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Hsa_circ_001193 regulates proliferation and apoptosis of nasopharyngeal carcinoma cells through targeting mir-496

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Abstract. – OBJECTIVE: To explore the effects of hsa_circ_001193 on the proliferation and apoptosis of nasopharyngeal carcinoma (NPC) cells.

MATERIALS AND METHODS: The messenger ribonucleic acid (mRNA) expression level of hsa_circ_001193 in three NPC cell lines (CNE-1, SUNE-1, and HONE-1) and human normal nasopharyngeal epithelial cell line (NP69) was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The expression of hsa_circ_001193 was silenced through transient transfection with small-interfering RNA (siRNA). Regulatory effects of hsa_circ_001193 knockdown on the proliferation and apoptosis of HONE-1 cells were determined using cell counting kit-8 (CCK-8) assay, colony formation assay, and flow cytometry. Potential miRNAs binding hsa_circ_001193 were predicted in the StarBase, which was further verified *via* Dual-Luciferase reporter assay and qRT-PCR. Moreover, the involvement of the predicted target miRNA in the proliferation of HONE-1 cells regulated by hsa_circ_001193 was determined by CCK-8 assay.

RESULTS: Compared with that in human normal nasopharyngeal epithelial cell line (NP69), the expression of hsa_circ_001193 was significantly upregulated in NPC cell lines ($p<0.05$). The results of CCK-8 assay and colony formation assay showed that knockdown of hsa_circ_001193 could significantly suppress the cell proliferation ability and colony formation ability compared with control group ($p<0.05$). The results of flow cytometry revealed that the apoptosis rate in hsa_circ_001193 knockdown group was remarkably higher than that in control group ($p<0.05$). Besides, according to the analysis of StarBase database, there were binding sites between hsa_circ_001193 and miR-496. The Dual-Luciferase reporter assay manifested that miR-496 bound hsa_circ_001193 ($p<0.05$).

CONCLUSIONS: Hsa_circ_001193 can serve as the miR-496 sponge, which regulates proliferation and apoptosis of NPC cells through up-regulating miR-496. Our findings provide a new therapeutic target for NPC.

Key Words:
Nasopharyngeal carcinoma, Hsa_circ_001193, MiR-496, Proliferation, Apoptosis.

Introduction

Nasopharyngeal carcinoma (NPC) is derived from nasopharyngeal epithelial cells, with a high grade of malignancy, high local metastasis rate, and proneness to early distant metastasis[1]. The pathogenesis of NPC is related to environmental factors, genetic susceptibility, and Epstein-Barr virus infection[2]. Currently, radiotherapy has been widely used in the treatment of NPC patients. Although the response rate of NPC patients greatly improves, their 5-year survival rate remains at about 70%. A number of NPC patients have tumor recurrence or distant metastasis within a few years after treatment[3]. Therefore, it is of significance to explore the key pathways and molecules in the pathogenesis of NPC for improving the early diagnosis and effective treatment of NPC.

A large number of non-coding ribonucleic acids (RNAs), especially micro RNAs (miRNAs) and long non-coding ribonucleic acids (lncRNAs), exert biological functions and diagnostic values in NPC[4,5]. The discovery of circular RNAs (circRNAs) further enriches the mechanism of post-transcriptional regulation[6]. Salzman[7] has previously proven that accumulated circRNAs participate in translational reg-

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ulation by controlling modification and gene expressions. The competing endogenous RNA (ceRNA) hypothesis was first proposed in 2011 and then, verified using bioinformatics, cytobiology, and animal models[8]. CeRNA molecules, including lncRNAs, pseudogenes, and circRNAs, competitively bind the target miRNAs with miRNA response elements (MREs), thereby regulating their expression levels[9]. The ceRNA hypothesis provides a new direction for clarifying the occurrence and development of tumors at the transcriptional level.

CircRNAs are a large class of endogenous non-coding RNAs, which, unlike linear RNAs, are characterized by covalently closed-loop structure and 5'→3' polarity, without poly(A) tails. CircRNAs are expressed in eukaryotic cell lines of different species[10] and play a key role in regulating a variety of cellular activities and pathological processes. They are involved in the occurrence and development of many types of cancer. MiRNAs are small non-coding RNAs that bind to the 3'-untranslated region (3'UTR) of the target mRNA and act as tumor promoters or inhibitors in various cancers, thereby potentially regulating the biological process[11]. In addition, some circRNAs are enriched in multiple conserved miRNA binding sites, and they can serve as ceRNAs to regulate downstream gene expressions at transcriptional and post-transcriptional levels[12]. The occurrence and progression of tumors are regulated by these epigenetic changes. Han et al[13] proved that circMTO1 inhibits the progression of hepatocellular carcinoma by promoting the p21 expression as a sponge for oncogenic miR-9, indicating that circMTO1 is a potential target in the treatment of hepatocellular carcinoma. Downregulated circMTO1 in hepatocellular carcinoma tissues can be used as a predictor for the low survival rate of patients. However, there have been no studies clarifying the effect of circ-001193 on NPC. It is necessary to further explore its biological function and potential mechanism in NPC.

Materials and Methods

Materials

The human normal nasopharyngeal epithelial cell line (NP69) and NPC cell lines (CNE-1, SUNE-1, and HONE-1) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640) high-glucose medium and fetal bovine serum (FBS; HyClone, South Logan, UT, USA); TRIzol reagent and Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA); reverse transcription kit and quantitative polymerase chain reaction (qPCR) kit (Toyobo, Osaka, Japan); cell counting kit-8 (CCK-8), apoptosis assay kit, and bicinchoninic acid (BCA) protein quantification kit (Yeasen Biotechnology, Shanghai, China); specific Has-circ-001193-targeted siRNAs (si-1#, si-2# and si-3#), negative control (si-NC), miR-496 mimics, and negative control (miR-NC; designed and chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China); Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA); Thermal Cycler CFX6 System real-time qPCR instrument (Bio-Rad, Hercules, CA, USA); FACScan flow cytometer (BD, Franklin Lakes, NJ, USA).

Cell Culture and Transfection

Cells were cultured in the RPMI-1640 medium containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37°C. HONE-1 cells were used for *in vitro* biological function experiments. HONE-1 cells in the logarithmic growth phase were collected and inoculated into a 6-well plate (2×10^5 cells/well), followed by transfection according to the instructions of Lipofectamine 2000.

Total RNA Extraction and QRT-PCR

The total RNA was first extracted from cells using TRIzol reagent, incubated with an appropriate amount of chloroform, and centrifuged. The supernatant was transferred into a new centrifuge tube. The RNA in supernatant was precipitated with isopropanol and washed with 75% ethanol. RNA was air dried, diluted in diethyl pyrocarbonate (DEPC)-treated water, and stored at –80°C. According to the instructions of the reverse transcription kit, the RNA extracted was reversely transcribed into complementary deoxyribonucleic acid (cDNA). Then, qPCR was performed in strict accordance with the instructions of the qPCR kit under the following conditions: pre-denaturation at 95°C for 15 min, denaturation at 94°C for 15 s, and 55°C for 30 s, and extension at 72°C for 30 s, for a total of 40 cycles. The relative RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized with GAPDH. Primer sequences used in this study were as follows: circ_001193, F: 5'-GGCACGCACGCACCATTACAC-3', R:

5'-CGTAGGCATATGGCCGACGA-3'; mir-496, F: 5'-GGGGTGTAAACATCCTCGACTG-3', R: 5'-ATTGCGTGTCGTGGAGTCG-3'; GAPDH: F: 5'- CCGGGCTCTCTGCACCTAGTATC-3', R: 5'-AGGTCCGTTGACATCCGAATC-AC-3'. U6: F: 5'-CTCGCTTCGGCAGCACATAT-3', R: 5'-TTGCGTGTCATCCTTGCG-3'

CCK-8 Cell Proliferation Assay

At 24 h after transfection, the cells were digested and inoculated into a 96-well plate (2×10^3 cells/well), with 6 replicates in each group. At 24, 48, 72, and 96 h after culture, the cell proliferation was detected *via* CCK-8 assay. Briefly, 10 μL of CCK-8 solution was added into each well for incubation in the dark at 37°C for 3 h. The absorbance of each well at 450 nm was measured using a microplate reader. Each experiment was repeated 3 times independently.

Colony Formation Assay

At 24 h after transfection, the cells were inoculated into the 6-well plate (5×10^2 cells/well), with 3 wells in each group, and cultured in RPMI-1640 medium containing 10% FBS in the incubator for 2 weeks. Then, the cells were washed with phosphate-buffered saline (PBS) for 3 times, fixed with methanol and stained with 0.1% crystal violet, followed by manual counting under a microscope.

Detection of Apoptosis Via Flow Cytometry

The apoptosis rate of the transfected cells was determined using flow cytometry. At 48 h after transfection, 1×10^6 cells were digested with 0.25% trypsin without EDTA (ethylenediaminetetraacetic acid), washed with pre-cooled PBS for 3 times, and centrifuged. Cells were suspended in the binding buffer, incubated in Annexin V-FITC and propidium iodide (PI) in the dark at room temperature for 15 min. Finally, the apoptosis rate was determined using the FACSCanto II flow cytometer.

Luciferase Activity Assay

The relation between hsa_circ_001193 and miR-496 was verified using the StarBase prediction software (http://starbase.sysu.edu.cn/). The wild-type (WT) and mutant-type (MUT) vectors corresponding to hsa_circ_001193 and miR-496 were synthesized, and inserted into the XhoI/NotI site of the empty psiCHECK-2 to construct the recombinant plasmids. Sequencing was conducted to test the constructed plasmids. Then, the cells were co-transfected with recombinant WT or MUT plasmids, and miR-496 mimics or miR-NC using Lipofectamine 2000. The cells were collected after 48 h, and the firefly and *Renilla* luciferase activity was continuously measured according to the instructions of the Dual-Luciferase reporter assay kit, with 3 replicates for each sample.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for the analysis of statistical data, and the data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The differences between the two groups were analyzed by using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ suggested the statistically significant difference.

Results

Hsa_circ_001193 Expression Was Upregulated in Human NPC Cell Lines

To explore the role of hsa_circ_001193 in the development of NPC, qPCR was performed to analyze the mRNA expression level of hsa_circ_001193 in three kinds of NPC cell lines (CNE-1, SUNE-1, and HONE-1) relative to that in human normal nasopharyngeal epithelial cell line (NP69). As shown in Figure 1, the expression of hsa_circ_001193 in human NPC cell lines was significantly higher than that in normal nasopharyngeal epithelial cell line ($p<0.05$). HONE-1 cells expressed the highest abundance of hsa_circ_001193, which were selected for subsequent exploration of biological function.

Effect of Inhibiting Hsa_circ_001193 Expression on Proliferation of HONE-1 Cells

In view of the up-regulated expression of hsa_circ_001193 in NPC cells, specific Has-circ-001193-targeted siRNAs were transfected to determine the effect of hsa_circ_001193 knockdown on the proliferation of NPC HONE-1 cells. First, the knockdown effect of hsa_circ_001193 was detected by qRT-PCR. As shown in Figure 2A, three designed siRNAs all could downregulate hsa_circ_001193 ($p<0.05$), and the si-2# showed

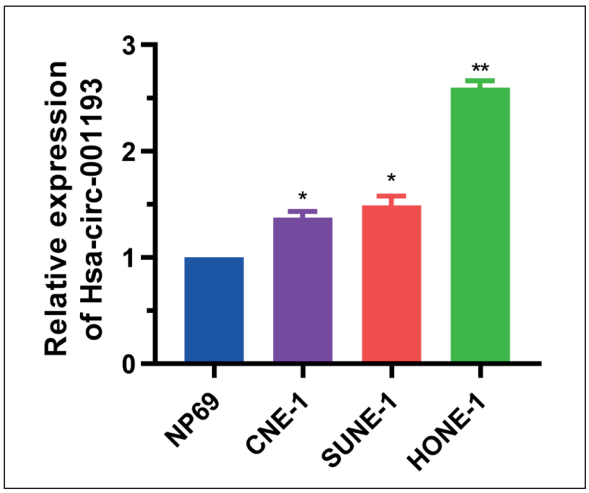

Figure 1. MRNA expression level of hsa_circ_001193 in three kinds of NPC cell lines (CNE-1, SUNE-1 and HONE-1) relative to that in human normal nasopharyngeal epithelial cell line (NP69) detected *via* qPCR. *p<0.05, **p<0.01.

the best efficacy. As shown in Figure 2B, the results of CCK-8 assay detected that knockdown of hsa_circ_001193 significantly suppressed the proliferation of HONE-1 cells (p<0.05).

Effect of Inhibiting Hsa_circ_001193 Expression on Colony Formation of NPC HONE-1 Cells

The colony formation assay was further used to explore the effect of hsa_circ_001193 on the colony formation of HONE-1 cells. It was found that knockdown of hsa_circ_001193 could significantly suppress the colony formation of HONE-1 cells (Figure 3).

Effect of Inhibiting Hsa_circ_001193 Expression on Apoptosis of HONE-1 Cells

The effect of hsa_circ_001193 on the apoptosis rate of HONE-1 cells was determined using flow cytometry. The results showed that the apoptosis rate in hsa_circ_001193 knockdown group was evidently higher than that in control group (Figure 4). Combined with the above results, it is concluded that knockdown of hsa_circ_001193 can inhibit the proliferation and induce the apoptosis of HONE-1 cells.

Hsa_circ_001193 Targeted MiR-496 to Inhibit its Expression

To explore the potential regulatory mechanism of hsa_circ_001193 on the proliferation of NPC cells, miRNAs potentially binding hsa_circ_001193 were predicted. According to the prediction of StarBase database, there were binding sites between hsa_circ_001193 and miR-496 (Figure 5A). To confirm this finding, hsa_circ_001193 sequences containing the predicted WT or MUT miR-496 sites were cloned into luciferase plasmids. Then, the changes in luciferase activity were detected using the Dual-Luciferase reporter gene system. The results revealed that WT luciferase activity was remarkably inhibited in miR-496 mimics group (p<0.05), but there were no evident changes in the MUT luciferase activity (Figure 5B). Besides, qPCR further proved

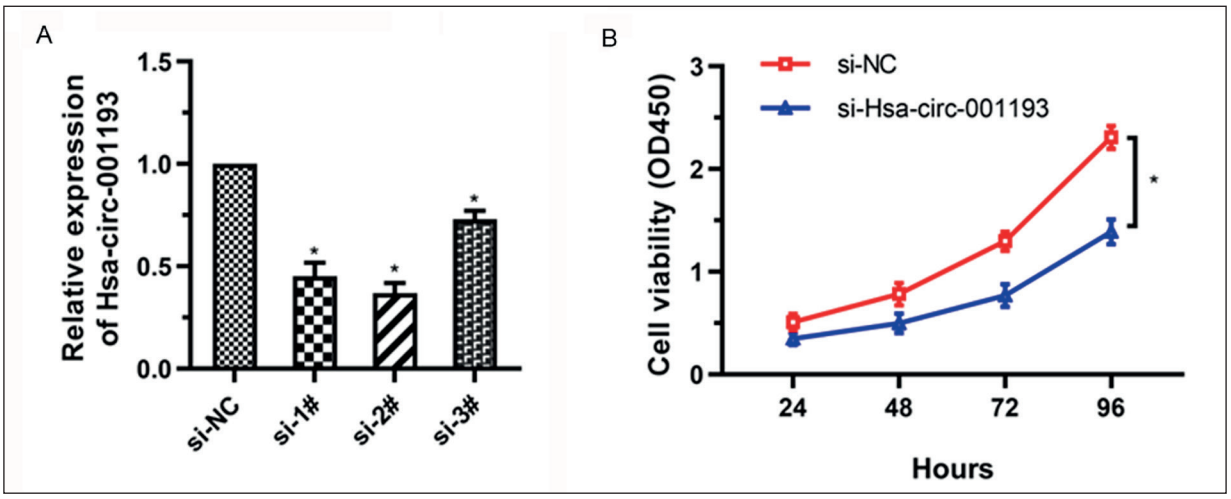

Figure 2. Effect of hsa_circ_001193 on proliferation of HONE-1 cells. **A,** Knockdown effect of hsa_circ_001193 detected *via* qPCR. **B,** Effect of hsa_circ_001193 on proliferation of HONE-1 cells detected *via* CCK-8 assay. *p<0.05.

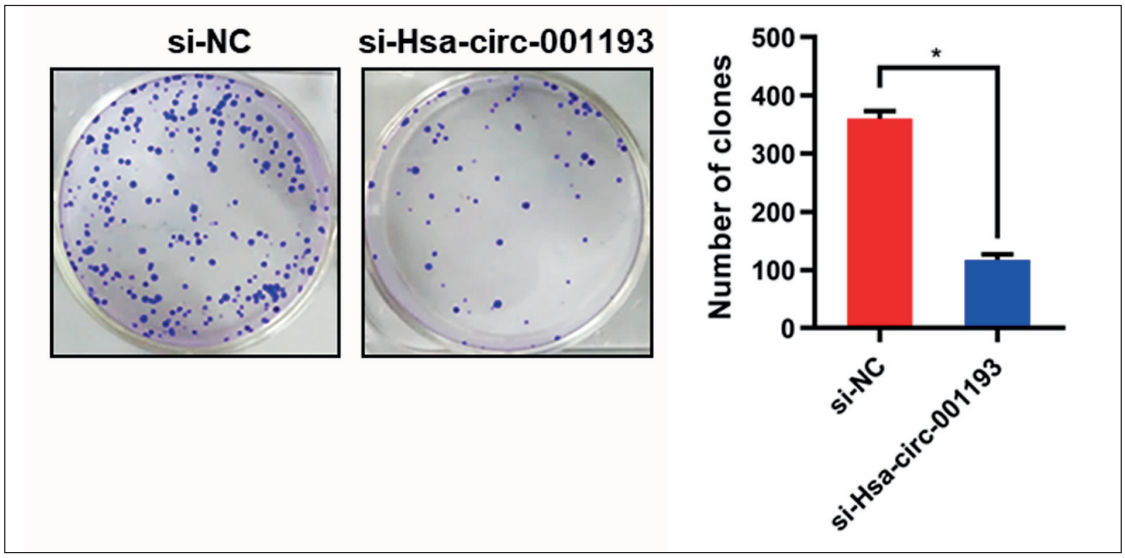

Figure 3. Effect of hsa_circ_001193 on colony formation of HONE-1 cells (magnification × 40). *p<0.05.

that knockdown of hsa_circ_001193 remarkably raised the expression of miR-496 (p<0.05). Similarly, miR-496 could also negatively affect the expression level of hsa_circ_001193 (p<0.05).

MiR-496 Was Involved in Hsa_circ_001193-Mediated Inhibitory Effect on Proliferation of HONE-1 Cells

To further verify whether miR-496 is involved in the hsa_circ_001193-mediated inhibitory effect on proliferation of HONE-1 cells, si-hsa_circ_001193 and miR-496 mimics were co-transfected into HONE-1 cells, and then proliferation changes of HONE-1 cells were observed using CCK-8 assay. As shown in Figure 6, knockdown of miR-496 weakened the inhibitory effect of silencing hsa_circ_001193 on proliferation of HONE-1 cells (p<0.05). It is suggested that knockdown of miR-496 can reverse the hsa_circ_001193-mediated inhibitory effect on proliferation of HONE-1 cells.

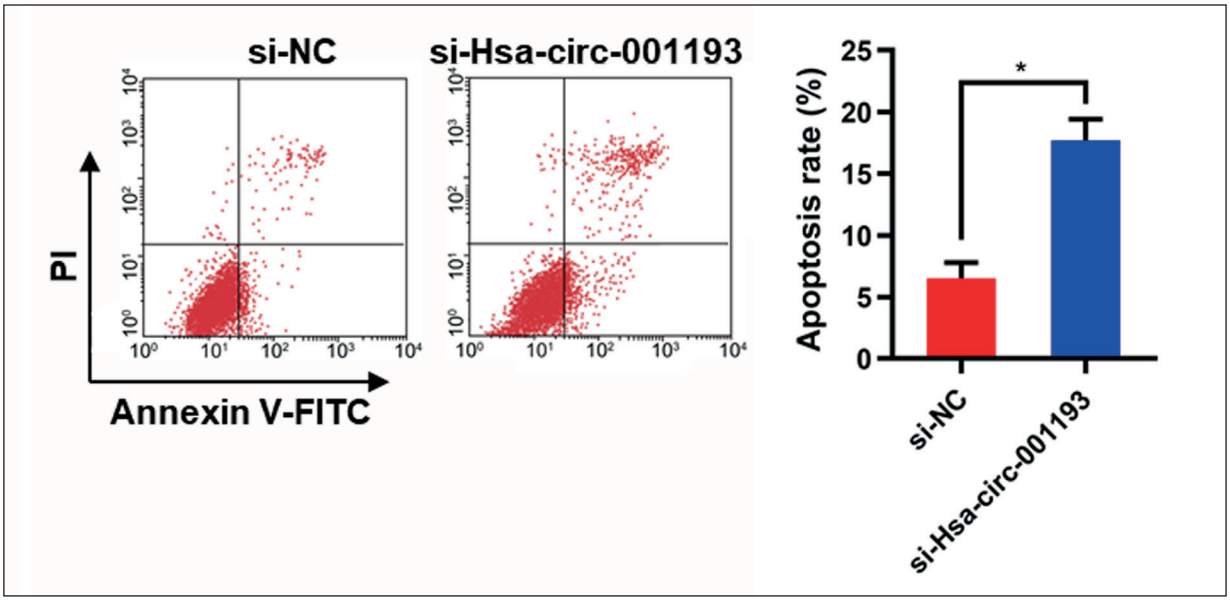

Figure 4. Effect of hsa_circ_001193 on apoptosis of HONE-1 cells. *p<0.05.

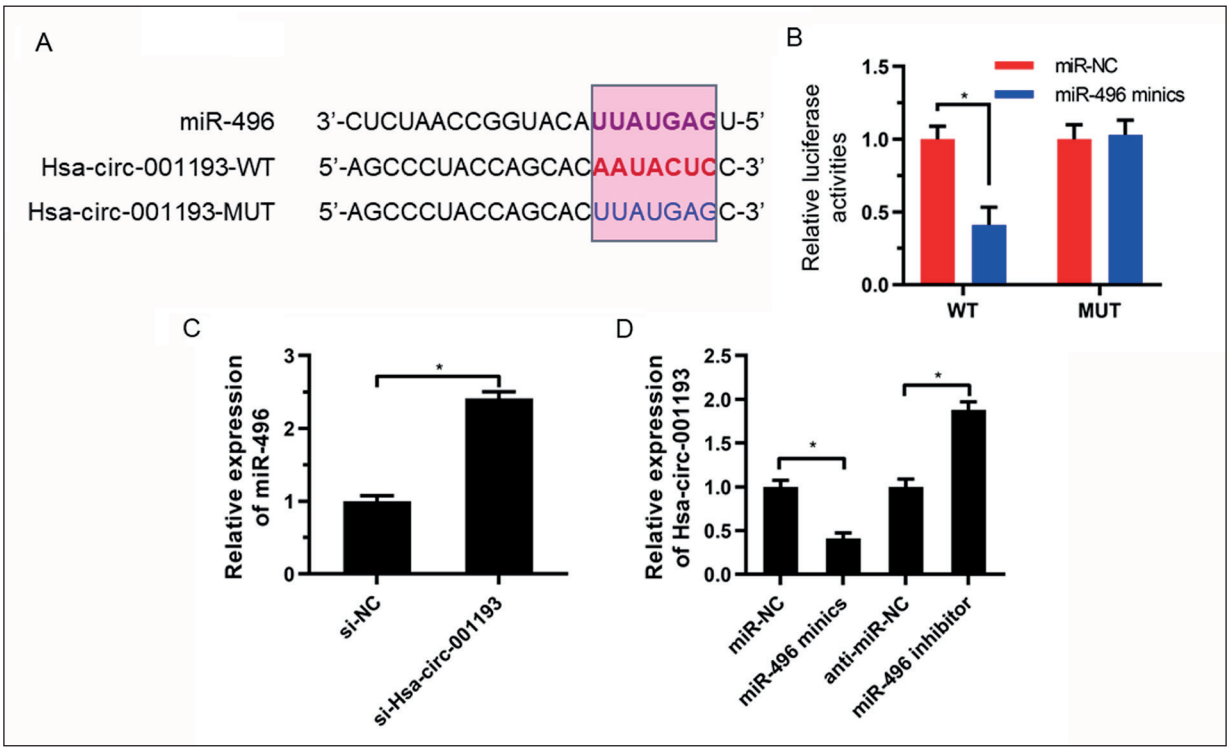

Figure 5. A, Binding sites between hsa_circ_001193 and miR-496. **B,** Luciferase activity assay confirms the interaction of has-circ-001193 with miR-496. **C, D,** QPCR confirms the regulatory relation between has-circ-001193 and miR-496. *p<0.05.

Discussion

NPC is characterized by poor differentiation and high metastasis. Most NPC patients have been in the advanced stage with cervical lymph node metastasis when they are diagnosed. Although the local control rate of NPC has been greatly improved after the combination therapy of radiotherapy and chemotherapy, early detection and effective intervention are lacking[14]. Therefore, exploring the underlying pathogenesis of NPC is helpful to develop new diagnostic and therapeutic targets.

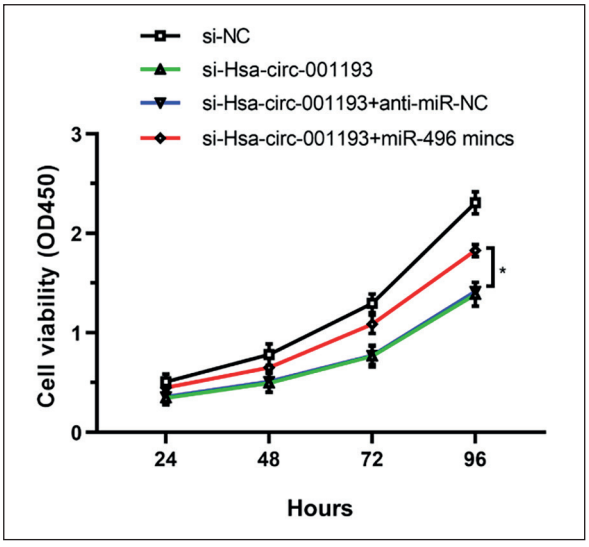

Figure 6. MiR-496 is involved in hsa_circ_001193-mediated inhibitory effect on proliferation of HONE-1 cells. *p<0.05.

CircRNAs are a kind of novel endogenous non-coding RNAs, which are considered as key regulators of gene expressions and pathological process recently. The abnormally expressed circRNAs play an important role in cancerization and tumor progression[15]. In recent years, circRNAs have become a rising star in the non-coding RNAs, which stand for a class of endogenous RNAs in mammalian cells. They are characterized as stable structure, high cell type specificity, tissue specificity, and specific expression in the developmental stage[16]. Li et al[17] found that circRNA 0000096 (hsa_circ_0000096) is significantly down-regulated in gastric cancer tissues, and its knockdown significantly inhibits cell proliferation, and migration *in vitro* and *in vivo*. Zhan et al[18] proved that hsa_circRNA_103809 is highly expressed in hepatocellular carcinoma patients, and its knockdown remarkably suppresses

proliferation, cycle progression, and migration of hepatocellular carcinoma cells. Moreover, hsa_circRNA_103809 can increase the protein expression of miR-377-3p, fibroblast growth factor receptor 1, by interacting with miR-377-3p, and reducing its expression level. In this paper, the biological function of hsa_circ_001193 in NPC cells and its underlying mechanism were mainly explored. It was found that the expression of hsa_circ_001193 was up-regulated in NPC cell lines. We speculated that hsa_circ_001193 may play a role as an oncogene, and its up-regulation may be closely related to the occurrence and development of NPC. Then, siRNA was transiently transfected to inhibit the expression of hsa_circ_001193, and the effects of hsa_circ_001193 knockdown on proliferation and apoptosis of HONE-1 cells were determined. The results of CCK-8 assay, colony formation assay, and flow cytometry showed that knockdown of hsa_circ_001193 could evidently inhibit the proliferation and promote the apoptosis of NPC cells. The above findings demonstrated that hsa_circ_001193 was capable of regulating proliferation and apoptosis of NPC cells, which may become a biomarker for the diagnosis and risk prediction of NPC.

It is well known that miRNAs play key roles in regulating the gene expressions and various biological processes, such as cell proliferation, invasion, and apoptosis. In recent years, the circRNA-miRNA axis and its effect on various cancers have been widely studied. It has been confirmed that circRNAs can act as miRNA sponges to regulate gene transcription and cellular activities in many processes[19]. Zhang et al[20] found that circTRIM33-12 is down-regulated in hepatocellular carcinoma tissues and cell lines. The down-regulation of circTRIM33-12 is significantly associated with malignant features of hepatocellular carcinoma. It serves as an independent risk factor for the overall survival and recurrence-free survival of patients with hepatocellular carcinoma after operation. They pointed out that circTRIM33-12 up-regulates TET1 expression through miR-191 sponge, thus leading to a significant decline in the 5-hydroxymethylcytosine level in hepatocellular carcinoma cells. In this study, the correlation between hsa_circ_001193 and miR-496 was predicted, and the hsa_circ_001193/miR-496 regulatory axis in NPC was confirmed. In a word, hsa_circ_001193, as miR-496 sponge, regulated proliferation and apoptosis of NPC cells.

Conclusions

This study indicates that hsa_circ_001193 can act as the miR-496 sponge to further suppress the proliferation of NPC cells. The research results are helpful to understand the molecular mechanism of hsa_circ_001193 in the occurrence and development of NPC cells. These findings offer a new perspective to the role of circRNAs in the development of NPC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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