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Original Article

Long non-coding RNA MG3 inhibits proliferation and migration of non-small cell lung carcinoma through regulating EGFR

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Abstract: Lung cancer is the most common and easy to recurrent respiratory tract malignant tumor around the world. Non-small cell lung cancer (NSCLC) shows the highest incidence. Although the molecular targeted therapy becomes a new hot spot, the molecular mechanism underlying the pathogenesis of lung cancer is still unclear. Long noncoding RNA (lncRNA) can participate in the regulation of gene expression through mediating transcription and epigenetics. As a member of lncRNA, maternally expressed gene 3 (MEG3) is involved in various tumors regulation, while its function and related mechanism in NSCLC have not yet been elucidated. Lung cancer cell line A549 and normal bronchial epithelial cell line 16HBE were cultured in vitro. A549 cells were randomly divided into three groups: normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected. MEG3 expression was detected by real time PCR. Cell invasive ability was determined by Transwell assay. Cell proliferation was tested by MTT assay. Cell apoptosis was evaluated by flow cytometry. EGFR expression was analyzed by Western blot. Our results showed that MEG3 level significantly declined in A549 cells compared with 16HBE cells ($P < 0.05$). Compared with control and empty plasmid group, pcDNA3.1-MEG3 plasmid transfection obviously elevated MEG3 expression, suppressed tumor cell proliferation, induced cell apoptosis, declined invasive ability, and downregulated EGFR expression ($P < 0.05$). In conclusion, LncRNA MEG3 may suppress NSCLC proliferation, promote cell apoptosis, and restrain cell invasion through regulating EGFR. It can be treated as a new biological target for NSCLC diagnosis and treatment.

Keywords: lncRNA, NSCLC, MEG3, EGFR, proliferation

Introduction

Lung cancer is the most common and easy to recurrent respiratory tract malignant tumor around the world. Non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers [1, 2]. In spite of medical technologies progress and more treatment methods appearance, lung cancer still has the largest hazard to human health due to high postoperative recurrence rate and short mean survival time [3, 4]. The incidence and mortality rate of lung cancer increase year by year in China [5]. Because of numerous related factors involved in lung cancer occurrence and development, complicated mechanism, few methods for early diagnosis, and poor curative effects to the advanced cases, most of the patients with lung cancer present poor prognosis [6, 7]. Therefore, seeking effective treatment method and inhibition of lung cancer invasion have become one of the challenges in medical field [8]. At present, the molecular targeted therapy has become a new hot spot for NSCLC, whereas the molecular mechanism underlying the pathogenesis and development of NSCLC is still unclear.

In recent years, a large amount of the transcription of non-coding RNA has been found in eukaryote. Non-coding transcriptions account for the vast majority of the human genome [9]. Long noncoding RNAs (lncRNAs) refer to the transcriptions with more than 200 nt that does not participate in protein coding. They were originally defined as transcription "noise". However, they were found to be involved in epi-

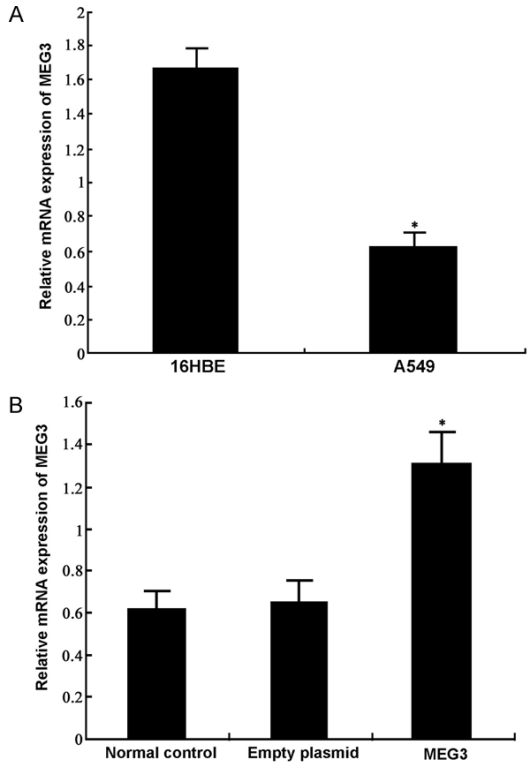

Figure 1. MEG3 expression in 16HBE cells and A549 cells transfected without or with MEG3 plasmid. RNA was extracted from 16HBE cells or A549 cells followed by measurement of the mRNA expression of MEG3 by real-time PCR (A). In addition, A549 cells in logarithmic phase were randomly divided into three groups: normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected followed by measurement of the mRNA expression of MEG3 (B). *P < 0.05, compared with 16HBE cells. *P < 0.05, compared with normal control.

genetics, transcriptional regulation, and post-transcriptional regulation [10, 11]. LncRNA is the most frequent protein noncoding sequence transcripted in genome [12]. Previous study on tumor mainly focused on protein coding gene regulation, whereas recent investigation revealed that lncRNAs also play an important role in tumor occurrence and development [13]. As a member of lncRNA, maternally expressed gene 3 (MEG3) has been confirmed to be involved in various tumors regulation [14, 15], while its function and related mechanism in NSCLC have not yet been elucidated.

Materials and methods

Main instruments and reagents

NSCLC cell line A549 and human normal bronchial epithelial cell line 16HBE were purchased from ATCC. DMEM medium, FBS, penicillin and streptomycin, and EDTA were from Hyclone. DMSO and MTT were from Gibco. Enzyme-EDTA was from Sigma. PVDF membrane was from Pall Life Sciences. Western blot related reagents were from Beyotime. ECL reagent was from Amersham Biosciences. Rabbit anti human NF-κB and EGFR monoclonal antibodies, and HRP-conjugated mouse anti rabbit IgG secondary antibody were from Cell Signaling. Transwell chamber was from Corning. PCR amplification kit and purification kit were from Promega. Plasmid extraction kit and restriction enzyme were from Roche. RNA extraction kit and reverse transcription kit were from Axygen. Annexin V-FITC apoptosis detection kit was from BD Bioscience. Other reagents were from Sangon. FACS Calibur flow cytometer was from BD. ABI 7700 Fast real time PCR amplifier was from ABI. Labsystem Version 1.3.1 spectrophotometer was from Bio-rad.

A549 cell culture and grouping

A549 cells stored in liquid nitrogen were revived at 37°C water bath. The cells in logarithmic phase at 3-8th passage were used for experiments. A549 cells were randomly divided into three groups: normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected.

MEG3 plasmid construction and transfection

The primers of MEG3 were designed by Primer 6.0 software according to MEG3 sequence and inserted to pcDNA3.1 carrier. Clonal cDNA library was applied as template to construct pcDNA3.1-MEG3 cDNA containing restriction enzyme cleavage site. The PCR production was purified and digested to connect to pcDNA3.1 plasmid. Recombinant pcDNA3.1-MEG3 was established by double-digestion and sequencing. pcDNA3.1 empty plasmid and pcDNA3.1-MEG3 plasmid were added to 200 μl serum free medium and incubated at RT for 15 min, respectively. Next, they were mixed with lipo2000 in 1.6 ml serum free medium and added to the cells at 37°C and 5% CO_2 for 6 h. Then the cells were changed medium and continuously cultured for 48 h for the following experiments.

Real-time PCR

Total RNA was extracted by Trizol according to the manual and reversely transcripted to cDNA.

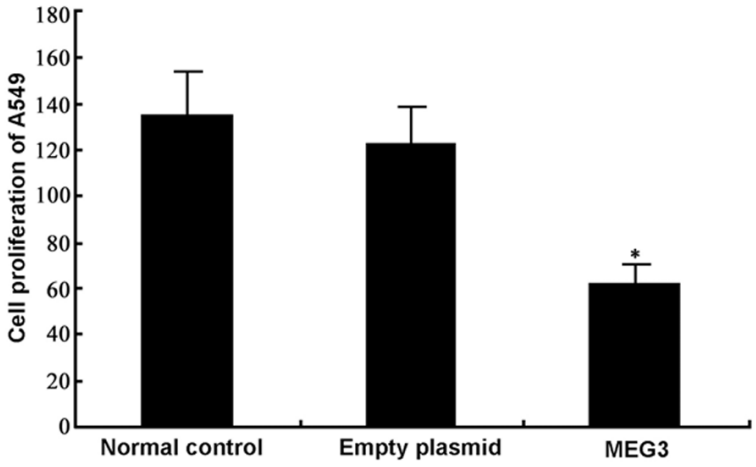

Figure 2. The effect of MEG3 overexpression on A549 cell proliferation. A549 cells in logarithmic phase were randomly divided into three groups: normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected followed by measurement of cell proliferation by MTT assay. *P < 0.05, compared with normal control.

The cDNA was used as a template for PCR amplification. The primer sequences used in the experiments were designed by Primer 6.0 software and synthetized by Invitrogen. The primer sequences were listed as follows: MEG3, forward, 5'-ACTCCATGGAGCCAGAAACG-3', reverse, 5'-ATGAAGCTGGTAGCGCAGT-3'. GAPDH, forward, 5'-GGGAGGTCCCAGTCAAT-3', reverse, 5'-GAGTCCTTCCACGATCAA-3'. PCR reaction was performed as follows: 55°C for 1 min, followed by 40 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as an internal reference. The relative expression level was calculated by $2^{-\Delta Ct}$ method.

MTT assay

A549 cells in logarithmic phase were randomly divided into three groups: normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected. The well was added with 20 μl MTT every 24 h with three replicates in each time point. After 4 h incubation, the well was added with 150 μl DMSO and the plate was read at 570 nm wave length. The experiment was repeated at least three times.

Transwell assay

Transwell chamber was coated with 50 mg/L Matrigel at bottom and the upper surface at 1:5. After air dried for 4 h, 500 μl DMEM containing 10% FBS was added to the lower chamber. A total of 100 μl cells suspension prepared by serum free medium was added to the upper chamber. The cells cultured in Transwell chamber without Matrigel coating were treated as control. After 48 h, the chamber was washed by PBS to remove the cells on the surface. Next, the membrane was fixed with absolute alcohol and stained by crystal violet to calculate the cell number on the lower surface of the membrane. The experiment was repeated at least three times.

Flow cytometry

A549 cells were digested and seeded in 50 ml culture bottle at a density of 5×10^5/mL. After treatment, the cells were fixed with pre-cooled 75% alcohol at 4°C overnight. After washed by the mixture of 800 μl 1×PBS and 1% BSA, the cells were treated with 100 μg/ml PI (3.8% SodiumCitrate, pH 7.0) and 100 μl RnaseA (10 mg/m) at 37°C for 30 min, the cells were analyzed by flow cytometry and calculated using FCSExpress 3.0 software.

Western blot

Total protein was extracted using RIPA and stored at -20°C after qualification. Then the protein was separated by Western blot. After transferring and blocking, the membrane was incubated with EGFR antibody (1 in 2000 dilution) at 4°C. Then the membrane was washed by PBS and 10% PBST was added. Next, the membrane was incubated with goat anti rabbit secondary antibody (1 in 2000 dilution) at RT for 30 min. At last, the membrane was developed and the data was analyzed by Quantity One software. The experiment was repeated at least four times.

Statistical analysis

SPSS 16.0 software was applied for data analysis. Measurement data was presented as mean ± standard deviation ($\overline{x}$ ± SD). One-way ANOVA was performed for multiple groups' comparison. P < 0.05 was considered as statistical significance.

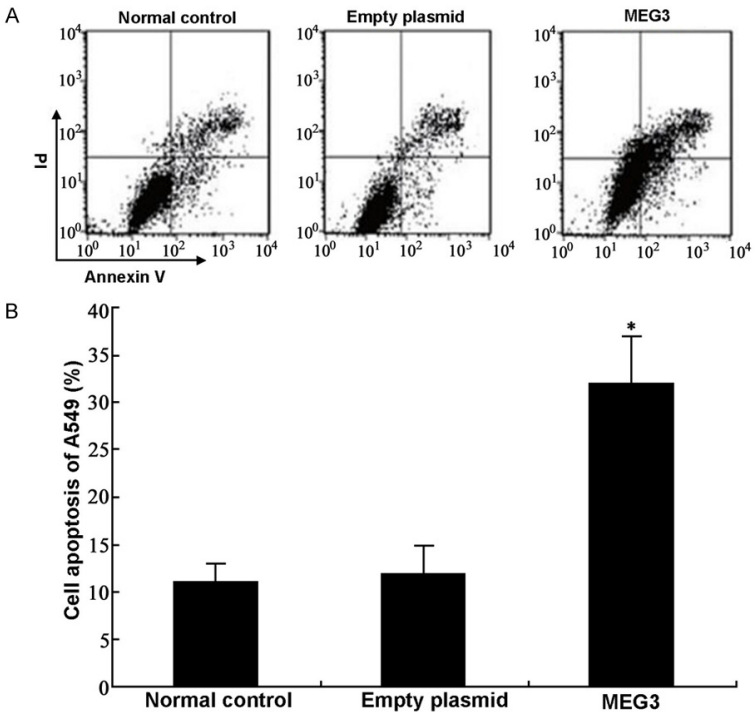

Figure 3. The impact of MEG3 on A549 cell apoptosis. A549 cells were randomly divided into three groups: normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected followed by measurement of cell apoptosis by flow cytometry (A, B). *P < 0.05, compared with normal control.

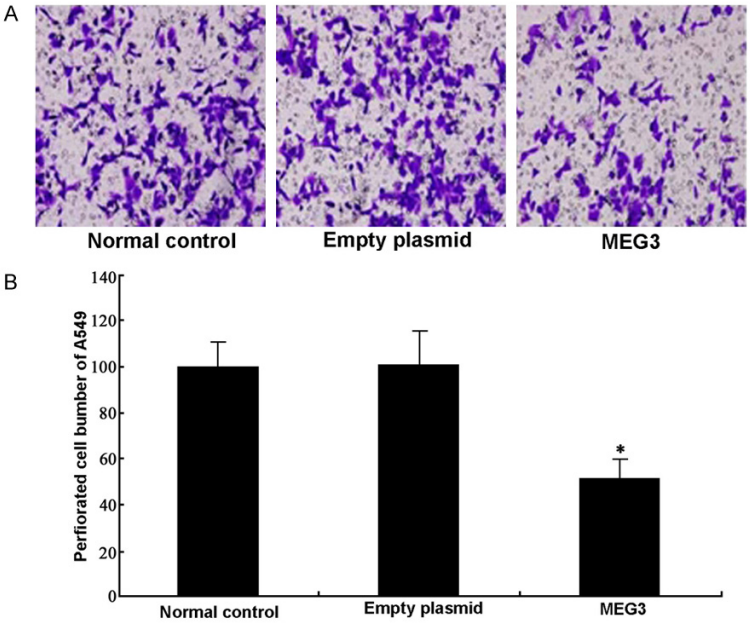

Figure 4. Effect of MEG3 overexpression on A549 cell invasion. A549 cells were randomly divided into normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected followed by measurement of cell invasion by transwell assay using crystal violet staining (A). Quantified cell numbers were shown in (B). *P < 0.05, compared with normal control.

Results

MEG3 expression in A549 and 16HBE cells

Real time PCR was applied to test MEG3 RNA expression in logarithmic A549 cells and 16HBE cells. It was showed that compared with 16HBE cells, MEG3 RNA expression was significantly lower in A549 cells (P < 0.05) (**Figure 1A**).

The impact of MEG3 plasmid transfection on MEG3 expression in A549 cells

Real time PCR was adopted to determine MEG3 expression in A549 cells after MEG3 plasmid transfection. It was revealed that pcDNA-MEG3 transfection obviously promoted MEG3 expression in A549 cells compared with normal control and empty plasmid group (P < 0.05) (**Figure 1B**).

The effect of MEG3 on A549 cell proliferation

MTT assay was performed to evaluate the impact of MEG3 transfection on A549 cell proliferation. The results demonstrated that after 48 h MEG3 transfection, A549 cell proliferation was markedly supp-r essed compared with control (P < 0.05) (**Figure 2**), suggesting that MEG3 had inhibitory effect on A549 cell proliferation.

The impact of MEG3 on A549 cell apoptosis

Flow cytometry was used to detect MEG3 transfection's effect on A549 cell apoptosis. It was found that MEG3 transfection for 48 h apparently induced lung cancer cell apoptosis compared with control and empty plasmid group (P <

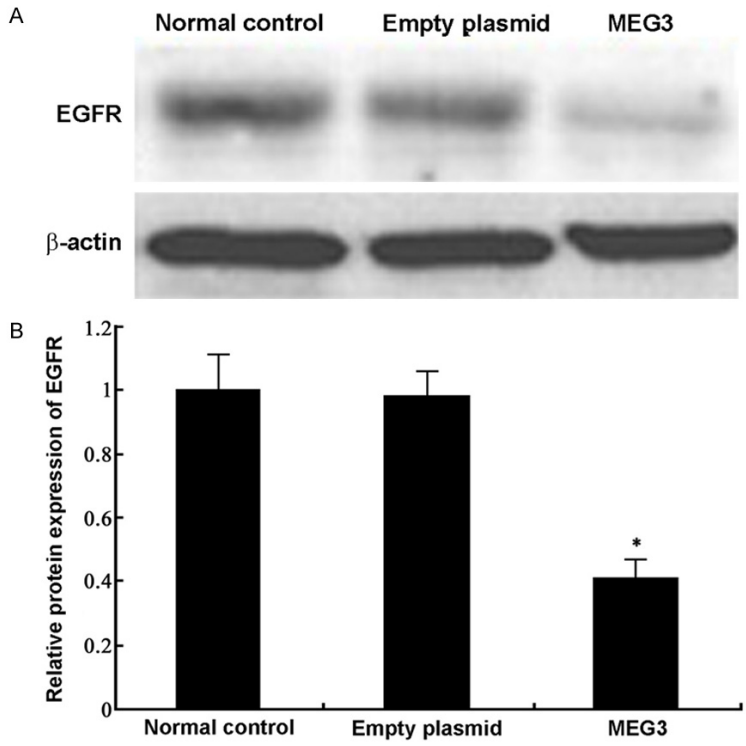

Figure 5. Effect of MEG3 overexpression on EGFR expression in A549 cells. A549 cells were randomly divided into normal control, empty plasmid group with pcDNA3.1 transfected, and MEG3 group with pcDNA3.1-MEG3 transfected. After that, proteins were extracted from A549 cells in different group followed by measurement of the EGFR protein level by western blot (A). Quantitative analysis of EGFR expression was shown in (B). *P < 0.05, compared with normal control.

0.05) (**Figure 3**), indicating that MEG3 may induce A549 cell apoptosis.

Upregulation of MEG3 affected A549 cell invasion

Transwell assay presented that MEG3 transfection for 48 h upregulated MEG3 and suppressed A549 cell invasion compared with control and empty plasmid group (P < 0.05) (**Figure 4**), revealing that MEG3 may affect tumor cell invasion.

Upregulation of MEG3 affected EGFR expression in A549 cells

Western blot was performed to analyze regulation of MEG3 impact on EGFR protein expression in A549 cells. It was demonstrated that after MEG3 transfection for 48 h, EGFR protein level was significantly downregulated compared with control and empty plasmid group (P < 0.05) (**Figure 5**). The results mentioned above suggested that MEG3 overexpression in A549 cells suppressed cell proliferation, promoted cell apoptosis, and inhibited cell invasion by regulating EGFR expression.

Discussion

The pathogenesis, treatment, and prognosis of NSCLC have been received much attention. Tumor invasion and metastasis are important reasons for the tumor recurrence and poor prognosis, and even cause of death. NSCLC is easy to occur metastasis and invasion, with lower 5-year survival rate [16]. NSCLC classification and judgment still mainly depend on pathological biopsy and light microscope observation, which might be influenced by sampling, sample size, differentiation degree, and all kinds of human factors, thus, effective early diagnosis and prognosis markers for NSCLC have important clinical significance [17].

It was found that lncRNA may participate in physiological activities through various ways of gene regulation, such as chromatin modification, genomic imprinting, intranuclear transport, chromosome gene silencing, and transcriptional activation, etc. LncRNAs can regulate cells under normal and pathological conditions, including growth, proliferation, cell cycle, and cell apoptosis, etc. Therefore, lncRNAs are important regulatory factors during human disease occurrence and development [18, 19]. In recent years, abnormal lncRNA expression was detected in the process of tumor formation and progress. The expression and function mechanism of some lncRNAs in cancer have been found [20]. Therefore, the relationship between lncRNAs and NSCLC is extremely close. LncRNA HOTAIR was found abnormally expressed in the early diagnosis of NSCLC. HOTAIR was the first discovered lncRNA with the trans transcription regulation effect. It is significantly upregulated in NSCLC, thus may act as a candidate diagnos-

tic marker for NSCLC [20, 21]. It was reported that some lncRNAs may be associated with NSCLC metastasis, invasion, and even drug resistance. However, there is lack of investigation to confirm the correlation between lncRNA and NSCLC occurrence and development.

LncRNA MEG3 was confirmed to be expressed in numerous normal tissues, however significantly downregulated or even undetectable was observed in a variety of tumors, including nasopharyngeal carcinoma, liver cancer, colorectal cancer, meningioma, and leukemia. In vitro studies revealed that MEG3 overexpression can inhibit multiple cancer cells proliferation, suggesting that lncRNA MEG3 may be treated as a tumor suppressor gene in tumor occurrence and development [22]. The function and mechanism of lncRNA MEG3 in NSCLC have not been elucidated. This study confirmed that compared with normal 16HBE cells, MEG3 expression in lung cancer A549 cells was significantly decreased, which was in consistent with previous report in other tumors [22]. LncRNA MEG3 overexpression inhibited tumor cell proliferation, elevated tumor cell apoptosis rate, and reduced cell invasion, indicating that lncRNA MEG3 may inhibit NSCLC proliferation and invasion. As an important member of the type I growth factor family, and also a member of the erbB family, EGFR has tyrosine kinase activity to promote tumor proliferation, infiltration, and angiogenesis [23]. Our results demonstrated that lncRNA MEG3 overexpression can inhibit EGFR expression, thus to affect NSCLC proliferation and invasion.

In conclusion, LncRNA MG3 can suppress NSCLC proliferation, facilitating cell apoptosis, and inhibit cell invasion through mediating EGFR. It can be used as a new diagnosis and molecular biological target for NSCLC.

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Disclosure of conflict of interest

None.

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