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LncRNA MEG3 promotes the sensitivity of vincristine by inhibiting autophagy in lung cancer chemotherapy

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Abstract. – OBJECTIVE: Lung cancer is one of the most common malignancies worldwide, the morbidity and mortality of which have been on rising in recent years. Moreover, lncRNAs have been implicated in the development of various cancers, as well as cancer treatment and prognosis. In this study, long non-coding RNA (lncRNA) MEG3, an identified tumor suppressor, was explored for its role in the chemotherapy of lung cancer.

MATERIALS AND METHODS: All cases were divided into (I+II) group and (III+IV) group according to different stages of tumor node metastasis (TNM), and were divided into sensitive group and insensitive group according to chemotherapy sensitivity. A549 and H292 cells were selected as the resistant cell and non-resistant lung cancer cells. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to detect the expression of MEG3. After transfection with overexpression plasmid pcDNA-MEG3 or/and different concentrations of vincristine, cell viability and proliferation were measured by cell counting kit-8 (CCK-8) assay and plate cloning assay, respectively. Western blotting was used to analyze the expressions of autophagy-related proteins.

RESULTS: *In vivo*, lncRNA MEG3 was significantly lower in III+IV group and insensitive group than that in I+II group and sensitive group. *In vitro*, MEG3 expression in resistant cells was significantly lower than that in non-resistant cells. Overexpression of MEG3 significant inhibited the viability and proliferation of both resistant and non-resistant lung cancer cells. Western blot results showed that autophagy level was higher in resistant cells than that in non-resistant cells, while overexpression of MEG3 significantly reduced the expression of autophagy-related proteins. CCK-8 results also indicated that the cell viability was negatively correlated with the dose of vincristine, while the viability of drug-resistant cells was higher than that of non-drug resistant cells after the treatment of vincristine. The vitality of both cells decreased in a concentration-dependent manner after combined treatment with vincristine and MEG3.

CONCLUSIONS: Our data indicated that lncRNA MEG3 showed a low expression in chemotherapy-sensitive lung cancer tissues, and overexpression of lncRNA MEG3 attenuated autophagy level, thus increasing the sensitivity of vincristine in chemotherapy of lung cancer.

Key Words:
Lung cancer, LncRNA MEG3, Vincristine, Drug resistance, Autophagy.

Introduction

Lung cancer is one of the most common malignancies with the highest increase in morbidity and mortality worldwide, which has become the greatest threat to human health and life. In China, the incidence of lung cancer increased at 1.63% per-year from 1991 to 2005[1]. Although many drugs have been developed to treatable lung cancer, patient's survival rate has not improved significantly. Besides, chemotherapy is palliative and mainly focused on extending the patient's survival and improving his quality of life. Therefore, it is very important to explore and clarify the mechanisms underlying chemotherapeutic drugs in lung cancer to improve the therapeutic effect of these clinical drugs.

Long non-coding RNA (lncRNA) was first found in mouse DNA transcripts by Okazaki et al[2]. LncRNAs are a type of transcripts longer than 200 nucleotides and lacking open reading frames. Numerous studies have shown that lncRNAs are involved in many biological processes such as chromosome silencing, genomic imprinting, chromatin modification and transcriptional activator interference[3]. In addition, aberrant expres-

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sion of lncRNA leads to tumor cell resistance, which is a huge challenge in cancer treatment[4]. Maternally expressed gene 3 (MEG3) has been reported to be expressed in many normal cells and tissues; loss of MEG3 was observed in a variety of tumors. Thus, MEG3 is often considered as a tumor suppressor. However, the relationship between MEG3 and drug resistance of lung cancer cells remains unclear.

Vincristine is an alkaloid extracted from the *Apocynaceae vinca*. Vincristine has been used as a broad-spectrum anti-cancer drug for more than 40 years for its good antitumor effect[5]. Although it is involved in various types of malignant tumor chemotherapy regimens, its efficiency varies greatly among different tumors, but tumor cells will gradually show resistance and curative effect will be greatly reduced after a period of usage[6]. Thus, research on the anti-tumor mechanisms of vincristine is particularly important.

As a highly conserved biological process in cells, autophagy is involved in the cellular energy metabolism, self-renewal and many other biological events[7,8]. Previous studies have revealed that autophagy inhibits the early stages of tumor formation by clearing harmful unfolded protein, dissolving damaged mitochondria and limiting inflammation reaction. Of note, some studies have also shown that autophagy may play a role in promoting tumor growth by counteracting the adverse microenvironment.

Materials and Methods

LncRNA MEG3 Expression Analysis

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to analyze the expressions of MEG3 in different stages of lung cancer patients and chemotherapy-sensitive and non-sensitive lung cancer patients, as well as the expressions of MEG3 in A549 and H292 cells treated with or without vincristine. This investigation was approved by the Ethics Committee of the First Hospital of Jilin University. Signed written informed consents were obtained from all participants before the study.

Cell Culture

A549 and H292 cell lines were cultured in 1640 medium (with 1% penicillin streptomycin) containing 10% fetal bovine serum (FBS), Gibco (Rockville, MD, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2. For plasmids transfection, cells were seeded in 6-well plates. 8 μL of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and 4 μg of pcDNA-lncrna MEG3 were dissolved in 500 μL of 1640 medium (Gibco, Rockville, MD, USA), which were added to each well when the density of adherent cells reached nearly 80%. The control group was added with the isodose Lipofectamine 2000 and pcDNA-NC as the experimental group. The medium was changed after 6 h-transfection.

Cell Counting Kit-8 (CCK8) Assay

Cells in logarithmic growth phase were digested with 0.25% trypsin and cell suspensions were harvested and seeded into 96-well plates, with 10 wells for each group. Each well contained at least 2×10^3 cells and 200 μL of culture medium. Incubation overnight allowed the cells to grow adherently, then the cell supernatant was washed with phosphate-buffered saline (PBS). A mixture containing 90 μL of pure Dulbecco's Modified Eagle Medium (DMEM) and 10 μL of CCK-8 solution (Beyotime Biotechnology, Shanghai, China) was added to each well. After incubation for 2 h, the absorbance (A) value of each well was read by microplate reader at 450 nm.

Plate Cloning Assay

After digestion with 0.25% trypsin, cell suspension was collected and adjusted to a concentration of 1×10^3/mL, and then seeded into 12-well plates with 4 wells per group. Each well contained at least 100 μL of cell suspension and 2 mL of 10% Dulbecco's modified eagle medium (DMEM) complete medium. 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 Blotting Analysis

Total protein was extracted after A549 and H292 cells were treated. We used 10% polyacrylamide gradient gels to separate our target proteins and these proteins were then transferred to 0.22 μm polyvinylidene difluoride (PVDF) membranes. All the membranes were incubated in blocking buffer (5% fat-free milk) before incubation with primary antibodies (Invitrogen, Carlsbad, CA, USA) at 4°C overnight. After incubation with the corresponding secondary antibody (Invitrogen, Carlsbad, CA, USA), these protein bands were subjected to enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA) and then imaged.

Statistical Analysis

SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis, GraphPad Prism (Version X; La Jolla, CA, USA) was used for picture editing. Statistical analysis was performed using *t*-test to evaluate the significance of the experimental data. The data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$ and (***) for $p < 0.001$.

Results

Different Expressions of lncRNA MEG3 Are Observed in Different Lung Cancer Tissues

QRT-PCR results showed that the expression of MEG3 was markedly lower in the lungs of patients with stage III+IV lung cancer than that in stage I+II lung cancer (Figure 1A), and was also significantly lower in chemotherapy-insensitive group than that in non-sensitivity group (Figure 1B).

MEG3 Inhibits Lung Cancer Cell Viability and Proliferation

We next detected the expression of MEG3 in H292 and A549 cells transfected with or without pcDNA-MEG3 by qRT-PCR. MEG3 showed a low level of expression in control cells and a significantly enhanced expression in cells transfected with high expression plasmids, indicating that our plasmid was successfully constructed and the transfection efficiency was satisfied (Figure 2A-C). In addition, overexpression of MEG3 significantly reduced the viability and proliferation ability of H292 and A549 cells (Figure 2D-G), suggesting that MEG3 may regulate lung cancer development by affecting tumor cells' viability and proliferation,

Vincristine Has Tumor Suppressor Effect and the Drug Resistance is Negatively Correlated With the Expression of MEG3

To determine the effect of vincristine on different lung cancer cells, CCK-8 assay was performed and the results suggested that both cells exhibited a marked decrease with the increase of vincristine concentration, while the vitality of drug-resistant cells was stronger than that of non-resistant cells (Figure 3A, C). Besides, qRT-PCR results revealed that MEG3 expression was significantly lower in drug-resistant cells than that in non-drug resistant cells (Figure 3B, D). The above results indicated that vincristine has a different suppressed effect on different lung cancer cells and the drug resistance of lung cancer cells to vincristine might have a negative correlation with the MEG3 level.

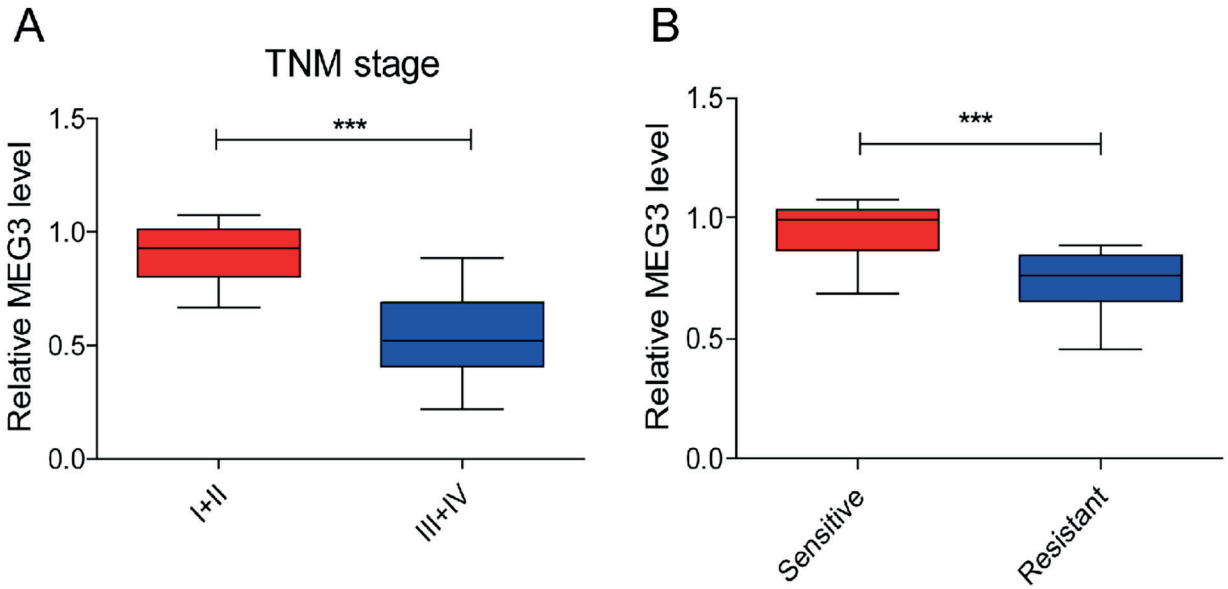

Figure 1. MEG3 is poorly expressed in lung cancer. ***A,*** MEG3 expressions in stage III+IV lung cancer tissues were lower than those in stage I+II. ***B,*** MEG3 was less expressed in tissues that were less chemosensitive than those in chemosensitive tissues.

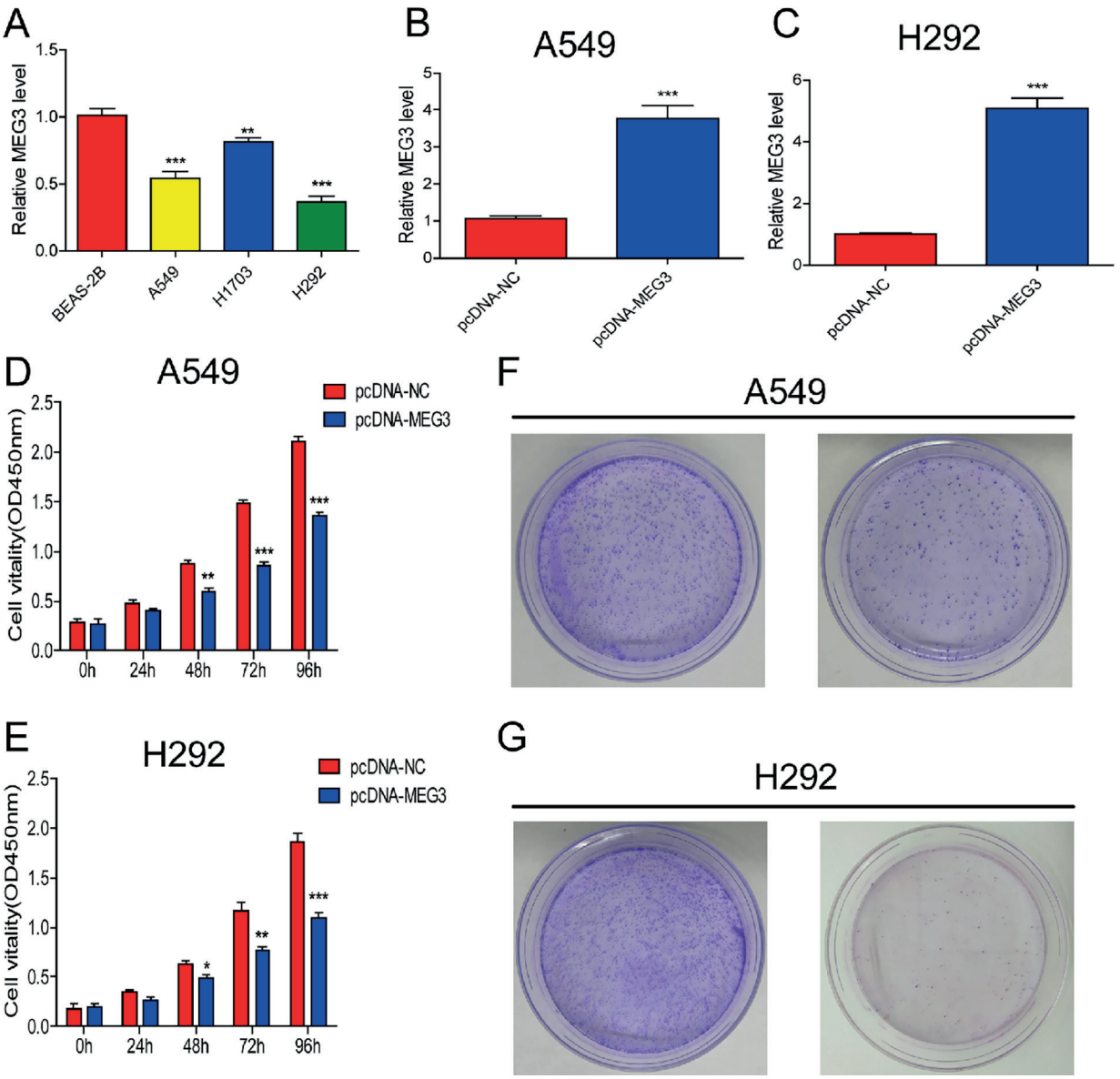

Figure 2. MEG3 inhibits lung cancer cell viability and proliferation. ***A,*** MEG3 level was detected in normal lung cell line BEAS-2B and lung cancer cell lines A549, H1703 and H292 by qRT-PCR. ***B-C,*** MEG3 expression was significantly increased in A549 and H292 cells after transfection of pcDNA-MEG3. ***D-E,*** Transfection of pcDNA-MEG3 resulted in a significant decrease in viability of A549 and H292 cells compared to cells transfected with pcDNA-NC. ***F-G,*** Transfection of pcDNA-MEG3 resulted in a significant decrease in cell proliferation capacity of A549 and H292 cells compared to cells transfected with pcDNA-NC.

MEG3 Can Inhibit the Formation of Autophagy In Lung Cancer Cells

Compared with non-sensitive tumor cells, the expressions of autophagy proteins were significantly increased in vincristine-sensitive cells (Figure 4A). However, autophagy levels were significantly down-regulated in both A549 and H292 cells after transfected with pcDNA-MEG3 (Figure 4B). These results suggested that autophagy is involved in the development of lung cancer, whereas MEG3 might partially regulate tumor cells by inhibiting autophagy level.

MEG3 and Vincristine Enhance the Effect on Lung Cancer Cells

In view of the above results, we further used vincristine and pcDNA-MEG3 to co-treat both A549 and H292 cells to examine whether MEG3 can potentiate the therapeutic effect of vincristine on lung cancer (Figure 4C, D). Our find-

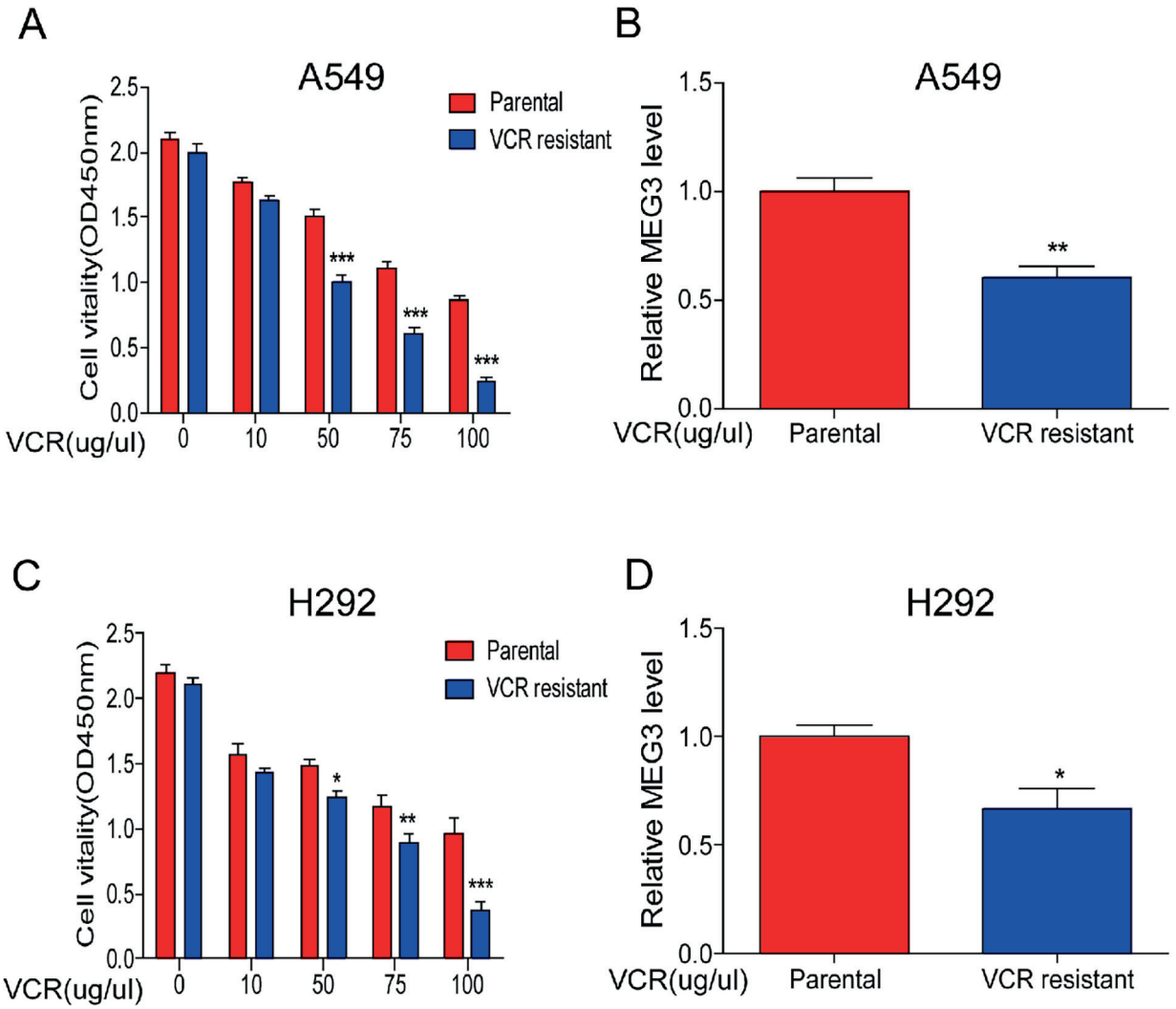

Figure 3. The expression of MEG3 is negatively correlated with the sensitivity of vincristine. ***A,*** After the treatment of different concentrations of vincristine, both A549 cells and drug-resistant A549 cells showed decreased viability with the increased concentration of vincristine, and the viability of the resistant A549 cells was higher than that of the non-drug resistant cells. ***B,*** MEG3 expression in resistant A549 cells was significantly lower than that in non-resistant cells. ***C,*** After the treatment of different concentrations of vincristine, both H292 cells and drug-resistant H292 cells showed decreased viability with the increase concentration of vincristine, and the viability of the resistant H292 cells was higher than that of the non-drug resistant cells. ***D,*** MEG3 expression in resistant H292 cells was significantly lower than that in non-resistant cells.

ingsshowed that after different concentrations of vincristine treatment combined with overexpression of MEG3, the activities of both resistance and non-resistant cells were significantly decreased with a concentration-dependent manner.

Discussion

Lung cancer is one of the most serious cancer diseases in the world, and its morbidity and mortality have been significantly increased during the last decades[9]. With the rapid development of molecular biology, molecular targeted therapy of various cancers has attracted more and more researchers' attention due to its significant advantages. However, the emergence of drug resistance in tumor cells has gradually become one of the important problems that hinder this treatment. Although researches on the mechanisms of tumor drug resistance have been greatly progressed, our understanding of the role of lncRNAs in this process is still quite limited. So far, more than 1,000 lncRNAs have been identified[10]. LncRNAs are very conservative in the evolutionary process, indicating that

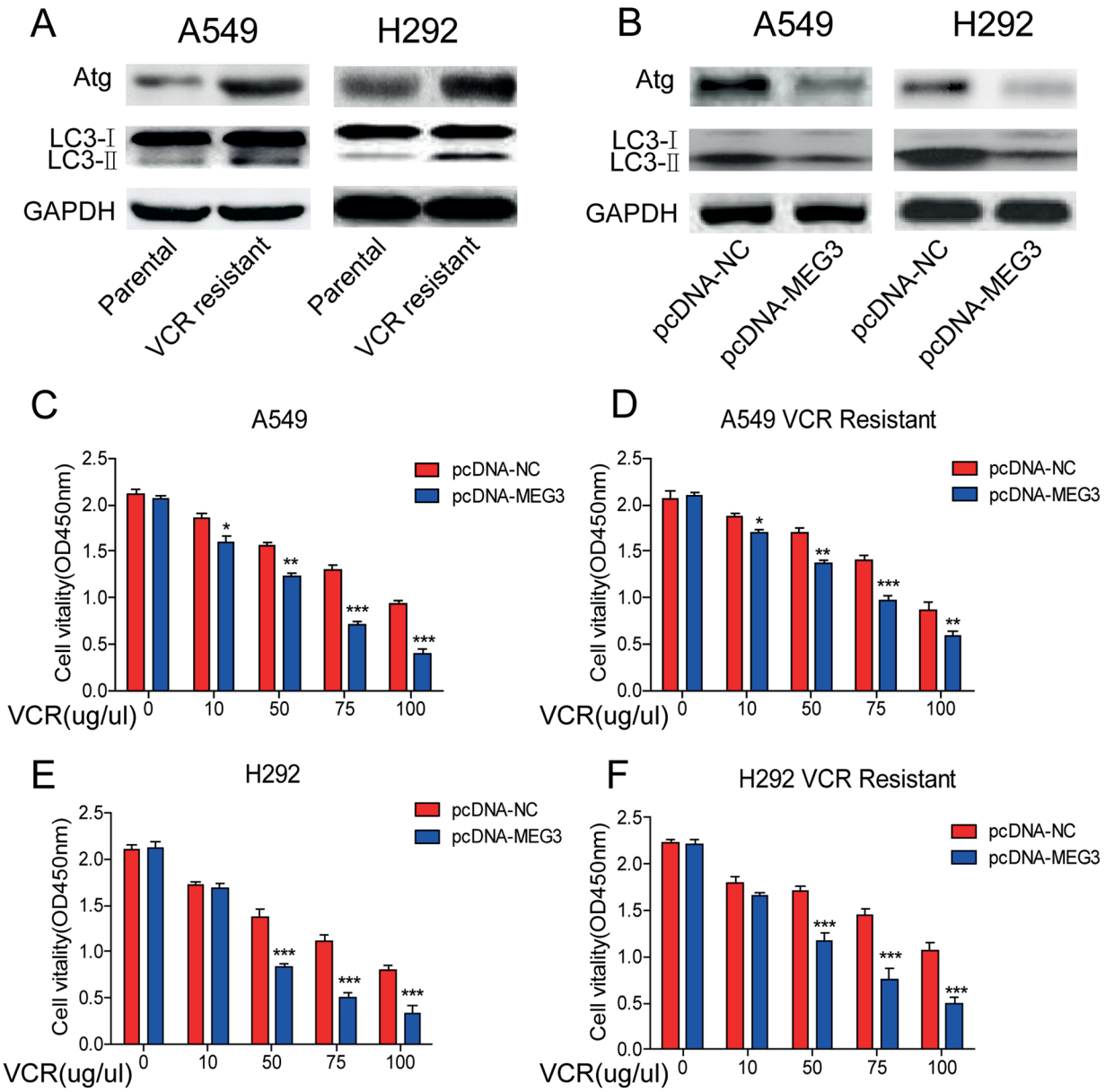

Figure 4. MEG3 inhibits autophagy level in cancer cells and decreases the viability of cells after vincristine treatment. ***A,*** The protein expressions of Atg, LC3-I and LC3-II were significantly increased in vincristine-resistant A549 and H292 cells. ***B,*** Transfection of pcDNA-MEG3 in A549 and H292 cells significantly decreased the expressions of Atg, LC3-I and LC3-II. ***C-D,*** A549 and resistant A549 cells were treated with pcDNA-MEG3 or pcDNA-NC combined with different concentrations of vincristine, and the viability of pcDNA-MEG3 transfected cells was significantly decreased than that of cells transfected with pcDNA-NC. ***E,*** H292 and resistant H292 cells were treated with pcDNA-MEG3 or pcDNA-NC combined with different concentrations of vincristine, and the viability of pcDNA-MEG3 transfected cells was significantly decreased than that of cells transfected with pcDNA-NC.

they may play an important role in multiple biological processes[11]. Abnormal lncRNA expressions have been shown to exert close relationship with the development, treatment, and prognosis of tumors in many studies[12]. MEG3 is a potential tumor suppressor expressed in many normal tissues of human internal organs. The loss or decrease of MEG3 expression was detected in many tumors, including meningioma, colon cancer, nasopharyngeal carcinoma and leukemia[13]. We observed a down-regulation of MEG3 expression in drug-resistant tissues and

cells of lung cancer. Enhanced expression of MEG3 inhibited viability and proliferation of lung cancer cells, and promoted the inhibitory effect of vincristine on tumors, which further explained the suppressive effect of MEG3 in lung cancer. Consistently, Xia et al[14] reported that knocking out of MEG3 activates the Wnt/β-catenin pathway, indicating that down-regulation of MEG3 expression increases the resistance of lung adenocarcinoma cells to cisplatin, which also supported the results in this work from the mechanism level.

Induction of tumor cell apoptosis is the main route of chemotherapeutic drugs killing tumor cells[15]. However, accumulated evidence indicated that chemotherapeutic drugs can also induce autophagy activation in many tumor cells except apoptosis initiation[16]. This work also found that autophagy-related proteins showed higher expression in drug-resistant cells than that in non-resistant cells, consistently with previous studies. Han et al[17] observed that gefitinib and erlotinib can induce a high level of autophagy in drug-resistant cells expressing wild-type epidermal growth factor in non-small cell lung cancer, but not in sensitive cells. Our results also showed a decreased autophagy level in A549 and H292 cells by up-regulating MEG3 expression. This investigation provided a new target for the development of therapeutic drugs targeting autophagy-related diseases and suggested new ideas and approaches for the prevention and treatment of diseases. The role of lncRNAs as new class of disease diagnostic markers and therapeutic target molecules will become increasingly prominent.

Conclusions

Our data suggested that MEG3 enhanced the sensitivity of vincristine to lung cancer by inhibiting autophagy in tumor cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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