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# Low expression of lncRNA MEG3 promotes the progression of oral squamous cell carcinoma by targeting miR-21

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**Abstract. – OBJECTIVE: The aim of this study was to explore whether maternally expressed gene 3 (MEG3) could facilitate the proliferation and migration of oral squamous cell carcinoma (OSCC) cells by selectively binding to miR-21, thereby participating in the progression of OSCC.**

**PATIENTS AND METHODS: The expression levels of MEG3 and miR-21 in OSCC tissues and normal control tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The effects of MEG3 and miR-21 on cell proliferation and migration were examined by cell counting kit-8 (CCK-8), transwell, and scratch assay, respectively. Meanwhile, cell cycle was detected using flow cytometry. The binding relationship between miR-21 and MEG3 was confirmed by dual luciferase assay. In addition, MEG3 and miR-21 were simultaneously knock-down to figure out whether MEG3 could regulate the proliferation and migration of OSCC cells through targeted binding to miR-21.**

**RESULTS: QRT-PCR results indicated that MEG3 expression in OSCC tissues was remarkably lower than that of normal control tissues. However, the expression of miR-21 was significantly higher in OSCC tissues. Meanwhile, it was found that inhibiting MEG3 expression in OSCC cell lines could significantly promote cell proliferation and migration, while the simultaneous inhibition of miR-21 showed the opposite effect. Dual Luciferase assay results revealed that MEG3 could selectively bind to miR-21. In addition, we demonstrated that the knockdown of MEG3 in Tca-8113 and CAL-27 cells partially reversed the inhibitory effect of downregulated-miR-21 on cell proliferation and migration. These results further suggested that MEG3 might regulate OSCC cell proliferation via selectively binding to miR-21.**

**CONCLUSIONS: Low expression of MEG3 can promote the proliferation and migration of OSCC cells through targeted binding to miR-21.**

*Key Words:*
MEG3, MiR-21, Cell proliferation, Cell migration, OSCC.

## Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor that occurs in the oral and maxillofacial region. Smoking, alcohol, local adverse stimuli, papillomavirus and biological factors are all risk factors for OSCC, among which the former two are at greater risk[1]. The predilection sites of oral cancer also change in different periods. In the late stage of OSCC, the tongue, larynx, jaw, neck, important blood vessels, functional nerves and skull base are all involved. Meanwhile, it is often accompanied by some dysfunctions, including language disorder, swallowing disorder, pain, and numbness. All of the disorders have a great impact on the quality of patients' life[2]. Some studies have shown that the incidence of malignant tumors is a complex biological process. Influenced by various factors, normal cells may undergo gene mutation, oncogene activation or tumor suppressor gene inactivation, eventually leading to abnormal division and proliferation[3]. Some non-coding RNAs can be used as oncogenes or tumor suppressor genes[4] to regulate cell proliferation, invasion and apoptosis, thus participating in the incidence and progression of OSCC[5,6].

Different RNAs have their own different biological functions. According to the length of RNA molecules, all non-coding RNAs can be classified as multiple types, including snoRNA, microRNA, piRNA, tRNA and snRNA[7,8]. Among them, long-chain non-coding RNA (1ncRNA) is defined as a type of non-protein coding RNA with

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over 200 nt in length. LncRNA is transcribed by RNA polymerase II/I, and only a small fraction is transcribed by RNA polymerase III. Meanwhile, lncRNA plays a crucial role in the growth and development of various diseases, especially malignant tumor[9]. With the increasing research on lncRNA, the biological role of lncRNA in cancer and the underlying molecular mechanism are becoming more and more explicit. Meanwhile, lncRNA can participate in a variety of biological processes, such as epigenetic modification, selective splicing, RNA degradation and transcription processes[10].

LncRNA-MEG3 (maternally expressed gene 3) is a non-coding RNA molecule containing no open reading frame, which is located on human 14q32. It has a total length of about 1700 nucleotides and 12 transcripts[11]. MEG3 plays a key role in the progression of various tumors through different mechanisms. Currently, it is recognized that MEG3 can increase the protein content of P53 in the human body and further target GDF15 promoter to increase its expression, eventually affecting the proliferation and differentiation of cancer cells. Furthermore, low expression of MEG3 can reduce the degradation of P53 by decreasing the content of MDM2, as well as increasing the overall level of P53[11,12].

MEG3 is significantly down-regulated in OSCC tissues[13], which predicts poor prognosis of OSCC patients. However, the specific underlying mechanism remains elusive. In this work, we explored the biological function of MEG3 in OSCC and further investigated the possible underlying mechanism.

## Patients and Methods

### Specimen Collection and Processing

Fresh OSCC tissues and normal control tissues were collected from 45 OSCC patients who received surgery in Yongchuan Hospital of Chongqing Medical University from 2014 to 2017. All subjects received no treatment before surgery. Patients volunteered to participate in the study and the signed informed consent was obtained from each patient before it. This investigation was approved by the Ethics Committee of the hospital.

### Cell Culture and Transfection

Normal control cell line (HIOEC) and OSCC cell lines (SCC25, CAL27, HB, and Tca-8113) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% newborn bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C incubator with 90% relative humidity and 5% $CO_2$. After the confluence of cells reached 70% to 80%, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). After removing PBS, the transfection reagent mixed with Lipofectamine 2000 was added to the culture dish according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After 6 hours, the culture medium was replaced with complete medium. The transfection efficiency was verified by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). MiRNA mimics and MEG3 siRNA used in this study were provided by Shanghai Jima Company (Shanghai, China).

### RNA Extraction

Total RNA in serum, cells or tissues was extracted as follows: 0.25 mL serum was added to 0.75 mL TRI Reagent LS. Subsequently, 0.2 mL chloroform was added in an Eppendorf (EP) tube, followed by incubation for 2-5 minutes. After centrifugation at 12000 g for 15 minutes at 4°C, the aqueous phase complex was transferred to another centrifuge tube. RNA was extracted by adding isopropanol to the mixture. After centrifugation, RNA precipitate was washed with 75% ethanol. Then, the RNA precipitate was dried in air for 5 minutes, dissolved in RNase-free water and stored at -80°C until use.

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

Reverse transcription reaction system was prepared on ice using the PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan). The reaction was terminated to obtain cDNA. Mature miRNAs were detected using TaqMan MicroRNA Assays. MiR-21 and U6 were used as templates; meanwhile, miR-21 and U6 fluorescent probes were used as primer amplification. The specific reaction was carried out in ABI Step OneTM Real-Time PCR System (Foster City, CA, USA). The PCR amplification conditions were: pre-denaturation at 94°C for 5 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and 30 s, for a total of 40 cycles. Prim-

ers used in this study were as follows: MEG3 (F: 5'-TCCATGCTGAGCTGCTGCCAAG-3', R: 5'- AGTCGACAAAGACTGACACCC-3'), GAPDH (F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'), miR-21 (5'-GATACCAAAATGTCAGACAGCC-3'), U6 (F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'). Relative expression of the target gene was calculated by the $2^{-\Delta\Delta CT}$ method. Each experiment was repeated 3 times.

### Cell Counting Kit-8 (CCK-8) Assay

Cells in logarithmic growth phase were selected seeded into 96-well plates at a density of $1\times10^6$/mL. After 6 h, 10 μL CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well, followed by incubation at 37°C for 2 hours in the dark. After the culture solution was aspirated, the absorbance of each well was measured at 450 nm by a microplate reader after 6 h, 24 h, 48 h, and 72 h, respectively.

### Transwell Assay

A total of $1\times10^5$ transfected Tca-8113 and CAL-27 cells were seeded into the upper chamber with serum-free medium. The complete medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) was added to the lower chamber as a chemotactic agent, and the cells were invaded at 37°C and 5% $CO_2$ for 48 h. The cells invading the lower surface of the filter were fixed with 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min. The number of cells that migrated to the lower chamber was counted under an inverted microscope. 5 fields were randomly selected for each sample. This experiment was repeated three times.

### Cell Scratch Test

Tca-8113 and CAL-27 cells in logarithmic growth phase were seeded into 6-well culture plates at a density of $1\times10^6$ cells/well, respectively. After 24 h, the tip of the pipette was used to scratch a line in the middle of each well. The width and length of the scratch were 1 mm and 20 mm, respectively. Then serum-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) was added to continue cell culture. At the end of the scratch, 2 points were selected per well to take pictures under an inverted microscope (0 h). Then the cells were cultured in an incubator for 24 h, followed by taking out to be photographed again.

### Flow Cytometry

Cells in each group were collected, and the concentration was adjusted to about $1\times10^5$/mL. Subsequently, the cells were fixed with 1 mL 75% ethanol pre-cooled at -20°C and stored in a 4°C refrigerator overnight. Afterward, the fixative was washed with PBS twice. 100 μL RNaseA was added, and the cells were incubated at 37°C water bath for 30 min in the dark. Next, 400 μL Propidium Iodide (PI) staining was added and mixed. After 4 min at 4°C, flow cytometry was performed to detect and record the cell cycle at the excitation light wave of 488 nm (red fluorescence).

### Luciferase Reporter Gene Assay

Bioinformatics methods were used to predict the potential binding sequences of miR-21 to MEG3. Human MEG3 3'-UTR wild sequence or a MEG3 3'-UTR mutant sequence containing a predicted target site was inserted into the pGL3 promoter vector. One day prior to cell transfection, Tca-8113 and CAL-27 cells were seeded into 24-well plates ($5\times10^5$ cells/well). After co-transfecting 0.12 μg Luciferase reporter vector and 40 nM miR-21 mimics or negative control into the two cell lines for 48 h, the Luciferase activity of each well was examined.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) and GraphPad Software (La Jolla, CA, USA) were used for all statistical analysis. Chi-square test was used for classification data and the Student's *t*-test was used for the measurement data. All measurement data were expressed as mean ± s. $p < 0.05$ was considered statistically significant.

## Results

### MEG3 Was Lowly Expressed in OSCC Tissues

We first detected the expression of MEG3 in 45 OSCC tissues and normal control tissues by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Results showed that the expression of MEG3 was significantly decreased in OSCC tissues (Figure 1A). Meanwhile, MEG3 expression in late OSCC tissues was remarkably lower than that of earlier stages (Figure 1B), suggesting that MEG3 might be involved in the

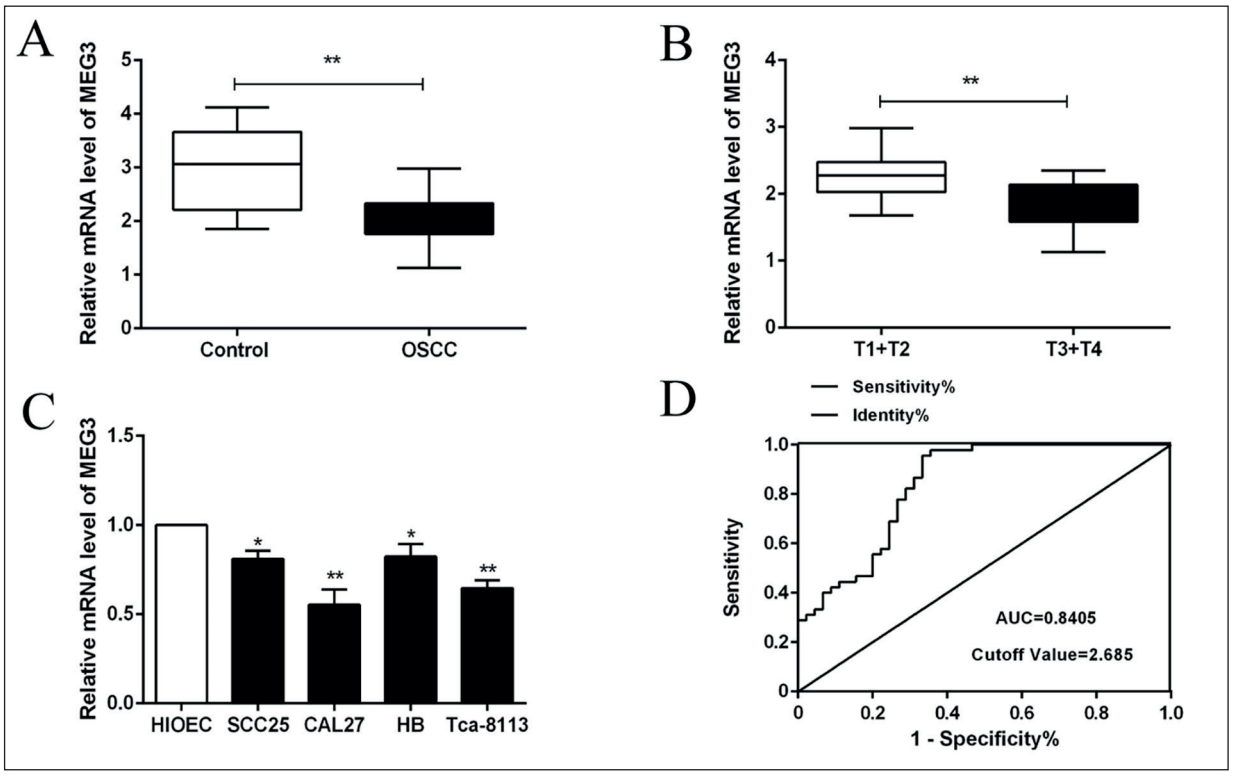


**Figure 1.** Low expression of MEG3 in OSCC tissues and cells. ***A,*** MEG3 was lowly expressed in OSCC tissues. ***B,*** The expression of MEG3 in tissues of OSCC patients in stage T3 and T4 was lower than those in stage T1 and T2. ***C,*** MEG3 was down-regulated in OSCC cell lines. ***D,*** MEG3 could be used as a diagnostic marker for OSCC (AUC=0.8405, Cutoff Value=2.685).

development of OSCC. Subsequently, we found that MEG3 was also significantly down-regulated in OSCC cells, including SCC25, CAL27, HB and Tca-8113 (Figure 1C). Particularly, Tca-8113 and CAL-27 cells expressed the lowest MEG3 level. ROC curve analysis found that MEG3 could be used as a marker for early diagnosis of OSCC (AUC=0.8405) (Figure 1D).

### *Knockdown of MEG3 Promoted OSCC Cell Proliferation and Migration*

MEG3 siRNA#1, MEG3 siRNA#2 and MEG3 siRNA#3 were transfected into the two selected OSCC cell lines. It was found that MEG3 siRNA significantly inhibited MEG3 expression (Figure 2A), of which MEG3 siRNA#2 presented the highest interference efficiency. Therefore, MEG3 siRNA#2 was selected for subsequent experiments. Then, we performed the CCK8 assay to detect the proliferation of OSCC cells after knocking down MEG3. CCK8 results showed that the MEG3 down-regulation significantly enhanced the proliferation of OSCC cells (Figure 2B). Subsequently, the effects of MEG3 on cell migration and healing ability were examined through transwell and scratch assay, respectively. Data indicated that the inhibition of MEG3 expression remarkably motivated cell migration and healing capacity (Figure 2C, 2D). Meanwhile, flow cytometry revealed a significantly accelerated cell cycle after MEG3 down-regulation (Figure 2E).

### *MEG3 Could be Targeted Bind to MiR-21*

We used bioinformatics to predict the miRNAs that could bind to MEG3 and performed functional analysis. Subsequently, miR-21 was selected (Figure 3A). Luciferase reporter gene assay showed that the Luciferase activity in cells of the MEG3-WT 3'UTR group was significantly reduced after transfection of miR-21. However, there was no significant difference in cells of the MEG3-MUT 3'UTR group (Figure 3B). This indicated that miR-21 could bind to MEG3. After interference with MEG3 in the two cell lines, the expression levels of miR-21

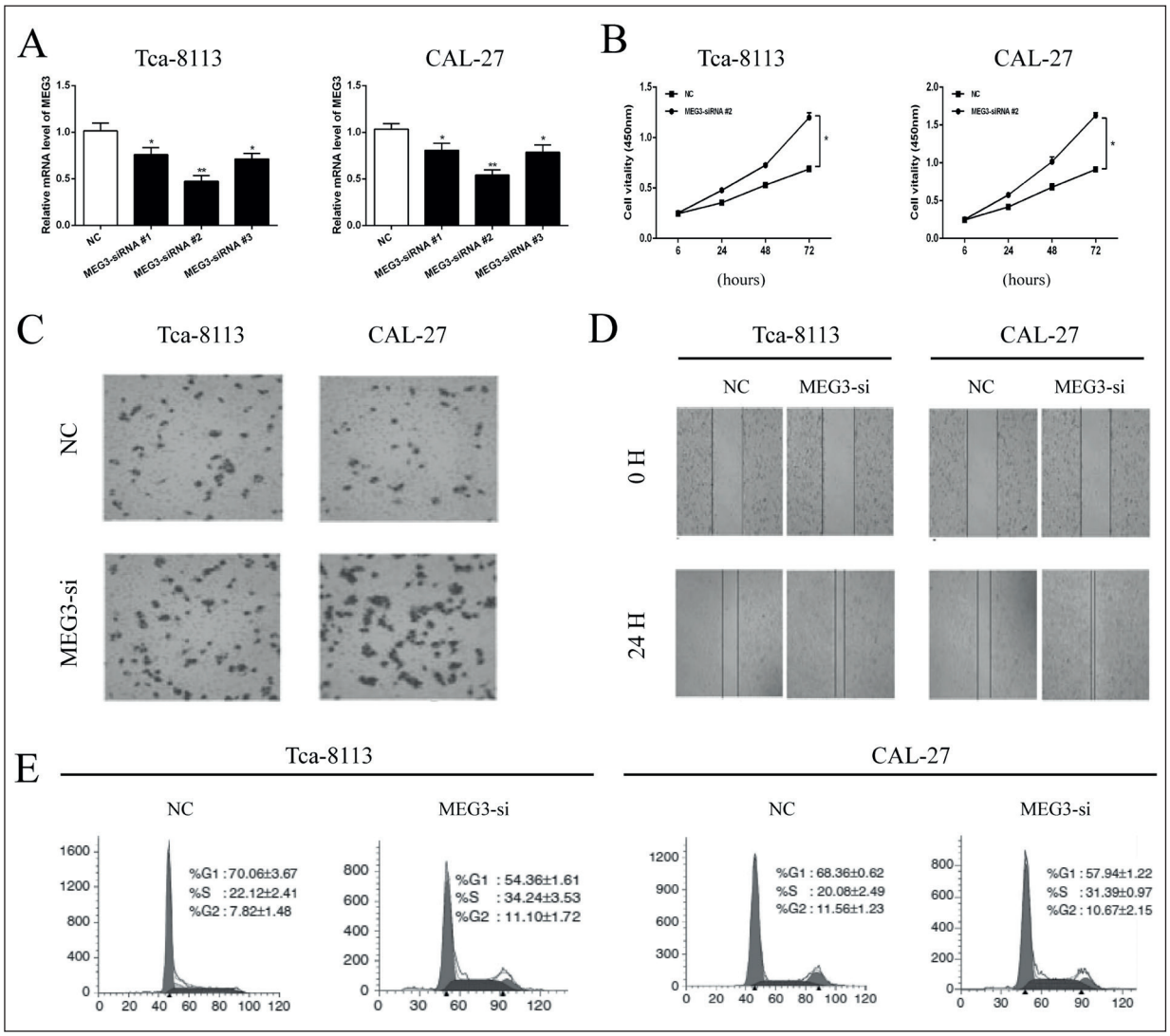


**Figure 2.** Knockdown of MEG3 promoted cell proliferation and migration. ***A,*** After MEG3 was interfered, MEG3 level in Tca-8113 and CAL-27 cell lines was reduced. ***B,*** Knockdown of MEG3 in Tca-8113 and CAL-27 cell lines promoted cell proliferation. ***C,*** Knockdown of MEG3 in Tca-8113 and CAL-27 cell lines promoted cell migration. ***D,*** Knockdown of MEG3 in Tca-8113 and CAL-27 cell lines enhanced cell healing ability. ***E,*** Knockdown of MEG3 in Tca-8113 and CAL-27 cell lines promoted cell cycle.

were significantly enhanced (Figure 3C). Subsequently, we examined the expression level of miR-21 in OSCC tissues and normal control tissues by qRT-PCR. The results showed that miR-21 was highly expressed in OSCC tissues (Figure 3D), especially in the advanced stage (Figure 3E). By analyzing the expressions of MEG3 and miR-21 in OSCC tissues, we found that MEG3 was negatively correlated with miR-21 (Figure 3F). Similarly, ROC curve analysis demonstrated that miR-21 could be used as a marker for early diagnosis of OSCC (AUC=0.8923) (Figure 3G).

### *MEG3 Promoted the Proliferation and Migration of OSCC Cells by Binding to MiR-21*

MiR-21 was downregulated in the two OSCC cell lines (Figure 4A). After interfering with the expression of miR-21 and MEG3, we tested the proliferation and migration of cells. It was found that the knockdown of miR-21 significantly inhibited cell proliferation and migration, while the simultaneous knockdown of MEG3 could reverse this inhibitory effect (Figure 4B, 4C). Meanwhile, the cell cycle showed the same trend (Figure 4D). The above

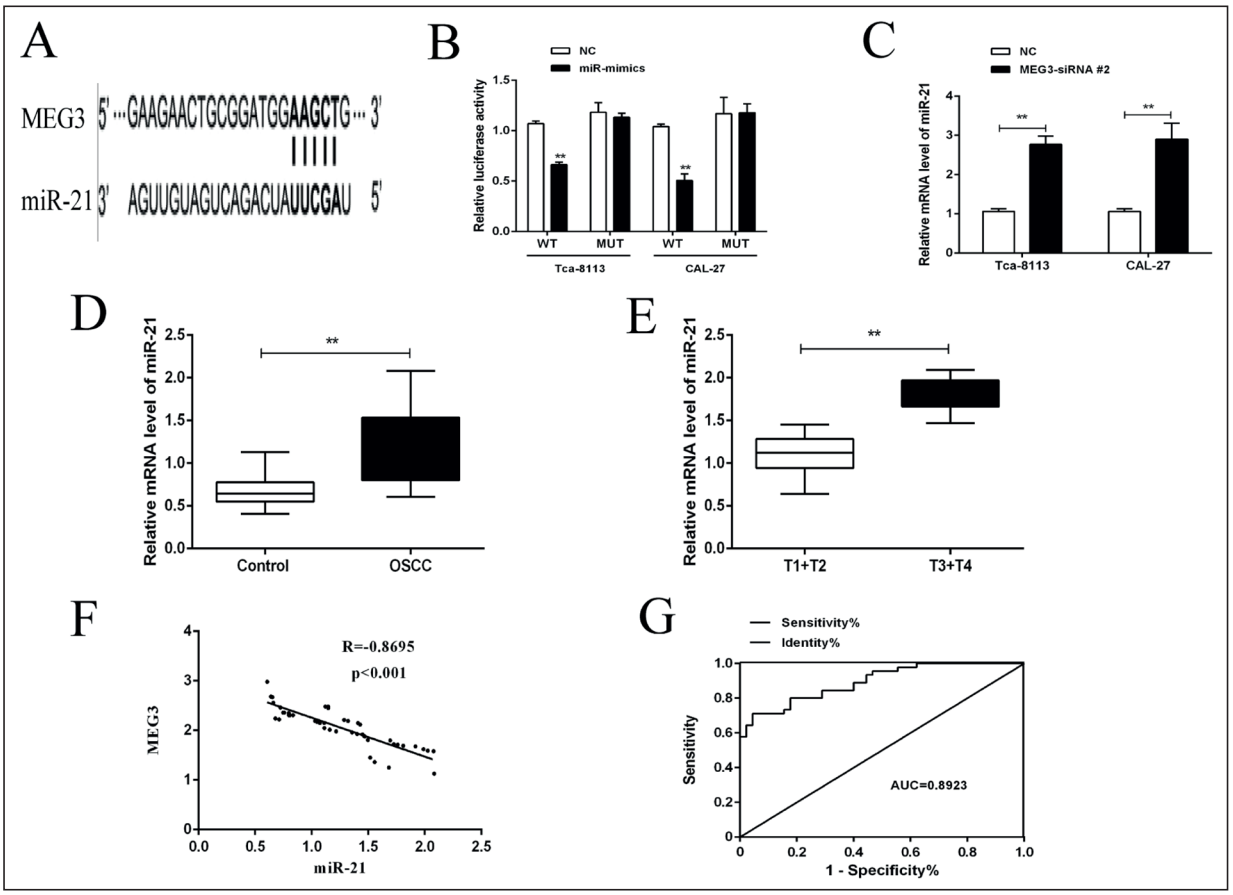


**Figure 3.** MEG3 could selectively combine with miR-21. ***A,*** The binding sites of MEG3 to miR-21 were predicted by bioinformatics. ***B,*** Dual Luciferase assay showed that MEG3 could bind to miR-21. ***C,*** knockdown of MEG3 significantly increased miR-21 expression. ***D,*** MiR-21 was highly expressed in OSCC tissues. ***E,*** The expression of miR-21 in OSCC patients in stage T3 and T4 was higher than those in stage T1 and T2. ***F,*** The expression of MEG3 was significantly negatively correlated with the expression of miR-21. ***G.*** MiR-21 could be used as a diagnostic marker for OSCC (AUC = 0.8923).

results indicated that MEG3 might promote the proliferation and migration of OSCC cells *via* binding to miR-21.

## Discussion

The 5-year survival rate of OSCC is less than 50%. Although epidemiological studies have revealed some common risk factors, the molecular mechanisms of genetics are still unclear[14]. At present, the treatment of OSCC is mainly based on surgical treatment. However, postoperative chemotherapy and radiotherapy are more likely to lead to serious complications, such as the destruction of the body's immunity and necrotizing osteomyelitis. This may eventually threaten patients' life. Therefore, it is necessary to investigate the incidence, development and regulation mechanism of OSCC[15].

MiRNAs are a class of non-coding RNAs that play a very important regulatory role in cell molecular biology. MiRNAs do not encode proteins themselves, but can function *via* base-pairing with complementary sequences within mRNA molecules. Consequently, these mRNA molecules are silenced[16]. The highly conserved, temporal and tissue-specific properties of miRNAs lay the foundation for clinical application. At present, reports have confirmed that the abnormal expression of miRNAs is closely related to the occurrence and development of malignant tumors. MiRNAs have different expressions in different tissues and even different histopathological types of tumors, thus affecting the proliferation and apoptosis of tumor cells[17].

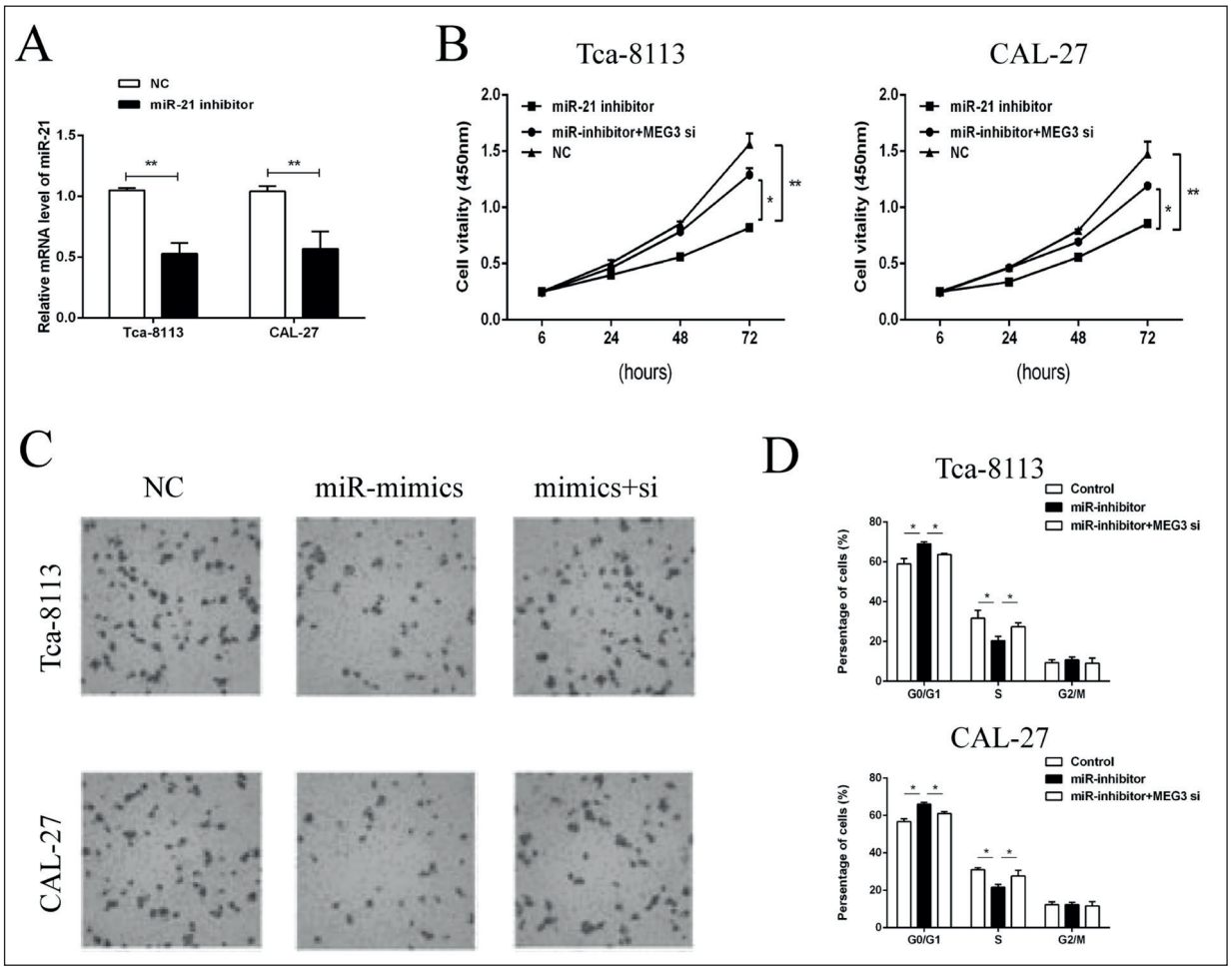


**Figure 4.** MEG3 promoted OSCC cell proliferation and migration by binding to miR-21. ***A,*** MiR-21 expression was decreased after interference with miR-21 in Tca-8113 and CAL-27 cell lines. ***B,*** Knockdown of miR-21 in Tca-8113 and CAL-27 cell lines could inhibit cell proliferation, while the simultaneous knockdown of MEG3 reversed this inhibitory effect. ***C,*** The knockdown of miR-21 in Tca-8113 and CAL-27 cell lines inhibited cell migration, while the simultaneous knockdown of MEG3 reversed this inhibitory effect. ***D,*** The knockdown of miR-21 in Tca-8113 and CAL-27 cell lines arrested cell cycle, while the simultaneous knockdown of MEG3 promoted cell cycle.

MEG3 was the first lncRNA found to have tumor suppressive effects, with approximately 1.6 kb in size. It is expressed in most normal human tissues to varying degrees, which is most expressed in brain tissues and pituitary. However, MEG3 expression is absent or extremely low in various human malignant tumor tissues and cells, such as gastric cancer, cervical cancer, prostate cancer and breast cancer[18-21]. The mechanisms leading to its low expression are mainly through promoter hypermethylation and gene deletion. MiR-21 is one of the human miRNAs discovered earlier. Meanwhile, it has become an important tool in the study of human miRNAs' function due to its clear background[22]. In addition, miR-21 is widely distributed in various mammalian tissues. Therefore, the research on its biological function is especially meaningful[23]. Although miR-21 can be used as a tool to study the mechanism and function of human miRNAs, the function of miR-21 itself has been further investigated. The most prominent results are shown in the relationship between miR-21 and malignant tumor. MiR-21 plays an important role in the pathogenesis of hepatocellular carcinoma, gastric cancer, ovarian cancer and other diseases[24-26].

In our work, we focused on the expression level and function of MEG3 in OSCC tissues and cell lines. We also explored its potential mechanisms that led to the occurrence and

progression of OSCC. It was found that MEG3 expression was remarkably reduced in tumor tissues. Meanwhile, similar results were further verified in OSCC cell lines. These findings suggested that MEG3 was closely related to some biological functions of OSCC. Based on the above studies, we selected Tca-8113 and CAL-27 cell lines for the functional study of MEG3. The findings revealed that low expression of MEG3 promoted the proliferation and migratory capacity of cells. Also, Luciferase reporter gene assay verified that MEG3 could bind to miR-21. The interaction played a vital role in the progression of OSCC. Meanwhile, it was found that the knockdown of miR-21 significantly inhibited the proliferation and migration of OSCC cells. However, the simultaneous knockdown of MEG3 exhibited the opposite results. Therefore, it was speculated that MEG3 might affect the proliferation of OSCC cells by regulating miR-21.

## Conclusions

We found that MEG3 could affect the proliferation and migration of OSCC cells by affecting the activity of miR-21. Our findings suggested that MEG3 might have a diagnostic and therapeutic value in the future treatment of OSCC.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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