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MiR-155 regulates oral squamous cell carcinoma Tca8113 cell proliferation, cycle, and apoptosis via regulating p27Kip1

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Abstract. – OBJECTIVE: P27Kip1 is the one of the negative regulators of the cell cycle that plays an important role in regulating cell cycle and inhibiting cell proliferation by restraining cell in G1 phase. P27Kip1 downregulation maybe related to the occurrence of oral squamous cell carcinoma (OSCC). It was found that miR-155 significantly upregulated in OSCC tissue. Bioinformatics analysis revealed that miR-155 may bind with the 3'-UTR of p27Kip1. This study investigated the role of miR-155 in regulating p27Kip1 and affecting Tca8113 cell proliferation, cycle, and apoptosis.

PATIENTS AND METHODS: A total of 46 cases of OSCC patients received treatment in our hospital were enrolled to obtain tumor tissue. Another 25 normal oral mucosa samples were selected as control to detect the relationship between miR-155 and p27Kip1 expressions. Dual luciferase assay was adopted to confirm the targeted relationship between miR-155 and p27Kip1. Flow cytometry was applied to test cell apoptosis and cell cycle. CCK-8 assay was used to evaluate cell proliferation. Caspase-3 activity was detected by spectrophotometry.

RESULTS: MiR-155 upregulated, while p27Kip1 declined in OSCC tissue compared with normal oral mucosa. Their expressions were related to TNM stage. MiR-155 targeted suppressed p27Kip1 expression. MiR-155 mimic and/or pEGFP-p27Kip1 transfection obviously declined p27Kip1 expression, blocked cell cycle in G1 phase, reduced cell proliferation, enhanced Caspase-3 activity, and increased cell apoptosis in Tca8113 cells.

CONCLUSIONS: MiR-155 increased, while p27Kip1 reduced in OSCC tissue. Inhibition of miR-155 upregulated p27Kip1 expression, blocked cell cycle in G1 phase, weakened cell proliferation, and induced cell apoptosis.

Key Words:
Oral cancer, miR-155, p27Kip1, Apoptosis, Proliferation, Cell cycle.

Introduction

Oral cancer is the most common malignant tumor in oral and maxillofacial region accounting for 3-5% of systemic malignant tumor. It accounts for about 20% of the head and neck malignant tumor[1]. The incidence of oral cancer is only second to nasopharyngeal carcinoma in head and neck malignant tumors[2]. Among all patients with oral cancer, oral squamous cell carcinoma (OSCC) is the main histopathological type with more than 90%[3]. The pathogenesis of oral cancer is complex, such as smoking, drinking, viral infection, malnutrition, and poor eating habits. It is currently considered that smoking and drinking exhibit the largest danger to oral cancer. P27Kip1, also known as Cyclin-dependent kinase inhibitor 1B (CDKN1B), can induce cell cycle G1 phase blockage and suppress cell mitosis and proliferation via inhibiting Cyclin E - CDK2 and Cyclin D - CDK4. As a tumor suppressor gene, the expression and function of p27Kip1 is related to the oncogenesis of laryngocarcinoma[4], prostate cancer[5], gastric cancer[6], and breast cancer[7]. It was showed that p27Kip1 level declined in OSCC tissue and was related to the survival rate and prognosis of patients with oral cancer[8,9]. MicroRNA is a type of non-coding single-stranded RNA molecule in eukaryotes at the length of 18-22 nucleotides. It participates in various biological processes including cell proliferation, cycle, differentiation, and apoptosis by promoting target mRNA degradation or suppressing translation via complete or incomplete complementary binding with target mRNA[9]. More and more researches[10,11] suggest that miRNAs play a critical role in the occurrence of oral cancer. It was showed that miR-155 increased in OSCC tissue, suggesting that miR-155 may be an oncogene in OSCC[8,10,12].

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Patients and Methods

Main Reagents and Materials

Human OSCC cell line Tca8113 was purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Lonza (Valais, Switzerland). Fetal bovine serum (FBS) was got from Gibco (Waltham, MA, USA). Penicillin-streptomycin was bought from CellGro (Corning, NY, USA). RNA extraction kit was bought from OMEGA (Norcross, GA, USA). Lipofectamine 2000 was got from Invitrogen (Carlsbad, CA, USA). ReverTra Ace qPCR RT Kit and SYBR green were from Takara (Dalian, China). Rabbit anti-human p27Kip1 was from Abcam (Cambridge, MA, USA). Mouse anti β-actin was from Santa Cruz (Santa Cruz, CA, USA). Horse radish peroxidase (HRP) labeled secondary antibody was from Jackson ImmunoResearch (West Grove, PA, USA). Annexin V/PI apoptosis detection kit, Cell Cycle Analysis Kit, and RIPA were from Beyotime (Shanghai, China). CCK-8 detection kit was from Dojindo (Tokyo, Japan). Luciferase reporter gene vector pLUC Luciferase vector was obtained form Ambion (West Grove, PA, USA). Dual-Luciferase® Reporter Assay System and pGL3-promoter plasmid were from Promega (Madison, WI, USA).

Clinical Information

A total of 46 OSCC patients received treatment in Jiangsu Cancer Hospital and Institute Affiliated to Nanjing Medical University between Nov 2015 and Apr 2016 were enrolled. Tumor tissue was extracted during the surgery. Another 25 normal oral mucosa samples were selected as control. All the samples were diagnosed by pathology, including 24 cases of tongue cancer, 6 cases of gum cancer, 7 cases of oral floor carcinoma, 5 cases of carcinoma of buccal mucosa, and 4 cases of carcinoma of soft palate. There were 24 males and 22 females with mean age at 61.8 (41-76) years old. According to the TNM staging published by Union for International Cancer Control (UICC), there were 12 cases in stage I, 9 cases in stage II, 15 cases in stage III, and 10 cases in stage IV. No patients received chemotherapy, radiotherapy, or other special therapy. The study was approved by Jiangsu Cancer Hospital and Institute Affiliated to Nanjing Medical University Ethics Committee and all the subjects had signed informed consent.

Cell Culture

Human oral cancer Tca8113 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and cultured in 37°C and 5% CO_2. The cells in logarithmic phase were used for experiments.

Luciferase Reporter Gene Vector Construction and Dual-luciferase Reporter Gene Assay

The full length or mutation fragments of 3'-UTR of p27Kip1 gene was amplified based on HEK293 cell genome. Polymerase chain reaction (PCR) product was recycled and cloned to pLUC to transform DH5α competent cells. The positive clone was screened and the plasmid with correct sequence was applied for cell transfection, named pLUC-P27Kip1-3'UTR-wt and pLUC-P27Kip1-3'UTR-mut, respectively. Lipofectamine 2000 was used to co-transfect pLUC-P27Kip1-3'UTR-wt (or pLUC-P27Kip1-3'UTR-mut) and miR-155 mimic to HEK293T cells for dual-luciferase activity detection.

P27Kip1 Gene Overexpression and Transfection

pEGFP-N3 was selected as plasmid vector, while EcolR1 and Sal1 were chosen as restriction endonuclease to amplify p27Kip1 gene. pEGFP-NC and pEGFP-p27Kip1 plasmids were transfected into Tca8113 cells upon calcium phosphate transfection method. Transfection efficiency was evaluated by flow cytometry. P27Kip1 expression was detected by qRT-PCR and Western blot.

Cell Transfection and Grouping

Tca8113 cells were divided into five groups, including inhibitor NC, miR-155 inhibitor, pEGFP-NC, pEGFP-P27Kip1, and miR-155 inhibitor + pEGFP-P27Kip1 groups. The cells were collected after 72 h for the following experiments.

qRT-PCR

Total RNA was extracted from cells using OMEGA kit. The cells were treated with TRK buffer. After washed by Wash Buffer I and II for twice, the RNA was dissolved in RNase-free

water. Then the RNA was reverse transcribed to cDNA using ReverTra Ace qPCR RT Kit. The reverse transcription system contained 2 μg total RNA, 2 μL RT Buffer (5×), 0.5 μL oligo dT+Random primer Mix, 0.5 μL RT Enzyme Mix, 0.5 μL RNase inhibitor, and ddH_2O. The PCR primers used were as follows. $miR\text{-}155P_F$: 5′-ACGCTCAGTTAATGCTAATCGTGATA-3′, $miR\text{-}155P_R$: 5′-ATTCCATGTTGTCCACTGTCTCTG-3′; $U6P_F$: 5′-ATTGGAACGATACAGAGAAGATT-3′, $U6P_R$: 5′-GGAACGCTTCACGAATTTG-3′; $p27Kip\text{-}1P_F$: 5′-CACTGCAGAGACATGGAA-3′, $p27Kip\text{-}1P_R$: 5′-GCTTCATCAAGCAGTGA-3′; $\beta\text{-}actin\text{-}P_F$: 5′-GAACCCTAAGGCCAAC-3′, $\beta\text{-}actinP_R$: 5′-TGTCACGCACGATTTCC-3′. The PCR reaction system was composed of 5 μl 2 × SYBR Green Mixture, 1 μl forward and reverse primer at 2.5 μM, 1 μl cDNA, and ddH_2O. The reaction was performed on Bio-Rad CFX96 at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

Western Blot

Total protein was extracted from the cells by RIPA on ice and quantified by BCA method. A total of 40 μg protein was separated by 10% SDS-PAGE for 3 h and transferred to PVDF membrane at 300 mA for 1.5 h. After blocked with 5% skim milk at room temperature for 60 min, the membrane was incubated with primary antibody at 4°C overnight (p27Kip1 1:200, β-actin 1:800). After washed with phosphate buffered saline tween (PBST), the membrane was further incubated in horse radish peroxidase (HRP) labeled secondary antibody at room temperature for 60 min (1:8000). At last, the membrane was treated by electrochemiluminescence (ECL) reagent for 3 min and developed. Quantity One was applied to analyze the protein bind.

Flow Cytometry Detection of Cell Apoptosis

The cells were digested by enzyme and collected. After resuspended in 100 μl binding buffer, the cells were added with 5 μl Annexin V-FITC and 5 μl PI at room temperature avoid of light for 15 min. Then the cells were tested on Beckman FC 500 MCL flow cytometry (Brea, CA, USA).

CCK-8 Assay

The cells were seeded in 96-well plate at 8×10^3/well and incubated for different times. A total of 10 μl CCK-8 solution was added to each well and incubated for 4 h. The plate was tested on microplate reader at 450 nm to obtain the absorption value (A450).

Flow Cytometry Detection of Cell Cycle

The cells were digested by enzyme and centrifuged at 1000 g for 5 min. After washed with PBS, the cells were fixed with 1 ml precooled 70% ethanol at 4°C for 24 h. Next, the cells were treated with propidium iodide (PI) and RNase A at 37°C for 30 min. Then the cells were tested on flow cytometry at 488 nm to test DNA content.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. Measurement data was presented as mean ± standard deviation and compared by *t*-test. $p < 0.05$ was depicted as significant difference.

Results

MiR-155 Upregulated, while p27Kip1 Decreased in OSCC Tissue

qRT-PCR revealed that miR-155 level upregulated in OSCC tissue compared with normal oral mucosa. Moreover, its expression elevated following TNM upstage (Figure 1A). p27Kip1 mRNA was significantly lower in OSCC tissue than that in normal oral mucosa. Also, its level declined following TNM upstage (Figure 1B). Western blot demonstrated that p21Kip1

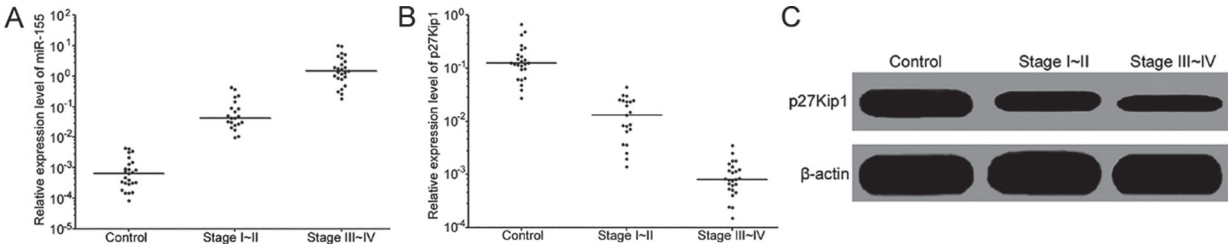

Figure 1. MiR-155 upregulated, while p27Kip1 decreased in OSCC tissue. ***A,*** qRT-PCR detection of miR-155 expression. ***B,*** qRT-PCR detection of P27Kip1 mRNA. ***C,*** Western blot detection of p27Kip1 protein.

protein expression markedly reduced in OSCC tissue in stage III-IV compared with in stage I-II (Figure 1C).

MiR-155 Targeted Regulated p21Kip1 Expression in Tca8113 Cells

Bioinformatics analysis demonstrated the targeted relationship between miR-155 and the 3'-UTR of p27Kip1 mRNA (Figure 2A). Dual-luciferase reporter gene assay indicated that miR-155 mimic transfection significantly reduced the relative luciferase activity of HEK293T cells transfected by wild-type p27Kip1-3'-UTR plasmid (Figure 2B) but not mutated p27Kip1-3'-UTR, suggesting that miR-155 targeted band with the 3'-UTR of p27Kip1 mRNA and suppressed its expression. MiR-155 mimic or anti-miR-155 transfection in Tca8113 cells inhibited or upregulated p27Kip1 mRNA and protein expressions, respectively (Figure 2C-D). It revealed that p27Kip1 was targeted regulated by miR-155 in oral cancer cells.

P27Kip1 Overexpression Efficiency Verification

Flow cytometry showed that EGFP positive rate reached 80% in Tca8113 cells transfected by pEGFP-p27Kip1 or pEGFP-NC, whereas no EGFP was detected in untransfected Tca8113 cells (Figure 3A). It suggested the high efficiency of virus transfection. Western blot demonstrated that p27Kip1 protein level was similar in pEGFP-NC group and untransfected group. p27Kip1 protein was higher in pEGFP-p27Kip1 group compared with pEGFP-NC group and untransfected group, indicating that p27Kip1 was successfully overexpressed in Tca8113 cells (Figure 3B).

Inhibition of miR-155 Induced Tca8113 Cell Cycle Blockage and Apoptosis, and Suppressed Proliferation

MiR-155 mimic and/or pEGFP-p27Kip1 transfection upregulated miR-155 and p27Kip1 levels (Figure 4A and B), blocked cell cycle in G1 phase (Figure 4C), reduced cell proliferation (Figure 4D), enhanced Caspase-3 activity (Figure 4E), and increased cell apoptosis in Tca8113 cells (Figure 4F).

Discussion

In recent years, the incidence of oral cancer keeps on increasing. The incidence of oral cancer in our country is as high as 48.1/100,000, including the male as 31.1/100,000 and the female as 16.9/100,000. Its mortality rate is up to 22.1/100,000, with 15.3/100,000 in male and 6.8/100,000 in female[11]. Following the promotion of comprehensive treatment including surgery, radiotherapy, and chemotherapy, the survival and prognosis of oral cancer have been greatly improved than before. However, the 5-year survival

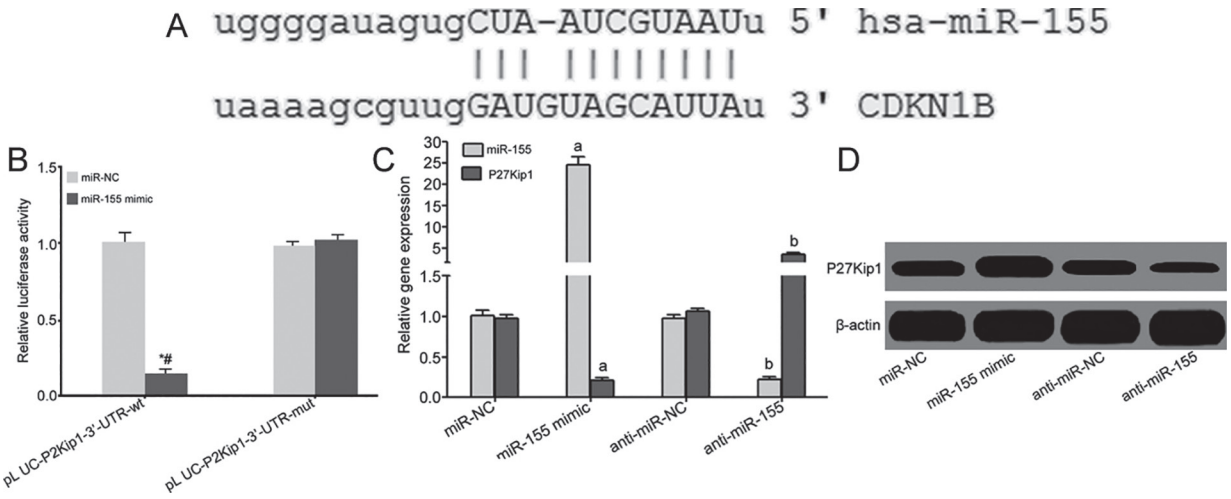

Figure 2. MiR-155 targeted regulated p21Kip1 expression in Tca8113 cells. ***A,*** The binding site between miR-155 and the 3'-UTR of P27Kip1 mRNA. ***B*** Dual luciferase reporter gene assay. ***C,*** qRT-PCR detection of miR-155 and p27Kip1 expressions in Tca8113. ***D*** Western blot detection of p27Kip1 protein level. $^{\#}p < 0.05$, compared with miR-NC. $^{\#}p < 0.05$, compared with pLUC-P27Kip1-3′-UTR-mut. $^{a}p < 0.05$, compared with miR-NC. $^{b}p < 0.05$, compared with anti-miR-NC.

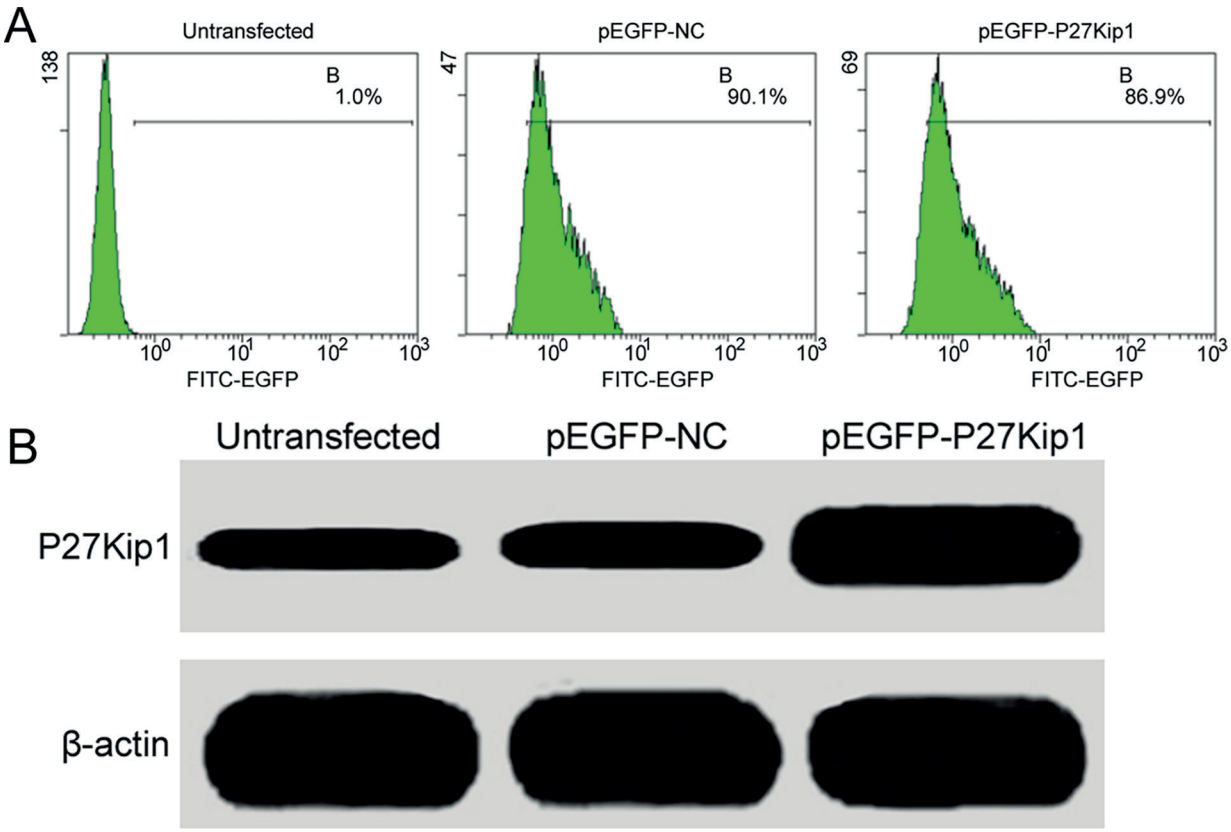

Figure 3. p27Kip1 overexpression efficiency verification. ***A,*** Flow cytometry detection of EGFP positive rate. ***B,*** Western blot detection of p27Kip1 protein expression.

rate of oral cancer still maintains at 50-60% within the last 20 years, and it was even lower in stage III and IV. Relapse and metastasis are the main reasons to limit the survival rate. Since the oral histological structure is complex, operation is usually difficult to achieve the radical effect, which is one of the important reasons leading to postoperative metastasis and recurrence. Also, because of the vascular structure and lymphatic drainage around the oral and maxillofacial region, local lymphatic metastasis occurs early in oral cancer. It was showed that the postoperative relapse rate was higher than 35%. There are about 30% of patients died of relapse and metastasis[13]. Cell growth depends on the balance between a variety of regulatory factors in the cell cycle, including the positive and negative regulation of cell cycle protein on cell growth. Cyclin D is a member of multiple cell cycle proteins that can form complex with Cyclin-dependent kinase 4/6 (CDK4/6). It can phosphorylate Rb via Cyclin E/CDK2 complex and trigger cell proliferation by promoting cell cycle in S phase as DNA synthesis[14]. Cyclin D plays a crucial regulatory role in the induction of cell cycle from G1 phase to S phase (G1/S transition). Cyclin D1 can shorten the G1 phase and induce cells to enter S phase to accelerate cell proliferation[15]. P27Kip1 gene locates on human chromosome 12p12.0-12p13.1, which encodes a protein peptide composed of 198 amino acids[16]. As a new discovered G1 checkpoint related gene, P27Kip1 is a member of Cyclin dependent kinase inhibitors (CDKIs) family. It plays a negative regulatory role in mitosis, cell cycle, and cell proliferation, thus acts as a tumor suppressor gene in tumorigenesis[17]. CDKIs mainly block cell cycle and suppress cell proliferation by inhibiting cyclin/CDK complex activation or activity. P27Kip1 protein increase in early and middle phases of G1, and binds with Cyclin E/CDK2 and/or Cyclin D/CDK4 complex via two serine loci on its N-terminal. It restrains ATP loading and blocks G1/S transition to playing a role in blocking cell cycle and inhibiting mitosis[18]. p27Kip1 downregulation may result in the dysfunction of CDK inhibition, leading to Rb

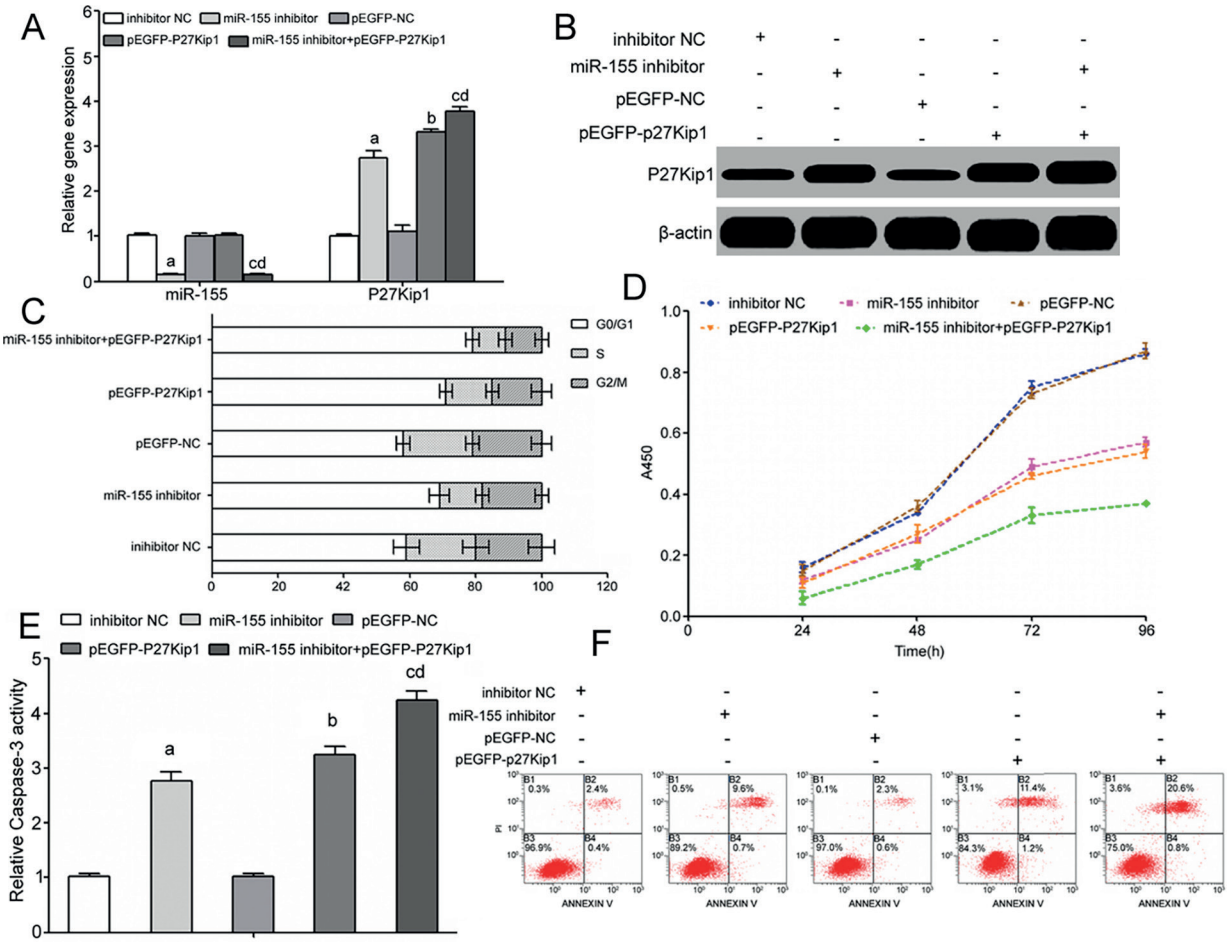

Figure 4. Inhibition of miR-155 induced Tca8113 cell cycle blockage and apoptosis, and suppressed proliferation. ***A,*** qRT-PCR detection of gene expression. ***B,*** Western blot detection of p27Kip1 protein expression. ***C,*** Flow cytometry detection of cell cycle. ***D,*** CCK-8 detection of cell proliferation. ***E,*** Spectrophotometry detection of Caspase-3 activity. ***F,*** Flow cytometry detection of cell apoptosis. $^{a}p < 0.05$, compared with inhibitor NC. $^{b}p < 0.05$, compared with pEGFP-NC. $^{c}p < 0.05$, compared with inhibitor NC. $^{d}p < 0.05$, compared with pEGFP-NC

phosphorylation in the excessive activation state. It further enhances the transcription activity of E2F, resulting in excessive cell proliferation and inducing cancer[19]. In addition to cell proliferation and cell cycle, p27Kip1 also participates in the regulation of cell migration, apoptosis[20], and autophagy[21]. Downregulation of p27Kip1 is associated with tumorigenesis, progression, and prognosis. It was showed that p27Kip1 was reduced in oral cancer tissue and related to survival rate and prognosis[8,9]. Moreover, miR-155 level significantly upregulated in oral cancer tissue, suggesting that miR-155 may be a oncogene of oral cancer[8,10,12]. Bioinformatics analysis revealed that miR-155 may bind with the 3'-UTR of p27Kip1. This study investigated the role of miR-155 in regulating p27Kip1 and affecting Tca8113 cell proliferation, cycle, and apoptosis.

Our result showed that miR-155 expression was higher in OSCC tissue compared with normal oral mucosa, and was associated with TNM stage. On the contrary, p27Kip1 mRNA and protein expressions significantly reduced in OSCC, and their levels were lower in advanced TNM stage. Kudo et al[22] reported that p27Kip1 downregulation was associated with the occurrence of OSCC. Zhang et al[23] revealed that p27Kip1 expression reduced in OSCC tissue and related to lower 5-year survival rate. Vallonthaiel et al[24] showed that the lower level of p27Kip1 in the nucleus, the worse of OSCC prognosis. Fillies et al[25] demonstrated that p27Kip1 could be treated as an independent prognostic factor in OSCC patients with lymph node metastasis. The prognosis of patients with positive p27Kip1 expression was better than with negative p27Kip1

expression. Gao et al[26] also demonstrated that p27Kip1 protein declined in OSCC tissue and associated with TNM staging, histopathological grading, and lymph node metastasis. Gao et al[27] reported that p27Kip1 downregulation was related to the poor prognosis. This study founds that p27Kip1 expression in OSCC tissue was lower than that of normal oral mucosa, which was similar to the results of Zhang et al[23] and Gao et al[26]. Baba et al[8] showed that miR-155 elevated in OSCC tissue and was related to lymph node metastasis. Chen et al[28] reported that miR-155 significantly increased head and neck cancer. Liu et al[29] also showed that miR-155 upregulation was related to the occurrence of head and neck cancer. Rather et al[12] exhibited miR-155 markedly elevated in oral squamous cancer cell line and OSCC tissue. This study observed miR-155 upregulation in OSCC tissue compared with normal oral mucosa, which was in accordance with Baba et al[8] and Rather et al[12]. Dual-luciferase reporter gene assay revealed that miR-155 mimic transfection significantly reduced luciferase activity in HEK293T cells, confirming that p27Kip1 was targeted regulated by miR-155. Further detection showed that miR-155 mimic and/or pEGFP-p27Kip1 transfection upregulated miR-155 and p27Kip1 levels, blocked cell cycle in G1 phase, reduced cell proliferation, enhanced Caspase-3 activity, and increased cell apoptosis in Tca8113 cells. Kudo et al[30] demonstrated that Skp2 siRNA apparently inhibited p27Kip1 protein degradation, thus restraining oral cancer cell proliferation. It indicated that p27Kip1 upregulation played a role in weakening the malignancy of oral cancer cells. Our results exhibited that suppression of miR-155 upregulated p27Kip1 level, reduced cell proliferation, and blocked cell cycle, which was similar to Kudo[30]. Baba et al[8] showed that miR-155 inhibitor markedly increased SOCS1 expression, downregulated STAT3 level, and enhanced E-cadherin to suppress OSCC cell line HSC-3 EMT and migration. Rather et al[12] also reported that miR-155 overexpression significantly reduced CDC73 expression, enhanced cell proliferation, and inhibited cell apoptosis. AntagomiR-155 transfection upregulated CDC73 level in oral cancer cell line KB, thus to promote cell apoptosis, attenuate cell proliferation, and weakened the tumorigenicity in nude mouse. This study revealed the role of miR-155 upregulation in reducing p27Kip1 expression and OSCC oncogenesis. Downregulation of miR-155 or increasing p27Kip1 restrained oral cancer cell proliferation and cell cycle, which may provide a new molecular target for the early diagnosis and treatment of OSCC in clinic.

Conclusions

MiR-155 significantly upregulated, while p27Kip1 reduced in OSCC tissue. Inhibition of miR-155 upregulated p27Kip1 level, blocked cell cycle in G1 phase, attenuated cell proliferation, and promoted cell apoptosis.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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