MEG3 promotes liver cancer by activating PI3K/AKT pathway through regulating AP1G1

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Abstract. – OBJECTIVE: **This study aims to explore the biological function of maternally expressed gene 3 (MEG3) in liver cancer and the potential mechanism of phosphatidylinositide 3-kinases/protein kinase B (PI3K/AKT) pathway in regulating proliferation and invasion of hepatoma cells.**

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was applied to examine the level of MEG3 in 72 pairs of liver cancer tissues and corresponding adjacent tissues. Expression levels of MEG3 and AP1G1 in hepatocellular carcinoma cell lines including SMMC-7721 and BEL-7402 were detected. After transfection of MEG3-siRNA or AP1G1 overexpression plasmid, the proliferative and invasive abilities of hepatoma cells were detected through cell counting kit-8 (CCK-8) and cell invasion assay. The effects of MEG3 and AP1G1 on the cell cycle of hepatoma cell lines were examined using flow cytometry. Western blot was conducted to estimate the changes in the protein levels of AP1G1, p-PI3K, p-AKT and VEGF before and after transfection.

RESULTS: The level of MEG3 in hepatoma cancer tissues and cell lines was significantly reduced, especially in patients with advanced liver cancer. Knockdown of MEG3 significantly promoted proliferation and invasion of hepatoma cells, but accelerated cell cycle. Western blot analysis revealed that knockdown of MEG3 reduced the level of AP1G1 and activated the PI3K/AKT pathway. In addition, rescue experiments demonstrated that overexpression of AP1G1 partially reversed the promotive effect of lowly-expressed MEG3 on cell proliferation and invasion, suggesting that low expression of MEG3 may activate PI3K/AKT pathway by inhibiting AP1G1 expression.

CONCLUSIONS: Low expression of MEG3 could promote the proliferative and invasive abilities of hepatoma cells and accelerate cell cycle. The mechanism may be related to the inhibition of AP1G1 expression and activation of PI3K/AKT pathway.

Key Words:
MEG3, Liver cancer, Cell proliferation, Cell invasion, AP1G1.

Introduction

Primary liver cancer is one of the most common malignancies, and recent studies have shown that its mortality rate ranks third among all malignant tumors[1]. Mastering the mechanism of liver cancer and improving the prognosis of patients are still important tasks in the field of liver disease researches[2]. As with other solid tumors, the occurrence of liver cancer is a long-term process involving a large number of genetic and epigenetic mutations[3]. Although accumulating studies have reported the occurrence and development of liver cancer, the detailed molecular mechanisms still remain elusive. Long non-coding RNA (lncRNA) was once considered as a by-product of RNA polymerase II transcription without any biological function[4]. However, in recent years, with the deepening of research on lncRNA, it has been found that it can participate in different biological processes such as cell proliferation, cell differentiation, cell cycle, and apoptosis. Multiple mechanisms are involved in the regulatory effects of lncRNAs, such as chromatin remodeling and histone modification, genomic imprinting, post-transcriptional regulation, cell cycle regulation, gene transcription and translation, cell differentiation, etc.[5,6]. It is thus speculated that lncRNA may participate in tumor formation, proliferation, metastasis, etc. Therefore, we believed that certain lncRNAs can be helpful to improve the prognosis or find targeted therapy of tumors[7]. The maternally expressed gene 3 (MEG3) is a maternal expression gene originally discovered by Miyoshi *et al*[8] in 2000 which is located on chromosome 14q32.3 with a length of about 1.6 kb. It is a human homolog of gene captures gene 2 (genetraplocus 2, Gtl2) and the first lncRNA to be found to possess tumor-suppressing function. MEG3 lacks a fixed open reading frame and is considered to be a non-coding RNA due to the absence of a Kozak sequence in its short open reading frame, which can only be transcribed into

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RNA[9]. MEG3 is expressed in a variety of normal tissues, but it is abnormally expressed in various tumors such as gastric cancer, cervical cancer, prostate cancer, and breast cancer[10-13], especially in liver cancer[14, 15]. Studies have shown that MEG3 can inhibit the growth of a variety of cancer cells[9,16], showing that it can act as a tumor suppressor. At present, few studies have reported the role of MEG3. In this study, we first examined the level of MEG3 in liver carcinoma tissues and hepatoma cell lines, and further verified that it could regulate phosphatidylinositide 3-kinases/protein kinase B (PI3K/AKT) pathway by binding to AP1G1. In order to further explore the biological function of MEG3, MEG3 small interfering RNA was transfected into liver cancer cells to inhibit MEG3 expression. Effects of MEG3 on cell growth, invasion and cell cycle were then analyzed to reveal its anti-cancer mechanism.

Patients and Methods

Subject and Sample Collection

The fresh liver cancer tissues and corresponding adjacent tissues were obtained from 72 patients who were diagnosed with liver cancer and treated with surgery in Qilu Hospital of Shandong University from 2014 to 2017. The specimens were stored in liquid nitrogen tanks. Enrolled patients did not receive any treatment before surgery and had no family history. They volunteered to participate in the study and signed written informed consent. This study has been approved by the Hospital Ethics Committee.

Cell Culture

The normal control cell line THLE-3 and liver cancer cell lines including SMMC-7721, Hep3B, BEL-7402 and LM3 were purchased from Shanghai ATCC Company (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle' medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% (penicillin + streptomycin) in a 37°C incubator with 5% CO_2. The medium was changed once every other day. When the cells reached to 80-90% of confluency, they were passaged at a ratio of 1:2 or 1:3.

Cell Transfection

According to Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) instructions, cells with a growth situation in logarithmic phase were selected and transfected with MEG3-siRNA, pcDNA-AP1G1 or corresponding negative control synthesized by GenePharma (Shanghai, China). Cells were collected 24 h after transfection for other experiments.

RNA Extraction

Cells and tissues required for the experiment were collected and lysed with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). Then 250 µL of chloroform was added to the samples, which were shaken for 30 seconds and centrifuged at 4°C. The aqueous phase was aspirated in a new Eppendorf Tube (EP) tube, and an equal volume of pre-chilled isopropanol was added. After centrifugation, the precipitate was gently purged with 75% ethanol and dissolved in 20 µL of Diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). The concentration of extracted RNA was measured using a spectrophotometer, which was then stored in a refrigerator at -80°C until use.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The reverse transcription reaction system was prepared on ice using the PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan), and cDNA was obtained after the reaction completed. MiRNA quantitative PCR procedures were performed according to the miScript SYBR Green PCR Kit instructions (TaKaRa, Otsu, Shiga, Japan), with 10 µL of the total reaction system. The PCR amplification conditions were: pre-denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The primer sequences were as follows: MEG3 (F: 5'-TCCATGCTGAGCTGCTGCCAAG-3', R: 5'-AGTCGACAAAGACTGACACCC-3'), AP1G1 (F: 5'-TGCAATCCGGTCATCTTTTAGAG-3', R: 5'- AACTGTCCAAAGTGAGCAGGG-3'), GAPDH (F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3').

Cell Counting Kit-8 (CCK-8) Experiment

Cells in logarithmic growth phase were cultured in 96-well plates at a cell density of 1×10^6/mL, with 100 µL per well. Subsequently, 10 µL of CCK8 (Dojindo, Kumamoto, Japan) was added to each well at 0 h, 24 h, 48 h, and 72 h, and then incubated at 37°C for 2 hours. Subsequently, the culture solution was removed, and the absorbance value of each well was measured 1 h later at 450 nm using microplate reader.

Cell Invasion Assay

The final concentration of Matrigel was adjusted to 1 mg/mL with serum-free medium pre-cooled at 4°C. The upper chamber of transwell was coated with Matrigel and placed at 37°C for 3-5 h. The gel was coagulated and set aside. Cells in logarithmic growth phase were digested, washed with phosphate-buffered saline (PBS) (Beyotime, Shanghai, China), suspended in serum-free medium, and counted to adjust the concentration to 2×10^5 /mL. 600 μL of medium containing 10% serum was added in the lower chamber while 100 μL of cell suspension was added in the upper chamber. After cell culture for 24 h, the liquid in the upper chamber was removed, and the cells were fixed with formaldehyde for 30 minutes and stained by crystal violet for 15-30 minutes.

Flow cytometry

Cell concentration was adjusted to about 1×10^5/mL. Then, they were fixed with 1 mL of 75% ice ethanol pre-cooled at -20°C, and stored in a refrigerator at 4°C overnight. In the next day, cells were washed with PBS twice. After discarding the supernatant, 100 μL of RNaseA was added in the cells, and placed in water bath for 30 min at 37°C in dark. Subsequently, 400 μL of Propidium Iodide (PI) was added and mixed. Lastly, cell cycle detected in red fluorescence at 488 nm was recorded by flow cytometry.

Western Blot

Total protein was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). A suitable concentration of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was selected according to the molecular weight of the target protein. After electrophoresis, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and subjected to conventional immunostaining. Primary antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and AP1G1 were used to incubate the membrane at 4°C overnight, which was incubated with secondary primary at 37°C for 2 h. At last, chemiluminescence method was applied to develop the proteins in the membrane.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and GraphPad statistical software (La Jolla, CA, USA), and the measurement data were analyzed by Student's *t*-test. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). All measurement data were expressed as (Mean ± SD). The difference was statistically significant at $p<0.05$.

Results

MEG3 Level Was Significantly Reduced in Liver Cancer Tissues and Cell Lines

To investigate the association between MEG3 and liver cancer, qRT-PCR was used to examine the expression of MEG3 in 72 pairs of liver cancer tissues and corresponding adjacent tissues. The results revealed that the level of MEG3 in liver cancer tissues was significantly lower than that in adjacent tissues (Figure 1A), especially in advanced cancer tissues (Figure 1B). Afterwards, we examined the level of MEG3 in the normal cell line THLE-3 and the hepatoma cell lines including SMMC-7721, Hep3B, BEL-7402 and LM3. It was found that MEG3 expression significantly decreased in hepatoma cells, especially in SMMC-7721 and BEL-7402 cells (Figure 1C), which were then used for subsequent experiments. Three MEG3 siRNAs (MEG3-siRNA-1, MEG3-siRNA-2, MEG3-siRNA-3) were transfected into the above two cells, respectively. After 24 h, knockdown efficiency was detected using qRT-PCR assay. MEG3-siRNA-1 showed the best efficiency (Figure 1D), which was chosen for follow-up studies.

Knockdown of MEG3 Promoted Proliferation and Invasion of Hepatoma Cells and Accelerated Cell Cycle

MEMC3-NC and MEG3-siRNA were transfected into SMMC-7721 and BEL-7402 cells respectively, and cell proliferative ability was detected by CCK-8 assay at 0, 24, 48 and 72 h after transfection. It was found that compared with MEG3-NC group, MEG3-siRNA significantly promoted cell proliferation, and the OD values of the two groups were statistically significant after 24 h of transfection (Figure 2A). At the same time, cell invasion experiments also demonstrated that knockdown of MEG3 promoted invasive ability of SMMC-7721 and BEL-7402 cells (Figure 2B). Further, we used flow cytometry to detect changes in cell cycle after MEG3 knockdown. As shown in Figure 2C, inhibition of MEG3 accelerated the cell cycle. These results indicated that

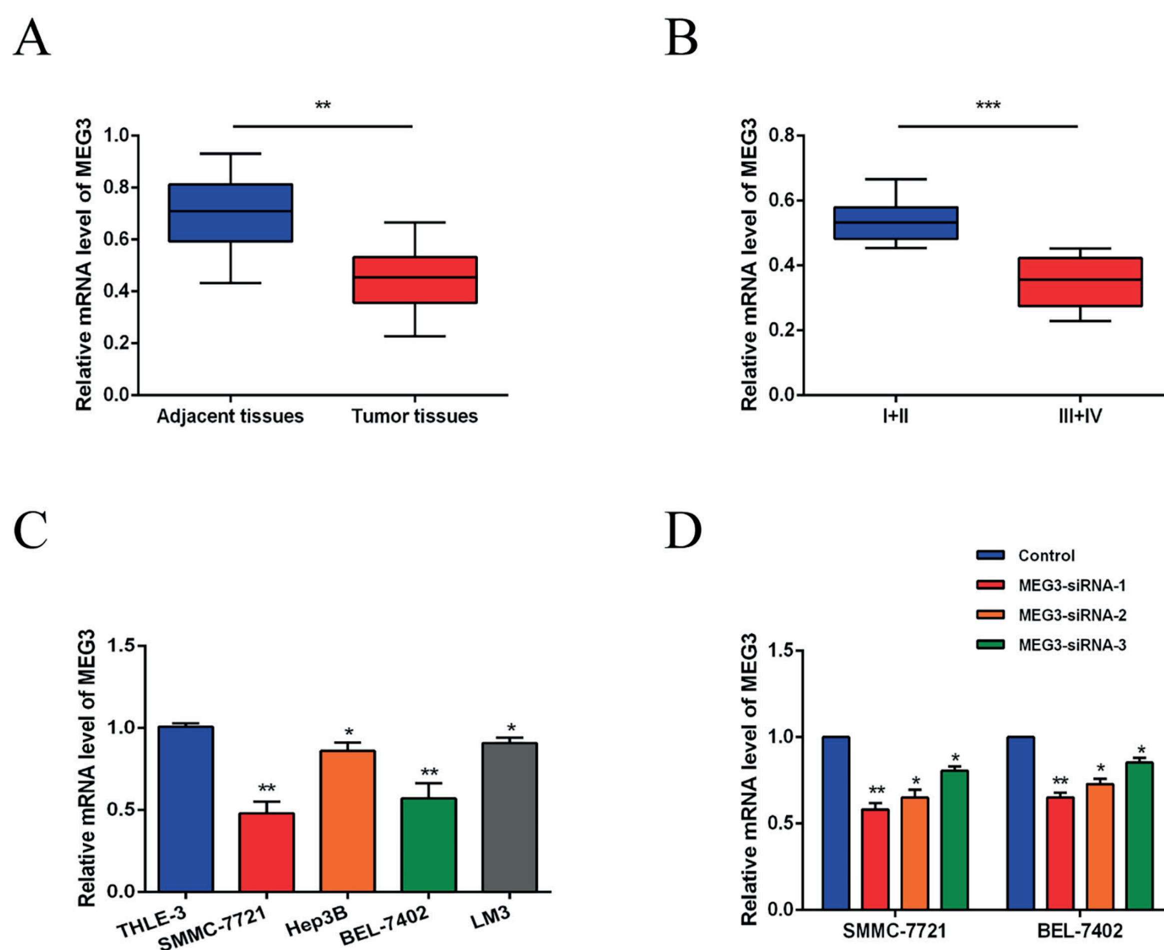

Figure 1. Expression of MEG3 in liver cancer tissues and cell lines. ***A,*** MEG3 expression significantly decreased in patients with liver cancer. ***B,*** Expression of MEG3 in liver cancer tissues of patients with different stages. ***C,*** MEG3 expression was significantly reduced in liver cancer cell lines. ***D,*** MEG3 expression was significantly down-regulated after knockdown of MEG3 in SMMC-7721 and BEL-7402 cells.

downregulation of MEG3 could promote proliferation and invasion, but accelerate cell cycle of the above two hepatoma cells.

Knockdown of MEG3 Inhibited AP1G1 Expression and Activated PI3K/AKT Pathway

Through literature review and online prediction, MEG3 can bind to AP1G1 and stabilize its expression. Subsequently, qRT-PCR analysis revealed that the level of AP1G1 in liver cancer tissues was remarkably lower than that in adjacent normal tissues (Figure 3A). In addition, MEG3 knockdown was found to strikingly inhibit AP1G1 expression at both mRNA and protein levels (Figure 3B, 3C), while the opposite result was observed by transfection of pcDNA-AP1G1 (Figure 3D, 3E). After transfection for 48 h, Western blot analysis revealed that inhibition of MEG3 remarkably downregulated the expression of AP1G1 in hepatoma cells while enhanced the levels of p-PI3K, p-AKT and VEGF. However, simultaneous transfection of pcDNA-AP1G1 partially reversed the expressions of AP1G1, p-PI3K, p-AKT and VEGF (Figure 3F). The above results suggested that MEG3 might play a role in the regulation of PI1K/AKT pathway by regulating AP1G1 expression in liver cancer.

Overexpression of AP1G1 Could Reverse the Effect of MEG3 on Inhibiting Cell Proliferation and Invasion

Cell proliferation was found to be enhanced after inhibition of MEG3 but was inhibited after simultaneous overexpression of AP1G1 (Figure 4A). At the same time, cell invasion experiments

also demonstrated that overexpression of AP1G1 reversed the enhanced invasion of SMMC-7721 and BEL-7402 cells induced by MEG3 knockdown (Figure 4B). Further, flow cytometry indicated that knockdown of MEG3 could accelerate cell cycle, while simultaneously overexpressing AP1G1 arrested cell cycle (Figure 4C). The above results illustrated that overexpression of AP1G1 could reverse the inhibitory effects of low expression of MEG3 on cell proliferation and invasion.

Discussion

Primary hepatocellular carcinoma is one of the common malignant tumors of the digestive system, and its incidence increases with social and economic development, lifestyle changes and environmental pollution. Although therapeutic approaches of liver cancer are diverse, the overall efficacy is poor and the mortality rate is high due to its high degree of malignancy, rapid progress,

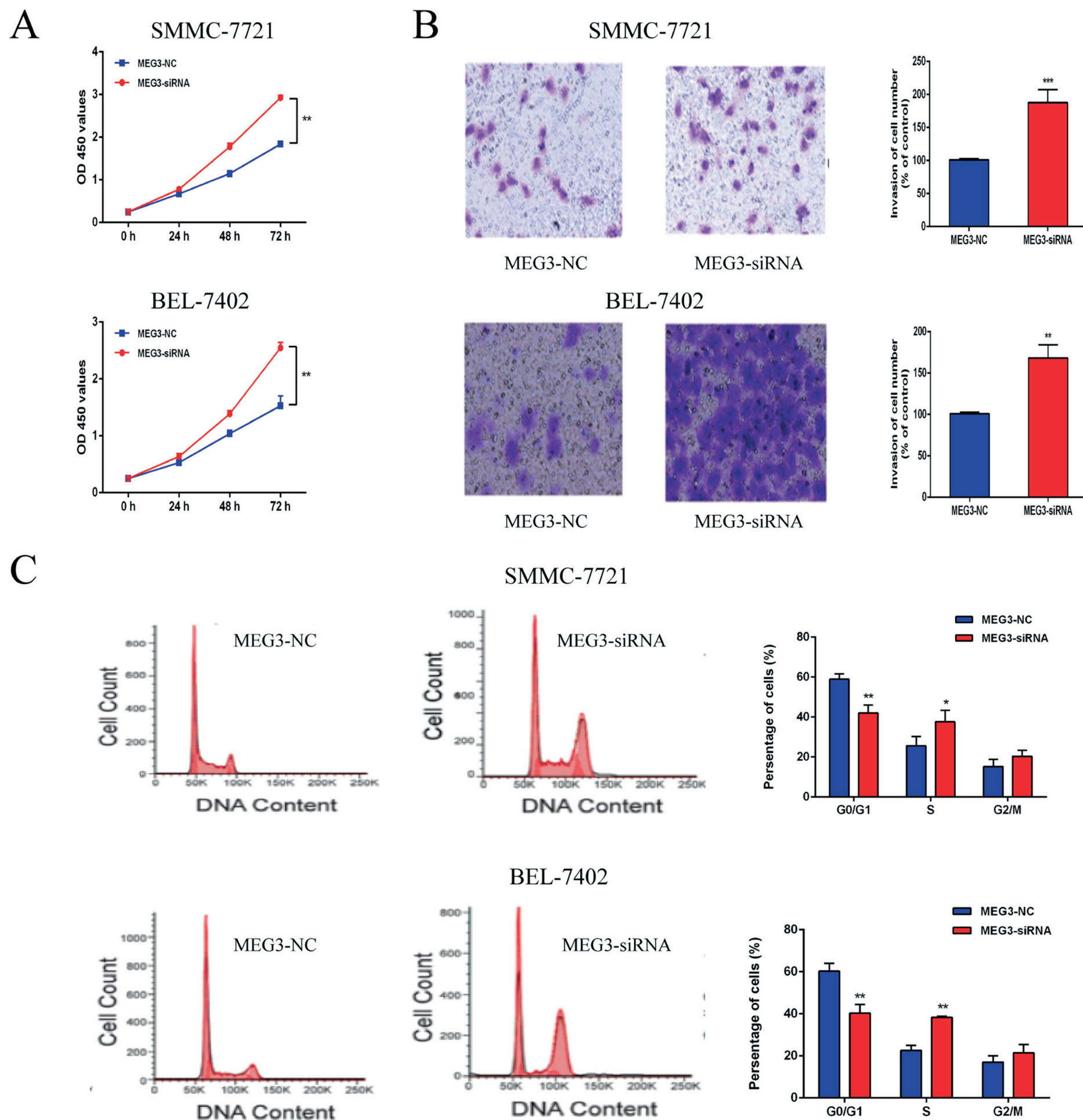

Figure 2. Low expression of MEG3 promoted cell proliferation and invasion. ***A,*** Knockdown of MEG3 in SMMC-7721 and BEL-7402 cells promoted cell proliferation. ***B,*** Low expression of MEG3 could promote SMMC-7721 and BEL-7402 cell invasion. ***C,*** Knockdown of MEG3 in SMMC-7721 and BEL-7402 cells promoted cell cycle.

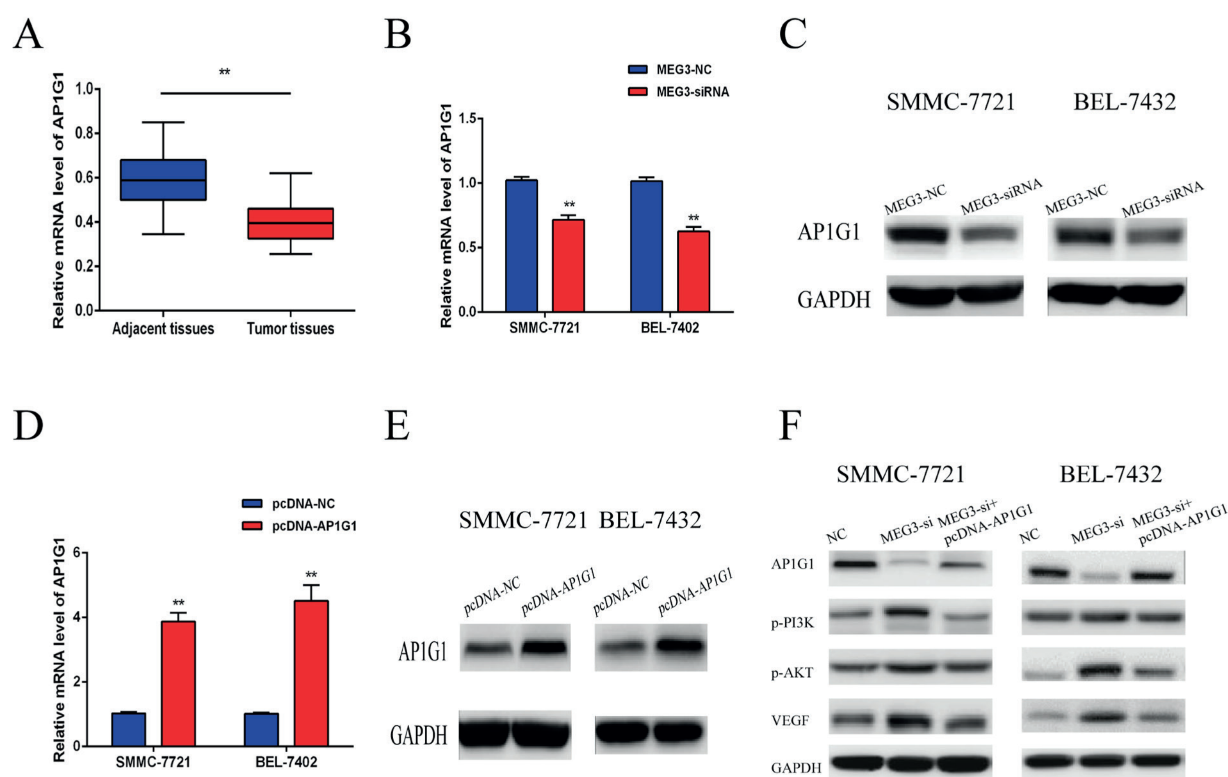

Figure 3. Low expression of MEG3 activated PI3K-AKT pathway by inhibiting AP1G1 expression. ***A,*** AP1G1 expression was significantly downregulated in patients with liver cancer. ***B,*** The mRNA expression of AP1G1 was significantly down-regulated after knockdown of MEG3 in SMMC-7721 and BEL-7402 cells. ***C,*** Knockdown of MEG3 in SMMC-7721 and BEL-7402 cells significantly inhibited the protein expression of AP1G1. ***D,*** After transfection of the AP1G1 overexpression plasmid in SMMC-7721 and BEL-7402 cells, the mRNA expression of AP1G1 significantly increased. ***E,*** Overexpression of AP1G1 significantly increased the protein expression of AP1G1. ***F,*** Low expression of MEG3 remarkably promoted the expression levels of proteins involved in PI3K-AKT pathway, which were downregulated after simultaneously inhibiting AP1G1 expression.

and resistance to drug resistance [17]. The molecular mechanism of the development of liver cancer remains unclear[18]. In recent years, the rapid development of molecular biology has advanced explorations of tumor development at the molecular level. Targeted therapy of liver cancer has gradually become a research hotspot. Although long non-coding RNA does not encode protein, it can regulate a variety of physiological cellular activities/ through transcriptional interference or activation and chromatin modification. It is a key factor in the development of tumors and neurodegenerative diseases[4]. In-depth studies on the relationship between long non-coding RNA and tumorigenesis are expected to provide new strategies for the prevention and treatment of tumors[19]. Evidences have shown that lncRNA is involved in many cellular activities and closely related to the occurrence and progression of tumors[20]. Recent studies have shown that lncRNA is involved in the development, progression and metastasis of liver cancer, and can be used as a molecular marker for early diagnosis, identifying tumor recurrence or prognosis of liver cancer. For example, H19 is highly expressed in hepatocarcinoma cells, and detection of H19 combined with alpha-fetoprotein is helpful for early diagnosis of liver cancer[21]. HOTAIR is also highly expressed in hepatoma tissues and closely related to the metastasis of hepatoma cells, which can be used as a predictor of postoperative tumor recurrence after liver transplantation[22,23]. It is reported that MEG3 can be expressed in many normal tissues. However, its expression is absent in some primary human tumors including gastric cancer, hepatocellular carcinoma, and oral squamous cell carcinoma[9,12,24], suggesting that lncRNA MEG3 has the potential to inhibit cell growth. In addition, exogenously expressed MEG3 can inhibit the growth of cancer cells, further indicating that MEG3 has a tumor suppressing effect. MEG3 is lowly expressed in most hepatocellular carcinoma tissues and can

strikingly inhibit cell proliferation and invasion, but has no significant effect on cell apoptosis. It is speculated that MEG3 may regulate other genes by binding to certain protein complexes, thereby exerting a role in inhibiting cell proliferation and invasion. AP1Gs encodes the Y(G) subunit of adaptor proteincornplex1 (APl). AP1 is a heterotetramer composed of four subunits, located in the trans-Golgi network structure and early endocytic bodies. AP1 is essential for cell secretion and endocytosis by mediating protein sorting[25,26]. Phosphatidylinositol 3-kinase (PI3K) / protein kinase B (Akt) signaling pathway is abnormally expressed in most human malignancies, exerting a vital function in cell proliferation, apoptosis, cell invasion and metastasis, cell cycle regulation, formation of tumor blood vessels, and chemoresistance of chemotherapy, etc.. Currently, PI3K/Akt pathway is a research hotspot of molecular targeted therapy for tumors[27-29]. The vascular en-

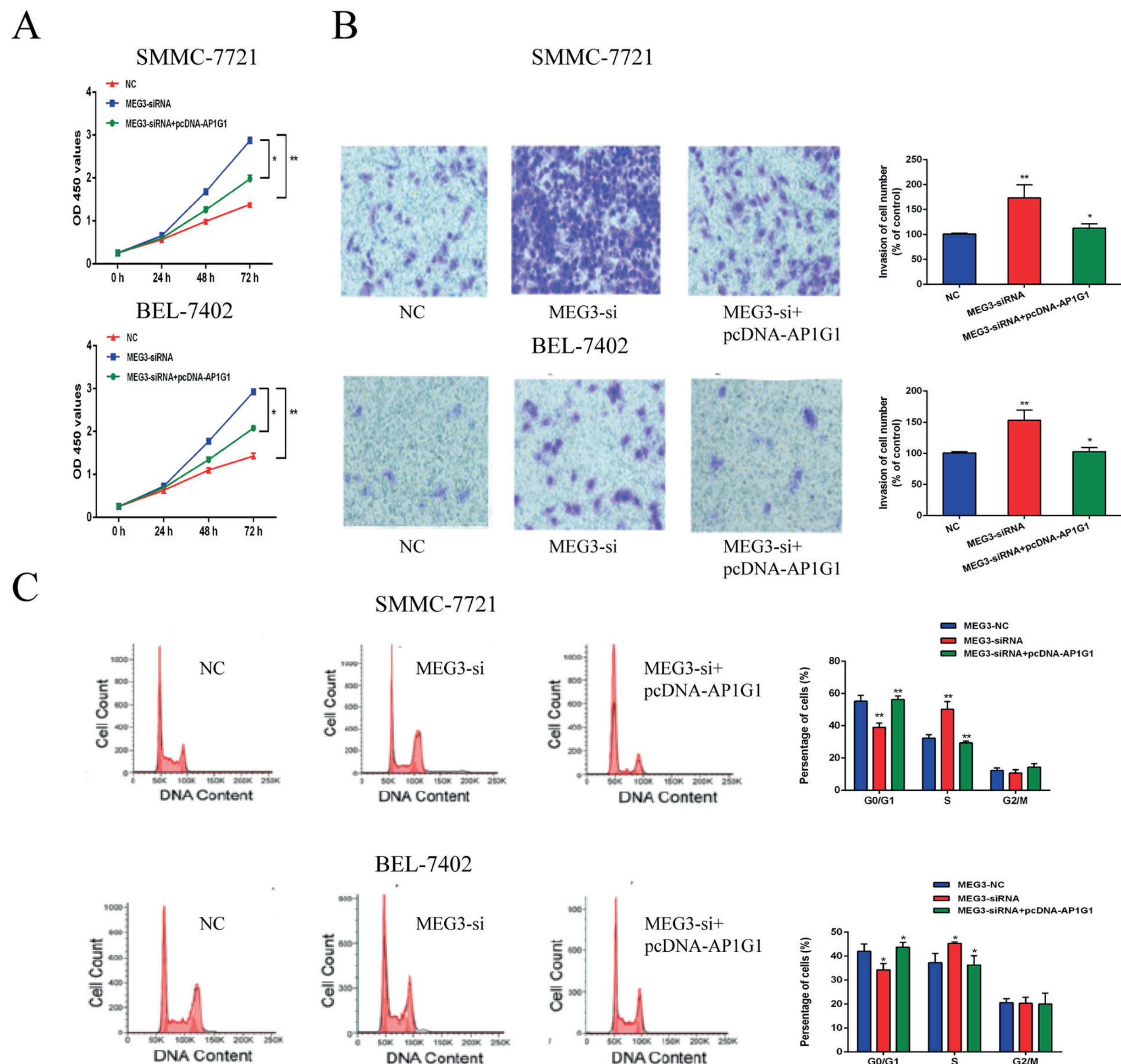

Figure 4. AP1G1 reversed the effect of MEG3 on cell proliferation and invasion. ***A,*** After inhibiting MEG3 expression, the cell proliferative ability was significantly enhanced, while simultaneous overexpression of AP1G1 weakened the cell proliferative ability. ***B,*** Inhibiting MEG3 expression significantly enhanced the cell invasive ability, while simultaneously overexpressing AP1G1 reduced it. ***C,*** Inhibiting MEG3 expression could promote cell cycle, while simultaneously overexpressing AP1G1 arrested the cell cycle.

dothelial growth factor (VEGF) in the endothelial growth factor/platelet-derived growth factor (VEGF/PDGF) family is involved in tumor angiogenesis[30]. Studies have shown that in tumor growth and invasion, the formation of new blood vessels provides the nutrients required for tumor activity, and therefore, VEGF plays a vital role in tumor metastasis[31]. In our research, MEG3 was lowly expressed in both HCC tissues and cell lines and could promote the proliferation and invasion of HCC cells. Subsequently, we predicted through bioinformatics that MEG3 can bind to AP1G1 and stabilize its expression. The mRNA and protein expressions of AP1G1 also decreased after MEG3 knockdown. Meanwhile, it was found that AP1G1 knockdown could activate PI3K/AKT pathway. Further rescue experiment revealed that overexpression of AP1G1 could restore the regulation of MEG3 knockdown on proliferation and invasion of hepatoma cells. Therefore, it is speculated that MEG3 may affect the proliferation and invasion of hepatoma cells by binding to AP1G1 and activating PI3K/AKT pathway.

Conclusions

Low expression of MEG3 may activate the PI3K/AKT pathway by inhibiting the expression of AP1G1, thereby promoting the proliferation and invasion of hepatocarcinoma cells and participating in the development of liver cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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