MiR-222 promotes proliferation, migration and invasion of lung adenocarcinoma cells by targeting ETS1

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Abstract. – OBJECTIVE: The lung adenocarcinoma is a type of lung cancer. This research is to investigate the effects of miR-222 on the proliferation, migration and invasion of the lung adenocarcinoma cells.
MATERIALS AND METHODS: At the beginning, MiR-222 and the controls were transfected to the lung adenocarcinoma cell line A549 for CCK-8 proliferation, transwell migration and Matrigel invasion, and then observed the effect of miR-222 on the proliferation, migration and invasion of lung adenocarcinoma cells. The miR-222 target was regulated by ETS1 downwards to participate in the regulation of the process by using the luciferase reporter assay, the Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) and the Western blotting.
RESULTS: According to CCK-8 proliferation assay, the Transwell migration and the Matrigel invasion assay, it discovered that MiR-222 can promote the proliferation, migration and invasion of the lung adenocarcinoma cells. Luciferase reporter assay, RT-qPCR and Western blot assay showed that miR-222 could regulate the expression of ETS1 downwards and ETS1 participated in the regulation of the process
CONCLUSIONS: ETS1 promotes proliferation, migration and invasion of lung adenocarcinoma cells by targeting the regulated miR-222 downwards.

Key Words:
    miR-222, ETS1, Proliferation, Migration, Invasion.

Introduction

The lung adenocarcinoma (LAC) is a histologic type of lung cancer, accounting for 30-35% of all cases. LAC is considered as a pathologic classification of non-small cell lung cancer[1]. There is a high frequency of occurrence worldwide, including China, and the malignant degree of lung adenocarcinoma is relatively high, with a five-year survival rate of about 15%[2,3]. The early adenocarcinoma is located at the periphery of the lung with clear boundaries; however, the characteristics of highly invasive and destructive growth of adenocarcinoma can easily invade the blood vessels and lymphatic vessel walls and cause blood and lymph node metastasis[3]. The occurrence and development of lung adenocarcinoma is a complex process, including the abnormal proliferation of cells, the epigenetic changes, the transfer of cancer cells and other processes[4,5]. The role of miRNAs in the occurrence of lung adenocarcinoma has widely been concerned, particularly the role of cell proliferation, the metastasis and the role of resistance to chemotherapy drugs. The expression levels of a variety of miRNAs in the lung cancer tissues and the patient's serums were changed; however, there were only a few studies[6] on the role of miRNAs in the lung adenocarcinoma. Previous researches[7-14] reported that miR-222

was highly expressed in many tumors, such as glioblastoma, non-small cell lung cancer, prostate cancer, breast cancer, colorectal cancer and bladder cancer, etc. Functional studies[7] showed that MiR-222 activated the PI3K/AKT signaling pathway by targeting the expression of phosphatase and the tension homology as deleted on chromosome ten (PTEN) and promoted the proliferation, migration and microtubule formation of tumor cells. Besides, miR-222 was highly regulated upwards in several solid tumors and can act on the transcription factor proto-oncogene ETS1 to promote the expression level of endothelial nitric oxide synthase (eNOS)[15]. In melanoma, miR-222 promoted the tumor metastasis by regulating the expression of ETS1 downwards[16]. To identify the role of miR-222 in the occurrence of lung adenocarcinoma, in this paper, we observed the effects of miRNA on proliferation, migration and invasion of lung adenocarcinoma cells via overexpression of miR-222, while the target genes involved in the process of miR-222 were also discovered.

Materials and Methods

Cells and Plasmids

Human lung adenocarcinoma cell line A549[16,17] and human embryonic kidney epithelial cell line 293T cells were purchased by Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), A549 cells among which were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL) and 293T cells were grown in 10% Dulbecco's Modified Eagle Medium Fetal Bovine Serum (DMEM FBS) medium. Ranilla luciferase expression plasmid pRL-TK was purchased from Promega (Madison, WI, USA). pGL3-control reporter vector was purchased from our hospital, the plasmids of ETS1 3 'UTR sequence were constructed in the downstream of the pGL3-control luciferase sequence, and then verified by sequencing and naming pGL3-ETS1 3 'UTR.

Reagents

The Trizol reagent, the protein molecular weight marker and the transfection reagent Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Protease inhibitor, phosphatase inhibitor and phenylmethylsulfonyl fluoride (PMSF) were purchased from Kan-

gcheng Biology Inc. (Shanghai, China) Anti ETS1 monoclonal antibody, anti α-tubulin monoclonal antibody and horseradish peroxidase labeled goat anti-mouse IgG were purchased from Merck Millipore (Billerica, MA, USA). CCK-8 chemical reagents were purchased from DOJINDO Chemistry Lab (Kumamoto, Japan). Transwell (8 μm) was purchased from Merck Millipore (Billerica, MA, USA). BD Matrigel™ Basement Membrane Matrix was purchased from BD Biosciences (San Jose, CA, USA). MiR-222 mimic and negative control (NC) were synthesized by Jima Biotech Co., Ltd. (Shanghai, China). Quantitative primers were synthesized by Shanghai Shenneng Bocai Biotech Co., Ltd. (Shanghai, China).

Luciferase Reporter Assay

293T was inoculated into the 48-well plate with 2×10^4/well and 0.2 ml DMEM medium was added. On the next day, miR-222 and the control NC were transfected with pGL3-Control or pGL3-ETS1 3 'UTR and pRL-TK plasmid into the cell; the cells were then collected by using GLOMAX multifunctional enzyme standard instrument Promega (Madison, WI, USA) for Luciferase Report Analysis 48 h later.

Detection of mRNA Expression on ETS1 Gene by Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

5×10^4 A549 was placed in a 6-well plate one day before the experiment and the cell adhesion was observed on the next day and the cell convergence rate reached 60-70%. The experimental procedures followed the recommended instruction of Lipofectamine™ 2000. MiR-222 and control NC were then transfected into A549 cells respectively. After 48 h, the trizol reagent was used to collect cells, extract RNA and reverse transcription. Thus, the reaction system was established according to the instructions of instSYBR Premix Ex Taq™ (Tli RNaseH Plus) Wuhan Kehaojia Biotech (Co., Ltd., Wuhan, Hubei, China). The RT-qPCR experiments were carried out by using ABI7300 (Guangdong Huayunyi Bio-Engineering Co., Ltd., Guangzhou, Guangdong, China) and the primer sequences used were shown in Table I. The reaction temperature: pre-denaturation at 95°C for 30 s, at 95°C for 5 s, at 60°C for 31 s for 40 cycles, and Ct value was obtained. Ct values were calculated and compared to the expression levels of the target genes in each sample according to the recommended instructions of ABI

PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA, USA).

Primer sequences are stated as follows:

ETSl: F: 5' GTG GTG AGG CAA GGA CCT AG 3', R: 5' TGA GTT GCC ATC TCA TCC CA 3';

β-actin: F: 5'- TTG CCG ACA GGA TGC AGA AGG A -3',

R: 5'- AGG TGG ACA GCG AGG CCA GGA T -3'.

Western Blot

We collected cell proteins and performed Western blot 48 h after MiR-222 and the control NC was respectively transfected into A549 cells. 20-μl-protein sample was added to each well, then the constant pressure 60 V electrophoresis was firstly carried out for 30 min. After the protein samples entered the separating gel, 110 V electrophoresis was then carried out for 90 min and afterwards the constant current 80 mA for 100 min to transfer the proteins into polyvinylidene difluoride film (PVDF) (Dongguan Dean Plastic Technology Co., Ltd., Dongguan, Guangdong, China). After the transfer was completed, the film with proteins was sealed with 5% skim milk at 37°C for 1 h, and then washed with TBS-T once in every 5 min. The anti ETS1 antibody was prepared by adding antibody dilution 1:1000 and placed at 4°C overnight and washed with tris buffered saline-tween (TBS-T) 3 times for 10 min. The horse radish peroxidase (HRP) labeled Goat anti-mouse Ig was prepared by adding antibody dilution 1:4000 and placed at 37°C for 1 h, and washed with TBS-T 3 times for 10 min followed by chemiluminescence detection. At meanwhile, the anti-α-tubulin prepared by the antibody dilution 1:1000 was considered as the first antibody, HRP labeled Goat anti-mouse antibody-by-antibody dilution 1:4000 as the second antibody, as reference of α-Tubulin detection.

CCK-8 Proliferation Assay

We transfected miR-222 and the control NC into A549 cells one day before experiment, and then treated with trypsin digestion cells in 96-well plate (3000 cells/well) on the next day, and incubated at 37°C with 5% CO_2 in incubation box. 10 μl CCK-8 was added to each well for 1 h after 1 d, 2 d, 3 d, 4 d and 5 d of incubation respectively. The OD-value on each well was measured by Biotech DR-3506 automatic enzyme mark reader (Vicenza, Italy) with the blank-well valued 0 and 450 nm wavelengths.

Transwell Migration Assay

We performed the same procedure of transfection of miR-222 and the control NC into A548 cells one day before experiment, and the transwell migration experiment on the next day. 500 μl 10% fetal bovine serum (FBS) 1640 medium was firstly added in the 24-well plate with transwell chamber on the wells, and then put in the incubation for 30 min. After digestion of the transfected miR-222 and the control NC cells, the cell suspension was prepared by the cells with pure 1640 medium into $5x10^5$/ml. On each chamber, we added 200-μl cell suspensions to make about 1×10^5 cells on each well. We set each group with 3 wells, put the 24-wells plate into cell incubator and removed the chamber for staining at 6 h and 12 h, and then observed the number of cells to determine the cell migration.

Matrigel Invasion Assay

We mixed the Matrigel and 1640 medium at the ratio 2:1 to a Matrigel-1640 mixture and placed them on ice for reserve. Transwell chambers were put in the 24-wells place, with 500-μl 10% fetal bovine serum (FBS) 1640 medium at the bottoms, and 60 μl Matrigel-1640 mixture in each chamber. Then, we put the chambers in incubation for 5 h at 37°C with 5% CO_2. After digestion of the transfected miR-222 and the control NC cells, the cell suspension was prepared by the cells with pure 1640 medium into $5x10^5$/ml. On each chamber, we added 200 μl cell suspension to make about 1×10^5 cells on each well. We set each group with 3 wells, put the 24-wells plate into the cell incubator and removed the chamber for staining at 6 h and 12 h, and then observed the number of cells to determine the cell migration.

Statistical Analysis

SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis and processing. The main observation data was the measurement data, which was expressed as x±S. The method for the comparison of the two groups was *t*-test, for partial state data was rank test with *p*<0.05 as statistically significant difference.

Results

Overexpression of miR-222 Promoted the Proliferation of Lung Adenocarcinoma Cells

MiR-222 and control NC were transfected into A549 cells respectively, and CCK-8 proliferation assay was carried out. Results showed that after
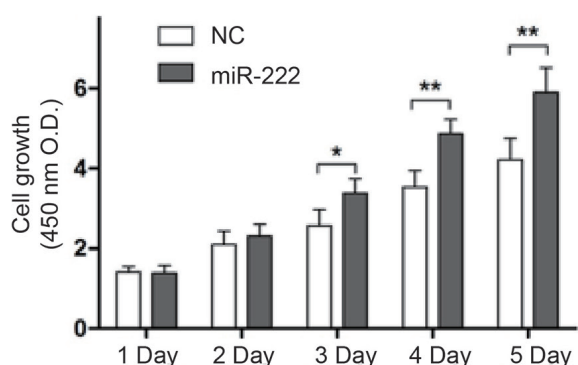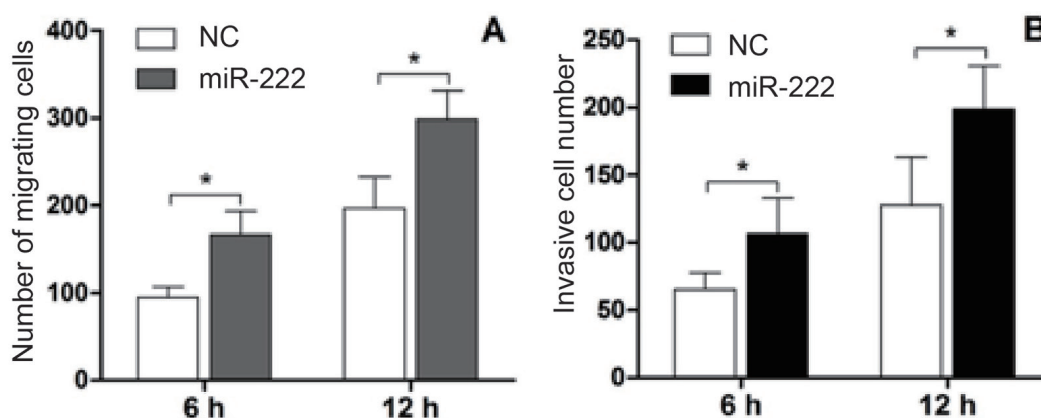
Figure 1. Overexpression of miR-222 promotes proliferation of lung adenocarcinoma cells after transfection into A549 cells. *$p<0.05$; **$p<0.01$.

day 3, day 4 and day 5, the proliferation of A549 cells was significantly higher than that in the control group and the difference was statistically significant (Figure 1).

Overexpression of miR-222 Promoted Migration and Invasion of Lung Adenocarcinoma Cells

Transwell migration and Matrigel invasion experiments were performed on A549 cells transfected with miR-222 and control NC respectively. Results showed, when compared with the control group, the transfected miR-222 cells at 6 h or 12 h to the number of cell migration and invasion chamber bottom increased significantly, and the difference was statistically significant (Figure 2A and Figure 2B). The overexpression of miR-222 significantly promoted the migration and invasion of A549 cells.

Effect of miR-222 Targeting on ETS1

To verify whether the ETS1 was a direct target gene for miR-222, a double reporter luciferase test was carried out. Results showed that miR-222 could significantly inhibit the activity of pGL3-ETS1 3'UTR luciferase reporter plasmid on the premise that the empty plasmid was not affected (Figure 3A, $p<0.01$). The result of microRNA acting on target gene was to inhibit the expression level of the target gene; therefore, we transfected miR-222 and the control NC into A549 cells respectively, and the expression level of ETS1 was detected by RT-qPCR and the Western blot assay. Results showed that the mRNA expression level of ETS1 was significantly decreased when compared with that in the control group after the expression of miR-222 (Figure 3B, $p<0.001$). The Western blot assay showed that the expression level of ETS1 was significantly lower than that of the control NC after transfection with miR-222 (Figure 4). These results indicated that ETS1 was a direct target gene of miR-222.

Overexpression of ETS1 Inhibited the Proliferation of Lung Adenocarcinoma Cells

To show whether ETS1 was involved in the proliferation of lung adenocarcinoma cell line miR-222, we transfected the ETS1 plasmid and the control group into the NC and miR-222 cells respectively to carry out the CCK-8 proliferation assay. Results showed that it could significantly inhibit the proliferation of A549 cells after the expression of ETS1 (Figure 5).

Figure 2. Overexpression of miR-222 promotes migration and invasion of lung adenocarcinoma cells. **A,** Transwell migration experiment showed that the number of migrating cells in miR-222 group was significantly higher than that in control group (*$p<0.05$); **B,** Matrigel invasion test showed that the number of invasive cells in miR-222 group was significantly higher than that in control group (*$p<0.05$).

Figure 3. MiR-222 targeting effect on ETS1. **A,** I: pGL3-control; II: pGL3-ETS1 3'UTR. Effect of miR-222 on pGL3-ETS1 3'UTR activity by luciferase reporter assay (** *p*<0.01). **B,** Detection of ETS1 mRNA expression level by RT-qPCR (** *p*<0.01)
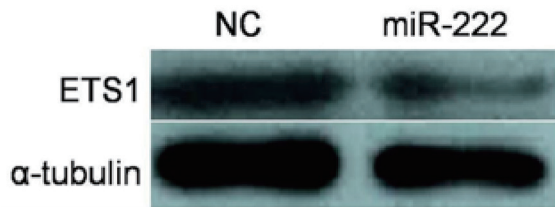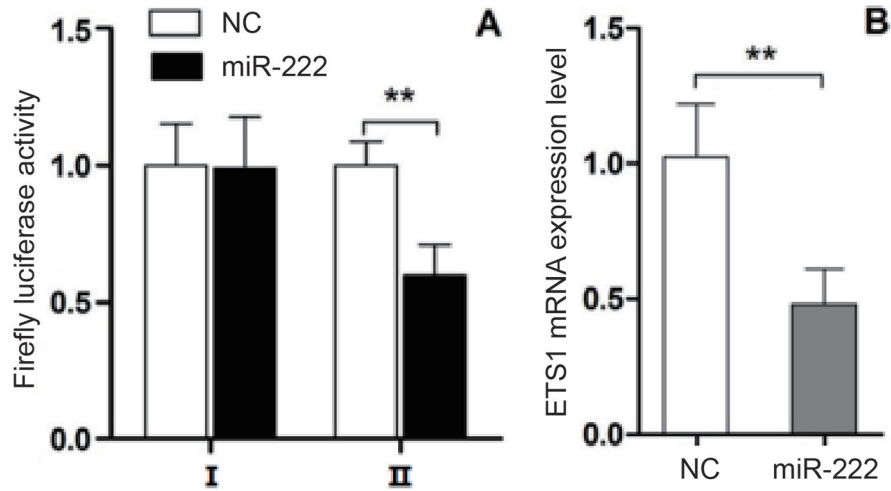
Figure 4. Detection of ETS1 protein expression by Western blot.

ma cell line miR-222, we transfected the ETS1 plasmid and the control group into the NC and miR-222 cells for Transwell migration and Matrigel invasion assay. Results showed that the migration and the invasion ability of A549 cells after ETS1 were significantly decreased in both 6 h and 12 h, and the difference was statistically significant (Figure 6). Results suggested that miR-222 promoted the migration and invasion of A549 cells by targeting ETS1.
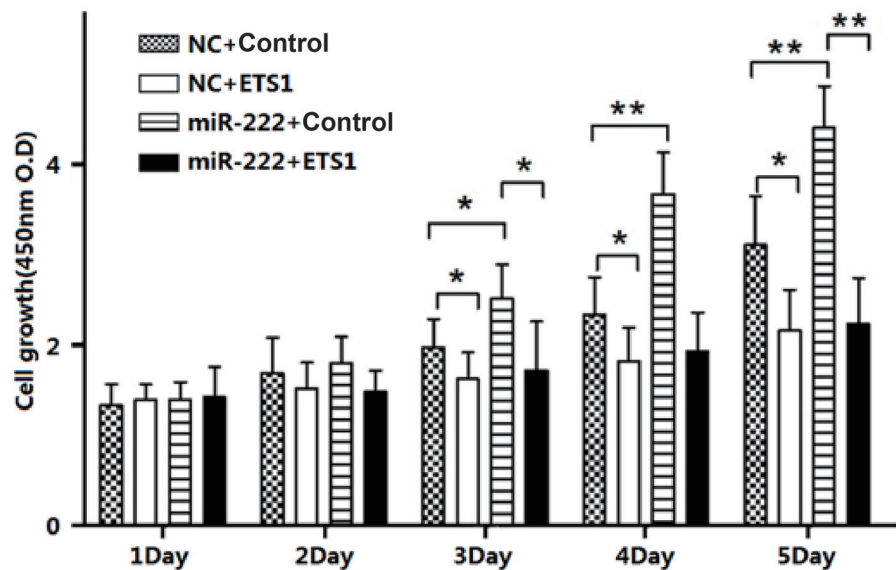
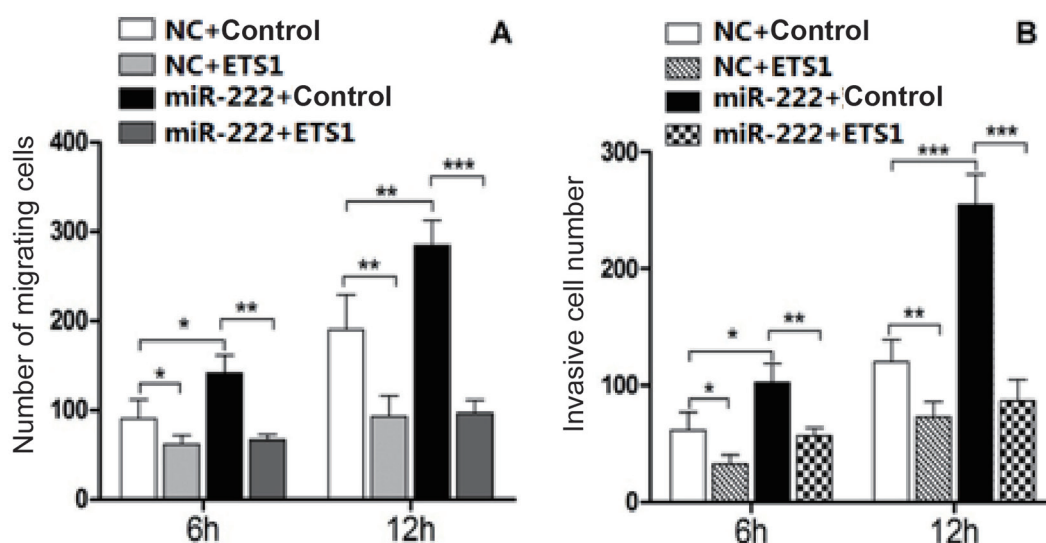## Overexpression of ETS1 Inhibited Migration and Invasion of Lung Adenocarcinoma Cells

To demonstrate whether ETS1 was involved in the migration and invasion of lung adenocarcino-

Discussion

Lung cancer is the most common malignant tumor with high mortality, and about 1.3 million

Figure 5. Overexpression of ETS1 inhibits proliferation of lung adenocarcinoma cells. * *p*<0.05; ** *p*<0.01.

Figure 6. Overexpression of ETS1 inhibits migration and invasion of lung adenocarcinoma cells. **A,** Overexpression of ETS1 significantly inhibited the migration ability of A549 cells (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). **B,** Overexpression of ETS1 significantly inhibited the invasiveness of A549 cells (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

people die of lung cancer each year in the world. Non-small cell lung cancer accounts for 85% of all lung cancers and 40% of non-small cell lung cancer is lung adenocarcinoma. Although the current diagnosis and treatment modalities continue to make innovation, the prognosis of lung adenocarcinoma is still poor; many patients have metastasis and the five-year survival rate is only 15%[2]. Therefore, it is necessary to have more researches on the mechanism of lung adenocarcinoma, in particular about metastasis, to provide a theoretical basis for further treatment. MiR-222 belongs to the miR-221/miR-222 family, which is highly expressed in many tumor-related microRNAs as involved in the proliferation and metastasis of many tumor cells. Mao et al[18] found that miR-222 has a higher expression level in the cancer tissues when compared with the non-small cell lung cancer tissues and the adjacent normal tissues. The high expression of miR-222 was significantly associated with clinical staging and lymph node metastasis and the patients with higher miR-222 expression had shorter survival time, which suggested that the miR-222 was involved in the occurrence and metastasis of non-small cell lung cancer. Another research group[19] found that the overexpression of miR-222 can significantly promote the proliferation of non-small cell lung cancer cell line H460, but there was no report about the study of miR-222 in the lung adenocarcinoma cell line A549. Thus, the effect of miR-222 on lung adenocarcinoma was studied in

this paper. In the lung adenocarcinoma cell line A549, the overexpression of miR-222 was found in functional studies. MiR-222 has significantly promoted the proliferation, migration and invasion of lung adenocarcinoma cells. MicroRNA is known to exert the biological function by regulating the expression of the target genes downward and the miR-222 was reported to be capable of targeting p27 to promote the proliferation of non-small cell lung cancer. As a matter of fact, a microRNA can act on multiple target genes, so that we can look for miR-222 target genes involved in the process. It was found that the miR-222 can be targeted to ETS1, but the interaction between miR-222 and ETS1 in lung cancer has not been reported. Hence, this work was confirmed by luciferase reporter assay and Western blot, and the ETS1 was indeed a target gene of miR-222, which was consistent with the study of Evangelista et al[14]. Then, based on the functional experiments, we found that after over expression of ETS1, the proliferation, migration and invasion capacity of A549 cells decreased significantly. These results suggested that miR-222 indeed promoted the proliferation, migration and invasion of A549 cells by inhibiting the expression of ETS1. ETS1, as a member of the transcription factor family, is involved in the regulation of transcription of many genes, and ETS1 plays multiple roles in the process of disease[20]. Low levels of ETS1 have been reported to be involved in the drug resistance of non-small cell lung cancer[21]. In this paper, it is

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suggested that miR-222 can inhibit the transcription of several genes by regulating the expression of ETS1 downwards, which is conducive to the proliferation, migration and invasion of lung adenocarcinoma cells.

Conclusions

Through the over-expression of miR-222 in lung adenocarcinoma cell A549, we found that miR-222 was expressed by targeting down-regulated transcription factor ETS1 to promote the proliferation, migration and invasion of A549 cells, which proved the role of miR-222 in lung cancer. This study provided more theoretical basis for biomarkers and new therapeutic targets for miR-222 in the diagnosis of lung cancer, especially the lung adenocarcinoma.

Conflict of interest
The authors declare no conflicts of interest.

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