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MAGI1-IT1 stimulates proliferation in non-small cell lung cancer by upregulating AKT1 as a ceRNA

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Abstract. – OBJECTIVE: This study aims to illustrate the potential role of MAGI1-IT1 in the progression of non-small cell lung cancer (NSCLC) and the underlying mechanism.

PATIENTS AND METHODS: The relative level of MAGI1-IT1 in normal lung tissues and NSCLC tissues was determined. Its level in NSCLC patients with different tumor sizes (<5 cm or >5 cm), metastatic statues (positive or negative), and tumor staging (stage I+II or stage III+IV) was detected as well. The prognostic potential of MAGI1-IT1 in evaluating the overall survival (OS) and progression-free survival (PFS) of NSCLC patients was assessed by the Kaplan-Meier method. In A549 and PC-9 cells, the regulatory effect of MAGI1-IT1 on the proliferative ability was examined by the cell counting kit-8 (CCK-8), colony formation, and 5-Ethynyl-2'-deoxyuridine (EdU) assay. The target miRNA of MAGI1-IT1 and the target gene binding to miRNA-512-3p were predicted using the Diana database. The interactions among MAGI1-IT1/miRNA-512-3p/AKT1 regulatory loop were tested by the Dual-luciferase reporter gene assay and RNA immunoprecipitation (RIP) assay. At last, the rescue experiments were carried out to uncover the regulatory effect of MAGI1-IT1/AKT1 axis on NSCLC progression.

RESULTS: MAGI1-IT1 was upregulated in NSCLC tissues. Its level was higher in NSCLC patients with larger tumor size, positive metastasis, or advanced stage. High level of MAGI1-IT1 predicted worse OS and PFS in NSCLC patients. The knockdown of MAGI1-IT1 remarkably attenuated the proliferative ability in A549 and PC-9 cells. MAGI1-IT1 could target miRNA-512-3p, and AKT1 was the target gene of miR-512-3p. The overexpression of AKT1 stimulated lung cancer cells to proliferate. Of note, the elevated proliferative rate in lung cancer cells overexpressing AKT1 was reversed by the silence of MAGI1-IT1.

CONCLUSIONS: MAGI1-IT1 is upregulated in NSCLC tissues and cell lines, and predicts a poor prognosis in NSCLC patients. MAGI1-IT1 stimulates proliferative ability in NSCLC by upregulating the AKT1 level by binding to miRNA-512-3p.

Key Words:
NSCLC, MAGI1-IT1, MiRNA-512-3p, AKT1.

Introduction

Lung cancer is a common malignant tumor in clinical practice. It is characterized as high malignancy and rapid development of the disease. The morbidity and mortality of lung cancer have risen in recent years, and it seriously affects the physical and mental health[1]. According to clinical data, non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer cases. The symptoms of early-stage NSCLC are atypical and concealed, leading to a low detective rate of early-stage and localized NSCLC. The 5-year survival of NSCLC is only about 7%[2]. The gene regulation is significant in the tumorigenesis of NSCLC. It is urgent to uncover the molecular mechanisms underlying the progression and metastasis of NSCLC.

As vital regulators, the discovery of long non-coding RNAs (lncRNAs) provides new directions in tumor research. LncRNAs are non-coding RNAs with over 200 nt in transcription length. They are closely linked to every aspect of the tumor cell behavior, serving as diagnostic, therapeutic, and prognostic hallmarks for tumors[3-5]. Functionally, lncRNAs are classified into oncogenes and tumor-suppressor genes[6,7]. Plenty of abnormally expressed lncRNAs in NS-

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CLC have been discovered, and they are involved in the pathological processes of NSCLC[8-10]. These lncRNAs are required for further exploration of their specific functions in influencing the progression of NSCLC.

LncRNAs exert their biological functions by interacting with the corresponding miRNAs[11,12]. A novel theory proposed that lncRNAs sponge miRNAs to further regulate the protein-encoding gene expressions through the transcription inhibition of the target RNAs, that is, ceRNA hypothesis[13,14]. Xu et al[15] demonstrated that lncRNA XIST sponges miR-374a to upregulate LARP1 level, thus stimulating NSCLC to proliferate, invade, and migrate. Wang et al[16] suggested that PVT1 is upregulated in hypoxic NSCLC cells. PVT1 upregulates HIF-1α via sponging miR-199a-5p, thereafter aggravating the hypoxia-induced damage in NSCLC. LncRNA MAGI1-IT1 is a newly identified lncRNA to be upregulated in epithelial ovarian cancer. In this paper, we mainly discussed the potential function of MAGI1-IT1 in the progression of NSCLC and the possible mechanism. Our findings may provide a new therapeutic target for the clinical treatment of NSCLC.

Patients and Methods

Clinical Samples

NSCLC and paracancerous tissues were surgically resected in Tumor Hospital, Peking University and immediately preserved in liquid nitrogen. Patients and their families in this study have been fully informed. This study was approved by the Ethics Committee of the Tumor Hospital, Peking University.

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

The cells were lysed to harvest RNAs using TRIzol method (Invitrogen, Carlsbad, CA, USA), and the extracted RNAs were subjected to reverse transcription according to the instructions of the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using a spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The relative level was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed as follows: MGAI1-IT1 (forward): TGATGCTGCTGATCTGGTCT; MGAI1-IT1 (reverse): GCCAAGTCTCTGCTCGTACC; AKT1 (forward): 5'-GCACCT-TCCATGTGGAGACT-3'; AKT1 (reverse):5'-GGGACACCTCCATCTCTTCA-3'; miRNA-512-3p (forward): 5'-TCGAGGATCCACCTTTCACTATTGAGCAACA-3'; miRNA-512-3p (reverse): 5'-TCGAGCTAGCTGAAGTCCAGTTTATGGCGCA-3'.

Cell Culture and Transfection

The human normal bronchial epithelial cell line (H1650) and lung cancer cell lines (H1975, PC-9, and A549) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. The fresh medium was replaced every 2-3 days and the cell passage was performed at 80-90% confluence. The transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfected cells for 24-48 h were harvested for the following experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells were seeded into 96-well plates with 3.0×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

The cells were seeded in the 6-well plate with 500 cells per well and cultured for one week. Subsequently, the cells were subjected to 1 h fixation in methanol and 20 min staining in 0.1% violet crystal. After removing the staining solution, the colonies were air dried and observed under a microscope.

Dual-Luciferase Reporter Gene Assay

The Luciferase vectors were constructed according to the binding sites in the promoter region. The cells were co-transfected with 50 pmol/L miRNA-512-3p mimics/NC and 80 ng wild-type/mutant-type plasmids, respectively. At 48 h, the cells were lysed to determine the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The cells were fixed in Phosphate-Buffered Saline (PBS) containing 3.7% formaldehyde. The rupture of the cell membranes was conducted using PBS containing 0.5% Triton X-100. Afterwards, the cells were labeled with EdU solution in the dark for 30 min and stained with Hoechst 33342 for other 30 min. The images of EdU-labeled and Hoechst-labeled cells were taken under fluorescence microscopy (magnification 40×).

RIP (RNA Immunoprecipitation)

The cells were lysed and 500 μg extraction was incubated with anti-IgG or anti-AGO2 at 4°C overnight. Meanwhile, 10 μg extraction was utilized as input. A protein-RNA complex was obtained when the intracellular specific proteins were captured by the antibody. Subsequently, the proteins were digested by proteinase K and the RNA molecules were extracted. During the experiment, the magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. The immunoprecipitant RNA was finally subjected to qRT-PCR to determine the relative level.

Western Blot

The total protein was extracted from the cells using radioimmunoprecipitation assay (RIPA) and quantified by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis. After being transferred on a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies at room temperature for 2 h. the bands were exposed by enhanced chemiluminescence (ECL) and analyzed by the Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (La Jolla, CA, USA) were used for data analysis. The data were expressed as mean ± standard deviation ($\bar{x} \pm$ SD). The intergroup data were compared using the *t*-test. The Kaplan-Meier method was introduced for the survival analysis, followed by the log-rank test to compare the differences between the two curves. $p<0.05$ considered the difference was statistically significant.

Results

MAGI1-IT1 Was Upregulated in NSCLC and Predicted a Poor Prognosis

Compared with paracancerous tissues, MAGI1-IT1 was upregulated in NSCLC tissues (Figure 1A). According to the collected pathological data, the MAGI1-IT1 levels in NSCLC patients with different tumor sizes (<5 cm or >5 cm), metastatic statues (positive or negative), and tumor staging (stage I+II or stage III+IV) were detected as well. MAGI1-IT1 level was higher in NSCLC patients with larger tumor size (Figure 1B), positive metastasis (Figure 1C), and advanced stage (Figure 1D). Moreover, the Kaplan-Meier method revealed a worse OS (Figure 1E) and PFS (Figure 1F) in NSCLC patients expressing a high level of MAGI1-IT1. Therefore, MAGI1-IT1 exerted a crucial role in NSCLC.

Knockdown of MAGI1-IT1 Suppressed Proliferative Ability in NSCLC

In vitro level of MAGI1-IT1 was identically upregulated in lung cancer cells relative to normal bronchial epithelial cells (Figure 2A). PC-9 and A549 cells expressed the highest abundance of MAGI1-IT1 among the three tested lung cancer cell lines, and were utilized for the following experiments. To further elucidate the role of MAGI1-IT1 in NSCLC, three MAGI1-IT1 shRNAs were constructed. The transfection efficacy of sh-MAGI1-IT11# and sh-MAGI1-IT12# were much pronounced, while sh-MAGI1-IT13# failed to remarkably downregulate the MAGI1-IT1 level in PC-9 and A549 cells (Figure 2B). CCK-8 assay revealed a viability decline in A549 and PC-9 cells transfected with sh-MAGI1-IT11# or sh-MAGI1-IT12# than those of the controls (Figures 2C and 2D). In the colony formation assay, the clonality was markedly attenuated after the knockdown of MAGI1-IT1 (Figure 2E). Moreover, the EdU-positive ratio decreased in lung cancer cells with MAGI1-IT1 knockdown (Figure 2F). The above data suggested the inhibited proliferative ability in NSCLC after the silence of MAGI1-IT1.

MAGI1-IT1/MiRNA-512-3p/AKT1 Regulatory Loop in NSCLC

In NSCLC tissues, a positive correlation was identified between MAGI1-IT1 level and AKT1 level (Figure 3A). AKT1 was found to be upregulated in NSCLC tissues compared with the adjacent normal ones (Figures 3B and 3C). Be-

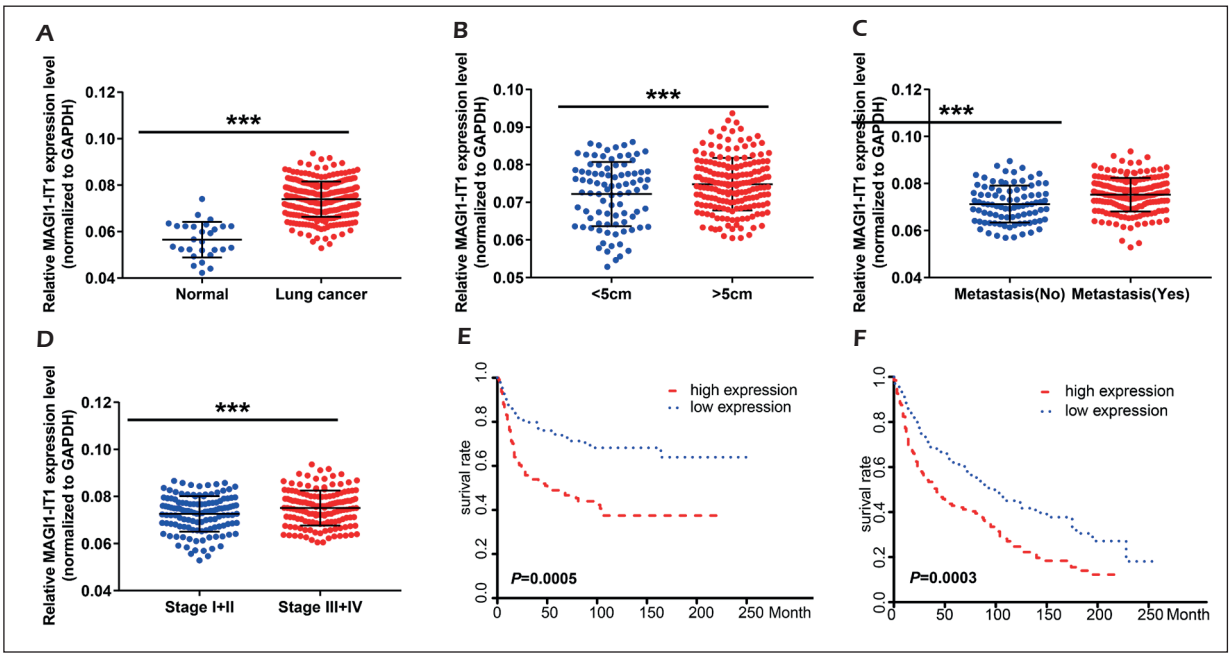

Figure 1. MAGI1-IT1 was upregulated in NSCLC and predicted a poor prognosis. **A,** MAGI1-IT1 level in NSCLC tissues and paracancerous tissues. **B,** MAGI1-IT1 level in NSCLC patients with <5 cm or >5 cm in tumor size. **C,** MAGI1-IT1 level in NSCLC patients either with metastasis or not. **D,** MAGI1-IT1 level in NSCLC patients in stage I+II or stage III+IV. **E,** Overall survival in NSCLC patients expressing high or low level of MAGI1-IT1. **F,** Progression-free survival in NSCLC patients expressing high or low level of MAGI1-IT1.

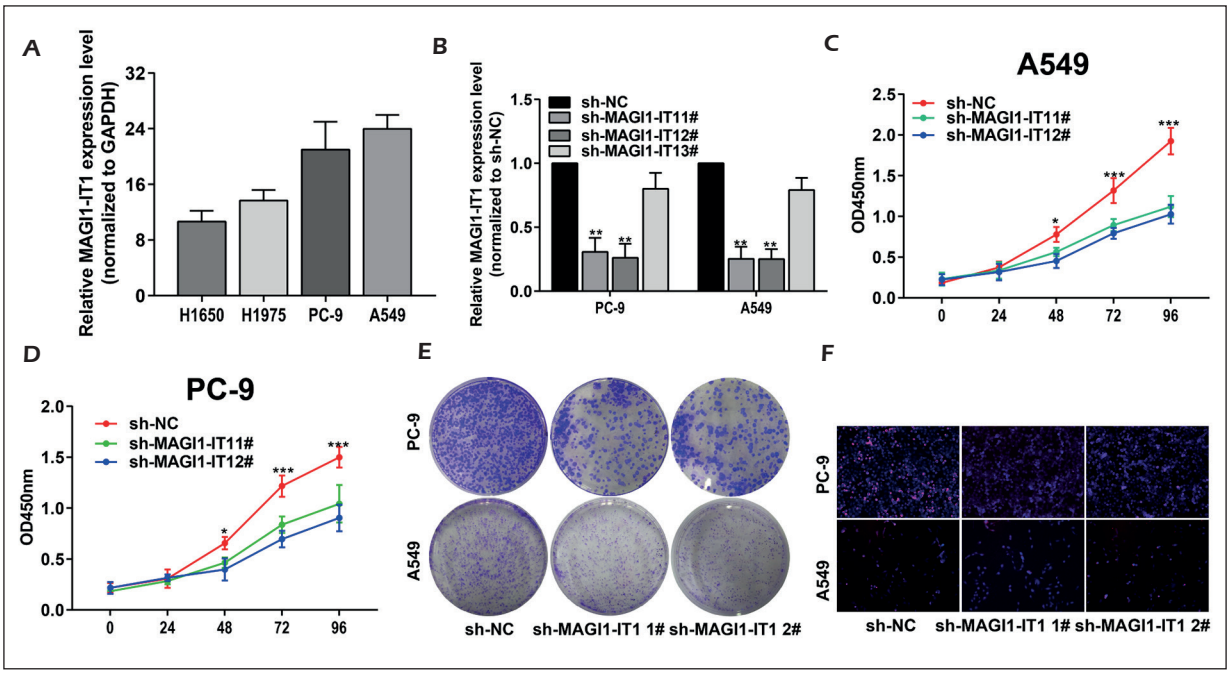

Figure 2. The knockdown of MAGI1-IT1 suppressed the proliferative ability in NSCLC. **A,** MAGI1-IT1 level in normal bronchial epithelial cell line (H1650) and lung cancer cell lines (H1975, PC-9, and A549). **B,** The transfection efficacy of sh-MAGI1-IT11#, sh-MAGI1-IT12#, and sh-MAGI1-IT13# in PC-9 and A549 cells. **C, D,** Viability in A549 and PC-9 cells transfected with sh-NC, sh-MAGI1-IT11#, or sh-MAGI1-IT12#. **E,** Clonality in A549 and PC-9 cells transfected with sh-NC, sh-MAGI1-IT11# or sh-MAGI1-IT12#. **F,** EdU-positive ratio in A549 and PC-9 cells transfected with sh-NC, sh-MAGI1-IT11# or sh-MAGI1-IT12# (Magnification: ×200).

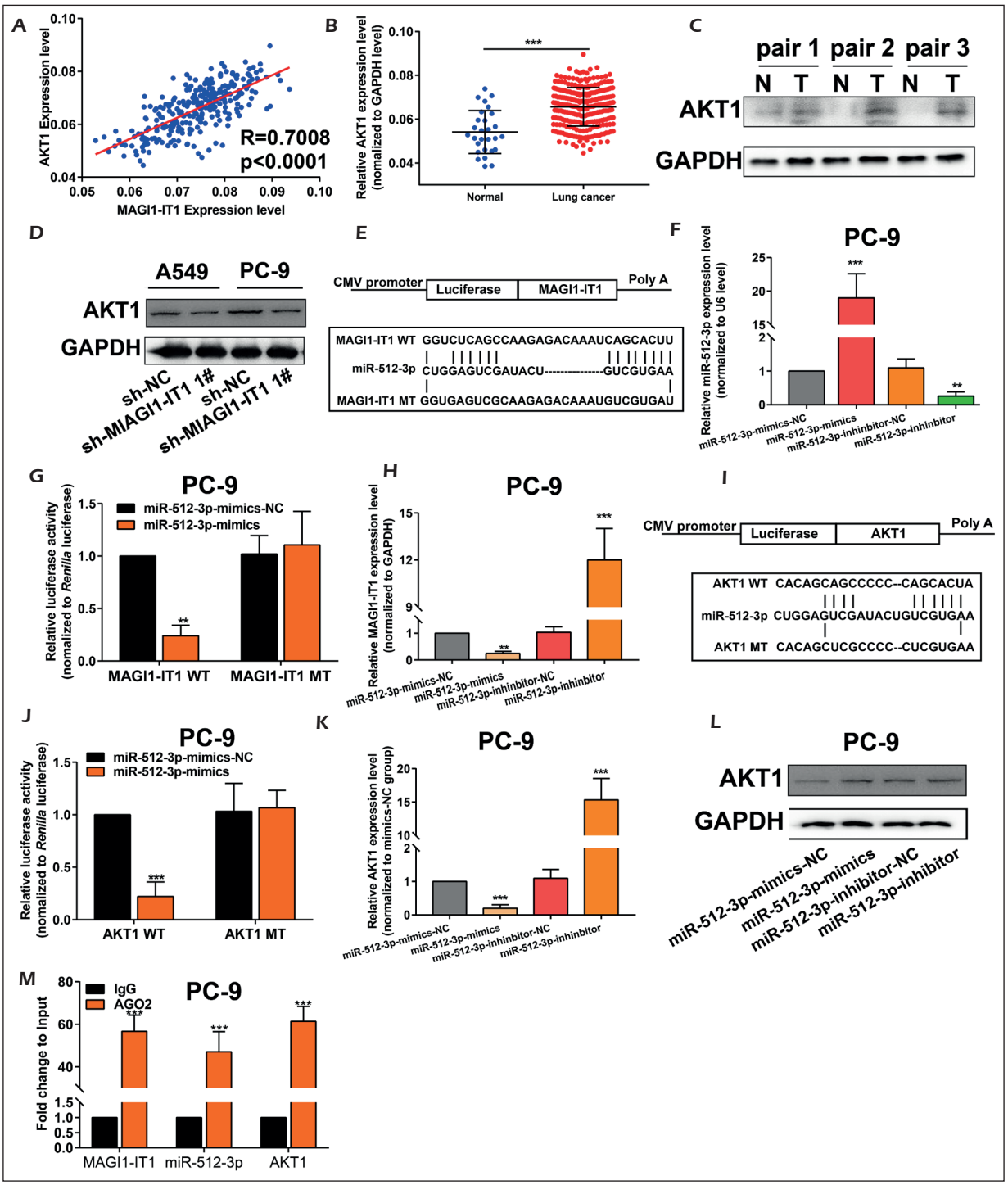

Figure 3. MAGI1-IT1/miRNA-512-3p/AKT1 regulatory loop in NSCLC. **A,** A positive correlation between expression levels of MAGI1-IT1 and AKT1 in NSCLC tissues. **B,** AKT1 level in NSCLC tissues and paracancerous tissues. **C,** Protein level of AKT1 in three paired NSCLC tissues and paracancerous tissues. **D,** Protein level of AKT1 in A549 and PC-9 cells transfected with sh-NC or sh-MAGI1-IT11#. **E,** Binding sequences between MAGI1-IT1 and miRNA-512-3p. **F,** MiRNA-512-3p level in PC-9 cells transfected with miRNA-512-3p mimics, inhibitor or NC. **G,** Luciferase activity in PC-9 cells co-transfected with miRNA-512-3p mimics/NC and MAGI1-IT1 WT/MAGI1-IT1 MT. **H,** MAGI1-IT1 level in PC-9 cells transfected with miRNA-512-3p mimics, inhibitor or NC. **I,** Binding sequences between AKT1 and miRNA-512-3p. **J,** Luciferase activity in PC-9 cells co-transfected with miRNA-512-3p mimics/NC and AKT1 WT/AKT1 MT. **K, L,** The mRNA and protein level of AKT1 in PC-9 cells transfected with miRNA-512-3p mimics, inhibitor or NC. **M,** Immunoprecipitants of MAGI1-IT1, miRNA-512-3p and AKT1 in anti-IgG and anti-AGO2.

sides, the transfection of sh-MAGI1-IT11# was able to downregulate the AKT1 level in both A549 and PC-9 cells (Figure 3D). Through prediction in the Diana database (www.microrna.gr/LncBase), miRNA-512-3p was believed to bind to MAGI1-IT1 (Figure 3E). Subsequently, miRNA-512-3p mimics and inhibitor were constructed (Figure 3F). The Dual-luciferase reporter gene assay uncovered a decline in the Luciferase activity after the co-transfection of miRNA-512-3p mimics and MAGI-IT1 WT, verifying the binding between miRNA-512-3p and MAGI-IT1 (Figure 3G). MAGI-IT1 level was negatively influenced by miRNA-512-3p in the PC-9 cells (Figure 3H). As a result, MAGI-IT1 targeted miRNA-512-3p and negatively regulated its level. Subsequently, the binding sites were observed between the promoter regions of miRNA-512-3p and AKT1 (Figure 3I). In the same way, the Dual-luciferase reporter gene assay confirmed the binding relationship between miRNA-512-3p and AKT1 (Figure 3J). The AKT1 level was negatively regulated by miRNA-512-3p at both mRNA and protein levels (Figures 3K and 3L). At last, the RIP assay illustrated that MAGI-IT1, miRNA-512-3p, and AKT1 were enriched in anti-AGO2 than those of the anti-IgG (Figure 3M). Collectively, we confirmed a regulatory loop MAGI1-IT1/miRNA-512-3p/AKT1 in NSCLC.

Overexpression of AKT1 Partially Reversed the Anti-Tumor Effect of MAGI1-IT1

To elucidate the potential role of AKT1 in NSCLC, pcDNA-AKT1 was constructed. The transfection of pcDNA-AKT1 markedly upregulated AKT1 level in PC-9 cells (Figure 4A). The functional assays all proved that the transfection of pcDNA-AKT1 stimulated the proliferative ability in PC-9 cells, manifesting elevated viability and colony formation number (Figures 4B and 4D). Notably, the accelerated proliferation in the PC-9 cells overexpressing AKT1 was partially attenuated by the co-transfection of sh-MAGI1-IT11# (Figures 4B and 4D).

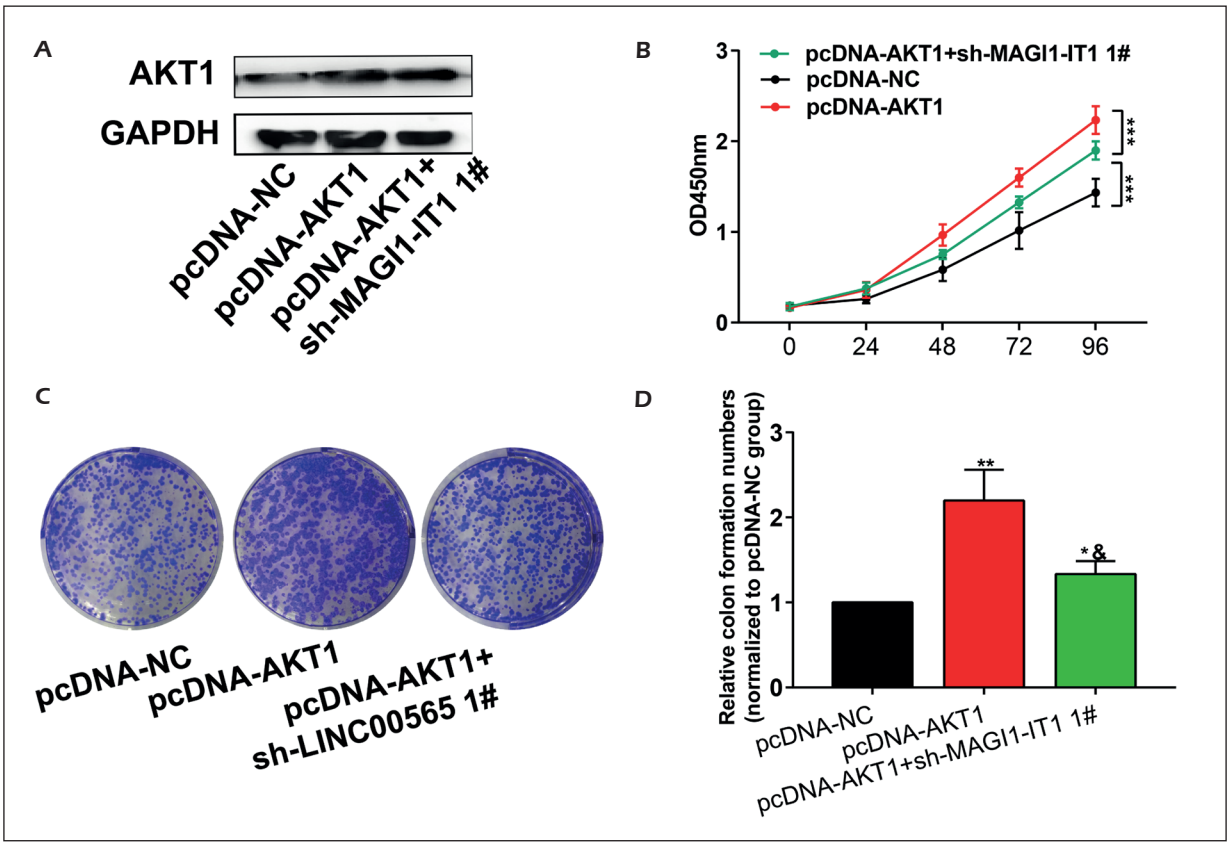

Figure 4. The overexpression of AKT1 partially reversed the anti-tumor effect of MAGI1-IT1. The PC-9 cells were transfected with pcDNA-NC, pcDNA-AKT1, or pcDNA-AKT1 + sh-MAGI1-IT11#. **A,** Protein level of AKT1. **B,** Viability. **C,** The formation of colonies. **D,** Relative number of colonies.

Discussion

NSCLC is a fatal malignancy that seriously endangers human lives[17]. Surgical resection is preferred for the treatment of early-stage NSCLC. However, over 50% of patients suffer postoperative metastasis within one year[18,19]. Hence, the target drugs for NSCLC are urgently required.

A great number of lncRNAs have been identified in regulating the progression of NSCLC. They are capable of mediating autophagy, apoptosis, proliferation, metastasis, and EMT of lung cancer cells[20-22]. A relevant study[23] pointed out that lncRNA could interact with the target miRNAs to further regulate the downstream gene expressions, thus participating in the progression of NSCLC. The investigations on the pathogenesis, metastasis, and drug resistance of NSCLC regulated by lncRNAs contribute to develop the therapeutic targets for NSCLC.

Our results found that MAGI1-IT1 was highly expressed in NSCLC tissues. Its level was higher in NSCLC patients with larger tumor size, positive metastasis, or advanced stage. Furthermore, MAGI1-IT1 presented a prognostic potential in NSCLC. A worse survival was predicted in NSCLC patients expressing a high level of MAGI1-IT1. In A549 and PC-9 cells, the knockdown of MAGI1-IT1 greatly attenuated viability, clonality, and EdU-positive ratio, indicating the inhibited proliferative ability.

Taken into consideration the ceRNA hypothesis, we speculated that MAGI1-IT1 may sponge the target miRNAs to exert its function in NSCLC[24,25]. Many oncogenic miRNAs in NSCLC could be regulated by lncRNAs. MiRNAs are non-coding RNAs with about 22 nucleotides. Mature miRNAs are capable of mediating mRNA expressions by binding to 3'UTR of target mRNAs[26]. Zhu et al[27] demonstrated that miRNA-512-3p suppresses cell adhesion and metastasis of lung cancer cells A549 and H1299 in NSCLC by targeting DOCK3. Hence, miRNA-512-3p is important in the pathological process of NSCLC. Our findings uncovered a negative interaction between miRNA-512-3p and MAGI1-IT1.

AKT, also known as protein kinase B (PKB), is a pleckstrin homology PH domain comprising a serine threonine kinase[28]. AKT consists of three members, AKT1, AKT2, and AKT3. As a vital subtype, AKT1 is closely related to many types of tumors[29-31]. Here, AKT was upregulated in NSCLC tissues, and positively correlated to MAGI1-IT1 level. AKT1 was verified to be the target gene of miR-412-3p, presenting a negative correlation between the two genes. The overexpression of AKT1 stimulated lung cancer cells to proliferate. Of note, the elevated proliferative rate in lung cancer cells overexpressing AKT1 was reversed by the silence of MAGI1-IT1. Our findings uncovered a novel therapeutic target for NSCLC.

Conclusions

MAGI1-IT1 is upregulated in NSCLC, and predicts a poor prognosis in NSCLC patients. MAGI1-IT1 stimulates the proliferative ability in NSCLC by upregulating the AKT1 level by binding to miRNA-512-3p.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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