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OTX1 is a novel regulator of proliferation, migration, invasion and apoptosis in lung adenocarcinoma

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Abstract. – OBJECTIVE: Orthodenticle Homeobox 1 (OTX1) has been found to be closely related to the development of several human tumours. However, the function and underlying molecular mechanisms of OTX1 in non-small cell lung cancer (NSCLC) are unclear. This research was performed to investigate the effects of downregulating OTX1 gene expression on the proliferation, migration, invasion, cell cycle and apoptosis of human NSCLC cell lines.

PATIENTS AND METHODS: Cultured NCI-H292 and XWLC cells were transfected with control small interfering RNA (siNC) or experimental siRNA (siOTX1). The mRNA levels were detected using a quantitative real-time PCR (RT-qPCR) assay. A Cell Counting Kit-8 (CCK-8) and a Real Time Cell Analyzer (RTCA) were used to determine cell activity. The RTCA and transwell chambers were used to assess cell migration and invasion. In addition, cell cycle progression and apoptosis were measured using flow cytometry, and the expression levels of key signalling pathway proteins were examined by Western blotting.

RESULTS: The results revealed that compared with the control group, the experimental group exhibited significantly decreased cell activity (*$p<0.001$), significantly decreased migration and invasion abilities (***$p<0.001$), and cell cycle arrest in G_2/M phase (*$p<0.05$). However, the number of apoptotic cells was higher in the experimental group than in the control group (*$p<0.05$). The Western blotting results were consistent with the functional experiment results.**

CONCLUSIONS: Silencing the OTX1 gene suppressed the proliferation, migration and invasion of NCI-H292 and XWLC cells, impeded the cell cycle transition from G_2 to M phase, and accelerated apoptosis, revealing OTX1, a regulator of NSCLC, as a potential new therapeutic target.

Key Words:
OTX1, NSCLC, Proliferation, Migration, Invasion, Cell cycle, Apoptosis.

Introduction

Globally, lung cancer is the type of cancer that causes the highest mortality; every year, more than 1 million people die from lung cancer worldwide[1]. Because lung cancer does not cause any signs or symptoms in the early stages, diagnosis is usually made in the late stages of the disease[2,3]. Non-small cell lung cancer (NSCLC) is a subtype of lung cancer[4], and approximately 85% of lung cancer cases are NSCLC[5]. Based on histopathology, NSCLC is divided into three types: large cell carcinoma (LCC), squamous cell carcinoma (SCC), and adenocarcinoma (ADC)[6]. Although modern surgical techniques and systemic chemotherapy have enabled significant progress, the prognosis of NSCLC patients is still poor, and the overall 5-year survival rate is only approximately 20%[7]. Therefore, it is urgent to explore the potential pathogenesis of NSCLC and identify new diagnostic biomarkers for the treatment of NSCLC, which may provide new strategies for clinical treatment.

Orthodenticle homeobox 1 (OTX1), an OTX family protein, is a transcription factor that specifically binds to TAATCC/T elements on target genes[8]. The OTX1 gene is located at 2p13 and contains 5 exons encoding 354 amino acids[9,10]. Initial research has shown that OTX1 is a key molecule in the process of axon thinning in the vertebrate cerebral cortex[10,11],

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and it is involved in embryonic development processes, including brain regionalization[12,13]; cortex formation; and sensory organ[14], retina[15] and breast development[16]. In mice, the OTX1 gene is necessary for neuronal differentiation in the forebrain and midbrain[14,17,18]. In the haematopoietic system, OTX1 is essential for the development of red blood cell compartments[19]. In addition, OTX1 plays significant roles in the development of auditory and visual sensory organs as well as in the transient control of pituitary growth hormone (GH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels[18]. As research has continued increasing amounts of evidence indicated that OTX1 is involved in the development of many malignancies, including hepatocellular carcinoma[16], colorectal cancer[20], medulloblastomas[21], breast cancer[10] and gastric cancer[22]. Qin et al[22] and Wang et al[23] have shown that OTX1 plays key regulatory roles in tumour growth, metastasis, and apoptosis of gastric cancer cells. Further research has shown that OTX1 promotes tumour proliferation and migration in colorectal[9,20] and hepatocellular carcinoma[16]. Therefore, OTX1 may be extensively involved in the development and progression of human cancers. However, the function of OTX1 expression in NSCLC is unclear.

We have observed that the OTX1 gene is significantly upregulated in NSCLC cells and tissues, which prompted us to explore the biological roles of OTX1 in NSCLC cells. In this study, the OTX1 gene was specifically silenced by small interfering RNA (siRNA), and the effects on NSCLC cell function (including cell viability, metastasis, cycle progression, and apoptosis) were observed. The present findings suggest that OTX1 plays key regulatory roles in the development and progression of NSCLC and may serve as a novel regulatory factor and a new target for the prevention or treatment of NSCLC. Previous studies[24,25] have shown that trans-retinoic acid can inhibit the expression of the OTX gene, which provides a promising approach for therapeutic targeting of this gene.

Patients and Methods

Patients and Sample Collection

Fifteen NSCLC specimens and adjacent normal samples were collected from patients who were undergoing surgery at Yunnan Cancer Hospital between October 2014 and December 2015. The cDNA of the 15 pairs of samples was sent to Shanghai OE Biotech Co. (Shanghai, China) for transcriptional microarray analysis, and the gene to be studied was analysed by using paired *t*-test results and fold change values as the screening criteria. The sample information can be obtained from previous studies[26]. In addition, 44 pairs of cancer and adjacent/normal lung tissue samples were collected from Yunnan Cancer Hospital between 2015 and 2016. These samples were used for PCR and Western blot experiments. The tissue sample information is shown in **Supplementary Table I**. These studies were approved by the Ethics Committee of the Yunnan Provincial Cancer Hospital and the Medical College of Yunnan University and were conducted in accordance with the standards stipulated in the Declaration of Helsinki. The patients did not receive any preoperative treatments (including chemotherapy, radiotherapy, and/or targeted therapy). Each subject in this study provided written informed consent. All samples were stored in liquid nitrogen or frozen at -80°C.

Database Analysis

First, to better compare the expression profiles of OTX1 between NSCLC tissues and adjacent normal lung tissues, we collected relevant data from the Oncomine website (https://www.oncomine.org/resource/login.html). Second, we analysed the OTX1 mRNA expression level differences between normal lung tissue and NSCLC tissue using the Ualcan and Gene Expression Profiling Interactive Analysis (GEPIA)[27] databases (http://ualcan.path.uab.edu/ and http://gepia.cancer-pku.cn/index.html, respectively). We further evaluated the results of OTX1 immunohistochemistry (IHC) based on the Human Protein Atlas (HPA) portal (https://www.proteinatlas.org/). Finally, the overall survival (OS) curve information of OTX1 from the Kaplan-Meier (KM) Plotter database (https://kmplot.com/analysis/) was analysed.

Cell Culture and Transfection

Human NSCLC cell lines (A549 and NCI-H292) and a normal lung cell line (the immortalized human bronchial epithelial cell BEAS-2B) were purchased from Kunming Institute of Zoology Center. Two local NSCLC cell lines (XWLC and GLC) were isolated from the cancer tissues of patients living in Xuanwei and Gejiu, Yunnan Prov-

ince, China. The A549, NCI-H292, and BEAS-2B cells were maintained in Roswell Park Memorial Insitute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA), while the XWLC and GLC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA). The cells were cultured in media supplemented with penicillin (100 microg/mL)/streptomycin (100 μg/mL) (Life Technologies, Carlsbad, CA, USA) and 10% foetal bovine serum (FBS; HyClone, South Logan, UT, USA). The cells were incubated at 37°C in an incubator containing 5% CO_2.

siRNAs used to silence human OTX1 (siOTX1s) and a negative control siRNA (siNC) were synthesized by GenePharma Technologies (Genepharma Inc., Shanghai, China). Three siRNAs targeting human OTX1 sequences (OTX1-homo-874: 5'-CCUAGCAACACCUCGUGUATT-3' (sense), 5'-UACACGAGGUGUUGCUAGGTT-3' (antisense); OTX1-homo-928: 5'- GCAGCCUCUUAUCCCAUGUTT-3' (sense), 5'-ACAUGGGAUAAGAGGCUGCTT-3' (antisense); and OTX1-homo-1101: 5'-CCAUCAUCACCACCCACAUTT-3' (sense), 5'-AUGUGGGUGGUGAUGAUGGTT-3' (antisense) and a non-specific siRNA sequence (5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGUAGAATT-3′ (antisense) were constructed (**Supplementary Table II**). The cells were added to a 6-well plate (Corning Costar, Corning, NY, USA) at a density of 2×10^5 cells/well and cultured in a humidified incubator with 5% CO_2 at 37°C. After reaching 80% confluence, the cells were transfected with siOTX1 or siNC using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, the efficacy of gene silencing was confirmed *via* RT-qPCR, and an siRNA was selected for subsequent functional experiments.

RT-qPCR

Total RNA was isolated from NSCLC cells and tissues using total RNA isolation reagent (TRIzol) (Invitrogen, Carlsbad, CA, USA), and genomic DNA residues were removed using deoxyribonuclease I (DNase I) (Invitrogen, Carlsbad, CA, USA). Then, the RNA was reverse-transcribed into cDNA using a PrimeScript™ RT-PCR Kit (Takara, Dalian, China). RT-qPCR was performed with a SYBR® Premix ExTaq™ Kit (TaKaRa, Dalian, China) on a CFX96™ Real-time PCR instrument (Bio-Rad, Hercules, CA, USA). The primer sequences (Sangon Biotech, Shanghai, China) used for PCR were as follows (**Supplementary Table III**): OTX1, 5'-CATGGCGGTTCCAGGTCTTGTG-3' (forward) and 5'-CAGCAGCAGCAACAGGATCGG-3') (reverse); GAPDH, 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (forward) and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (reverse). The PCR conditions were as follows: pre-deformation at 95°C for 2 min followed by 40 cycles at 95°C for 15 s and final generation of a dissolution curve. For each sample, the gene expression was normalized to that of GAPDH and calculated with the relative quantification ($2^{-\Delta\Delta Ct}$) method. All samples were analysed in triplicate.

Cell Proliferation Assays

Cell viability was evaluated in two ways. For CCK-8 assays, transfected cells were collected at 24 h post transfection and seeded into 96-well plates at a density of 3×10^3 cells per well (Corning Costar, Corning, NY, USA). After culture for 0, 24, 48 and 72 h, 10 mL of CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the cells were incubated for another 2.5 h. The optical density (OD) values were measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 450 nm.

For RTCA assays, the cells were seeded into an 16-well E-Plate (ACEA Biosciences, San Diego, CA, USA) at a density of 5×10^3 cells per well, and then, an RTCA Instrument (ACEA Biosciences, San Diego, CA, USA) was used for label-free, real-time and automated monitoring with proprietary microelectronic sensor technology. After 24 h, the cells were transfected with siOTX1 or siNC. The cell index analysis (30 min interval detection) lasted for 92 h.

Colony Formation Assay

After 48 h of transfection, the cells were digested with trypsin (Thermo Fisher Scientific, Waltham, MA, USA), seeded into 6-well plates (5×10^3 cells per well) and incubated at 37°C for ten days until most single colonies contained more than 100 cells. Subsequently, the naturally formed colonies were washed twice with phosphate-buffered saline (PBS; HyClone, South Logan, UT, USA), fixed with 4% paraformaldehyde (Sangon Biotech, Shanghai, China) for 1 h, and stained with 0.25% crystal violet (Solarbio, Beijing, China) for 1 h. Finally, photos were taken to count the colonies.

Migration and Invasion Assays

Cell migration assays were performed using transwell chambers (8 μm pore size) (Corning Costar, Corning, NY, USA) according to the manufacturer's instructions. After 48 h of transfection, the cells were washed with PBS, resuspended in serum-free medium and seeded in upper chambers at a density of 1×10^5 cells. Then, 800 μL of medium containing 20% FBS as a chemoattractant was added to the lower chambers. Following 24 h of incubation, the cells that migrated to the lower chambers were fixed with 4% paraformaldehyde and stained using 0.25% crystal violet. Finally, five randomly selected visual fields under a microscope at 100× magnification were imaged, and the number of migratory cells was counted using a Nikon TE2000 microscope (Nikon, Tokyo, Japan). The cell invasion assay was similar to the cell migration assay except that the transwell chambers were coated with growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

The ability of cells to migrate and invade was also analysed by RTCA assays. Cells in serum-free medium (100 μL) were seeded in 16-well CIM-Plate (1×10^5 cells per well) (ACEA Biosciences, San Diego, CA, USA) upper chambers, and 165 μL of medium containing 20% FBS was added to the lower chambers. Then, the RTCA DPlus Instrument (ACEA Biosciences, San Diego, CA, USA) was used to test the cell index (30-minute interval detection) for 72 h. An invasion assay was performed in the same manner as the migration assay except that the upper chamber was precoated with 1 mg/mL growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Flow Cytometric Analysis

For cell cycle analysis, cells were collected and fixed in 70% ethanol at 4°C overnight. Then, the cells were treated with ribonuclease (RNase; KeyGEN BioTECH, Nanjing, China) and stained with propidium iodide (PI; KeyGEN BioTECH, Nanjing, China) (PI:RNase=9:1) for 30 min at 37°C after washing with PBS. The distribution of cell cycle phases was determined by a BD FACS Jazz flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The phase ratio (%) was calculated as the percentage of cells in $G_1/S/G_2$ phase.

For apoptosis analysis, an Annexin V/PI Apoptosis Assay Kit (Vazyme, Nanjing, China) was utilized. Briefly, the cells were incubated with Annexin V-fluorescein isothiocyanate (Vazyme, Nanjing, China) and PI for 15-20 min in the dark at room temperature. After washing with PBS twice, a FACS Jazz flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was employed to detect apoptosis, and the results were analysed with Flow Jo 7.6.1 (TreeStar Software, San Carlos, CA, USA).

Western Blot Analysis

Cells were lysed in lysis buffer (Biomiga Inc., San Diego, CA, USA) containing phosphatase inhibitor and protease inhibitor (Roche, Mannheim, Germany) on ice for 1 h. To fully lyse tissues, the tissues were incubated in radioimmunoprecipitation assay (RIPA) lysis buffer (Biomiga Inc, San Diego, CA, USA) after grinding with liquid nitrogen. The subsequent procedures were the same for cells and tissues. The samples were centrifuged at 12,000 × g for 15 min at 4°C, and the supernatants were collected. The protein concentrations were determined according to a bicinchoninic acid (BCA) Protein Quantitative Kit (Biomiga Inc., San Diego, CA, USA), and denaturation at high temperature was performed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (TaKaRa, Dalian, China). The proteins were separated by 10% SDS-PAGE (20 μg per lane) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Then, the membranes were blocked with 5% non-fat dry milk or 5% bovine serum albumin (BSA; Sangon Biotech, Shanghai, China) in Tris-Buffered Saline with Tween-20 (TBST; Solarbio, Beijing, China) for 1 h and incubated overnight at 4°C with the primary antibodies anti-OTX1 (OM287107, 1:1000, Omnimabs, Alhambra, CA, USA), anti-N-cadherin (ab18203, 1:1000, Abcam, Cambridge, UK), anti-Vimentin (ab8978, 1:1000, Abcam Cambridge, UK), anti-Cyclin B1 (ab33911, 1:1000, Abcam Cambridge, UK), anti-PARP1 (ab32138, 1:1000, Abcam, Cambridge, UK), anti-cleaved PARP1 (ab32064, 1:1000, Abcam, Cambridge, UK), anti-p-ERK (ab17942, 1:1000, Abcam, Cambridge, UK), anti-ERK (ab76299, 1:5000, Abcam, Cambridge, UK), anti-Caspase3 (ab13585, 1:1000, Abcam, Cambridge, UK), anti-active Caspase3 (ab32042, 1:1000, Abcam, Cambridge, UK), anti-GAPDH (ab181602, 1:5000, Abcam, Cambridge, UK), and β-Tubulin (MA5-16308, 1: 10000, Invitrogen, Carlsbad, CA, USA). The membranes were then incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:500, Santa Cruz Biotechnology, Santa Cruz,

CA, USA) according to the manufacturers' instructions at room temperature for 60 min. The residual primary and secondary antibodies were washed away with TBST three times. Finally, the protein bands were detected by Western blot chemiluminescence (Protein Simple Jess, Biotechne, San Diego, CA, USA) after washing with TBST. The data were normalized to β-Tubulin or GAPDH loading controls. All experiments were performed in triplicate.

Statistical Analysis

GraphPad Prism software version 7.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. Student's *t*-test was used to determine the statistical significance of differences in OTX1 expression between groups. All data are expressed as the mean ± standard error. For each test, a two-sided p-value < 0.05 was considered to indicate a significant difference. *, **, and *** denote significance at the 0.05, 0.01 and 0.001 levels, respectively. Kaplan-Meier curves data were obtained from TCGA database and plotted with SPSS 21 software (IBM Inc., Armonk, NY, USA), logrank $p<0.05$ was considered statistically significant.

Results

OTX1 Expression Was Upregulated, As Revealed by RNA Microarray and Database Analyses

First, 15 pairs of microarrays of NSCLC/adjacent tissues available from previous scholars[26] (**Supplementary Figure 1**) were analysed. Microarray gene expression analysis was performed on mRNA, and genes with significant differences were screened by comparing gene expression in normal versus adjacent tumour tissues. The microarray analysis revealed 2741 differentially expressed genes, including 623 upregulated genes and 2118 downregulated genes. We found that OTX1 was among the upregulated genes (Figure 1A). Second, to further verify the relationship between OTX1 and tumours, we used the GEPIA portal to detect the relative expression of OTX1 transcripts in various cancer tissues compared to normal corresponding tissues. OTX1 was elevated in a number of cancers, including NSCLC. In addition, the expression of OTX1 was continuously upregulated in all stages of NSCLC, indicating the importance of this gene in disease progression (Figure 1B, 1C and **Supplementary Figure 2**). Finally, we assessed OTX1 mRNA expression levels in cancer and adjacent tissues based on The Cancer Genome Atlas (TCGA) through the Oncomine website and the Ualcan portal and found that OTX1 mRNA expression was upregulated in the cancer tissues (Figure 1D and 1E). Taken together, our findings reveal that the expression of the OTX1 gene has potential significance in the progression and expansion of NSCLC.

OTX1 mRNA and Protein Were Upregulated in NSCLC Tissues and Cells

To gain insight into the role of OTX1 in NSCLC tumorigenesis, we analysed the expression levels of OTX1 mRNA in NSCLC tissues through RT-qPCR. OTX1 mRNA was significantly upregulated in 80% (36/44) of cancer *vs.* normal tissues and 70% (31/44) of cancer *vs.* adjacent tissues (Figure 2A and 2B). Next, we performed Western blot analysis to evaluate the expression of OTX1 protein in cancer versus normal tissues (n=10/30) and in cancer versus adjacent tissues (n=10/29) (Figure 2C and 2D). Consistently, the results showed that OTX1 protein was also highly expressed in cancer tissues compared to adjacent/normal tissue (**Supplementary Figure 3**). In addition, OTX1 mRNA expression in all four NSCLC cell lines was higher than that in normal cells, with the highest expression levels in NCI-H292 and XWLC cells (Figure 2E). For protein expression, the results illustrated that OTX1 was highly expressed in all four NSCLC cell lines compared to the normal BEAS-2B cell line. Among the cells, NCI-H292, GLC, and XWLC cells exhibited the highest expression of OTX1 (Figure 2F). The results were consistent with the PCR results. Therefore, two highly expressing cell lines, NCI-H292 and XWLC, were selected for further experiments. The findings of these experiments further confirm that the OTX1 gene is highly expressed in NSCLC tissues and cells, affecting the occurrence and development of NSCLC.

OTX1 Expression Was Downregulated After Transfection With siOTX1

Since OTX1 was upregulated in NSCLC, we further investigated the effects of silencing OTX1 on NSCLC function. We performed three siOTX1 knockout experiments on the cells. Given the PCR results (Figure 3A) from the two cell lines, the experiments showed that siOTX1-1101 had

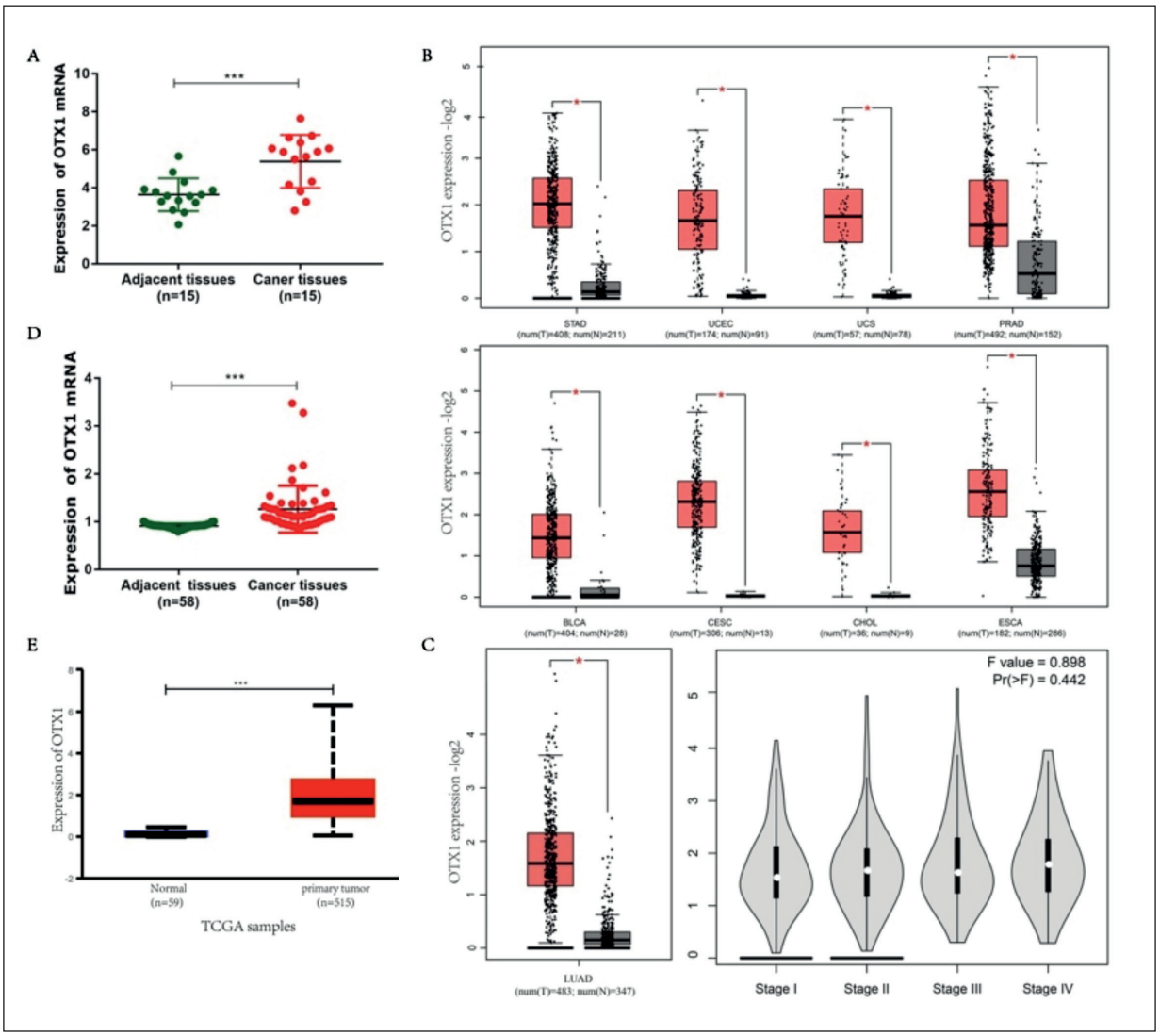

Figure 1. Expression levels of OTX1 mRNA in NSCLC tissues. **A,** Expression of OTX1 mRNA in 15 pairs of NSCLC/adjacent tissue samples. **B,** OTX1 mRNA expression in various normal versus cancerous tissues and **(C)** OTX1 mRNA expression in normal tissues versus NSCLC tissues at various stages from the GEPIA portal. **D,** OTX1 mRNA expression in 58 pairs of normal and NSCLC samples based on the Oncomine database. **E,** Expression of OTX1 mRNA in 59 normal and 515 NSCLC tissues from TCGA on Ualcan's website. *p<0.05, ***p<0.001.

the best knockout effect. Therefore, we selected siOTX1-1101 (hereinafter referred to as siOTX1) and verified its effects on OTX1 protein levels in the two cell lines, and the results revealed that the protein expression levels in both cell lines were decreased (Figure 3B). Thus, this siRNA was effective in knocking down mRNA and protein expression.

Downregulation of OTX1 Significantly Inhibited the Proliferation of NSCLC Cells

To survey the effects of OTX1 on the activity of NSCLC cells, three methods were used to measure cell proliferation. In the CCK-8 assay, significant inhibition was observed in both types of cells after transfection with siOTX1 for 48 h (Figure 4A). In the RTCA assay, the siOTX1-transfected experimental group showed increasingly evident separation from the siNC group, and the final result was consistent with the CCK-8 experimental results (Figure 4B). Finally, the colony formation experiments showed that cells transfected with siOTX1 formed smaller and fewer colonies than control cells (Figure 4D and 4E). Similarly, Western blot analysis revealed reductions in the levels of the proliferation-relat-

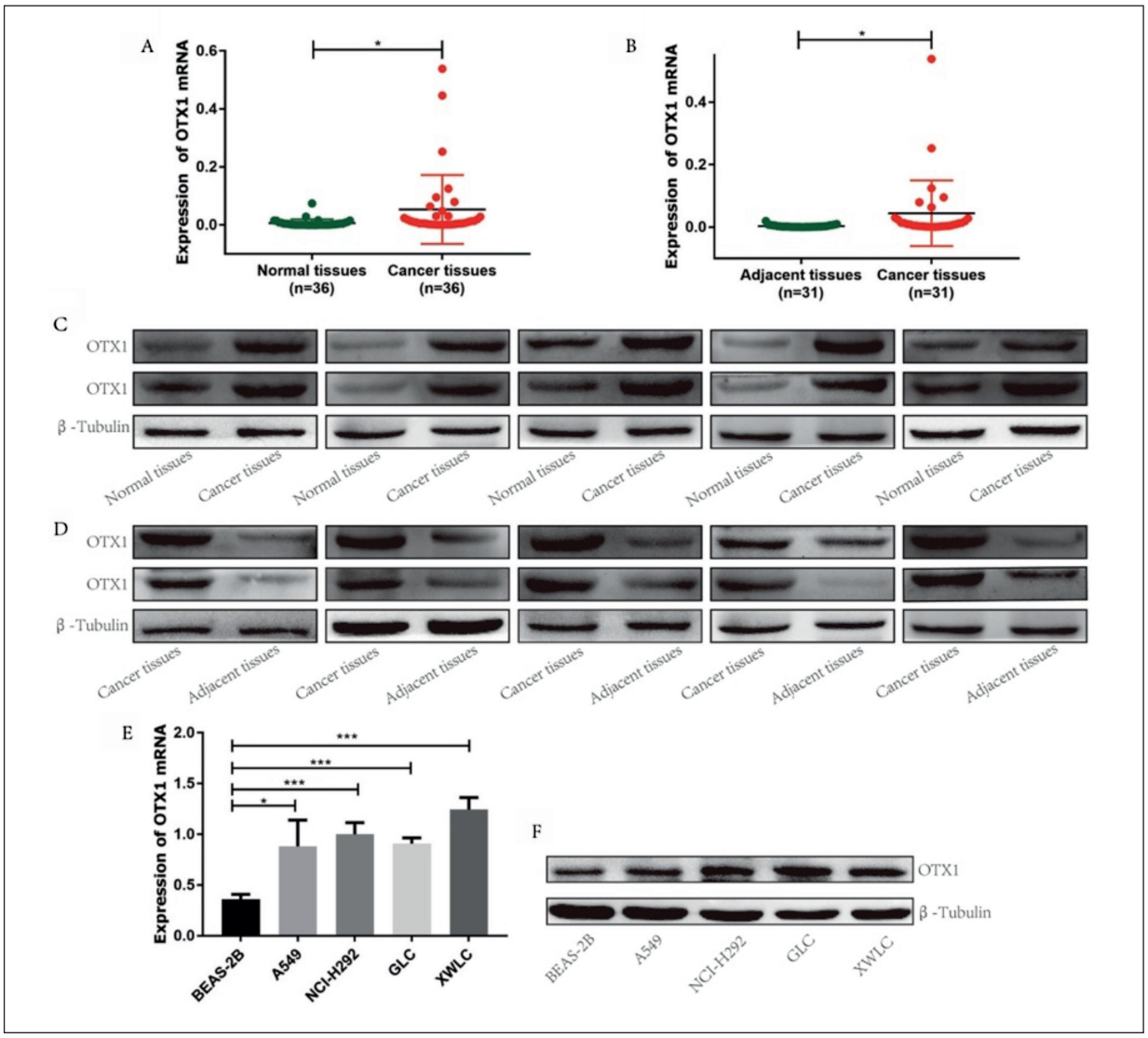

Figure 2. Expression levels of OTX1 in NSCLC tissues and cells. **A,** Thirty-six pairs of cancer samples overexpressing OTX1 mRNA versus normal samples. **B,** Thirty-one pairs of cancer samples overexpressing OTX1 mRNA versus adjacent samples. **C,** OTX1 protein was highly expressed in 30 cases of cancer and normal tissues (10 pairs). **D,** OTX1 protein was highly expressed in 29 cases of cancer and adjacent tissues (10 pairs). **E,** Expression levels of OTX1 mRNA in five cell lines. **F,** Expression levels of OTX1 protein in five cell lines. β-Tubulin was used as an internal control. *p<0.05, **p<0.01, ***p<0.001.

ed protein p-ERK in both cell lines (Figure 4C). These data indicate that the proliferation of NSCLC cells is promoted by OTX1.

Downregulation of OTX1 Inhibited the Migration and Invasion of NSCLC Cells

Due to the high metastatic rates of lung cancer, we used transwell chambers and RTCA assays to detect the migration and invasion abilities of NCI-H292 and XWLC cells after transfection with siOTX1. In the transwell assay, the number of cells that migrated into the lower compartment after siOTX1 transfection was remarkably lower in the experimental group than in the control group. In addition, quantitative analysis of the numbers of migrating and invading cells was performed (Figure 5A, 5B). The RTCA assay results illustrated that the cell curves for the two cell lines in the experimental group showed a steady trend, while those for the two cell lines in the control group showed an upward trend. The results were significantly dif-

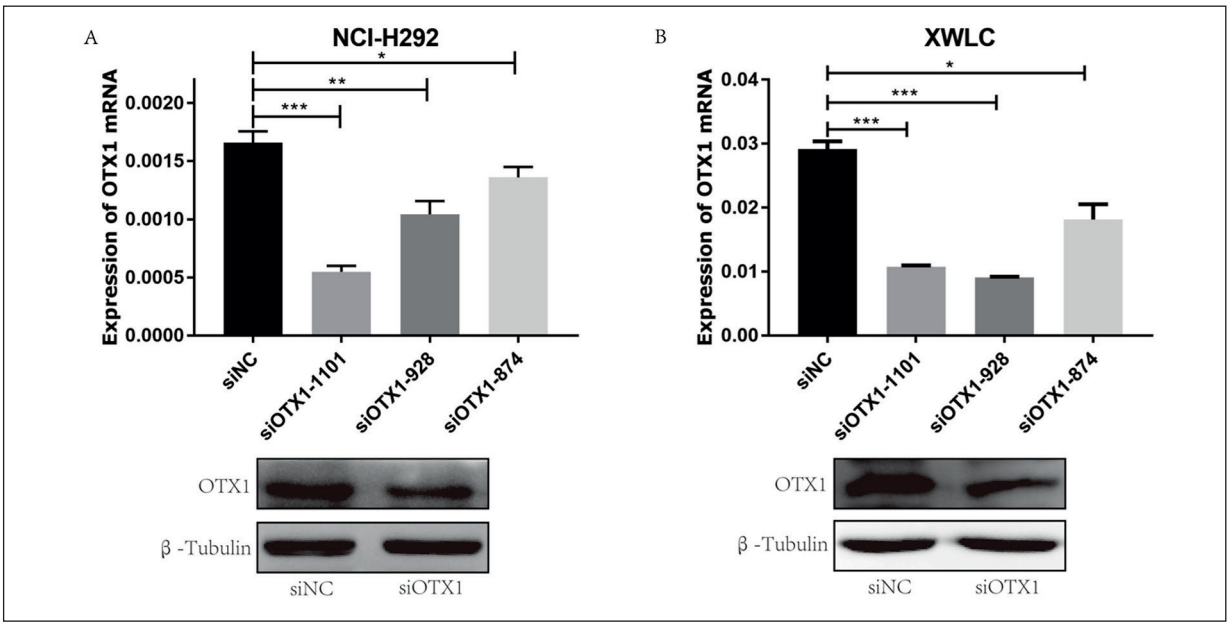

Figure 3. Expression of OTX1 mRNA and protein in NSCLC cells after siRNA transfection for 48 h. Expression levels of OTX1 mRNA and protein in NCI-H292 **(A)** and XWLC **(B)** cells after siOTX1 transfection for 48 h. β-Tubulin served as an internal control. The statistics are based on at least 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

ferent for the different groups (Figure 5C, 5D). Therefore, transfection of siOTX1 significantly reduced the metastatic capacity of the two cell lines. Subsequently, Western blot analysis was used to determine whether the downregulation of siOTX1 was associated with epithelial-mesenchymal transition (EMT)-related proteins. Consistent with the results at the cell level, we found that silencing OTX1 caused both cell lines to show decreases in N-cadherin and Vimentin levels (Figure 5E). The above results indicate that OTX1 may promote the migration and invasion of NSCLC cells by changing the expression of EMT-related proteins.

Downregulation of OTX1 Blocked Cell Cycle Progression in NSCLC Cells

To determine the effect of siOTX1 on cell growth, cell cycle distribution was examined using flow cytometry. As displayed in Figure 6A, after transfection of NCI-H292 and XWLC cells with siOTX1, the proportions of G_0/G_1-phase cells among the three groups were decreased. However, the ratios of G_2/M-phase cells were significantly higher. The inhibitory effect of siOTX1 on the growth of H292 and XWLC cells was due to G_2/M phase arrest. Consistently, the expression of Cyclin B1 in the G_2/M phase was markedly downregulated in the two cell lines after transfection with siOTX1 (Figure 6B). Overall, these results suggest that OTX1 knockdown inhibits NSCLC cell growth by inducing arrest in the G_2/M phase.

Downregulation of OTX1 Promotes Apoptosis in NSCLC Cells

To investigate the underlying mechanism of growth inhibition after OTX1 downregulation, we analysed apoptosis by flow cytometry. As distinctly indicated in Figure 7A, the number of apoptotic cells in the siOTX1-transfected group was significantly higher than that in the control group. Subsequently, the effect of OTX1 on the expression of apoptosis-related proteins was further investigated using Western blotting. As shown in Figure 7B, depletion of OTX1 lead to upregulation of active Caspase3 and cleaved PARP1, further supporting that OTX1 knockdown induces apoptosis in NSCLC cells. Collectively, these data indicate that inhibition of OTX1 can induce apoptosis by regulating apoptosis-related proteins, thereby modulating the development of NSCLC.

High Expression of OTX1 Is Associated With Poor Prognosis

In light of the results above, we investigated the expression of the OTX1 protein in NS-

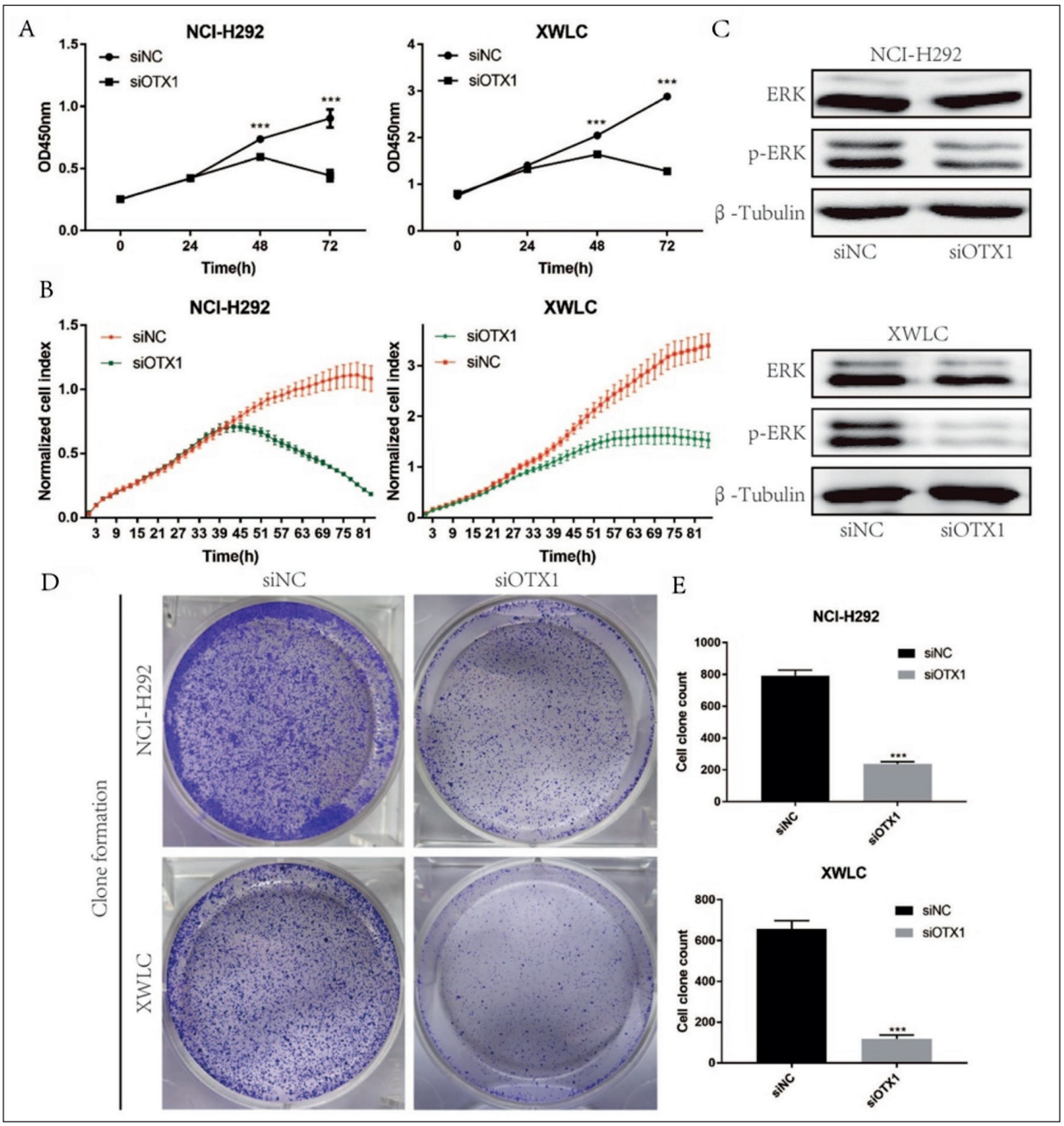

Figure 4. OTX1 knockdown inhibits the growth of NSCLC cells. The proliferation of NCI-H292 and XWLC cells was determined by CCK-8 assay **(A)** and RTCA assay **(B). C,** Western blot analysis of ERK and P-ERK protein expression levels; β-Tubulin was used as an internal reference. **D,** The colony-forming ability of NSCLC cells was detected after knocking down OTX1. Representative images of crystal violet-stained colonies are shown. **E,** Statistical analysis of the number of colonies. The statistics are based on at least three independent experiments. ***$p<0.001$.

CLC tissues determined using IHC through the HPA website. The results suggested that there was partial staining in the NSCLC tissues, indicating that OTX1 was expressed in these tissues (Figure 8A). Next, to evaluate the association between OTX1 gene expression and the prognoses of NSCLC patients, we downloaded Kaplan-Meier curves from KM Plotter for analysis, and the results showed that high OTX1 expression was associated with poor prognosis (Figure 8B). In summary, OTX1 is highly expressed in lung cancer tissues and is related

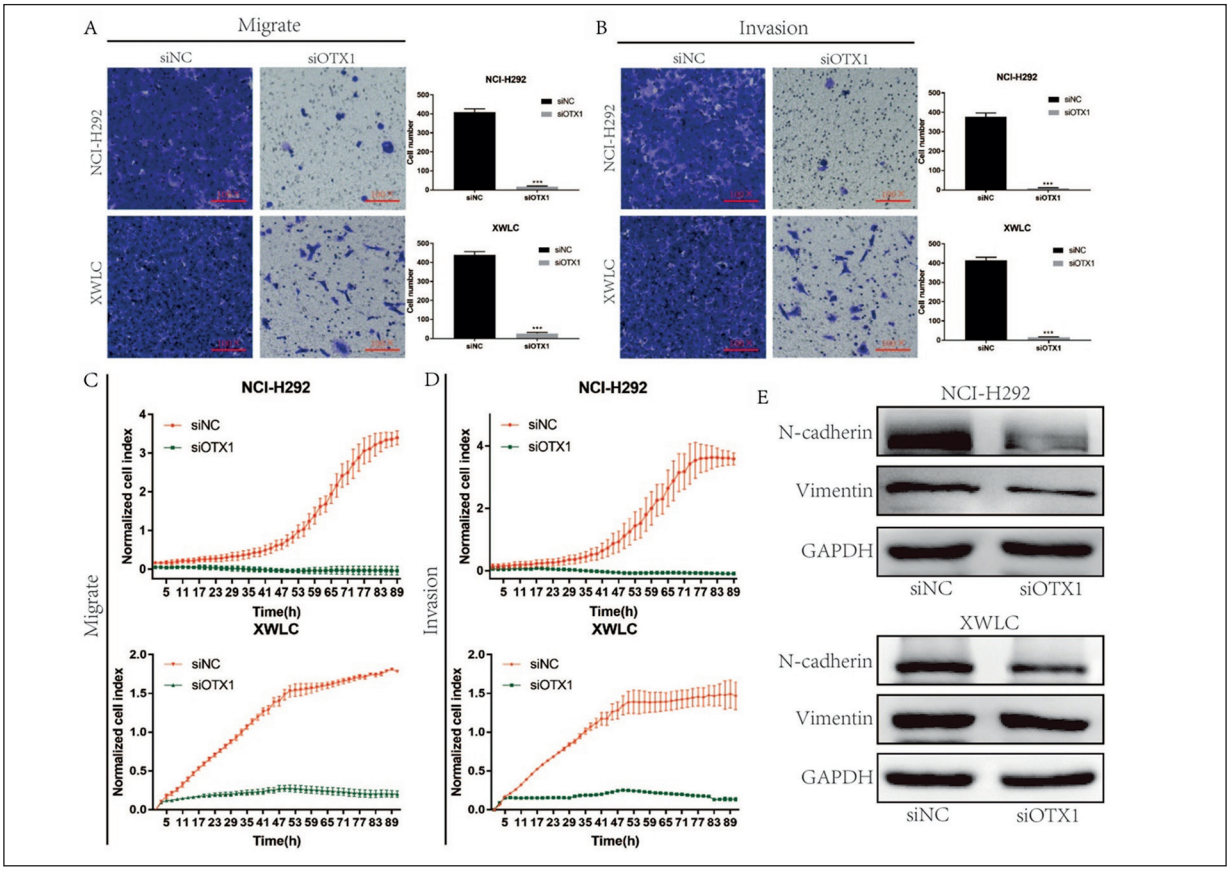

Figure 5. NSCLC cell migration and invasion decline after downregulating OTX1. The transwell results assay showed the impacts of downregulating OTX1 on the migration **(A)** and invasion **(B)** abilities of NCI-H292 and XWLC cells (magnification: 100×). The RTCA results showed the effects of downregulating OTX1 on the migration **(C)** and invasion **(D)** abilities of NCI-H292 and XWLC cells. **E,** Western blot analysis of EMT-related protein expression levels in NCI-H292 and XWLC cells after transfection with siOTX1. GAPDH was used as the internal control. ***p<0.001.

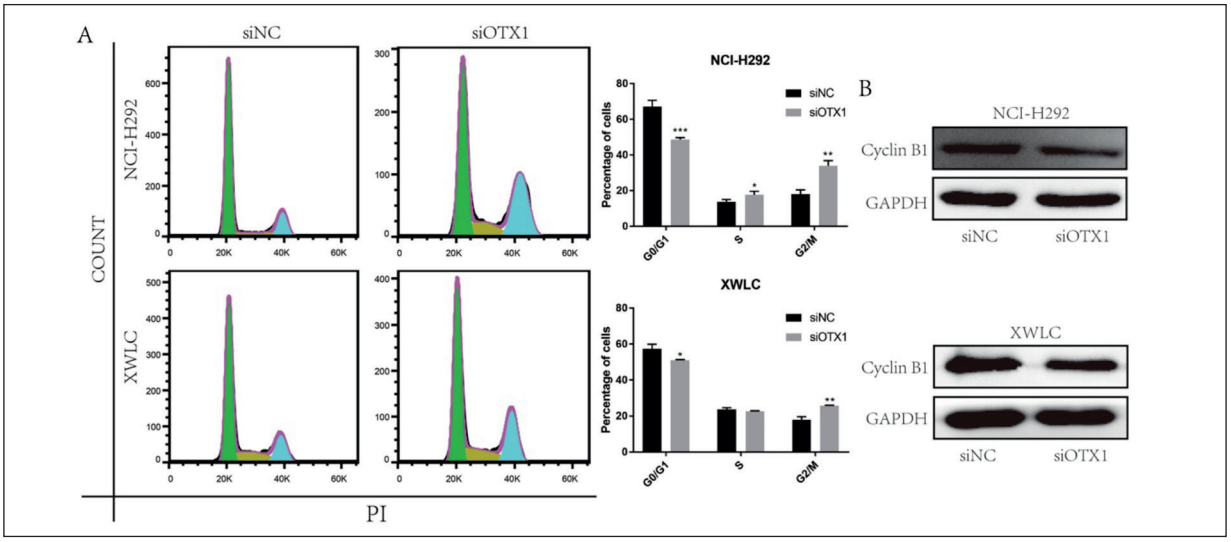

Figure 6. OTX1 accelerates progression from G_2 to M phase. **A,** Representative images of flow cytometry analysis of NCI-H292 (top) and XWLC (bottom) cells and quantitative analysis of the G_0/G_1, S, and G_2/M phase distribution 48 h after siOTX1 transfection. **B,** After transfection with siOTX1, the expression levels of Cyclin B1 in both cell lines were analysed by Western blotting. GAPDH was used as the loading control. *p<0.05, **p<0.01, ***p<0.001.

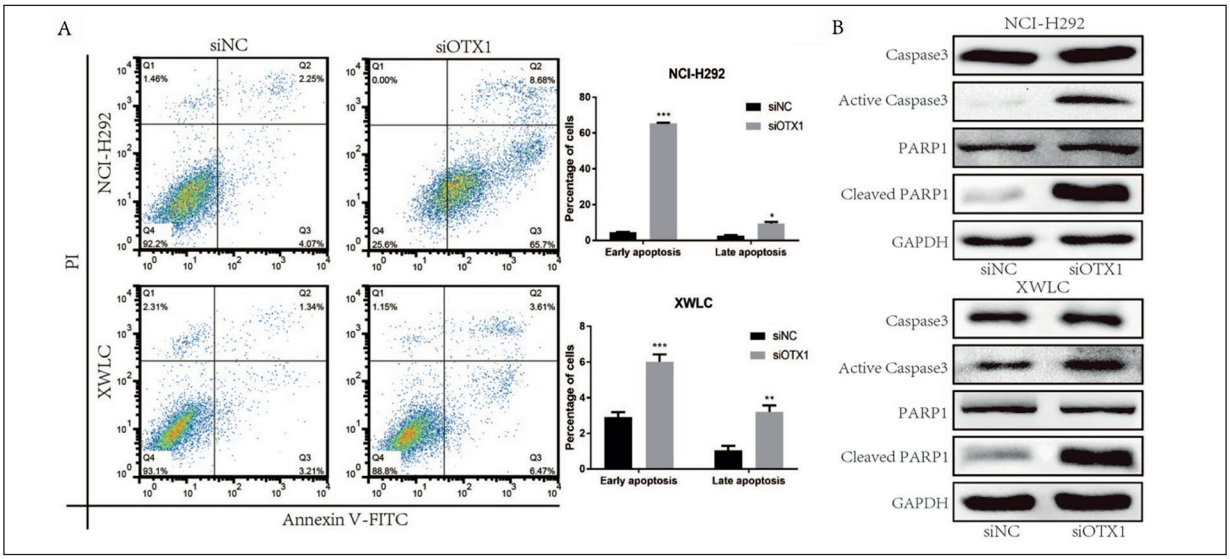

Figure 7. OTX1 inhibits apoptosis in NSCLC cells. **A,** Representative images of apoptosis in NCI-H292 (top) and XWLC (bottom) cells, as detected by flow cytometry. The proportions of NSCLC cells in early and late apoptosis were quantitatively analysed 48 h after siOTX1 transfection. **B,** Western blotting was used to analyse the expression of apoptosis-related pathway proteins. GAPDH served as an internal control. *p<0.05, **p<0.01, ***p<0.001.

to poor OS; thus, OTX1 is a potential NSCLC treatment target.

Discussion

Lung cancer accounts for 25% of all cancer deaths and remains one of the most challenging and deadly tumour diseases[28]. How to improve the survival rate and quality of life is an urgent question to be resolved. Targeted drug therapy, which is very specific, has achieved definite results for cancer treatment in recent years. Therefore, we sought to help find a good therapeutic target.

OTX1 is a transcription factor that is the vertebrate homologue of the Drosophila orthodenticle gene and contains a bicoid-like homology do-

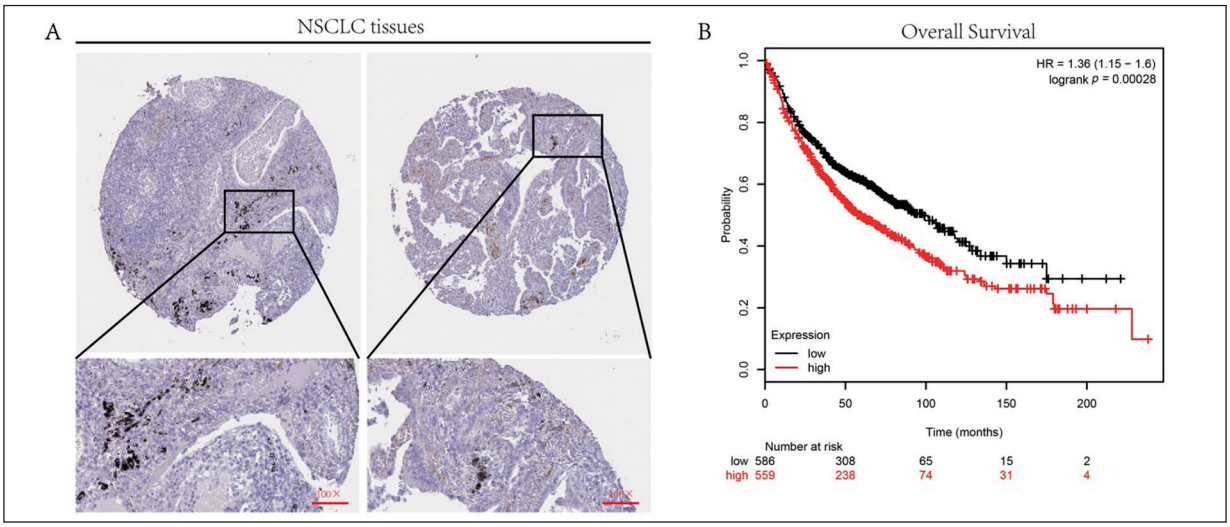

Figure 8. Relationship between high expression of OTX1 and prognosis. **A,** Representative image of NSCLC staining using an anti-OTX1 antibody from the HPA portal (antibody, HPA052122; magnification, 100×). **B,** Kaplan-Meier survival curves for different OTX1 expression levels. High expression is represented by the red curve, and low expression is represented by the black curve.

main[29]. OTX1 was previously considered to be a neuroectodermal marker[30] and has only recently been found to be expressed in various tumours, suggesting a potential role of OTX1 beyond neuroectodermal activity. Thus far, OTX1 has been reported as an oncogene for human tumours[31]. In recent years, increasing attention has been paid to the occurrence and progression of OTX1 in tumours. However, the biological roles of OTX1 in NSCLC cells and the underlying mechanisms of this factor remain largely unclear. Through further exploration in this experiment, we found that OTX1 is highly expressed in NSCLC and that its expression is negatively correlated with patient OS. We verified the expression of OTX1 in NSCLC cells and tissues for the first time *in vitro* and downregulated the expression of OTX1 using siRNA technology.

Tumours are characterized by their ability to proliferate indefinitely. As an integration point of various biochemical signals, ERK is involved in a variety of cellular processes, including differentiation, proliferation, transcriptional regulation and development[32]. Activation of this signal requires gradual phosphorylation by a kinase. When the expression of OTX1 was silenced, both the cell proliferation ability and the expression level of the related protein p-ERK were reduced, indicating that the role of OTX1 in NSCLC may depend on p-ERK activation. Another characteristic of tumours is a high rate of metastasis, and we found a reduced ability of cells to migrate and invade after downregulation of OTX1. The reductions in the levels of the related molecules N-cadherin and Vimentin may have been responsible for the decrease in cell metastatic ability, because N-cadherin and Vimentin are important molecular markers of the EMT[33]. In addition, cell cycle dysregulation is a hallmark of tumorigenesis. We determined through cell cycle distribution analysis that downregulating OTX1 leads to G_2/M phase arrest. Downregulation of the related Cyclin B1 protein can suppress the phase transition from G_2 to M[34], thereby inhibiting uncontrolled cell proliferation and malignant transformation. It is well known that the elimination of apoptosis leads to the development of cancer. The key executor of Caspase is Caspase3, and activation of Caspase3 leads to cleavage of PARP, which is considered a central indicator of apoptosis[35]. In this study, we confirmed that downregulation of OTX1 led to increases in cleaved PARP1 and active Caspase3 protein levels that corresponded to increases in the apoptosis rates of cells.

Conclusions

In summary, the knockdown of the OTX1 gene repressed the proliferation and metastasis of NSCLC cells. Blocking the progression of the G_2/M phase cell cycle increased the proportions of cells in early and late apoptosis, thereby promoting apoptosis. All these changes are expected to slow the progression and expansion of malignant tumours. Consequently, OTX1, a novel biomarker, is a potential therapeutic target for NSCLC. This research provides basic experimental support for the exploration of therapies targeting the OTX1 gene and provides evidence for the development of new therapeutic methods. However, the specific mechanisms of OTX1 in NSCLC need further research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

ZSY proposed research ideas and designed research schemes. YCY, WL and MDC were responsible for gene screening, experimental operations and data collection. LFF, RPZ, SH and LWY were in charge of data analysis and drafting of the paper. WJH, MJA and YXR Yang were involved in the whole experiment process and in the revision of the paper. All authors approved the submitted and final versions.

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