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MiR-16 inhibits proliferation of cervical cancer cells by regulating KRAS

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of micro ribonucleic acid (miR)-16 on the proliferation and apoptosis of cervical cancer (CC) cells and its related regulatory mechanism.

MATERIALS AND METHODS: The downstream regulatory targets of miR-16 were analyzed based on the miRNA online database. HCC94 cells were selected as experimental objects. Subsequently, the cells were transfected with miR-16 mimic (miR-16 mimic group), miR-16 small interfering RNA (siRNA) (miR-16 siRNA group) and only Lipofectamine 2000 transfection reagent [blank control group and miR-16 normal control (NC) group]. The expression level of miR-16 in HCC94 cells was measured via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cell counting kit-8 (CCK-8) assay, 5-Ethynyl-2'-deoxyuridine (EdU) staining assay and flow cytometry were then conducted to detect the effects of miR-16 on the viability, proliferation and apoptosis of HCC94 cells, respectively. Additionally, the effect of miR-16 on the protein expression level of kirsten rat sarcoma viral oncogene homolog (KRAS) in HCC94 cells was determined via Western blotting.

RESULTS: MiRNA online database analysis showed that KRAS was the downstream target of miR-16. Compared with miR-16 NC group, the viability and proliferation ability of HCC94 cells increased significantly in miR-16 siRNA group but decreased significantly in miR-16 mimic group ($p<0.05$). However, the apoptosis rate evidently declined in miR-16 siRNA group while increased remarkably in miR-16 mimic group ($p<0.05$). In addition, the protein expression level of KRAS in HCC94 cells was significantly higher in miR-16 siRNA group but significantly lower in miR-16 mimic group when compared with miR-16 NC group ($p<0.05$).

CONCLUSIONS: MiR-16 is lowly expressed in HCC94 cells. Moreover, highly expressed miR-16 represses the viability and proliferation of HCC94 cells and promotes their apoptosis by targeted regulation on KRAS.

Key Words:
MiR-16, Cervical cancer (CC), Cell proliferation, Apoptosis, KRAS.

Introduction

Cervical cancer (CC) is one of the most common malignancies in women, whose incidence is only second to breast cancer. The mortality rate of CC is relatively high, especially in developing countries where the incidence rate is higher than in developed countries. Currently, CC is becoming more common in young people[1,2]. Surgery is the major treatment approach for CC in clinical practice. After treatment, the 5-year survival rate is about 65% for patients with stage II or above CC and only 10% (poor prognosis) for those with locally advanced CC[3,4]. With the advance of science and technology, certain progress has been made in the prevention and diagnosis of CC by researchers in China and foreign countries. However, no safe and effective treatment methods or plans have been discovered so far. Therefore, it is urgent to improve the therapeutic effect and prolong the survival time of patients for medical workers.

Nowadays, researchers have found that the Kirsten rat sarcoma viral oncogene homolog (KRAS) plays a crucial role in the development and progression of malignant tumors at the genetic level[5]. KRAS is abnormally expressed or has site-directed mutations in many tumors, including gastric cancer, lung cancer and prostate cancer. Meanwhile, dysregulation of KRAS participates in such pathological processes as tumor cell proliferation, migration and invasion[6]. KRAS mostly mutates in codon 12, 13 and 61, leading to decreased ability of KRAS to hydrolyze GTP. Be-

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sides, more and more KRASs are activated due to the declined hydrolysis ability of GTPase, eventually promoting tumor development[7,8]. These findings all indicate that inhibiting KRAS expression may be a potential new method for the treatment of tumors.

Non-coding single-stranded small-molecule RNAs are a kind of RNA molecules with about 20-24 nucleotides in length, namely, microRNAs (miRNAs). With the progression of molecular biology, it has been discovered that miRNAs also exert biological functions[9]. MiR-16 was the first miRNA found to have a correlation with malignant tumors. MiR-16 is lowly expressed in various tumors like leukemia, prostate cancer, colon cancer and lymphoma[10,11]. Zubillaga-Guerrero et al[12] discovered in 2015 that miR-16 is lowly expressed in CC. It can target and modulate the cell cycle positive regulator Cyclin E1 that is also deemed as an indicator of poor prognosis in CC. Meanwhile, elevated expression of miR-16 up-regulates cell cycle to some extent, thus repressing the proliferation of CC cells. The above findings suggest that miR-16, a tumor suppressor gene, plays a vital role in the development and progression of CC. However, whether miR-16 can suppress the proliferation and apoptosis of CC cells by regulating KRAS has not been fully elucidated.

In this study, therefore, HCC94 cells were used as research objects to investigate the regulatory effect of miR-16 on cell proliferation and apoptosis, and to explore the underlying mechanism.

Materials and Methods

Reagents

MiR-16 mimic and small interfering RNA (siRNA) (RiboBio, Guangzhou, China), penicillin/streptomycin (P/S), fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA), cell counting kit-8 (CCK-8) kits (Beyotime Biotechnology, Shanghai, China), 96-well culture plates and culture dishes (Corning, Corning, NY, USA), miR-16 and β-actin primers (Thermo Fisher Scientific, Waltham, MA, USA), Annexin V/ propidium iodide (PI) apoptosis kits and 5-Ethynyl-2'-deoxyuridine (EdU) staining kits (Nanjing Genechem Co., Ltd., Nanjing, China), KRAS and β-actin primary antibodies and horseradish peroxidase (HRP)-labeled secondary antibodies (Abcam, Cambridge, MA, USA), enhanced chemiluminescence (ECL) kits (Solarbio, Beijing, China), SYBR Green polymerase chain reaction (PCR) MasterMixture kits (ABI, Applied Biosystems, Foster City, CA, USA) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Instruments

A pure water system (Millipore, Billerica, MA, USA), a microplate reader (Biotek, Winooski, VT, USA), a high speed centrifuge (Eppendorf, Hamburg, Germany), a CO_2 incubator and an ice machine (SANYO, Osaka, Japan), a quantitative PCR (qPCR) instrument and a electrophoresis apparatus (Beijing Liuyi Instrument Factory, Beijing, China), a super clean bench (Suzhou Antai Equipment Co., Ltd., Suzhou, China), and a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Culture

HCC94 cells purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in DMEM containing 1% P/S and 10% FBS. The culture medium was replaced regularly, followed by sub-culture. Next, the cells were divided into three groups, including blank control group [miR-16 normal control (NC) group], miR-16 mimic group and miR-16 inhibitor group (miR-16 siRNA group).

Cell Transfection and Quantitative Reverse Transcription PCR (qRT-PCR)

HCC94 cells in the logarithmic growth phase were collected and seeded into 6-well plates at a density of 5×10^5 cells per well. Next, miR-16 NC, miR-16 mimic and miR-16 siRNA were prepared into 20 μM solution with Lipofectamine 2000 transfection reagent. The mixture was then transfected into HCC94 cells when cell density reached 80%, followed by culture in an incubator for 48 h. Next, the expression level of miR-16 in transfected HCC94 cells was measured *via* quantitative reverse transcription PCR (qRT-PCR) assay. U6 was used as an internal reference. SYBR reaction was carried out using a 10 μL system for 35 cycles according to relevant instructions. 3 replicates were set for each sample. Primers used in this study were shown in Table I. The expression level of miR-16 was calculated based on the $2^{-\Delta\Delta Ct}$ value.

Detection of the Viability of HCC94 Cells Through CCK-8 Assay

Transfected HCC94 cells were re-seeded into 96-well plates at a density of 5×10^4 cells/mL, with

Table I. Primer information.

List	Primer
MiR-16	F: GCGGATCCAGCACATCATGGTTTACA
	R: GCGYCGACAAAAATGTTACCTTAAAGGG
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT

3 replicates for each group. Then, the cells were cultured for 24 h. Next, 10 μL of CCK-8 reagent was added to the cells, followed by incubation for 4 h in the dark. Absorbance at 450 nm was finally detected by a micro-plate reader.

Detection of the Proliferation of HCC94 Cells Via EdU Staining Assay

HCC94 cells were seeded into 6-well plates, and transfected with miR-16 NC, siRNA and mimic for 24 h. Next, 1 mL of EdU staining solution was added to each well for 30 min of incubation, followed by addition of anti-quenching fluorescent agent in drops. Thereafter, an inverted fluorescent microscope was employed to observe the staining.

Detection of the Apoptosis of HCC94 Cells by Flow Cytometry

HCC94 cells were first washed with phosphate-buffered saline (PBS) and trypsinized. The resulting cell suspension was then collected and centrifuged. After re-suspension with 195 μL of Annexin V-FITC binding solution, the cells were incubated with 5 μL of Annexin V-FITC in the dark for 15 min and with 10 μL of propidium iodide (PI) staining solution for 5 min. Cell apoptosis rate in each group was finally determined by a flow cytometer.

Prediction of Downstream Targets of MiR-16 Using TargetScan

The attribute "Human" was firstly selected in the TargetScan (http://www.targetscan.org), an online database. "KRAS" was searched using the "Gene" search box, and "miR-16" was searched in the search results. Finally, the binding site was queried.

Detection of the Protein Expression of KRAS in HCC94 Cells Via Western Blotting

HCC94 cells were re-inoculated in a 6-well plate, and transfected with miR-16 NC, siRNA and mimic using Lipofectamine 2000 for 24 h. After digestion with trypsin, radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was added, followed by lysis *via* oscillation at 4°C. Then, the protein lysate was collected and centrifuged at 4°C. Next, the supernatant was collected, added with 5× loading dye and subjected to metal bath for 10 min for protein degeneration. Protein samples were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gels and transferred onto membranes. After blocking with skimmed milk for 1 h, the membranes were incubated with KRAS primary antibody (1:1000) overnight. On the next day, the membranes were incubated with secondary antibody. Color development was conducted through enhanced chemiluminescence (ECL) method. Optical density of bands was finally analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

GraphPad 5.0 software (La Jolla, CA, USA) was utilized for systematical analysis of experimental results. Differences between two groups were analyzed by using Student's *t*-test. Comparison among multiple groups was done using One-way ANOVA, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Expression Level of MiR-16 in HCC94 Cells Via qRT-PCR

The expression level of miR-16 in HCC94 cells was measured *via* qRT-PCR. The results (Figure 1) showed that compared with miR-16 NC group, the level of miR-16 decreased significantly in miR-16 siRNA group ($^{*}p<0.05$) but increased markedly in miR-16 mimic group ($^{\#}p<0.05$).

Overexpression of MiR-16 Inhibited the Viability of HCC94 Cells

Cell viability in each group was detected by CCK-8 assay. It was found that the viability of HCC94 cells was remarkably higher in miR-16 siRNA group ($^{*}p<0.05$), while distinctly lower in miR-16 mimic group ($^{\#}p<0.05$) than miR-16 NC group after 24 h. These findings suggested that overexpression of miR-16 significantly repressed the viability of HCC94 cells (Figure 2).

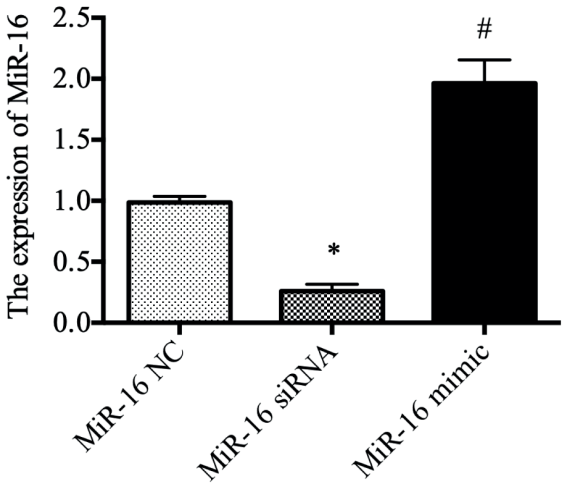

Figure 1. Expression level of miR-16 in HCC94 cells in each group ($^{*}p<0.05$: miR-16 siRNA group *vs.* miR-16 NC group, $^{\#}p<0.05$: miR-16 mimic group *vs.* miR-16 NC group).

Overexpression of MiR-16 Inhibited the Proliferation of HCC94 Cells

The results of EdU staining assay were shown in Figure 3A. In comparison with miR-16 NC group, miR-16 siRNA group had a significantly elevated proliferation rate of HCC94 cells ($^{*}p<0.05$). However, miR-16 mimic group displayed a clearly declined proliferation rate of HCC94 cells ($^{\#}p<0.05$) (Figure 3B). All these findings implied that overexpressing miR-16 substantially inhibited the proliferation of HCC94 cells.

Overexpression of MiR-16 Promoted the Apoptosis of HCC94 cells

Flow cytometry was employed to determine the effect of miR-16 on the apoptosis of HCC94 cells (Figure 4A). The results demonstrated that compared with miR-16 NC group, the apoptosis rate of HCC94 cells was markedly reduced in miR-16 siRNA group ($^{*}p<0.05$) but evidently raised in miR-16 mimic group ($^{\#}p<0.05$) (Figure 4B). The above results indicated that overexpression of miR-16 significantly promoted the apoptosis of HCC94 cells.

KRAS Was a Downstream Target of MiR-16

After searching "KRAS" in the online miRNA database TargetScan (Figure 5), miR-16 was screened out, with a high score in the database. Meanwhile, miR-16 had binding sites to the 1894-1900 in KRAS 3'-UTR, suggesting that KRAS was the downstream target of miR-16.

Overexpression of MiR-16 Down-regulated the Protein Expression Level of KRAS In HCC94 Cells

The band results of Western blotting were shown in Figure 6A. Compared with miR-16 NC group, the protein level of KRAS was significantly elevated in miR-16 siRNA group ($^{*}p<0.05$) while signifcantly reduced in miR-16 mimic group ($^{\#}p<0.05$) (Figure 6B). This demonstrated that overexpression of miR-16 inhibited the protein expression of KRAS in HCC94 cells.

Discussion

CC endangers the health of patients and brings financial burden to the families and the society[13]. With the widespread application of CC screening, CC is early detected and treated in recent years. However, most patients tend to be at the advance stage and have poor prognosis after treatment due to missed or wrong diagnosis caused by no evident clinical symptoms and signs at an early stage[14]. The development of CC has been found mainly associated with abnormal expression of oncogenes, filling of tumor angiogenesis, and abnormal proliferation of tumor cells. However, its pathogenesis remains unclear[15]. Therefore, the mechanism of CC, especially the pathogenesis in young patients, is worthy of investigation by researchers.

Highly evolutionarily conserved miRNAs are closely involved in regulating many physiological

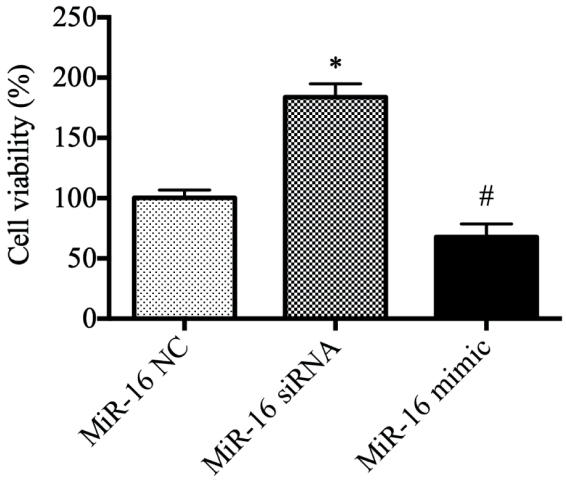

Figure 2. Viability of HCC94 cells in each group ($^{*}p<0.05$: miR-16 siRNA group *vs.* miR-16 NC group, $^{\#}p<0.05$: miR-16 mimic group *vs.* miR-16 NC group).

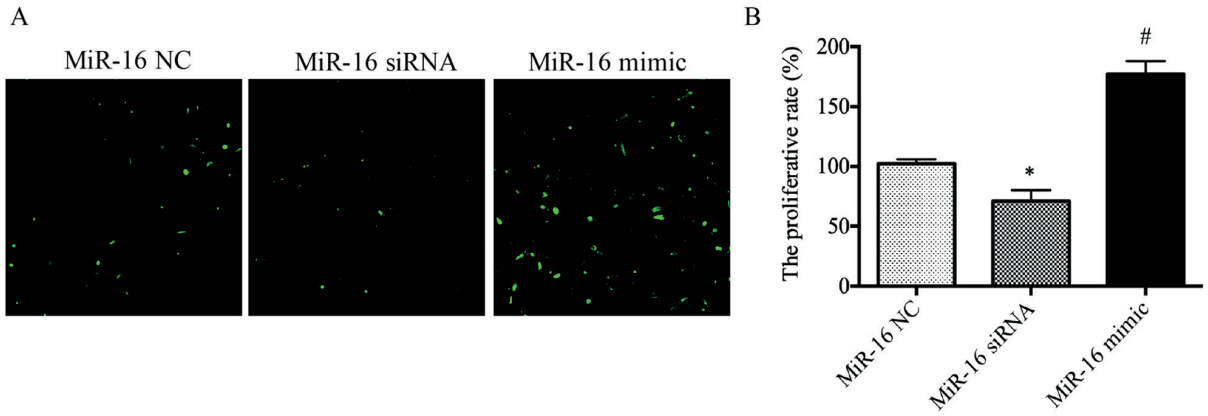

Figure 3. Proliferation of HCC94 cells in each group. **A,** EdU staining map (magnification: 200×), and **B,** Proliferation rate of cells ($^{*}p<0.05$: miR-16 siRNA group *vs.* miR-16 NC group, $^{\#}p<0.05$: miR-16 mimic group *vs.* miR-16 NC group).

and pathological processes of cells[16]. Though miRNAs are not involved in the regulation of encoded proteins, they are vital players in the regulation of post-transcriptional genes. Meanwhile, they can bind to the 3′ non-coding region of target genes to inhibit the degradation of target genes or translate them into proteins[17]. Scholars have manifested that abnormal expression of miR-16, a key tumor-suppressing miRNA, is associated with the development and progression of various tumors. Notably, Jiang et al[18] have found that miR-16 represses the proliferation and invasion of gastric cancer cells *via* targeted regulation of SALL4. Han et al[19] have discovered that miR-16 is able to target Bcl-2 to participate in modulating drug resistance of glioma cells. This suggests that miR-16 may be a new therapeutic target for glioma and exerts important regulatory effects on the development and progression of other tumors. However, the expression and mechanism of action of miR-16 in CC have not been fully expounded. Hence, the aim of this study was to explore the regulatory effects of miR-16 on the proliferation and apoptosis of HCC94 cells, as well as its possible regulatory mechanism.

HCC94 cells were firstly transfected with miR-16 NC, mimic and siRNA separately according to the instructions of Lipofectamine 2000. QRT-PCR assay was conducted to detect the expression of miR-16 in cells. The results (Figure 1) showed that compared with miR-16 NC group, the level of miR-16 decreased overtly in miR-16 siRNA group but increased significantly in miR-16 mimic group. This indicated that transiently transfected HCC94 cells with lowly expressed and highly expressed miR-16 were successfully obtained. Secondly, CCK-8 and EdU staining assays were carried out to detect the effects of miR-16 on the viability and proliferation of HCC94 cells. It was uncovered that both the viability and proliferation capability of HCC94 cells were significantly inhibited after overexpression of miR-16 (Figures 2-3). These findings suggested that overexpression of miR-16 inhibited the viability and proliferation of HCC94 cells. Next, the effects of miR-16 on cell apoptosis were examined using flow cytometry. The results (Figure 4) revealed that the apoptosis of HCC94 cells was enhanced after over-expression of miR-16. The above results indicated that miR-16 played an important role in the proliferation and apoptosis of HCC94 cells. To further investigate the mechanism of action of miR-16, the online miRNA database TargetScan was used to predict the downstream targets of miR-16. Finally, KRAS was screened out to have binding sites to miR-16. You et al[20] have found that low expression of miR-16 is capable of facilitating the development of colon cancer *via* targeted regulation on the miR-16-KRAS axis. In our study, Western blotting was employed to detect the protein expression of KRAS so as to explore the regulatory effects of miR-16 and KRAS. The results (Figure 6) uncovered that compared with miR-16 NC group, miR-16 siRNA group exhibited significantly up-regulated protein expression level of KRAS. However, miR-16 mimic group exerted a markedly down-regulated protein expression level of KRAS. All the above findings implied that the possible regulatory mechanism of miR-16 overexpression in impeding the proliferation and promoting the apoptosis of HCC94 cells might be related to its inhibition on KRAS expression.

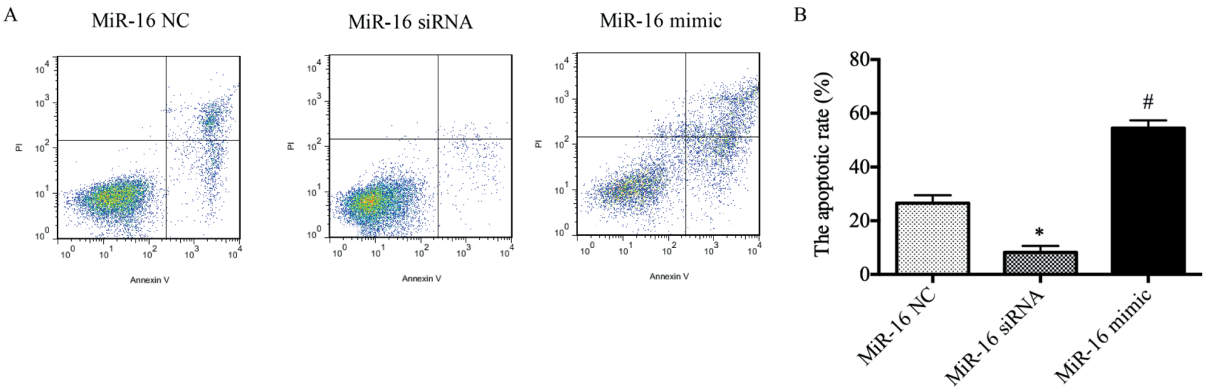

Figure 4. Apoptosis of HCC94 cells in each group. **A,** Results of flow cytometry, and **B,** Apoptosis rate of cells ($^{*}p<0.05$: miR-16 siRNA group *vs.* miR-16 NC group, $^{\#}p<0.05$: miR-16 mimic group *vs.* miR-16 NC group).

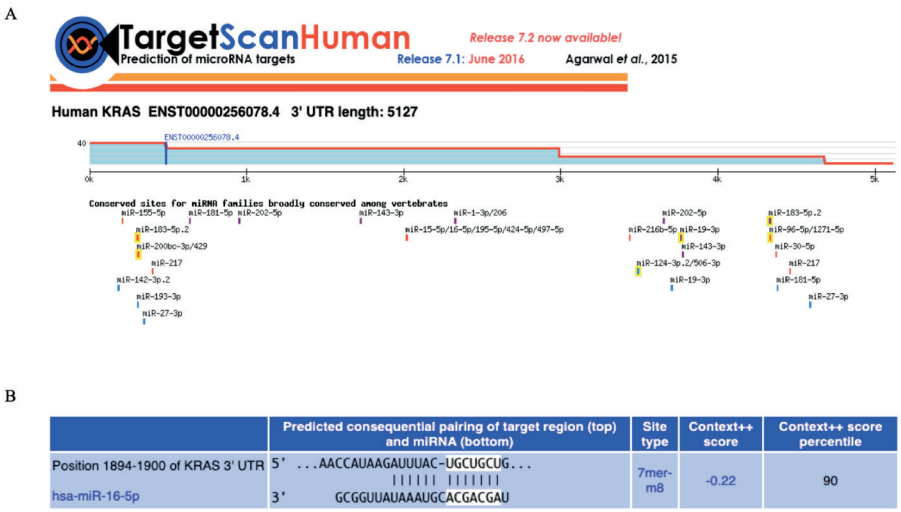

Figure 5. Search results in the online database TargetScan. **A,** MiRNAs with potential binding sites to KRAS, and **B,** Binding sites of miR-16 to KRAS.

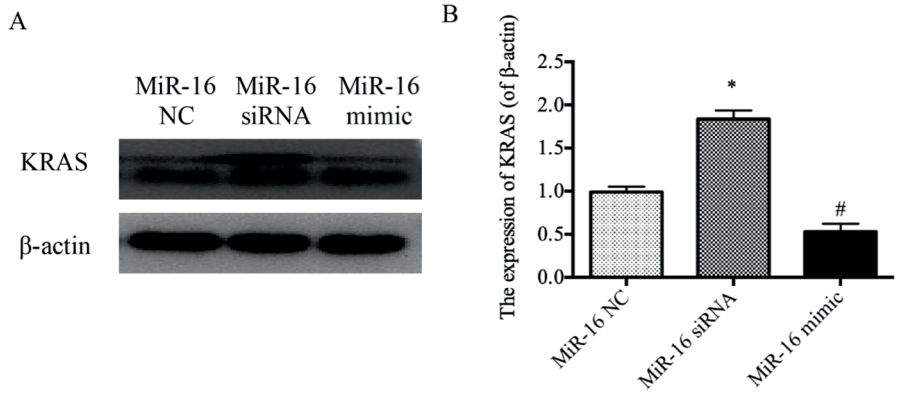

Figure 6. Protein expression level of KRAS in HCC94 cells. **A,** Results of Western blotting, and **B,** Relative expression of KRAS ($^{*}p<0.05$: miR-16 siRNA group *vs.* miR-16 NC group, $^{\#}p<0.05$: miR-16 mimic group *vs.* miR-16 NC group).

Conclusions

MiR-16, a tumor suppressor gene, is closely involved in modulating the proliferation and apoptosis of CC cells. Highly expressed miR-16 suppresses the proliferation and facilitates the apoptosis of CC cells by down-regulating KRAS expression. The novelty of this study was that our findings are of great significance for the treatment of CC and may provide new ideas for the application of miR-16 as a biological marker in the treatment of CC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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