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MiR-146a-5p inhibits proliferation and promotes apoptosis of oral squamous cell carcinoma cells by regulating NF-κB signaling pathway

F.-Y. ZHU[1], C.-W. GAN[2], M.-X. WANG[2], B.-C. SUN[2], F.-J. LI[2], Y.-H. QIU[3], K. WANG[2]

[1]Oral Medical Center, Xiangya Hospital, Central South University, Changsha, China
[2]Department of Oral and Maxillofacial Surgery, The Second Xiangya Hospital of Central South University, Changsha, China
[3]Department of Stomatology, Hainan Provincial People's Hospital, Haikou, China

Feiya Zhu and Chengwen Gan contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to investigate the function and potential mechanism of micro ribonucleic acid (miR)-146a-5p in oral squamous cell carcinoma (OSCC).

MATERIALS AND METHODS: The expression of miR-146a-5p in OSCC tissues and cell lines was examined by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis. Then, the role of miR-146a-5p in proliferation was analyzed by Cell Counting Kit-8 (CCK-8) assay. Besides, the proliferation and apoptosis of OSCC cells were detected by the colony formation assay and flow cytometry, respectively. Finally, the regulatory effect of miR-146a-5p/nuclear factor-kappa B subunit 1 (NF-κB1) was determined by Western blotting assay and Luciferase reporter assay system.

RESULTS: The expression of miR-146a-5p was markedly upregulated in OSCC cell lines. In addition, the silence of miR-146a-5p inhibited the proliferation and promoted the apoptosis of OSCC cells. According to the results of the Western blotting analysis and Luciferase reporter gene assay, NF-κB1 was identified as a direct target of miR-146a-5p. Moreover, the downregulation of NF-κB1 restored the inhibitory effect of silenced miR-146a-5p on the proliferation of SCC-9 cells.

CONCLUSIONS: MiR-146a-5p can inhibit the proliferation and accelerate the apoptosis of OSCC cells by directly targeting NF-κB1, and it plays a carcinogenic role in OSCC.

Key Words:
Oral squamous cell carcinoma, MiR-146a-5p, NF-κB1, Proliferation, Apoptosis.

Introduction

Oral squamous cell carcinoma (OSCC), is one of the most commonly diagnosed cancer types around the world, with more than 500,000 new cases every year[1]. It has the highest morbidity rate among head and neck malignancies, and the patients are usually prone to suffer from tumor invasion and metastasis. Besides, it is the sixth most common cancer in the world, accounting for approximately 5% of all the malignant tumors[2]. In spite of many therapeutic methods available for OSCC at present, including surgical resection, radiotherapy, and chemotherapy, the prognosis of the patients is still relatively poor. These treatments also tend to produce severe side effects, such as postoperative recurrence and hematoma, tooth decay, radiation necrosis, gastrointestinal reaction, and systemic diseases after chemotherapy. Therefore, it is necessary to elaborate the molecular mechanism of OSCC, so as to identify the new therapeutic targets and diagnostic markers.

Notably, more studies[3-5] have manifested that micro ribonucleic acids (miRNAs/miRs) are closely associated with the occurrence and development of OSCC. As a category of small non-coding RNAs with a length of 19-24 nucleotides, miRNAs exert their effects by targeting messenger RNAs (mRNAs). Over 2,500 kinds of human miRNAs have been reported since they were discovered[6]. In the comparison of normal tissues with cancer tissues, the abnormal expression of miRNAs has close correlations with the biological processes of tumors, including cell cycle, proliferation, differentiation, growth, and apoptosis[7]. MiRNAs can act as oncogenes or tumor suppressor genes[8], but the involvement of miRNAs in the development and progression of OSCC needs to be deeply understood. In the exploration of the role of miR-10a in oral cancer, Chen et al[9] found that both miR-10a and glucose transporter-1 (GLUT1) are upregulated in the tumor tissues of OSCC, and that the overexpression of miR-10a promotes the

Corresponding Author: Kai Wang, MD; e-mail: 1043157922@qq.com

glucose uptake and upregulates GLUT1 in OSCC cells. The knockdown of GLUT1 attenuates the enhancement of miR-10a overexpression on cancer cell proliferation, illustrating that miR-10a can stimulate OSCC cell proliferation by upregulating GLUT1 and promoting glucose metabolism. Long et al[10] elucidated that miR-146a-5p participates in modulating the growth and invasion of human breast cancer cells and revealed that miR-146a-5p is prominently downregulated in breast cancer tissues and cells. In addition, the overexpression of miR-146a-5p remarkably represses the proliferation, invasion, and migration of breast cancer cells. Moreover, miR-146a-5p serves as a tumor suppressor in breast cancer by directly targeting IRAK1. However, the role of miR-146a-5p in OSCC has not been clarified yet, so the influences of miR-146a-5p on the proliferation and apoptosis of OSCC cells and its potential mechanism were investigated in this study.

Materials and Methods

Materials

The materials used in this study were: Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS; Hangzhou Every Green Bio-Engineering Co., Ltd., Hangzhou, China), miRNA isolation kit, TaqMan miRNA reverse transcription kit and fluorescence quantitative Polymerase Chain Reaction (qPCR) kit for miRNA (Thermo Fisher Scientific, Waltham, MA, USA), siRNA transfection reagent and apoptosis assay kit (Sigma-Aldrich, St. Louis, MO, USA), miR-146a-5p mimic or miR-146a-5p inhibitor and negative controls (NCs; miR-NC and anti-miR-NC) (Suzhou GenePharma, Suzhou, China), Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), mirGLO plasmids and Dual-Luciferase assay system (Promega, Madison, WI, USA), and flow cytometer (BD, Franklin Lakes, NJ, USA).

Cell Culture and MiRNA Transfection

Normal oral epidermal keratinocyte line HOK and OSCC cell lines CAL-27, HSC4, and SCC-9 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All the cells were cultured in a DMEM containing 10% FBS and 1% penicillin/streptomycin in an incubator with 5% CO_2 at 37°C. The cell transfection was strictly performed according to the procedures of the transfection kit (Sigma-Aldrich, St. Louis, MO, USA), with a transfection concentration of 50 nM.

RNA Extraction and Quantitative Reverse Transcription (qRT)-PCR

The TaqMan miRNA isolation kit was utilized to extract miRNA, and the TaqMan miRNA reverse transcription kit was used for RT of miRNA. Then, miR-146a-5p was subjected to real-time quantification using the fluorescence qPCR kit for miRNA, with 1 µL of complementary deoxyribose nucleic acid (cDNA) as the template. The primers of miR-146a-5p and endogenous control U6 were synthesized by BGI. The primer sequences are as follows: miR-146a-5p: 5'-TGAGAACTGAATTCCATGGGTT-3'. U6: forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', and reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The PCR was conducted in a real-time PCR system (Bio-Rad, Hercules, CA, USA) according to the following conditions: 95°C for 55 s, 95°C for 18 s and 60.5°C for 35 s, 40 cycles in total, and the Ct value was standardized *via* $2^{-\Delta\Delta Cq}$ method.

Analysis of Cell Proliferation

The cell proliferation was evaluated *via* CCK-8 at 24 h after transfection. In short, the cells were seeded into a 96-well plate (2×10^3 cells/well) and cultured in the incubator with 5% CO_2 at 37°C. Next, 10 µL of CCK-8 solution was added at 24, 48, 72, and 96 h separately. Later, the cells were cultured for the other 3 h, and the optical density at 450 nm was measured using a microplate spectrophotometer.

Analysis of Cell Apoptosis Rate

The cell apoptosis level was detected using the apoptosis assay kit. After the cells were digested with trypsin and collected, they were washed with cold phosphate-buffered saline (PBS) twice. Then, the cells were resuspended in 500 µL of binding buffer, and 5 µL of Annexin V-fluorescein isothiocyanate (FITC) and 5 µL of Propidium Iodide (PI) were added for incubation in the dark at room temperature for 20 min. Finally, the cell apoptosis was determined *via* flow cytometry within 1 h.

Analysis of Colony Formation

The colony formation of the single cell was examined by means of colony formation assay. The cells in different groups were added into each

well of a 6-well plate and cultured in the DMEM with 10% FBS for 10 d. When the macroscopic colonies were formed, the cells were washed with PBS twice and added with 4% paraformaldehyde for 30 min for cell fixation, followed by staining with crystal violet for 30 min. Next, the colonies were counted, and the colony formation rate was calculated according to the formula: colony formation rate = (number of colonies/number of cells seeded) ×100%.

Western Blotting Assay

The total proteins were extracted from the cells using a total protein extraction kit. After that, 40 μg of proteins in each lane underwent electrophoresis with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel to separate the degenerated proteins. Next, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) which was blocked in 5% skim milk at room temperature for 2 h, followed by Western blotting assay through incubation with primary antibodies at 4°C. Then, the membrane was washed with Tris-Buffered saline and Tween (TBST) for 3 times, incubated with secondary antibodies at room temperature for 2 h, and detected using enhanced chemiluminescence (ECL) color development system.

Detection of Luciferase Activity

According to prediction, there is a binding site of miR-146a-5p in the 3'-untranslated region (UTR) of nuclear factor kappa B subunit 1 (NF-κB1), and the binding site or mutation site was inserted into the pmirGLO vectors, which were named as pmirGLO-NF-κB1 wild type (WT) and pmirGLO-NF-κB1 mutant type (MUT), respectively. Next, the negative control (NC) or miR-146a-5p mimic was transiently co-transfected with the pmirGLO-NF-κB1 WT or pmirGLO-NF-κB1 MUT vector. At 48 h after transfection, the activity of firefly and Renilla Luciferases was determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

The data were presented as mean ± standard deviation and analyzed *via* Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA). Paired t-test was employed for analysis of difference between the groups, and $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of MiR-146a-5p in OSCC Cells

Firstly, the expression of miR-146a-5p in OSCC cells and normal oral cells was investigated. It was shown in qRT-PCR that, compared with that in the normal oral epidermal keratinocyte line HOK, the expression level of miR-146a-5p was remarkably elevated in all OSCC cell lines (CAL-27, HSC4, and SCC-9) ($p<0.05$, Figure 1). It was conjectured that miR-146a-5p may be a type of carcinogenic factor for OSCC, and that the increase in its expression may be correlated with the progression of OSCC.

Impact of Silenced MiR-146a-5p on Proliferation of SCC-9 Cells

The miR-146a-5p inhibitor was transfected into SCC-9 cells. The results of qRT-PCR indicated that the expression level of miR-146a-5p in the cells transfected with miR-146a-5p inhibitor was notably lower than that in the cells transfected with anti-miR-NC ($p<0.05$, Figure 2A). The CCK-8 assay was adopted to analyze the influence of silenced miR-146a-5p on the proliferation of SCC-9 cells, and it was manifested that the cells transfected with miR-146a-5p inhibitor exhibited lower proliferation rate than those transfected with anti-miR-NC ($p<0.05$, Figure 2B).

Impact of Silenced miR-146a-5p on Colony Formation of SCC-9 Cells

SCC-9 cells transfected with miR-146a-5p inhibitor and anti-miR-NC were inoculated into the 6-well plate, and it was revealed that the number

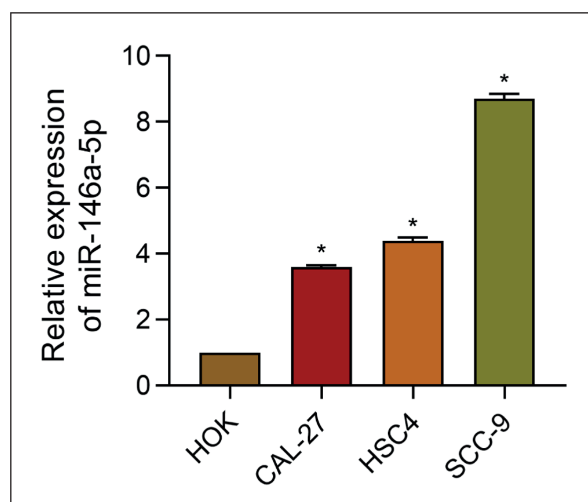

Figure 1. Expression of miR-146a-5p in OSCC cells in contrast with that in oral epidermal keratinocytes detected via qRT-PCR. *$p<0.05$.

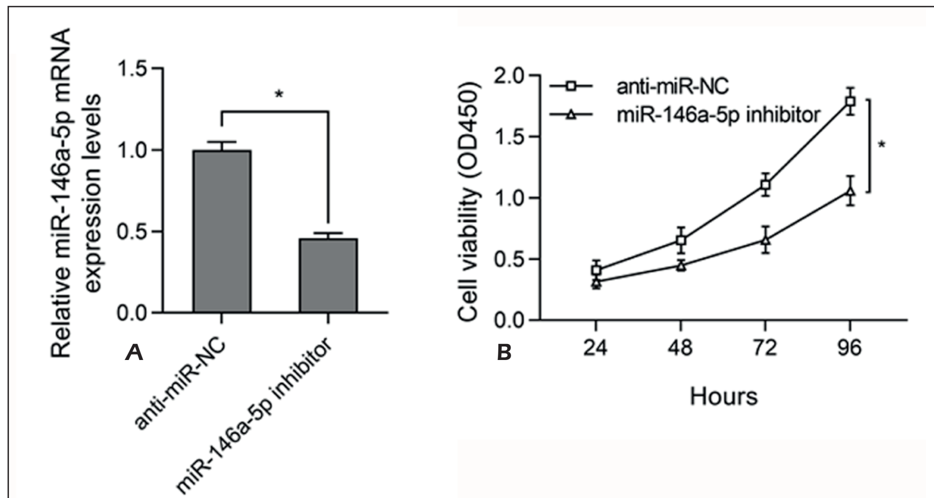

Figure 2. A, Effect of miR-146a-5p silencing determined by qRT-PCR. **B**, Cell proliferation after silencing of miR-146a-5p measured via CCK-8 assay. (*$p<0.05$).

of colonies after transfection with miR-146a-5p inhibitor was significantly smaller than that after transfection with anti-miR-NC ($p<0.05$, Figure 3).

Impact of Silenced miR-146a-5p on Apoptosis of SCC-9 Cells

SCC-9 cells transfected with miR-146a-5p inhibitor and anti-miR-NC were seeded into the 6-well plate, and the influence of silenced miR-146a-5p on apoptosis of SCC-9 cells was determined *via* flow cytometry 24 h later. According to the results, the apoptosis rate of the cells transfected with miR-146a-5p inhibitor was remarkably increased compared with that of the cells transfected with anti-miR-NC ($p<0.05$, Figure 4).

MiR-146a-5p Directly Targeted NF-κB1 Gene

It was predicted by TargetScan that NF-κB1 gene is a direct target of miR-146a-5p in the 3'-UTR (Figure 5A). Hence, attention was firstly paid to NF-κB1 gene, a component of the NF-κB signaling pathway. In order to confirm whether NF-κB1 is a direct target of miR-146a-5p, the Luciferase reporter plasmids containing partial 3'-UTR of NF-κB1 were constructed, which had wild-type or deleted target sites of miR-146a-5p, and the luciferase reporter molecules were detected in SCC-9 cells. As shown in Figure 5B, the activity of the Renilla Luciferase reporter gene containing wild-type miR-146a-5p was inhibited in miR-146a-5p

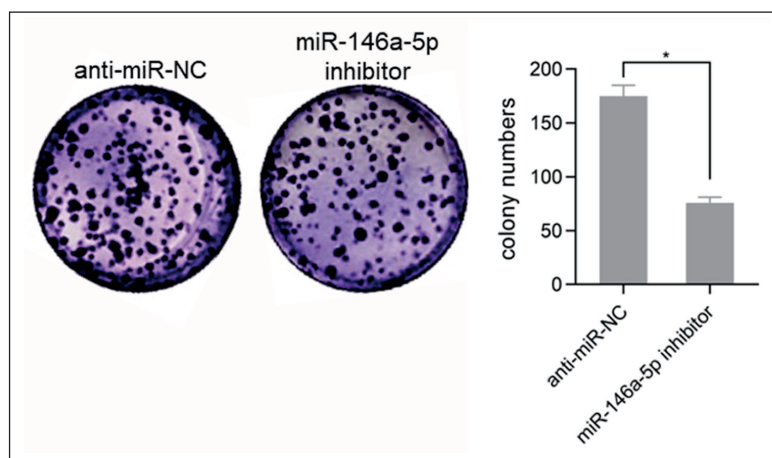

Figure 3. Impact of silenced miR-146a-5p on colony formation of SCC-9 cells analyzed through colony formation assay (magnification: 10×). (*$p<0.05$).

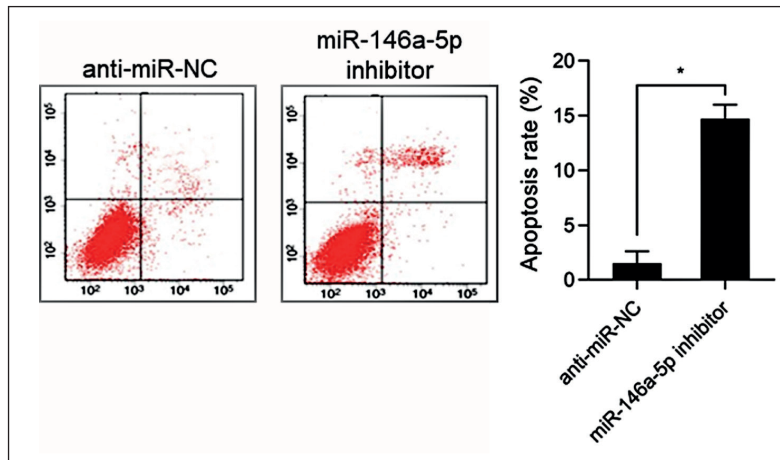

Figure 4. Impact of silenced miR-146a-5p on apoptosis of SCC-9 cells determined via flow cytometry (*$p<0.05$).

mimic transfection group ($p<0.05$), while that of the reporter plasmid containing deleted miR-146a-5p was not remarkably decreased. Moreover, it was testified by qPCR and Western blotting that the mRNA and protein levels of NF-κB1 were up-regulated after silencing of miR-146a-5p ($p<0.05$).

NF-κB1 Participated in the Inhibitory Effect on SCC-9 Cell Proliferation Mediated by miR-146a-5p

For the purpose of further verifying whether NF-κB1 is involved in the miR-146a-5p-mediated inhibitory effect on SCC-9 cell proliferation, si-NF-κB1 and miR-146a-5p inhibitor were co-transfected into SCC-9 cells to knock down NF-κB1 and miR-146a-5p. CCK-8 assay was conducted to detect the impacts on the proliferation of SCC-9 cells. The results manifested that the downregulation of NF-κB1 restored the inhibitory effect of silenced miR-146a-5p on the proliferation of SCC-9 cells (Figure 6) (*$p<0.05$).

Discussion

As the sixth most common cancer globally, OSCC accounts for about 90% of malignant oral tumors[11]. Although progress has been made in the diagnosis and treatment of the disease over the past decades, the 5-year overall survival rate of OSCC remains at approximately 50%[12]. A high proportion of OSCC patients are diagnosed with the disease in the advanced stage, with positive LMN and even distant metastasis, resulting in adverse clinical outcomes of the disease[13]. Hereon, it is extremely important to clarify the molecular mechanism of OSCC.

MiRNAs, small non-coding RNAs, cause the degradation of the target mRNAs at a relatively low frequency by inhibiting mRNA translation. They are involved in the post-transcriptional inhibition of the target genes in a sequence-specific manner[14]. Besides, they regulate up to 30% mRNAs in human cells and play roles in cell proliferation, differentiation, and apoptosis. A single miRNA is capable of targeting various mRNAs, some of which may exert carcinogenic or tumor-suppressive effects[15]. In addition, the growth and metastasis of cancer is a combination of cancer cells' own functions and interactions of numerous external factors with the cancer cells, and they also affect their behaviors. MiRNAs mainly influence a variety of external factors, such as the immune system, tumor-stromal cell, treatment, and tumor virus[15]. MiR-146a is a pleiotropic regulator of carcinogenesis as polymorphisms and altered expressions have correlations with cancer risk, as well as invasive and metastatic abilities of many cancers[16]. MiR-146a-5p is overexpressed in SCC of the head and neck, cervix and lung, and related to the carcinogenicity of OSCC[17]. To date, the role of miR-146a-5p in OSCC has not been elucidated. The novelty of this study was that we first investigated the effect of miR-146a-5p on proliferation and apoptosis of OSCC and its potential mechanism. In this paper, the relationship between miR-146a-5p and OSCC and the possible mechanism were primarily explored. Similarly, it was discovered that the expression of miR-146a-5p in human OSCC cell lines was notably higher than that in normal oral epidermal keratinocyte line. It was conjectured that miR-146a-5p may be a carcinogenic factor for human OSCC, and that the increase in its expression is probably associated with the progression of OSCC. Then, miR-146a-5p inhibitor was transfected into SCC-9 cells to silence miR-146a-5p, and the impacts of silenced

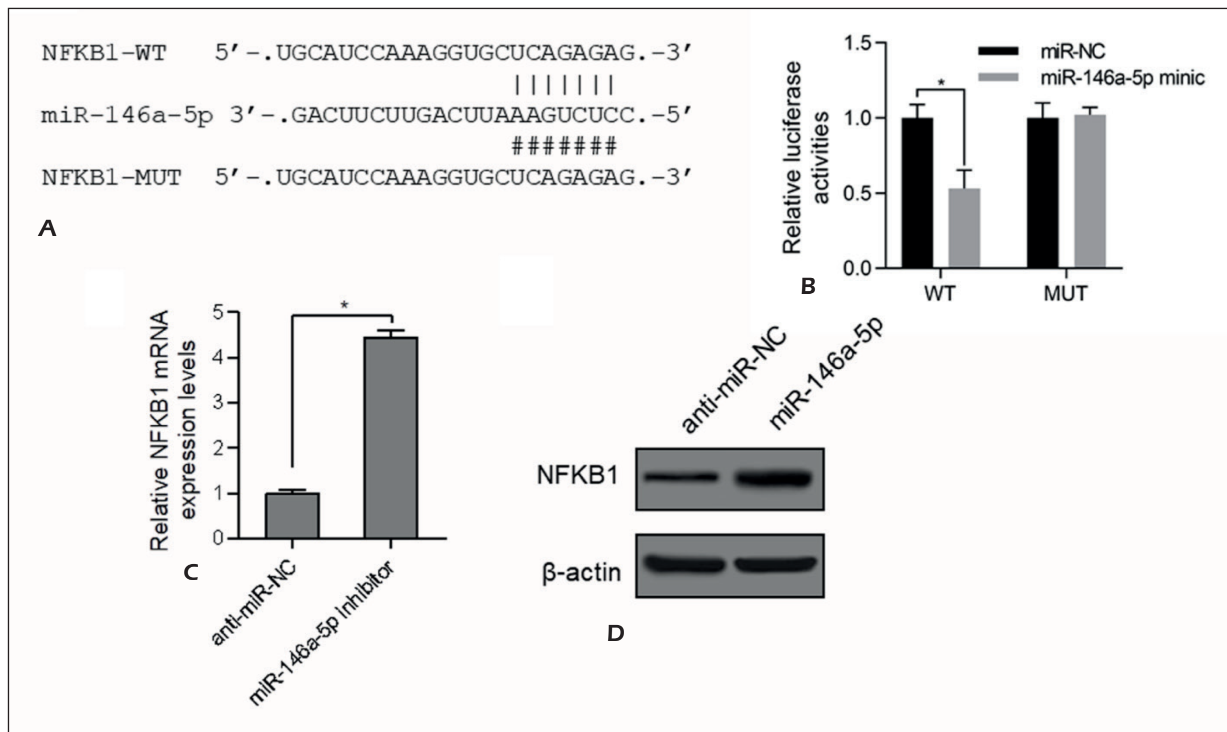

Figure 5. A, There is a binding site between miR-146a-5p and NF-κB1. **B**, There is an interaction between miR-146a-5p and NF-κB1 examined through qPCR. **C-D**, Regulatory effect of miR-146a-5p on NF-κB1 examined Western blotting. (*p<0.05).

miR-146a-5p on the proliferation and apoptosis of SCC-9 cells were examined. The results indicated that the silence of miR-146a-5p could evidently repress the proliferation and induce the apoptosis of OSCC cells, suggesting that miR-146a-5p acts as an oncogene during the development of OSCC.

The NF-κB transcription factor family regulates numerous biological processes, including many aspects of immune function[18]. This family consists of transcription factors with homologous structures, such as NF-κB1, NF-κB2, RelA, RelB, and c-Rel, which can conjugate with κB enhancers into homodimers or heterodimers. NF-κB protein is generally isolated in the cytoplasm by specifically suppressing proteins. The characteristic of IκB, the inhibitor of NF-κB lies in the repeated structure of ankyrin. As a prototypic member of IκB, IκBα is a major player in mediating the activation of canonical (or non-canonical) NF-κB signaling pathway[19]. Some other members of the IκB family exist and seemingly modulate more specific functions of NF-κB. NF-κB1 and NF-κB2 are translated into precursor proteins p105 and p100, respectively, which also act as IκB-like molecules[20]. Their C-termini have structural homology to IκB and inhibit nuclear translocation of related NF-κB members. The processing of proteasome-mediated p105 and p100 not only generates the corresponding mature proteins p50 and p52, but also leads to the nuclear translocation of chelate NF-κB[20]. In this research, it was revealed that NF-κB1 gene was the direct target of miR-146a-5p, which repressed the transcriptional activity. Besides, NF-κB1 was involved in the inhibitory effect on SCC-9 cell proliferation mediated by miR-146a-5p.

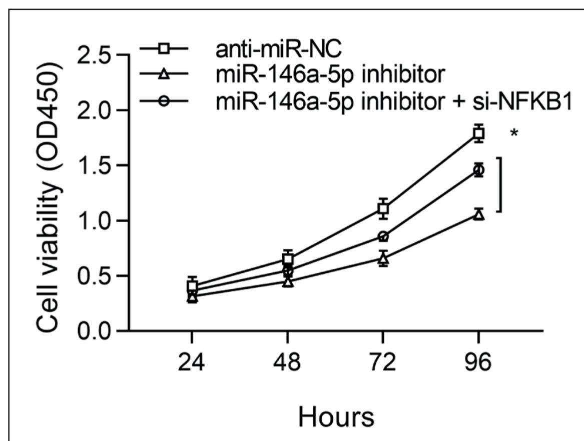

Figure 6. Proliferation activity in different groups of cells determined via CCK-8 assay. (*p<0.05).

Conclusions

In summary, the results demonstrate that miR-146a-5p exhibits upregulated expression in OSCC, and it can inhibit the transcriptional activity of NF-κB1 by directly targeting NF-κB1, thereby performing a carcinogenic function in OSCC.

Conflict of Interests

The authors declare that they have no conflict of interest.

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