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LncRNA MSTO2P promotes proliferation and autophagy of lung cancer cells by up-regulating EZH2 expression

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Abstract. – OBJECTIVE: This study was designed to investigate the specific mechanism underlying the regulatory effect of long non-coding ribonucleic acids (lncRNAs) MSTO2P on lung cancer (LCa) cell proliferation and autophagy via regulating enhancer of zeste homolog (EZH2) expression.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to analyze the levels of MSTO2P and EZH2 in 40 pairs of LCa tissues and corresponding adjacent tissues, as well as in LCa cell lines (H1299, H23, A549) and human bronchial epithelial cells (BEAS-2B). Besides, the effect of MSTO2P on cell proliferation ability was detected by cell counting kit-8 (CCK-8) and plate cloning experiments. The interaction between MTS02P and EZH2 as well as their effects on cell autophagy ability were examined by qRT-PCR and Western blot.

RESULTS: The qRT-PCR results showed that MSTO2P expression in LCa tissues was remarkably higher than that in adjacent tissues. Meanwhile, compared with human bronchial epithelial cells, the level of MSTO2P was remarkably up-regulated in LCa cells. After down-regulating MSTO2P, the cell proliferation ability was weakened, and the protein levels of autophagy-related genes including Agt5, LC-3I, and LC-3II were remarkably down-regulated. At the same time, EZH2 expression in LCa tissues was also remarkably up-regulated relative to adjacent tissues, and it was positively correlated with the expression of MSTO2P. In addition, after down-regulating MSTO2P, the EZH2 level was also remarkably reduced. Further experimental results revealed that EZH2 down-regulation could impair the cell proliferation ability and down-regulate the expressions of autophagy genes such as Agt5, LC-3I, and LC-3II.

CONCLUSIONS: LncRNA MSTO2P promotes LCa cell proliferation and autophagy by up-regulating EZH2. Therefore, MSTO2P may be a potential therapeutic target for LCa.

Key Words:
MSTO2P, EZH2, Lca, Cell proliferation, Autophagy.

Introduction

Lung cancer (LCa) is one of the most malignant tumors. In clinical practice, LCa is divided into non-small cell lung cancer and small cell lung cancer, of which the former accounts for about 80% and the latter accounts for about 20%[1]. Although the clinical treatment of LCa has made some progress, its prognosis is still not satisfactory. Clinical symptoms of early-stage LCa are not very obvious. By the time LCa is diagnosed in the advanced stage of LCa, the tumor cells have migrated and invaded, and there lacks effective treatment measures for advanced LCa, which is the main reason for the high mortality of patients with LCa[2]. Recent studies have shown that lncRNA is related to the proliferation, migration, and invasion of LCa cells, and it is expected to provide new targets for the therapy of LCa.

Cell autophagy is a series of conservative homeostasis in eukaryotic cell process. By forming autophagosomes or proteasomes, the damaged proteins and their organelles in the cells are phagocytized and degraded. If necessary, cell particles are recycled to produce the necessary protein materials, thereby ensuring the survival advantage of the cells under adverse conditions[3]. The interference of autophagy will lead to the occurrence of many acute and chronic diseases, such as tumors and neurodegenerative diseases[4]. In

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recent years, studies have shown that long non-coding ribonucleic acids (lncRNAs) have become a major research hotspot in the field of the tumor by regulating the occurrence, inhibition and chemotherapy-drug sensitivity of autophagy[5].

Only 20% of deoxyribonucleic acids (DNAs) in the human genome are used to encode proteins, while the remaining are transcribed into RNA, which could not be translated into proteins. Such RNA that does not encode any protein is called non-coding RNA. Among them, non-coding RNAs with length >200 nucleotides are named lncRNAs[6]. At present, lncRNA is considered as an important regulatory molecule in the human genome and plays a corresponding biological function. Studies have found that it not only mediates target recognition through base pairing, but also combines DNAs, RNAs, and proteins at the three-dimensional structure level to form a more complex regulatory network. It can be used as a signal molecule or a decoy molecule to participate in the regulation of gene expressions and transcription, and also as a guiding molecule or scaffold molecule, affecting the function and stability of protein molecules[7]. LncRNA is also involved in various important biological processes such as chromatin modification, gene expression regulation, transcriptional activation, transcriptional interference, and nuclear transport[8]. Researchers have found that lncRNA can regulate apoptosis, tumor migration and drug resistance in LCa[9]. As a promoter of gastric cancer, lncRNA MSTO2P has been gradually attached importance to[10,11], and studies have found that MSTO2P can affect the sensitivity of gastric cancer to chemotherapy by regulating autophagy[12]. However, the mechanism of MSTO2P in regulating the progression of LCa has not been reported. This work aims to further study the mechanism of its action in the development of LCa.

Patients and Methods

Sample Collection

From February 2017 to October 2018, 40 cases of LCa tissue specimens and corresponding adjacent ones from the Affiliated Hospital of Shandong University of Traditional Chinese Medicine were collected and stored in liquid nitrogen for use. All tissue samples were verified by pathology. Patients and their families signed informed consent, and this study was approved by the Affiliated Hospital of Shandong University of Traditional Chinese Medicine Ethics Committee.

Cell Culture

LCa cell lines (H1299, H23, A549) and human bronchial epithelial cells (BEAS-2B) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in complete medium (RPMI-1640; Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and incubated in a humidified incubator at 37°C with 5% CO_2.

Cell Transfection

MSTO2P siRNA and EZH2 siRNA used in this study and the corresponding negative controls were purchased from GenePharma (Shanghai, China). The cells used in the experiment (5×105 cells/well) were inoculated in a 6-well plate (Corning, Corning, NY, USA). After the cell density reached 80%, the transfection reagent was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and incubated at room temperature for 30 min. The culture dish was added and transfected for 24-48 h, and the transfection efficiency was detected by quantitative real-time polymerase chain reaction (qRT-PCR).

RNA Extraction and QRT-PCR

Purified total RNA was obtained from tissues or cells using a TRIzol (Invitrogen, Carlsbad, CA, USA) solution. The complementary deoxyribonucleic acid (cDNA) was obtained by reverse transcription (RT) in a 20 μL reaction system using a PrimeScript RT reagent Kit (TAKaRa, Tokyo, Japan) according to the manufacturer's instructions. QRT-PCR was carried out based on the following conditions: 10 min at 92°C, then 40 cycles of 10 seconds at 90°C and 1 min at 60°C. The primer sequences were as follows: MSTO2P (F: 5'-GTTCTGGATGGTTCCCCCAA-3'; R; 5'-TCTGCTACGTCATCGTGCTC-3'); EZH2 (F: 5'-AGAGGTACCG-GACGAAGA-ATAATCATGG-3'; R; 5'- TAGCTCGAGG-GTAGCAGATGTAAGG-3'); Agt5 (F: 5'-ATCGCTAATTAGATcGTCTGAA-3'; R; 5'-TGTCAGGGACTCCGATACGAGTG-3');LC-3I (F: 5'- GATGCGGTCGGATAAGTCCT-3'; R: 5'-GCTGAGCGGGTCATCATCCACA-3'); LC-3II

(F: 5'-CACACCCATCGCTGACATCTA-3'; R: 5'-CATCTCTCCTAATCCACCCG-3'); GAPDH (F: 5'-GGAATCCACTGGCGTCTTCA-3'; R: 5'- GGTTCACGCCCATCACAAAC -3')

Cell Counting Kit-8 (CCK-8) Assay

Cells were digested with 0.25% trypsin (Beyotime, Shanghai, China), and the cell suspension was harvested and seeded into 96-well plates with 6 replicates. Each well contains at least 2×103 cells and 200 μL of medium. Cells were allowed to adhere to growth overnight, and then the cell supernatant was washed with phosphate-buffered saline (PBS). A mixture containing 90 μL of pure Roswell Park Memorial Institute (RPMI-1640, Hyclone, South Logan, UT, USA) medium and 10 μL of CCK-8 solution (Beyotime, Shanghai, China) was added to each well. After 2 h of incubation, the absorbance of each well was read at 450 nm by a microplate reader.

Plate Cloning Experiment

After cell digestion with 0.25% trypsin, the cell suspension was collected and adjusted to a concentration of 1×10 3 /mL, and then seeded into 6-well plates with 3 replicates. Each well contained 100 μL of cell suspension and 2 mL of RPMI-1640 complete medium containing 10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA). The cells were continuously cultured for 2 weeks, and the medium was changed every 5 to 7 days. After 2 weeks, the cells were photographed and counted.

Western Blot

Total protein was extracted from transfected A549 and H23 cells. Target proteins were separated using a 10% concentration gradient polyacrylamide gel and then transferred the proteins to a 0.22 μm polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). All membranes were incubated for 2 h in blocking buffer (5% non-fat milk) and then incubated with primary antibodies (Invitrogen, Carlsbad, CA, USA) overnight at 4°C. After incubation with the corresponding secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature, these protein bands were visualized by enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA).

Statistical Analysis

Data analysis was performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA), and picture editing was performed using Graph-Pad Prism (Version X; La Jolla, CA, USA). Statistical analysis was performed using a Student's t-test to assess the difference in experimental data. Differences in $p<0.05$ were considered to be statistically significant.

Results

High Expression of MSTO2P in LCa Tissues and Cells

We found by qRT-PCR that MSTO2P expression was remarkably elevated in LCa tissues compared to adjacent tissues (Figure 1A). Meanwhile, MSTO2P was also remarkably increased in LCa cell lines (H1299, H23, A549) compared to human bronchial epithelial cells (BEAS-2B), and A549 and H23 cell lines were selected for subsequent experiments (Figure 1B). To further investigate the specific mechanism of MSTO2P in influencing LCa development, we transfected MSTO2P siRNA in A549 and H23 cells to knock down of MSTO2P, and qRT-PCR results showed a significant decrease in MSTO2P expression (Figure 1C and 1D). These results suggested that MSTO2P was highly expressed in LCa tissues and cells.

Down-regulation of MSTO2P Inhibits LCa Cell Proliferation and Autophagy

To further investigate the specific mechanism of MSTO2P in LCa progression, we silenced MSTO2P in LCa cells and detected the cell viability with CCK-8 assay. The data showed that the cell proliferation ability was remarkably inhibited after down-regulating MSTO2P expression in A549 and H23 cells (Figure 2A and 2B). We found the same results by plate cloning experiments (Figure 2C). At the same time, in order to evaluate its effect on autophagy of LCa cells, qRT-PCR was performed to detect the levels of autophagy-related genes. The results showed that after down-regulating the level of MSTO2, the expressions of autophagy-related genes including Agt5, LC-3I and LC-3II were remarkably reduced (Figure 2D and 2E). The same results were found at their protein levels by Western blot assay (Figure 2F). The above results showed that down-regulation of MSTO2P could inhibit LCa cell proliferation and autophagy abilities.

MSTO2P can Up-regulate EZH2 Level

Given the predominant role of EZH2 in tumors, we detected the expression of EZH2 in

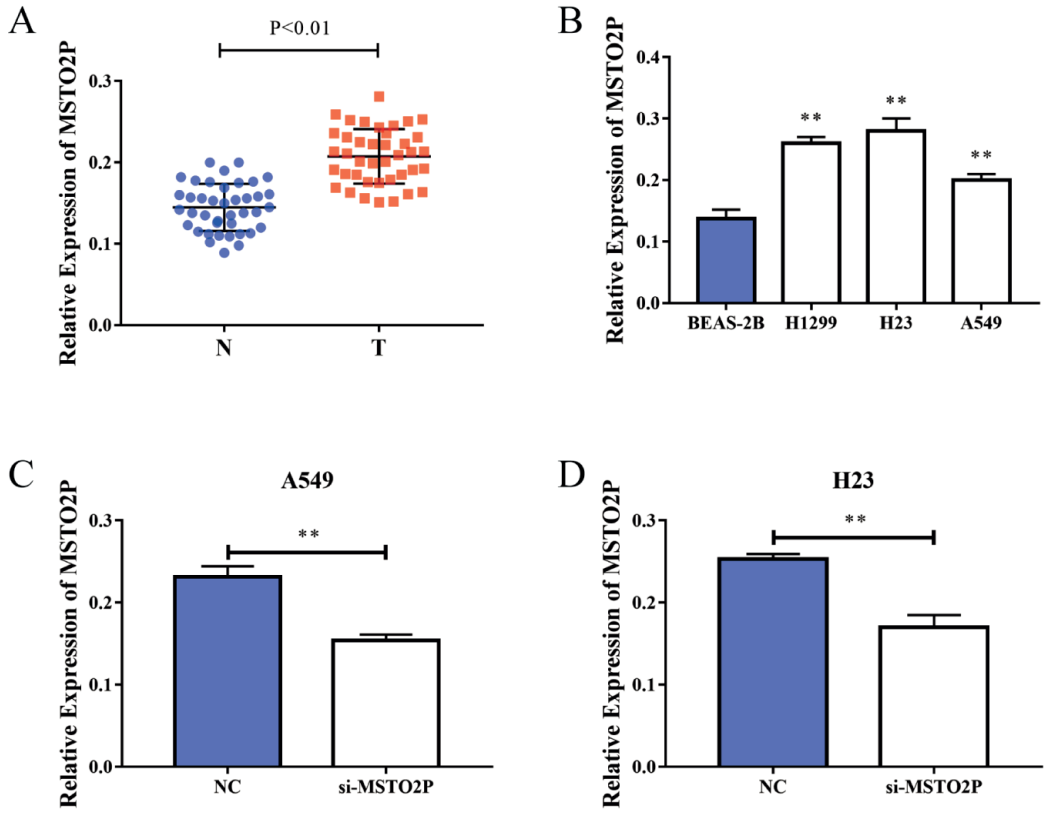

Figure 1. MSTO2P was highly expressed in lung cancer tissues and cells. ***A,*** qRT-PCR assay showed that MSTO2P expression was significantly up-regulated in lung cancer tissues compared with adjacent tissues; ***B,*** qRT-PCR assay showed that the expression level of MSTO2P significantly increased in lung cancer cell lines (H1299, H23, A549) compared with human bronchial epithelial cells (BEAS-2B); ***C,*** Transfection efficiency of si-MSTO2P in A549 cells; ***D,*** Transfection efficiency of si-MSTO2P in H23 cells. ($^{**}p<0.01$).

LCa and found that EZH2 expression was remarkably up-regulated in LCa tissues relative to adjacent tissues (Figure 3A). By using statistical tools, we found a positive correlation between EZH2 expression and MSTO2P expression in LCa tissues (Figure 3B). To further investigate their intermodulation relationship, EZH2 level was found to be remarkably down-regulated after silence of MSTO2P expression in A549 and H23 cells (Figure 3C). Therefore, we silenced EZH2 in LCa cells and detected the transfection efficiency by qRT-PCR and Western blot (Figure 3D and 3E). These above results showed that MSTO2P can positively regulate EZH2 expression in LCa cells.

Down-regulation of EZH2 Expression Can Inhibit LCa Cell Proliferation and Autophagy

To further study the mechanism of EZH2 in the development of LCa, CCK-8 assay was performed and we found that cell proliferation was remarkably attenuated after EZH2 knockdown in A549 and H23 cells (Figure 4A and 4B). The same results were obtained from the plate cloning experiment (Figure 4C). At the same time, in order to further study whether MSTO2P can affect the autophagy of LCa cells by regulating EZH2 expression, we used qRT-PCR to detect the levels of autophagy-related genes. The results showed that after down-regulating EZH2 expression, the mRNA and protein levels of autophagy-related genes including Agt5, LC-3I and LC -3II were remarkably reduced (Figure 4D-4F). The results indicated that down-regulation of EZH2 expression remarkably inhibited LCa cell proliferation and autophagy.

Discussion

LCa is one of the most common malignant tumors, and its mortality rate accounts for 28%

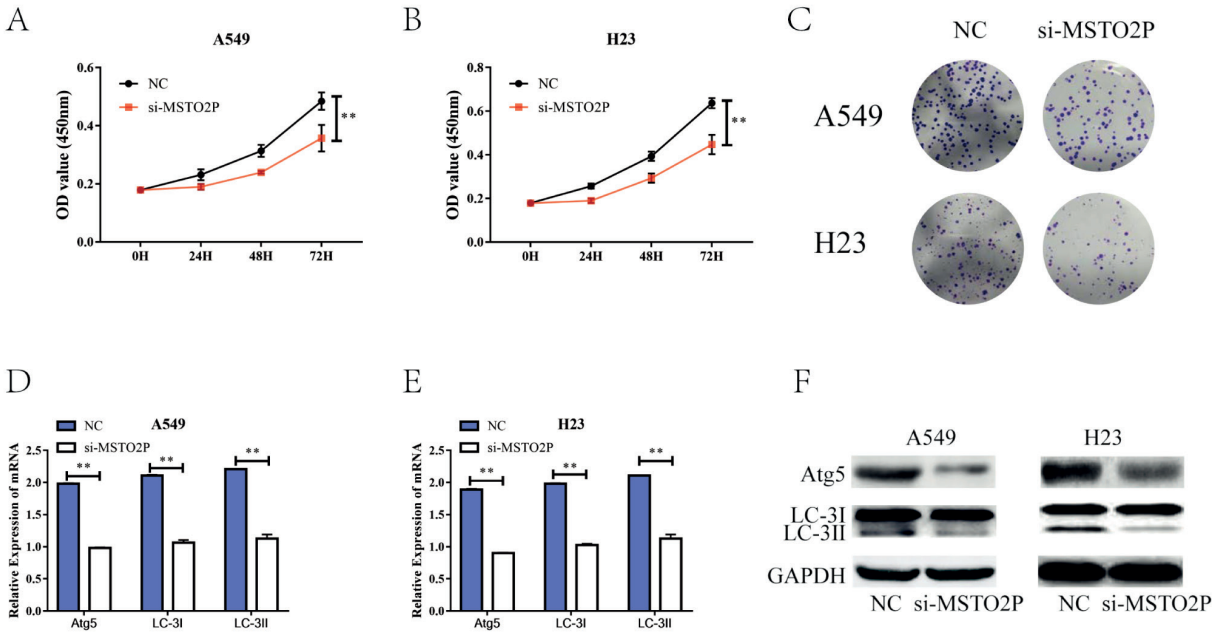

Figure 2. Down-regulation of MSTO2P inhibited cell proliferation and autophagy. ***A,*** The results of CCK-8 assay showed that the expression of MSTO2P was down-regulated in A549 cells, and the cell proliferation ability was weakened; ***B,*** Down-regulation of MSTO2P expression in H123 cells, cell proliferation ability decreased; ***C,*** The results of plate cloning experiments showed that the expression of MSTO2P was down-regulated in A549 and H23 cells, and the cell proliferation ability was weakened (magnification 20x); ***D,*** qRT-PCR analysis showed that the expression of MSTO2P was down-regulated in A549 cells, and the expression levels of autophagy-related genes Agt5, LC-3I and LC-3II significantly decreased; ***E,*** qRT-PCR detection showed that the expression of MSTO2P was down-regulated in H23 cells, and the expression levels of autophagy-related genes Agt5, LC-3I and LC-3II significantly decreased; ***F,*** Western blot analysis showed that the expression of MSTO2P was down-regulated in A549 and H23 cells, and the levels of autophagy-related genes Agt5, LC-3I and LC-3II decreased. ($^{**}p<0.01$).

of the global death rate of male tumor patients and 26% of the death rate of female tumor patients, which is the primary cause of worldwide tumor-related death[13]. In China, LCa has become the leading cause of death for male patients over 75 years old and female patients over 60 years old1. Among them, squamous and adenocarcinoma dominated non-small cell LCa account for more than 80% of the total LCa cases. Although surgical operation, radiotherapy and chemotherapy have been remarkably improved, the 5-year survival rate of LCa remains only 15%[14]. About 70% of patients with LCa present varying degrees of metastasis and local tumor recurrence after LCa resection. Genetic testing and screening of tumor molecular markers related to disease prognosis can better guide clinical medication, thus extending the survival time of patients15. With the development of molecular biology, scholars have found that in tumor tissues, in addition to specific gene locus changes and abnormal signal pathway activation, some special epigenetic traits will also be changed[16,17].

Zeste enhancer of Zeste homolog 2 (EZH2) is an important epigenetic regulatory gene with histone methyltransferase (HMT) activity, which can catalyze the H3K27me3 lysine of histone H3 to inhibit the expression of target genes[17]. EZH2 gene was first discovered in drosophila melanogaster, and is identified to be a key inhibitory regulator of drosophila melanogaster development gene[18]. With the deepening of researches, EZH2 gene is also present in human body, and the abnormal expression of EZH2 is related to the poor prognosis of solid tumors (including prostate cancer, breast cancer, kidney cancer and LCa)[19]. EZH2 gene is highly expressed in NSCLC, and the level is closely related to the prognosis of patients. Many investigators[20-24] also detected that the application of EZH2 inhibitor can effecti-

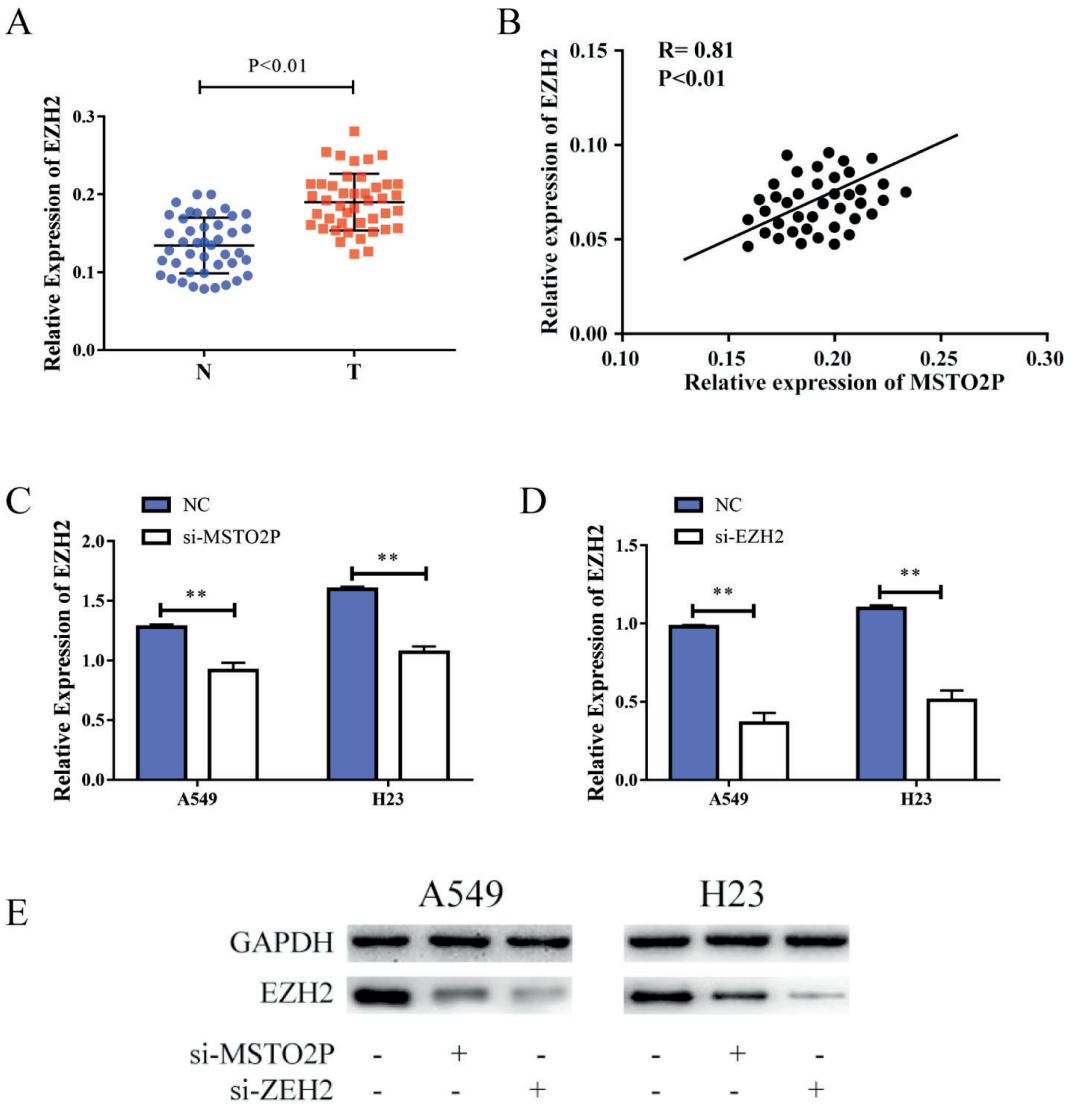

Figure 3. MSTO2P could up-regulate EZH2 expression. ***A,*** qRT-PCR analysis showed that EZH2 expression was significantly up-regulated in lung cancer tissues relative to adjacent tissues; ***B,*** There was a positive correlation between the expression level of EZH2 and the expression level of MSTO2P in lung cancer tissues; ***C,*** qRT-PCR assay showed that MSTO2P expression was down-regulated and EZH2 expression was inhibited; ***D,*** Transfection efficiency of si-EZH2 in A549 and H23 cells; ***E,*** Western blot analysis showed that EZH2 protein levels decreased after down-regulation of MSTO2P expression and transfection of si-EZH2 in A549 and H23 cells. ($^{**}p<0.01$).

vely reduce the invasion and migration ability of NSCLC and induce tumor cell apoptosis, indicating that EZH2 inhibitor has a good application prospect and clinical value.

In this report, we found that the level of MSTO2P in LCa tissues was remarkably higher than that in para cancer tissues. At the same time, MSTO2P level in LCa cell lines was also remarkably higher than that in human bronchial epithelial cells. After MSTO2P was down-regulated, the cell proliferation ability was weakened, and the protein levels of the autophagy related genes such as Agt5, LC-3I and LC -3II were remarkably reduced. Further study revealed that EZH2 expression was also remarkably up-regulated in LCa tissues compared with paracancererous tissues, and was positively correlated with MSTO2P expression. At the same time, when MSTO2P expression was knocked down in A549 and H23 cells, EZH2 level was remar-

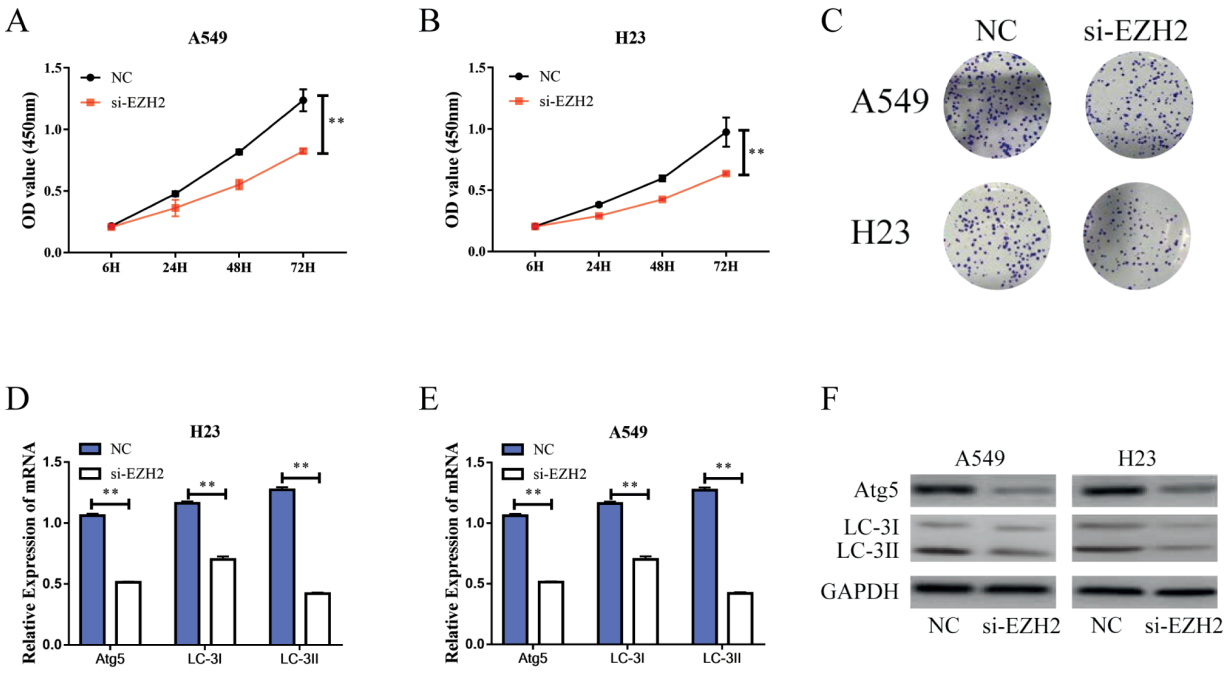

Figure 4. Down-regulation of EZH2 inhibited cell proliferation and autophagy. ***A,*** The results of CCK-8 assay showed that EZH2 expression was down-regulated in A549 cells, and cell proliferation ability was weakened; ***B,*** Down-regulation of EZH2 expression in H123 cells, cell proliferation ability decreased; ***C,*** The results of plate cloning experiments showed that EZH2 expression was down-regulated in A549 and H23 cells, and cell proliferation ability was weakened (magnification 20x); ***D,*** The qRT-PCR assay showed that the expression of EZH2 was down-regulated in A549 cells, and the expression levels of autophagy-related genes Agt5, LC-3I and LC-3II significantly decreased; ***E,*** The qRT-PCR assay showed that the expression of EZH2 was down-regulated in H23 cells, and the expression levels of autophagy-related genes Agt5, LC-3I and LC-3II significantly decreased; ***F,*** Western blot analysis showed that EZH2 expression was down-regulated in A549 and H23 cells, and the levels of autophagy-related genes Agt5, LC-3I and LC-3II decreased. ($^{**}p<0.01$)

kably reduced. Further researches found that cell proliferation was also remarkably inhibited after EZH2 down-regulation, and the protein levels of autophagy related genes including Agt5, LC-3I and LC -3II were remarkably suppressed. Therefore, we concluded that MSTO2P may promote the proliferation and autophagy of LCa cells through up-regulation of EZH2 expression.

Conclusions

We found that MSTO2P was involved in the development of LCa by up-regulating EZH2 expression, thereby promoting cell proliferation and autophagy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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