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ANXA3 deletion inhibits the resistance of lung cancer cells to oxaliplatin

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Abstract. – OBJECTIVE: The purpose of this study was to explore the role of ANXA3 in lung cancer cell resistance to oxaliplatin (OXA).

MATERIALS AND METHODS: After adding different concentrations of Ox, A549, and A549/Ox cell viability were examined using cell counting kit-8 (CCK-8) assay, and the mRNA and protein expressions of ANXA3 were analyzed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. After treating cells with 5 μg/mL and 15 μg/mL Ox for 24 hours and knocking down ANXA3, qRT-PCR, CCK8, flow cytometry, transwell, and BrdU assays were performed to examine ANXA3 expression level, cell viability, apoptosis, migration, and proliferative capacities, respectively. In addition, Western blot was performed to detect the protein expression of c-caspase 3.

RESULTS: The higher the concentration of Ox added, the worse the cell viability. Meanwhile, ANXA3 expression in A549/Ox cells was found remarkably higher than that in normal A549 cells. After treated with different concentrations of Ox for 24 hours, the cell viability, migration capacity and cell proliferation of A549 cells were found remarkably decreased, while the opposite results were observed in cell apoptosis and C-caspase 3 protein expression, and the Ox treatment group was evidently lower than control group.

CONCLUSIONS: Knockdown of ANXA3 may be able to inhibit the resistance of LCa cells to OXA.

Key Words:
Lung cancer, ANXA3, Oxaliplatin, Drug resistance.

Introduction

Lung cancer (LCa) is one of the most common malignant tumors in the world. It is also the greatest threat to human health and life safety, with the morbidity and mortality growing at the fastest speed. At present, the morbidity and mortality of LCa among all types of cancers rank first among males and second among females[1]. Although there have been many drugs for the treatment of LCa at present, the survival rate of patients has not been remarkably improved. Chemotherapy is palliative, with the main purpose of just extending the survival period of patients and improving their quality of life. In addition, drug resistance will gradually develop during the chemotherapy[2]. Therefore, it is of great significance to explore and clarify the mechanism of action of chemotherapy drugs for LCa and improve the therapeutic effect of this cancer.

Oxaliplatin (OXA) is one of the platinum chemotherapeutic drugs. The relative molecular weight of OXA is large, with the same relative molecular weight, and the number of molecules contained is less than cisplatin. However, OXA has stronger cytotoxic effect, which is speculated to be caused by its special molecular conformation. The structure of OXA is slightly different from that of other platinum drugs. The tight binding of OXA atoms to an oxalate group and 1,2-diaminocyclohexane enables OXA to initiate cell apoptosis through a variety of mechanisms[3]. There are three isomers of the OXA structure, but they do not act in exactly the same way as DNA. After OXA enters the nucleus, it can bind to DNA and take guanine (G) on the DNA strand as its target to form a variety of cross-linked structures (intrasinals and intrasinals), resulting in damage to DNA replication and RNA transcription[4]. When DNA damage caused by OXA occurs, cells are mainly repaired by intracellular nucleotide excision and repair, but the balance between apoptosis and proliferation will be destroyed if the damage cannot be repaired[4,5]. Several studies[6,7] have found that changes in the expression level of tumor-related proteins are closely related to

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the drug resistance of OXA in tumors. At present, there is still no report on OXA resistance in LCa.

Annexin is a family of phospholipid-binding proteins that are Ca^{2+} dependent, which can be involved in cytoskeleton formation and the regulation of cell-extracellular matrix interactions[8]. Annexin families include five classes, namely, A, B, C, D, and E. We humans belong to Annexin A, and 12 Annexin, including A1-A13 (Annexin A12 deletion) have been discovered. Annexin family members are involved in a variety of biological activities, such as cell proliferation, differentiation, apoptosis, anti-inflammatory, and intracellular and extracellular signal transduction[9]. Structural characteristics of the Annexin family of proteins are as follows. Approximately 70-helix amino acid residues constitute the C terminal with 4 highly similar structural regions, while the N terminal is highly variable, which is the main marker for distinguishing different Annexin family members and is composed of 20-200 amino acid residues[10,11]. Besides, the disorder of Annexin expression level is correlated with many tumors, and its expression level may be correlated with the tissue grade, clinical stage and metastasis, and invasion ability of tumors. Therefore, Annexin has attracted more and more attention in current studies, and has become a research hotspot. Annexin A3 (ANXA3) is a member of the Annexin family and plays a pivotal role in tumor formation, proliferation, apoptosis, and signal transduction. Changes in the expression level of ANXA3 have important effects on tumor development, drug resistance, and metastasis[12]. Currently, whether ANXA3 can act on LCa cells and thus affect its resistance to OXA has not been reported. Therefore, LCa cells were taken as the research object to explore whether ANXA3 can promote the drug resistance of LCa cells and provide a new idea for the study of drug resistance of this cancer.

Materials and Methods

Cell Culture

Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and penicillin/streptomycin at 37°C with 5% CO_2. Then, the cells in good growth were selected for subsequent research. A549 cells were treated with Ox at 0, 4, 8, 16, 32 μg/mL, respectively.

Cell Transfection

The cells in the logarithmic growth phase were digested and plated into a 6-well plate and cultured in an incubator until the cell density reached 80% confluence for transfection. After treated with 5 μg/mL or 15 μg/mL Ox for 24 hours, si-ANXA3 was transfected into A549 cells with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Medium was replaced after transfection for 6 h. Then, the cells were collected after 24-48 h for subsequent experiments. (si-ANXA3 F 5′-GGACAAGCAGGCAAAUGAATT-3′, R: 5′-UUCAUUUGCUUGUCCTT-3′).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Detection

The total RNA of the cells was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), and the relative expression of ANXA3 was detected using PCR assay kit. ANXA3 primers: forward 5′-CAAATTCACCGAGATCCTGT-3′ and reverse 5’-TGCTGGAGTGCTGTACGAAA-3’, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5’-CGAGATCCCTCCAAAATCAA-3’, reverse: 5’-TTCACACCCATGACGAACAT-3’.

Cell Counting Kit-8 (CCK-8) Assay

The cells in the logarithmic growth phase were seeded in a 96-well plate, each containing 1' 10^4 cells with 100 μL of culture medium. One hour before test, 10 μL of CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to each well. After incubating the plate for 1 to 4 hours in an incubator, the optical density (OD value) at 450 nm was measured with a microplate reader. The experiment was repeated three times.

Transwell Assay

Cells in each treatment group were separately trypsinized, and then plated into a 24-well plate of transwell chamber. 100 μL (cell density: 1×10^5 cells/mL) of cell suspension was added to the upper chamber, and 250 μL medium supplemented with 10% FBS was added to the lower chamber. Subsequently, after the cells were cultured for 48 h at 37°C, the chamber was taken out and cells in the upper chamber of the microporous membrane were wiped off with a cotton swab. After that, the chamber was carefully rinsed with phosphate-buffered saline (PBS) for 2 times. The cells adhering to the microporous membrane of the

chamber were fixed with 4% paraformaldehyde for 15 min, and then stained with crystal violet for 15 min. Afterwards the chamber was washed with PBS, dried, and observed under a 100-fold inverted microscope.

Flow Cytometry

After 24 h of transfection, cells were collected for cell apoptosis rate detection by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

BrdU Assay

After seeded in 96-well plates at 5×10^3 cells/well, cells were cultured for 24 h and then incubated with 10 μmol/L uridine for 4 h. Subsequently, the cells were fixed with 4% paraformaldehyde and incubated with BrdU antibody for 1 h. After washing cells with PBS, horseradish peroxidase (HRP)-labeled secondary antibody was added to incubate the cells for 30 min. Then, the cells were washed and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 15 min. Finally, the absorbance was measured at a wavelength of 450 to 540 nm in 30 min.

Western Blot

The cells in each group were collected for protein sample extraction with radioimmunoprecipitation assay (RIPA) cell lysate (Beyotime, Shanghai, China), and total protein concentration was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). 50 μg of sample protein was taken and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked with 5% skim milk powder for 1 h at room temperature. Next, antibodies against PTEN and GAPDH were added for incubation overnight at 4°C shaker. On the next day, the membranes were rinsed 3 times with Tris-Buffered Saline and Tween-20 (TBST) and incubated with second antibody for 1 h at room temperature. After that, the protein samples on the membranes were finally developed and analyzed with enhanced chemiluminescence (ECL) luminescence kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Data were statistically analyzed using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). The measurement data were expressed as mean ± standard deviation and plotted with GraphPad Prism 5.0 software (La Jolla, CA, USA). The mean comparison between the two groups was performed by independent sample *t*-test. The number comparison was analyzed by analysis of variance of repeated measures design. $p<0.05$ was considered statistically significant.

Results

Detection of Resistance to OXA in A549 Cells

The higher the concentration of Ox added, the worse the viability of A549 and A549/Ox cells (Figure 1A). The expression of ANXA3 in A549/Ox cells was remarkably higher than that in normal A549 cells (Figure 1B, 1C), suggesting that A549 was resistant to OXA.

Low Expression of ANXA3 Promoted Cell Apoptosis While Inhibited Proliferation and Migration of A549 Cells With or Without Ox Treatment

To explore the mechanism of cell resistance to OXA, A549 was treated with 5 μg/mL Ox for 24 hours, and then inhibited ANXA3 expression. It was found that ANXA3 level and cell viability, as well as migration ability were decreased remarkably after knocking down ANXA3, and the Ox treatment group was remarkably lower than the non-addition group (Figure 2A, 2B, 2D). On the contrary, cell apoptosis was significantly increased (Figure 2C), indicating that ANXA3 could inhibit apoptosis of A549 cells while promote proliferation and migration.

For further validation, A549/Ox was treated with 15 μg/mL Ox for 24 hours and also knocked down ANXA3. Similarly, ANXA3 expression, cell viability, as well as cell proliferation was found remarkably decreased, and the Ox treatment group was remarkably lower than the non-addition group (Figure 3A, 3B, and 3C). At the same time, cell apoptosis and C-caspase 3 protein expression showed the opposite trend, and the Ox treatment group was remarkably higher than the non-addition group (Figure 3D, 3E). The above results further demonstrate that ANXA3 is associated with cell resistance to Ox.

Low Expression of ANXA3 Inhibited Migration of A549/Ox Cells With or Without Ox Culture

After knocking down ANXA3, the cell migration ability was remarkably reduced, and the

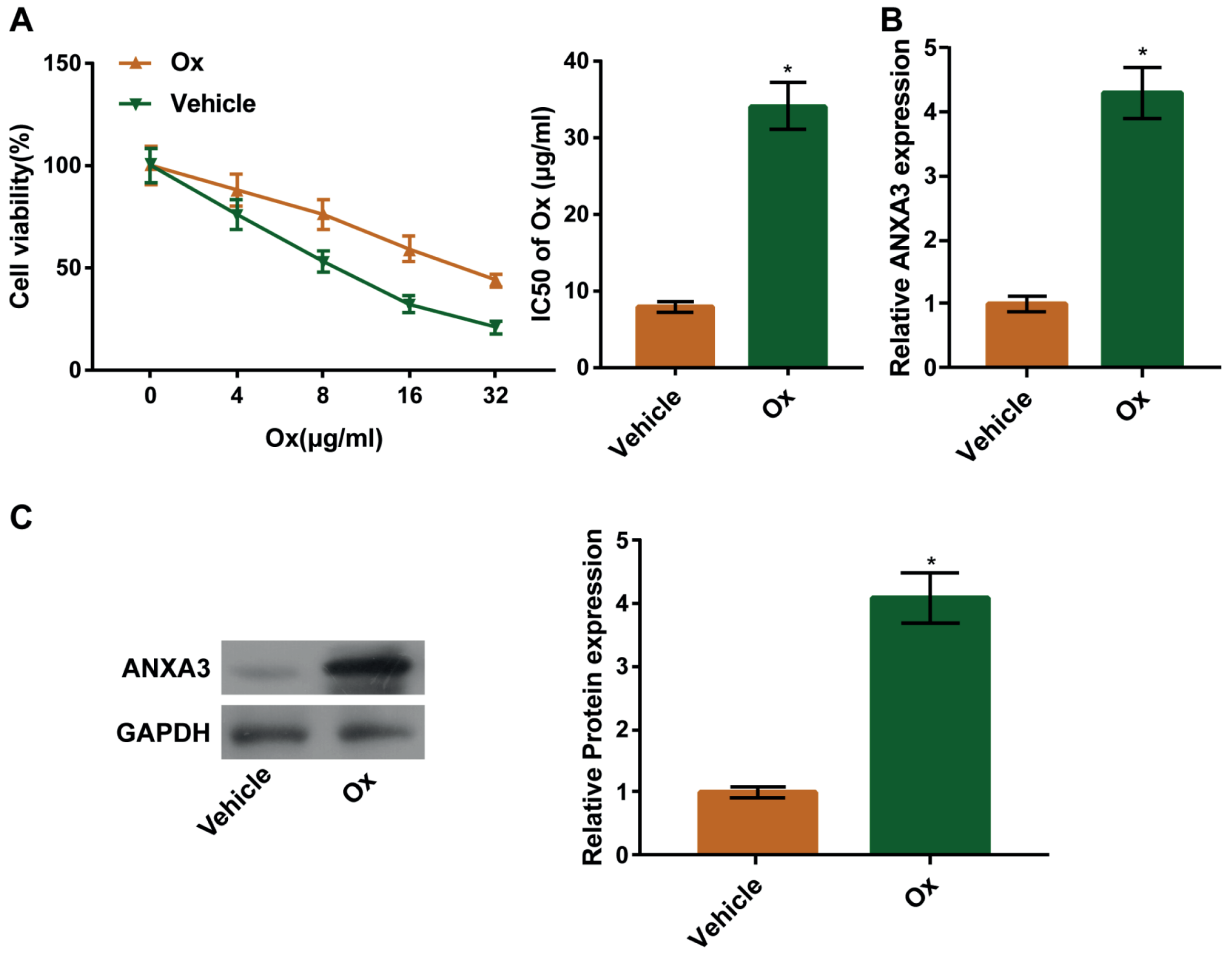

Figure 1. Detection of resistance to OXA in A549 cells. **A,** CCK8 assay shows that the higher the concentration of Ox added, the worse the viability of A549 and A549/Ox cells. **B,** The expression of ANXA3 in A549/Ox cells is significantly higher than that in normal A549 cells detected by qRT-PCR. **C,** The expression of ANXA3 protein in A549/Ox cells is significantly higher than that in normal A549 cells detected by Western blot.

Ox treatment group was remarkably lower than the non-addition group (Figure 4), indicating that ANXA3 deletion inhibited LCa cell migration and affected the role of OXA in LCa cells.

Discussion

LCa is one of the most common malignant tumors in the world and the leading cause of death of male malignant tumors. It ranks first among all malignant tumors in men, while in women, its incidence rate ranks fourth, and mortality ranks second[13]. Current standard therapies for LCa include surgical resection, platinum-based dual chemotherapy, and radiation therapy. However, these therapies rarely achieve the goal of curing LCa, and the 5-year survival rate is still only 17%[14]. Cisplatin is a common drug used in clinical NSCLC chemotherapy, but the resistance of tumor cells to cisplatin is a common clinical problem, which seriously affects the therapeutic effect[15]. Therefore, the study of drug resistance in LCa has important clinical significance.

Annexins are a class of calcium-dependent protein superfamilies that are structurally associated with phospholipids, accounting for approximately 2% of total cellular proteins[16], and they can participate in cytoskeletal composition and regulate cell-to-extracellular matrix interactions. ANXA3 is one of the members of Annexins family. The ANXA3 gene is located on

the human chromosome 4q13-q22 and consists of 323 amino acid residues[17]. Tong et al[18] demonstrated by mass spectrometry that the protein expression levels of ANXA3 in Bel cells, a kind of Hepatic cancer cell line, and its parent-resistant Bel/Fu cells were remarkably different, and ANXA3 protein was highly expressed in Bel/Fu cells. Subsequently, Pan et al[19] verified that ANXA3 can participate in and promote chemotherapy resistance of liver cancer. At the same time, Tong et al[20] revealed that the expression of ANXA3 can be regulated by JNK signaling pathway. Consistent with these studies, this research showed that ANXA3 was highly expressed in the Ox-resistant cell line A549/Ox, indicating that ANXA3 might be associated with Ox resistance of LCa cells.

OXA is a third-generation platinum drug commonly used in chemotherapy for colorectal cancer, gastric cancer, and pancreatic cancer. It is actively transported into cells and binds to nucleophilic molecules in the cell, mainly DNA, and also binds to RNA and protein. An intrachain adduct is formed between two adjacent guanines or between guanine and adenine, disrupting DNA replication and transcription[20].

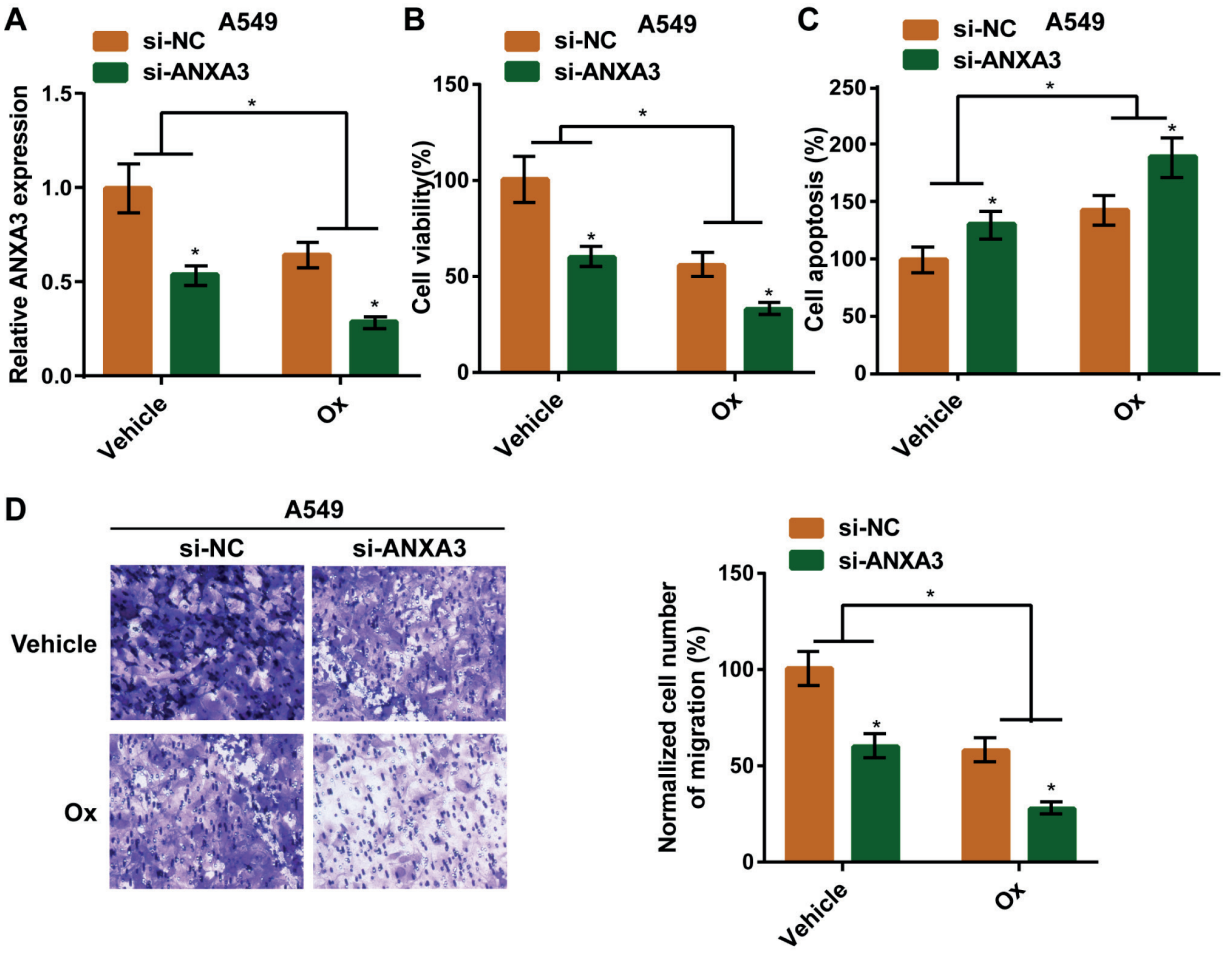

Figure 2. Low expression of ANXA3 promotes apoptosis inhibition and proliferation of A549 cells with or without Ox culture. After 5 hours of treatment with Aug 5 μg/mL Ox, the ANXA3 is knocked down and cultured for 48 hours. **A,** After quantification of ANXA3 by qRT-PCR, the expression of ANXA3 is markedly decreased, and the Ox treatment group is remarkably lower than the non-addition group. **B,** CCK-8 result shows that the cell viability is decreased significantly, and the Ox treatment group is significantly lower than the non-addition group. **C,** Flow cytometry reveals that apoptosis is decreased obviously after knockdown of ANXA3, and Ox treatment group is significantly higher than non-addition group. **D,** Transwell assay shows that the cell migration ability is significantly decreased after knocking down ANXA3, and the Ox treatment group is significantly lower than the non-addition group (magnification: 40×).

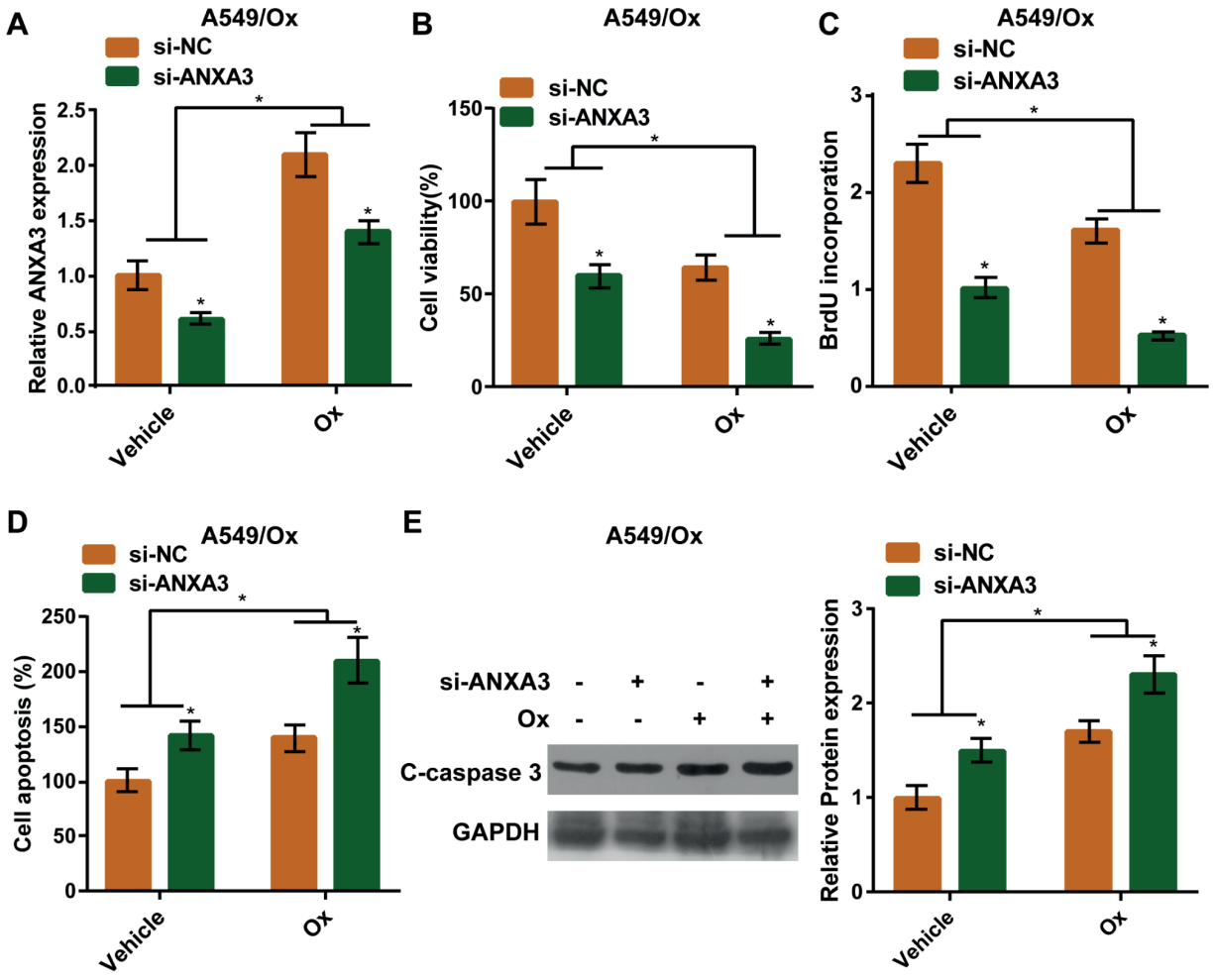

Figure 3. Low expression of ANXA3 promotes apoptosis-inhibiting proliferation and migration of A549/Ox cells with or without Ox culture. After 24 hours of treatment of A549/Ox with 15 μg/mL Ox, the ANXA3 is knocked down and cultured for 48 hours, **A,** QRT-PCR detection indicates that the expression of ANXA3 is significantly decreased after knockdown of ANXA3. **B,** CCK8 detection indicates that the cell viability is decreased significantly, and the Ox treatment group is significantly lower than the non-addition group. **C,** BrdU detection shows that the cell proliferation is significantly reduced, and the Ox treatment group is significantly lower than the non-addition group. **D,** Flow cytometry shows that apoptosis is decreased significantly after knockdown of ANXA3, and Ox treatment group is significantly higher than non-addition group. **E,** Western blot result indicates that the expression of C-caspase 3 protein is significantly increased, and the Ox treatment group is significantly higher than the non-addition group.

Interference with HOX8 expression will inhibit cell cycle, cell proliferation, and reverse OXA resistance in hepatocellular carcinoma cells[21], while the opposite result was observed when HOX8 was overexpressed. In addition, recent studies[22] have shown that ANXA3 can inhibit the resistance of colorectal cancer cells to OXA through the MAPK signaling pathway. The novelty of this study is that we first explored the regulatory role and mechanism of ANXA3 in oxaliplatin resistance in lung cancer cells. In this study, it was found that ANXA3 deletion inhibited the resistance of LCa cells to OXA, which might provide new ideas and ways for clinical treatment of LCa. However, the related molecular mechanisms should need further research and exploration.

Conclusions

We found that ANXA3 is able to promote the resistance of LCa cells to OXA.

Conflict of Interests

The Authors declare that they have no conflict of interests.

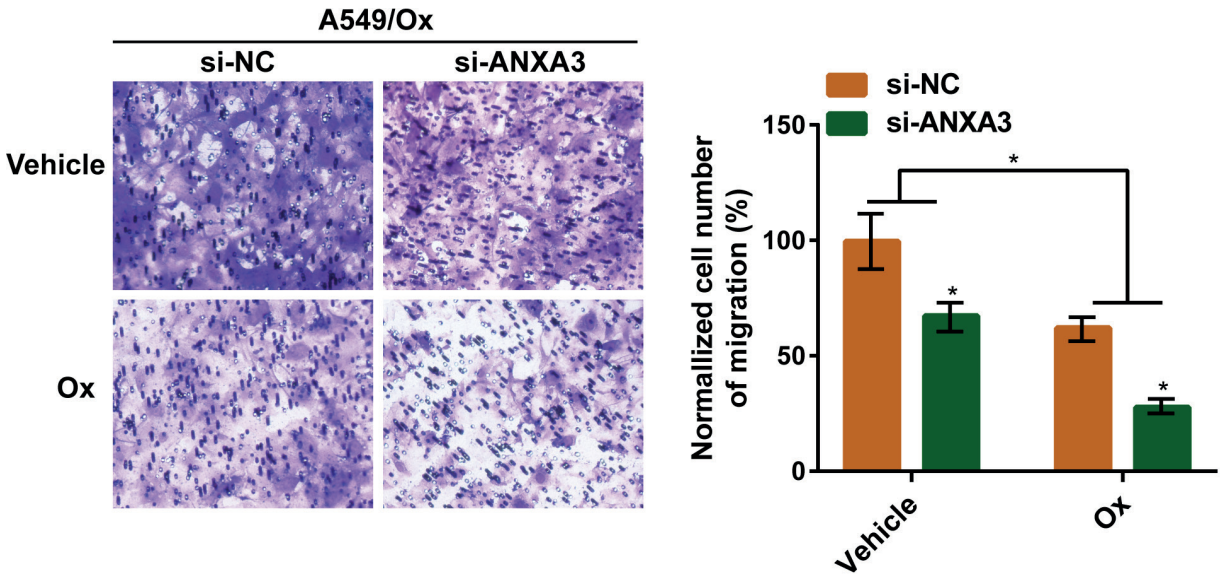

Figure 4. Low expression of ANXA3 inhibits migration of A549/Ox cells with or without Ox culture. Transwell assay shows that the cell migration ability is significantly decreased after knocking down ANXA3, and the Ox treatment group is significantly lower than the non-addition group (magnification: 40×).

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