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MicroRNA-646 inhibits proliferation and cell cycle progression of nasopharyngeal carcinoma cells by targeting mTOR

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Abstract. – OBJECTIVE: To clarify whether microRNA-646 could regulate the proliferative potential and cell cycle progression of nasopharyngeal carcinoma cells through targeting mammalian target of rapamycin (mTOR). It, therefore, could influence the occurrence and progression of nasopharyngeal carcinoma.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was used to detect expression of the levels of microRNA-646 and mTOR in tumor tissues and paracancerous tissues of patients with nasopharyngeal carcinoma. Besides, their expressions in nasopharyngeal carcinoma cell lines were determined by qRT-PCR. Survival analysis was conducted to evaluate the sensitivity and specificity of microRNA-646 in diagnosing nasopharyngeal carcinoma. The overall survival of patients with nasopharyngeal carcinoma was calculated based on their expression levels of microRNA-646. The regulatory effects of microRNA-646 and mTOR on proliferative potential and cell cycle progression were explored by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Dual-luciferase reporter gene assay was conducted to verify the relationship between microRNA-646 and mTOR, which was further confirmed by Pearson correlation analysis. Finally, gain-of-function experiments were carried out to determine whether microRNA-646 could regulate the proliferative potential and cell cycle progression of nasopharyngeal carcinoma cells by targeting mTOR.

RESULTS: MicroRNA-646 was lowly expressed in nasopharyngeal carcinoma tissues and cell lines. Survival analysis confirmed the diagnostic value of microRNA-646 in nasopharyngeal carcinoma. Besides, the nasopharyngeal carcinoma patients with high level of microRNA-646 were expected to have a longer 5-year survival time compared with those with low level. Overexpression of microRNA-646 inhibited the proliferative potential and cell cycle progression of HONE1 and SUNE1 nasopharyngeal carcinoma cells. Dual-luciferase reporter gene assay detected the binding of microRNA-646 to mTOR. Moreover, mTOR was highly expressed in nasopharyngeal carcinoma tissues and cell lines. A negative correlation was found between microRNA-646 and mTOR. That is, the overexpression of mTOR could reverse the inhibitory effects of microRNA-646 on the proliferative potential and cell cycle progression of HONE1 and SUNE1 cells.

CONCLUSIONS: MicroRNA-646 remains a low level in nasopharyngeal carcinoma. It inhibits the proliferative potential and cell cycle progression of nasopharyngeal carcinoma cells by targeting mTOR. It can, therefore, inhibit the development of nasopharyngeal carcinoma.

Key Words:
Nasopharyngeal carcinoma, MicroRNA-646, mTOR, Proliferation, Cell cycle.

Introduction

Nasopharyngeal carcinoma is a common malignancy in the head and neck. Its incidence accounts for 50% of the primary malignancies originating from the head and neck epithelium. The incidence rate of nasopharyngeal carcinoma is extremely high in southern China and immigrants and their descendants overseas, seriously endangering human health. It is currently believed that Epstein-Barr (EB) virus infection, chemical carcinogens and genetic susceptibility genes are the leading pathogenic factors for nasopharyngeal carcinoma[1,2]. Despite many effects have been devoted, the specific pathogenesis of nasopharyngeal carcinoma remains unclear[3]. Hence, searching for molecular markers accurately reflects the occurrence, development and prognosis of nasopharyngeal carcinoma. It is particularly important for early diagnosis and individualized treatment

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of nasopharyngeal carcinoma[4]. MicroRNAs, as non-coding, small RNAs, are newly discovered and highly conserved. They exert a crucial effect on the regulation of gene expressions at post-transcription[5]. MicroRNAs completely or incompletely pair with the 3' untranslated region of the target gene mRNA. It can, therefore, degrade or inhibit the translation of target genes. In the biological processes, microRNAs[6] can regulate cellular behaviors and serve as oncogenes or tumor suppressors in tumors. Chen et al[7] have identified the close relationship between the abnormally expressed microRNAs and nasopharyngeal carcinoma. MiRNA-17-92 and miRNA-155 are highly expressed in nasopharyngeal carcinoma, while miRNA-143 and miRNA-145 are downregulated[7]. Liu et al[8] have found that miR-29c participates in the progression of nasopharyngeal carcinoma by inhibiting the proliferation and invasion of nasopharyngeal carcinoma cells. In this study, we showed that microRNA-646 was lowly expressed in nasopharyngeal carcinoma, suggesting the potential role in the development of nasopharyngeal carcinoma. Pan et al[9] have indicated that microRNA-646 is a negative regulator of the epidermal growth factor receptor (EGFR) pathway, inhibiting the proliferation and migration of lung cancer cells. MicroRNA-646 inhibits the progression of gastric cancer by targeting FOXK1. Low expression of microRNA-646 suppressed proliferation and migration of rectal cancer cells by targeting NOB1[10]. These researches demonstrated the significant function of microRNA-646 in tumors with different mechanisms. The biological function of microRNA-646 in nasopharyngeal carcinoma, however, has been rarely reported. In this study, we focused on the possible role of microRNA-646 in nasopharyngeal carcinoma and its potential mechanism.

Patients and Methods

Sample Collection

Nasopharyngeal carcinoma was pathologically diagnosed. Fresh tumor tissues and paracancerous tissues of 24 patients who underwent surgery for nasopharyngeal carcinoma were harvested. All patients were followed up, and their clinical data were recorded, including sex, age, tumor size, and tumor number. None of enrolled patients had preoperative treatment and family history. Patients volunteered to participate in the study and signed written informed consent. This study has been approved by the Ethics Committee of Seventh People's Hospital of Jinan (Jinan, China). The harvested tissues were preserved in liquid nitrogen.

Cell Culture

The normal cell line NP69 and the nasopharyngeal carcinoma cell lines CNE1, CNE2, HONE1 and SUNE1 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Nasopharyngeal carcinoma cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Cell Transfection

Cells were transfected with microRNA-646 mimics, pcDNA-mTOR (mammalian target of rapamycin) or negative control at 70-80% of confluence Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection solution was maintained for 20 min and slowly added in the culture medium. After 4 hours, the fresh medium was replaced. Transfected cells were harvested at different time points for functional experiments.

RNA Extraction

5×10^6 cells or 50 mg tissues were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) and thoroughly mixed with 0.2 mL of chloroform. After centrifugation at 4°C, 12,000 rpm for 10 minutes, the precipitate was incubated with isopropanol of same volume for another centrifugation. At last, the precipitate was washed with 75% ethanol, air-dried and dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA sample was quantified, purified and then preserved in a -80°C refrigerator.

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

The extracted RNA was reversely transcribed on ice to obtain complementary deoxyribose nucleic acid (cDNA). U6 was considered as the internal reference. QRT-PCR was carried out according to the instructions of the SYBR Green PCR Kit (TaKaRa, Dalian, China) with a total reaction system of 10 μL. Parameters of qRT-PCR were: pre-denaturing at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extending at 72°C for 7 min. Primers of microRNA-646 and U6 were as follows: MicroRNA-646, F:

5'-ACACTCCAGCTGGGAAGCAGCTGCCTC-3', R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGACUCCG-3'; U6, F: 5'-CTCGCTTCGGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'.

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with 20 nmol/L microRNA-646 mimics or were negatively controlled with 600 ng mTOR WT or mTOR MUT. After 24 hours, cells were lysed and centrifuged at 10,000 g for 5 min. 100 μL of suspension was taken to determine luciferase activity. The relative light units of Firefly (RLU-1) and Renilla (RLU-2) of target gene were recorded.

Determination of Cell Cycle Progression

Cells transfected for 72 hours were fixed with the 70% ice ethanol at -20°C overnight. After centrifugation, cell density was adjusted to 1×10^6/mL. The resuspended cells in 1 mL of suspension were then incubated with 1 mL of RNase A (20 μg/mL) and propidium iodide (PI) (50 μg/mL) at 37°C for 30 min in the dark. At last, cell cycle progression was finally determined by using flow cytometry (Partec AG, Arlesheim, Switzerland).

Cell Proliferation Assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well. 10 μL of cell counting kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added at 6, 24, 48, 72 and 96 h, respectively. About 1 h later, the absorbance at 450 nm was recorded and proliferative rate was calculated.

Western Blot

The total protein was extracted with the cell lysate to determine the protein expression. After the quantification of the total protein by bicinchoninic acid (BCA) (Abcam, Cambridge, MA, USA), the sample was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. The transferred polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) was blocked with 5% skim milk. Membranes were then incubated with the primary antibody and the corresponding secondary antibody. Band exposure was developed by chemiluminescence.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used for analyzing the measurement data. All measurement data were expressed as mean±SD. Survival analysis was conducted by introducing receiver operating characteristic (ROC) curve. Overall survival of patients with nasopharyngeal carcinoma was calculated using Kaplan-Meier method. The relation between microRNA-646 and mTOR was evaluated by Pearson correlation analysis. The difference was statistically significant at $p<0.05$.

Results

MicroRNA-646 Showed a Low Expression in Nasopharyngeal Carcinoma

First, we determined the expression of microRNA-646 nasopharyngeal carcinoma tissues and paracancerous tissues in 24 cases by qRT-PCR. The results showed that microRNA-646 was lowly expressed in nasopharyngeal carcinoma tissues (Figure 1A). Identically, microRNA-646 was also lowly expressed in nasopharyngeal carcinoma cell lines compared with controls (Figure 1B). Based on the collected follow-up data, survival analysis showed the potential diagnostic value of microRNA-646 in nasopharyngeal carcinoma (AUC=0.895, cut-off value=0.01643, Figure 1C). Moreover, the 5-year survival of nasopharyngeal carcinoma patients with different expression levels of microRNA-646 was analyzed. Nasopharyngeal carcinoma patients with a high level of microRNA-646 were expected to have a longer survival than those with a low level (p=0.0167, HR =0.4184, Figure 1D). We may conclude that there is a close relationship between microRNA-646 and nasopharyngeal carcinoma.

MicroRNA-646 Inhibited Proliferative Potential and Cell Cycle Progression of Nasopharyngeal Carcinoma

The microRNA-646 is closely related to the progression of nasopharyngeal carcinoma. We, therefore, further explored its role in regulating the biological behaviors of nasopharyngeal carcinoma. HONE1 and SUNE1 cell lines were selected for the following experiments. We first verified the transfection efficiency of microRNA-646 mimics in HONE1 and SUNE1 cells. QRT-PCR data showed markedly upregulated microRNA-646 after transfection of microRNA-646 mimics. Besides, the data also revealed a great transfection efficacy (Figure 2A and 2B).

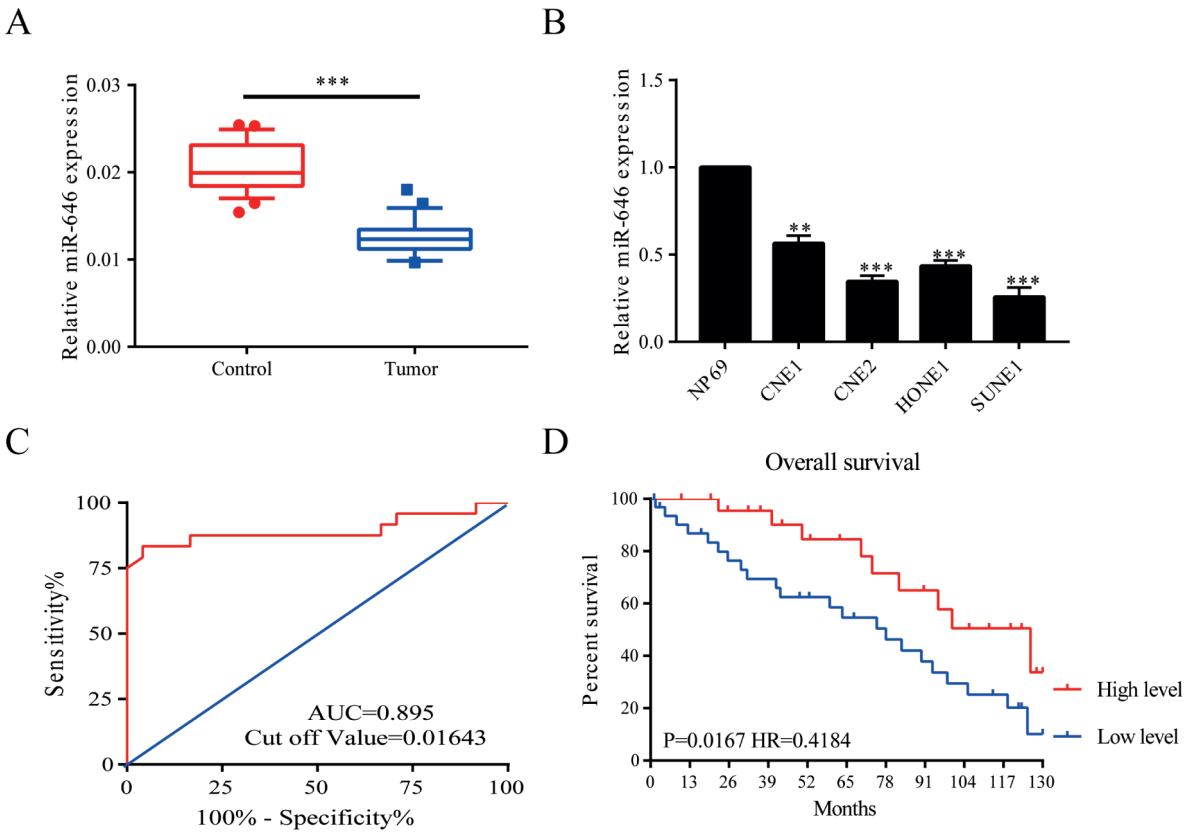

Figure 1. MicroRNA-646 showed a low expression in nasopharyngeal carcinoma. **A,** MicroRNA-646 was lowly expressed in nasopharyngeal carcinoma tissues than paracancerous tissues. **B,** MicroRNA-646 was lowly expressed in nasopharyngeal carcinoma cell lines than NP69 cell line. **C,** Survival analysis of microRNA-646 in nasopharyngeal carcinoma (AUC=0.895, cut-off value=0.01643). **D,** Nasopharyngeal carcinoma patients with a high level of microRNA-646 were expected to have a longer survival than those presenting a low level (p=0.0167, HR=0.4184). ***p<0.001.

Subsequently, the effect of microRNA-646 on the proliferative potential of nasopharyngeal carcinoma cells was tested by CCK-8 assay. The transfection of microRNA-646 mimics inhibited the viabilities of HONE1 and SUNE1 cells (Figure 2C and 2D). Moreover, nasopharyngeal carcinoma cells overexpressing microRNA-646 were mainly arrested in G0/G1 phase, indicating an arrested cell cycle progression (Figure 2E and 2F). We, therefore, considered that microRNA-646 inhibited the proliferative potential and cell cycle progression of nasopharyngeal carcinoma cells.

mTOR Was the Target Gene of MicroRNA-646

Through online prediction and biological analysis, mTOR was found to be a potential target gene for microRNA-646. It showed that there were potential binding sites between microRNA-646 and mTOR (upper panel, Figure 3A). Moreover, the validation by dual-luciferase reporter assay further showed the binding condition between them (bottom panel, Figure 3A). We also detected the expression level of mTOR in nasopharyngeal carcinoma tissues. Higher protein and mRNA levels of mTOR were seen in nasopharyngeal carcinoma tissues than those in paracancerous tissues (Figure 3B). Further Pearson correlation analysis showed a negative correlation between the expressions of microRNA-646 and mTOR (R=-0.6374, p=0.0008, Figure 3C). By transfection of microRNA-646 mimics, both mRNA and protein levels of mTOR were downregulated in HONE1 and SUNE1 cells (Figure 3D). To sum up, mTOR was the target gene of microRNA-646 and its expression was negatively regulated by microRNA-646.

mTOR Reversed the Regulatory Effects of MicroRNA-646 on Cellular Behaviors of Nasopharyngeal Carcinoma

Since mTOR was the target gene of microRNA-646 we speculated that the function of microRNA-646 in regulating cellular behaviors of nasopharyngeal carcinoma was achieved by mTOR. To verify our speculation, we conducted a series of gain-of-function experiments. First, pcDNA-mTOR was constructed, and its transfection efficacy in HONE1 cells and SUNE1 cells were successfully verified (Figure 4A). Then, cells were co-transfected with pcDNA-mTOR and microRNA-646 mimics. CCK-8 assay showed that the overexpression of mTOR partially reversed the inhibitory effect of microRNA-646 on the proliferative potential of HONE1 and SUNE1 cells (Figure 4B and 4C). Similarly, the inhibitory effect of microRNA-646 on the progression of cell cycle was reversed by the overexpression of mTOR as well (Figure 4D and 4E). The above results proved that microRNA-646 regulated nasopharyngeal carcinoma cells by targeting mTOR.

Discussion

Due to the important regulatory effects of microRNAs on gene expression or protein translation, they are closely related to tumorigenesis. Studies[11] have shown that microRNAs are differentially expressed in many tumors when serving as proto-oncogenes or tumor-suppressor genes. MiR-141 and miR-200c maintain an interaction with ZEBI in pancreatic cancer cells, which is a promotive factor for EMT. Therefore, they keep the progression of EMT in a dynamic balance. The progression of pancreatic cancer would be accelerated by external stimuli once the balanced EMT progression breaks by external stimuli[12]. MiR-21 enhances invasiveness and migration of colon cancer cells by targeting the tumor suppressor PTEN. It can thus promote the phosphorylation of FAK and expressions of matrix metalloproteinases 2 and 9[13]. HMGA2 is involved in EMT pancreatic cancer tissues, whereas let-7 microRNA-mediated HMGA2 downregulation do not prevent the transformed phenotype in these cells, suggesting a complexity of microRNA

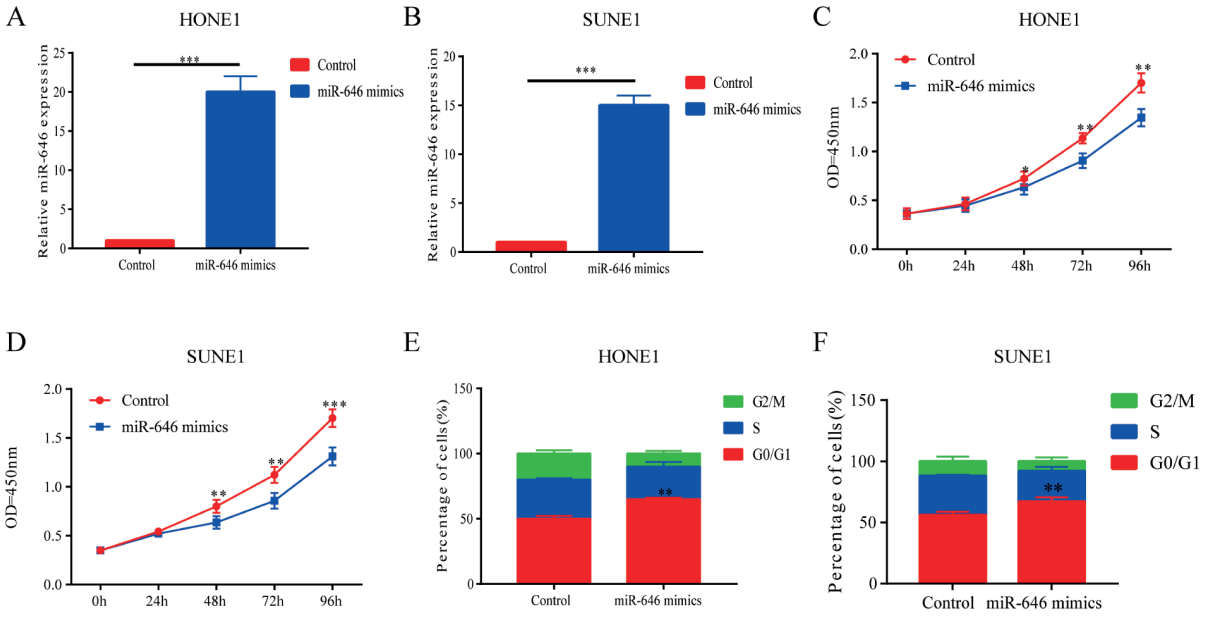

Figure 2. MicroRNA-646 inhibited proliferative potential and cell cycle progression of nasopharyngeal carcinoma. **A,** Transfection efficiency of microRNA-646 mimics in HONE1 cells. **B,** Transfection efficiency of microRNA-646 mimics in SUNE1 cells. **C,** CCK-8 assay showed that transfection of microRNA-646 mimics in HONE1 cells inhibited cell viability. **D,** CCK-8 assay showed that transfection of microRNA-646 mimics in SUNE1 cells inhibited cell viability. **E,** Cell cycle was arrested in G0/G1 phase after transfection of microRNA-646 mimics in HONE1 cells. **F,** Cell cycle was arrested in G0/G1 phase after transfection of microRNA-646 mimics in SUNE1 cells. $^{**}p<0.01$, $^{***}p<0.001$.

function[14]. In this study, we found that microRNA-646 was lowly expressed in nasopharyngeal carcinoma. A series of functional researches have confirmed the inhibitory effects of microRNA-646 on the proliferative potential and the cell cycle progression of nasopharyngeal carcinoma cells. It was firstly discovered in the analysis of *Saccharomyces cerevisiae* mutant resistance that the mammalian target of rapamycin (mTOR) is a silk/threonine protein kinase [15]. PI3K/AKT/mTOR pathway exerts a crucial role in cellular behaviors and tumorigenesis in multiple malignancies[16-18]. The activated mTOR would lead to massive proliferation of tumor cells, increased secretion of oncogenes, accelerated cell cycle, and shortened G1 phase, which are conducive to the rapid development of tumors[19]. mTOR shows a promotive role in the development and progression of pancreatic cancer. Rapamycin, the mTOR inhibitor, remarkably alleviates the tumor growth and metastasis in mice with pancreatic cancer[20]. In the present study, mTOR was screened out to be a target gene of microRNA-646 by bioinformatics analysis, which further verified for their binding condition. MicroRNA-646 could negatively regulate the mTOR expression in nasopharyngeal carcinoma cells. Furthermore, it is concluded that mTOR overexpression partially reverses the inhibitory effect of microRNA-646 on the proliferative potential and the cell cycle progression of nasopharyngeal carcinoma cells. We believed that microRNA-646 exerted its regulatory function in nasopharyngeal carcinoma through targeting mTOR. Based on the above results, we concluded that microRNA-646 inhibits the proliferation and arrests cell cycle of nasopharyngeal carcinoma cells by targeting mTOR. Our results provide a new theoretical basis for the prevention and treatment of nasopharyngeal carcinoma.

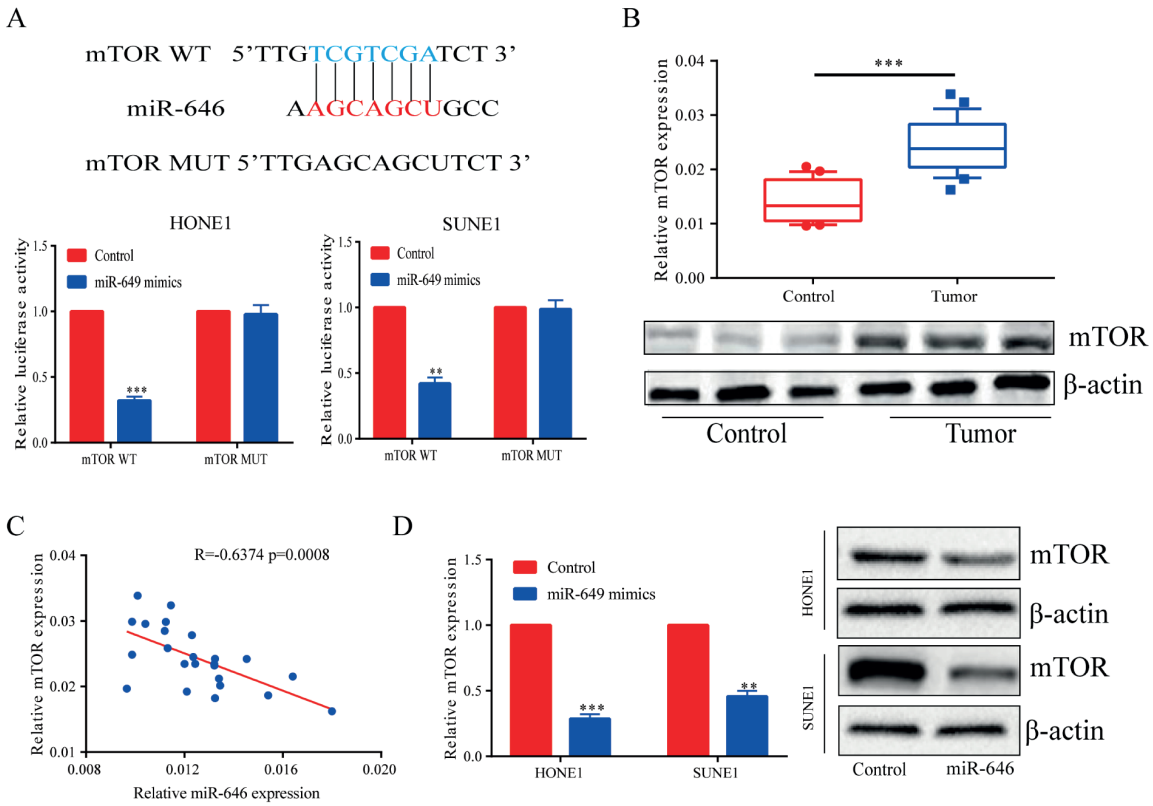

Figure 3. mTOR was the target gene of microRNA-646. **A,** A potential binding sites between microRNA-646 and mTOR. **B,** mTOR was highly expressed in nasopharyngeal carcinoma at both mRNA and protein level. **C,** Pearson correlation analysis showed a negative correlation between expressions of microRNA-646 and mTOR (R=-0.6374, p=0.0008). **D,** Transfection of microRNA-646 mimics downregulated both mRNA and protein levels of mTOR in HONE1 and SUNE1 cells. **p<0.01, ***p<0.001.

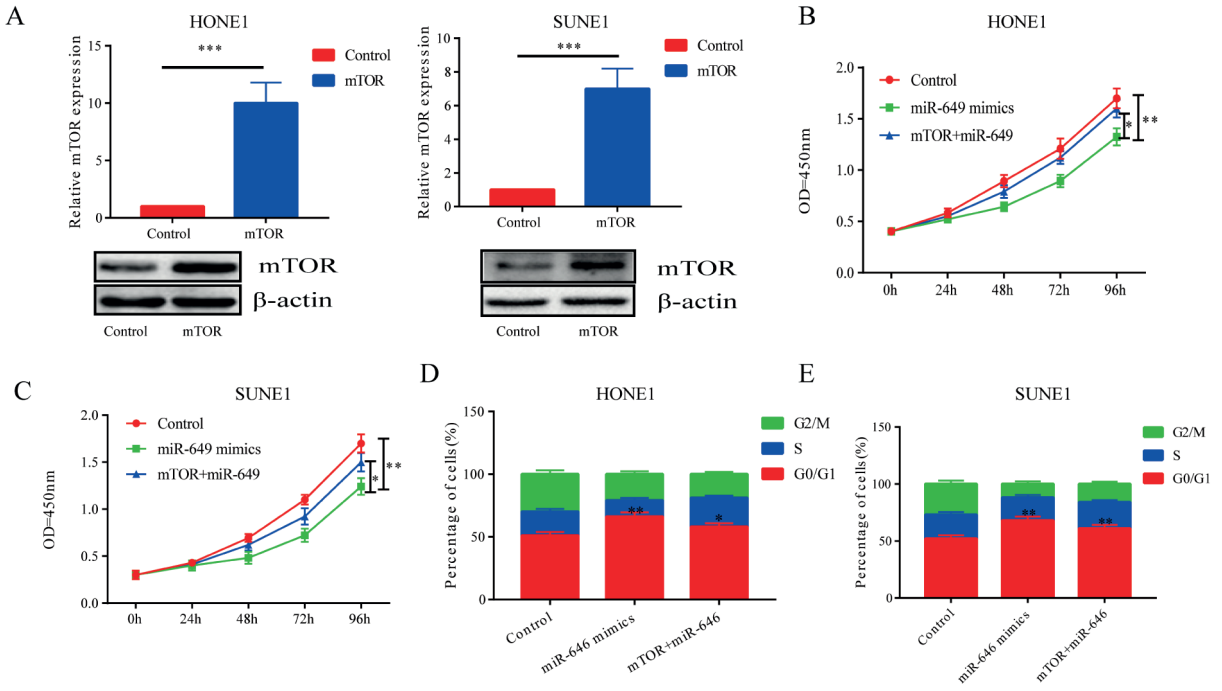

Figure 4. mTOR reversed the regulatory effects of microRNA-646 on cellular behaviors of nasopharyngeal carcinoma. **A,** Transfection efficacy of pcDNA-mTOR in HONE1 and SUNE1 cells. **B,** CCK-8 assay showed that mTOR overexpression partially reversed the inhibitory effect of microRNA-646 on the proliferative potential of HONE1 cells. **C,** CCK-8 assay showed that mTOR overexpression partially reversed the inhibitory effect of microRNA-646 on the proliferative potential of SUNE1 cells. **D,** The inhibitory effect of microRNA-646 on cell cycle progression of HONE1 cells was reversed by mTOR overexpression. **E,** The inhibitory effect of microRNA-646 on cell cycle progression of SUNE1 cells was reversed by mTOR overexpression. *p<0.05, **p<0.01, ***p<0.001.

Conclusions

microRNA-646 remains a low level in nasopharyngeal carcinoma. It inhibits proliferative potential and cell cycle progression of nasopharyngeal carcinoma cells by targeting mTOR, thereafter inhibiting the development of nasopharyngeal carcinoma.

Conflicts of interest

The authors declare no conflicts of interest.

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