, lncRNA XIST promotes proliferation, invasion, and migration of human malignant glioma stem cells through adsorption of miR-152[24]. In addition, lncRNA EANCR inhibits cell proliferation and metastasis in NSCLC by affecting EMT, thereby functioning as a tumor suppressor gene[25]. In the present study, we found that NBR2 was decreased in NSCLC tissues and cells, and its low expression was also associated with poor prognosis of patients with NSCLC. At the same time, we demonstrated that overexpression of NBR2 could inhibit migration and proliferation of NSCLC cells. These results indicated that NBR2 might serve as a tumor suppressor in NSCLC.

Previous reports have shown that one of the important features of malignant transformation of tumors is the invasion and metastasis of cells. EMT is a process that epithelial cells transform into mesenchymal phenotype cells, which plays a significant role in cancer metastasis and multifibrotic diseases[26-28]. Biological studies have demonstrated that during EMT, epithelial cells

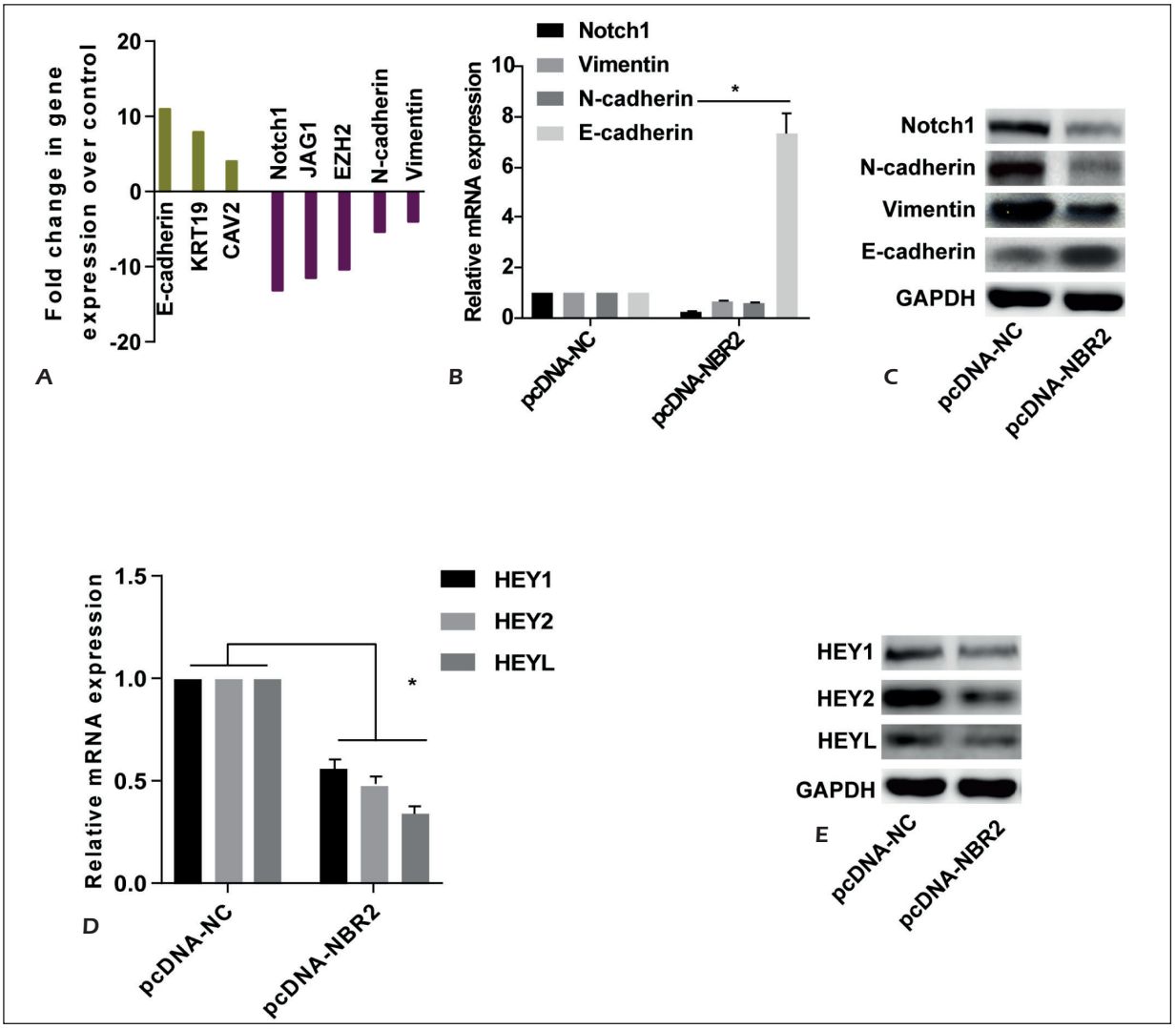

Figure 3. Effect of NBR2 on Notch1 signaling and EMT in non-small cell lung cancer cells. **A**, Determination of EMT-related gene expression using EMT PCR arrays in AsPC-1 non-small cell lung cancer cells transfected with pcDNA3.1-NBR2 or pcDNA3.1. **B**, After transfection of pcDNA3.1-NBR2 by qRT-PCR, the mRNA levels of Notch1, N-cadherin, and Vimentin were decreased and the expression of E-cadherin mRNA was increased in AsPC-1 cells. **C**, After transfection of pcDNA3.1-NBR2 by Western blot, the protein levels of Notch1, N-cadherin, and Vimentin in AsPC-1 cells were significantly decreased, and the protein expression level of E-cadherin was significantly increased. **D**, qRT-PCR detection of transfection of pcDNA3.1-NBR2, AsPC-1 cells decreased mRNA expression levels of HEY1, HEY2, and HEYL. **E**, Western blot analysis showed that the protein expression levels of HEY1, HEY2, and HEYL were decreased in AsPC-1 cells after transfection of pcDNA3.1-NBR2.

re-encode gene expression, for example, as epithelial cell marker molecules, E-cadherin and a-catenin were down-regulated, resulting in loss of polarity, loss of attachment to the basement membrane and the occurrence of other epithelial phenotypes. In turn, interstitial phenotypes such as higher migration and invasion ability, including increased expression of interstitial cell markers such as N-cadherin and Vimentin, can result in an increased cell invasion and migration[29-31]. Tumor cells can diffuse away from the primary tissue after obtaining stromal cell-like properties, a key step in cancer metastasis[32,33]. Although EMT has been shown to promote the development and metastasis of a variety of tumors, its mechanism of action in NSCLC metastasis has not been fully elucidated.

The Notch family is composed of a single transmembrane receptor that, in mammals, can interact with ligands (DLL1, 3, 4, and Jagged1-2) and eventually translocate into the nucleus to activate transcription of target genes[34]. The Notch signaling pathway is one of the significant pathways involved in the EMT process. This pathway is

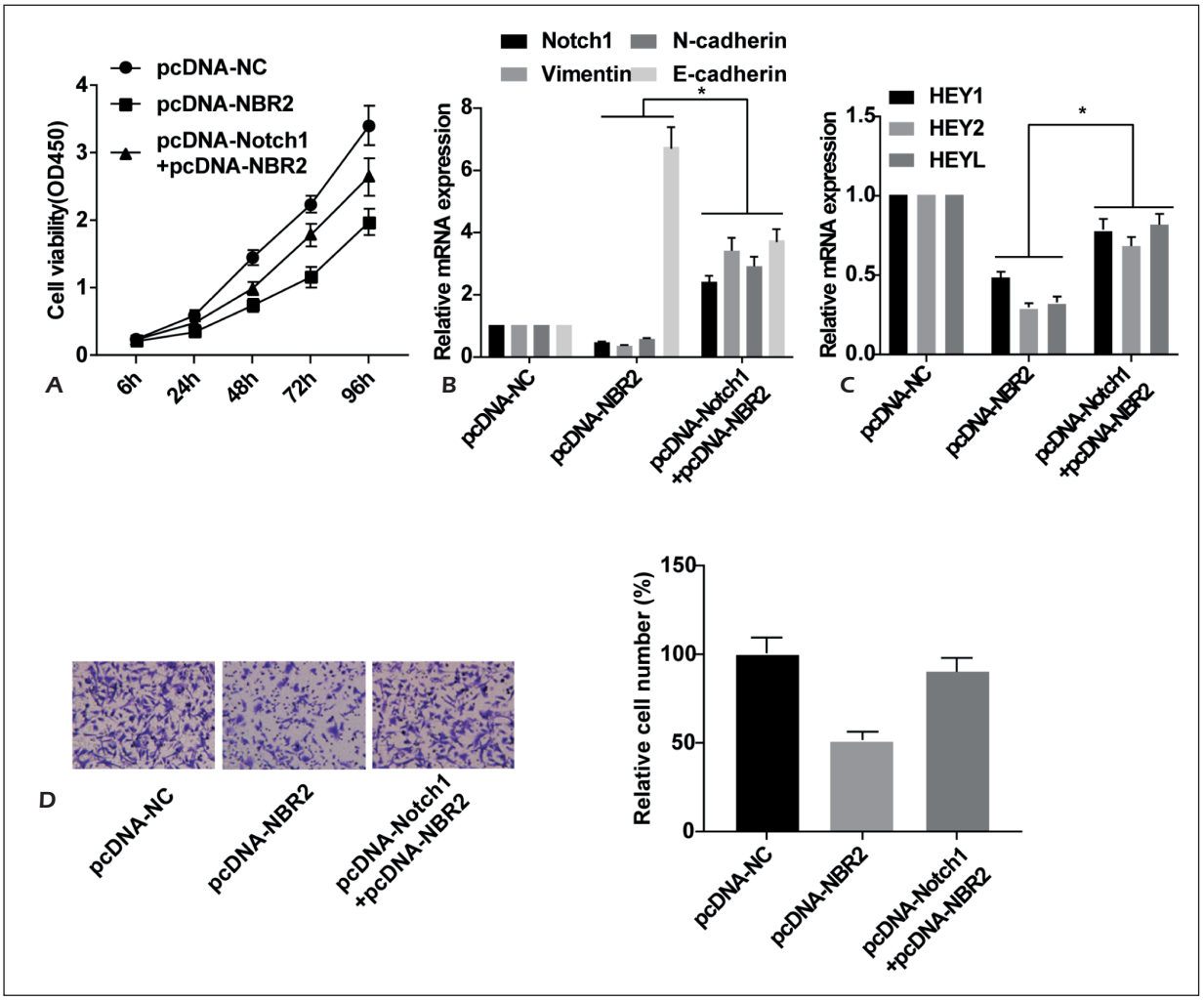

Figure 4. Overexpression of Notch1 reverses the inhibitory effect of NBR2 on proliferation and migration of non-cell lung cancer cells. Overexpression of NBR2 in AsPC-1 cells simultaneously overexpresses Notch1. ***A***, CCK8 assay can reverse the decrease in cell viability caused by overexpression of NBR2 alone. **B**, qRT-PCR assay can detect the decrease of mRNA levels of Notch1, N-cadherin, E-cadherin and the increase of Vimentin mRNA level caused by overexpression of NBR2. **C**, qRT-PCR assay detects the ability to reverse the expression of HEY1, HEY2, and HEYL caused by overexpression of NBR2. **D**, The transwell assay detects the ability to reverse the ability of cells to migrate after overexpression of NBR2 (magnification: 40×).

highly conserved that reflects the mechanism of intercellular interaction, and Notch1 is one of the major factors in Notch signaling pathway[35]. We explored how NBR2 regulated the Notch pathway in NSCLC to influence EMT progression. We performed an EMT RT2 PCR sequence analysis and found that Notch1, N-cadherin, and Vimentin were down-regulated in AsPC-1 cells that had been overexpressed with NBR2. Also, we found that overexpression of Notch1 reversed the inhibitory effect of NBR2 on migration and proliferation, and EMT of non-cellular lung cancer cells, confirming that NBR2 overexpression could inhibit the Notch1-EMT signaling pathway. These indicated that NBR2 could inhibit the progression of EMT through the Notch1 pathway and thereby inhibit the progression of non-small cell carcinoma. This may provide new evidence for the development of NBR2 in cancer therapy.

Conclusions

LncRNA NBR2 was down-regulated in NSCLC tissues and cells, and the low expression of NBR2 was associated with poor prognosis of patients. NBR2 could inhibit proliferation, migration, and EMT progression of NSCLC cells by regulating the Notch1 pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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