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MicroRNA-19b Promotes Nasopharyngeal Carcinoma More Sensitive to Cisplatin by Suppressing KRAS

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Abstract

MicroRNAs have been reported to play a vital role in diverse biological processes and tumorigenesis. MicroRNA-19b-5p has been observed to be downregulated in other cancers, but the function of microRNA-19b-5p in human nasopharyngeal carcinoma has not been well investigated. In our study, these results demonstrated that microRNA-19b-5p was significantly downregulated in 37 pairs of nasopharyngeal carcinoma tissues when compared to normal tissues. Enforced expression of microRNA-19b-5p inhibited activity of cell proliferation and cell migration of nasopharyngeal carcinoma cancer cells, CNE1 and HNE1. Furthermore, microRNA-19b-5p targeted KRAS proto-oncogene, GTPase in cancer cells. In human clinical specimens, KRAS was higher expressed in cancer tissues when compared with normal tissues, which was inversely correlated with the expression of microRNA-19b-5p. More interestingly, microRNA-19b-5p sensitizes CNE1 cells to cisplatin by inhibiting its target KRAS. Finally, microRNA-19b-5p inhibits tumorigenesis *in vivo*. Thus, our results investigated that microRNA-19b-5p functioned as a tumor suppressor and indicated its potential application for the treatment of human nasopharyngeal carcinoma in future.

Keywords

miRNA-19b, KRAS, cisplatin, chemosensitivity, nasopharyngeal carcinoma

Abbreviations

CDDP, cisplatin; FBS, fetal bovine serum; GTP, guanosine triphosphate; miR-NC, miRNA-negative control; miRNA, microRNA; miRNA-19b, microRNA-19b-5p; NPC, nasopharyngeal carcinoma; RAS, rat sarcoma; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; WHO, World Health Organization; 3′-UTR, 3′-untranslated region

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Introduction

Nasopharyngeal carcinoma (NPC) is one of endemic malignancy cancers in China.[1] Every year in China, there are over 60 000 new cases, causing 34 000 deaths.[2] In clinical trials, intensity-modulated radiation therapy and in combination with chemotherapy have greatly improved the treatment of NPC, whereas tumor recurrence and metastasis remain as the main urgent problems related to death after treatment.[3-5] Thus, there is urgent need to investigate new molecular mechanisms that underlie the progression of NPC.

MicroRNAs (miRNAs) are a large class of endogenous 21 to 23 nucleotides noncoding RNAs, which regulate about 30% of gene expression of human.[6] The miRNAs could inhibit gene expression by binding to the 3′-untranslated regions (3′-UTRs) of corresponding mRNAs, thus modulating various biological

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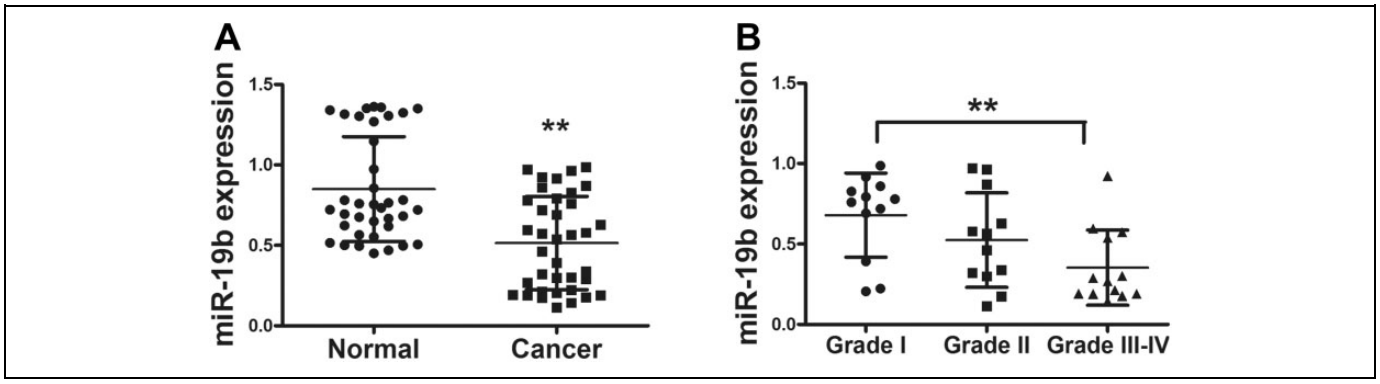

Figure 1. MiR-19b is significantly downregulated in cancer tissues. A, Relative miR-19b expression levels were analyzed by qRT-PCR in 37 pairs of human cancer tissues and adjacent normal tissues. U6 RNA level was used as an internal control. B, The miR-19b expression in 3 different grades of cancer samples. According to the pathological classification, the 37 pairs of human cancer tissues were divided into 3 groups: WHO grade I, grade II, and grade III–IV. Data represent mean (SD) of 3 replicates. **Significant difference at $P < .01$. miR-19b indicates microRNA-19b-5p; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; WHO, World Health Organization.

processes, including cell differentiation, cell proliferation, cell cycle, cell migration, and cell apoptosis.[7,8] As a tumor regulator in several cancers including gastric cancer, colon cancer, breast cancer, melanoma, and NPC, miRNA-19b-5p (miR-19b) can affect tumor cell growth, cell migration, and chemoresistance. Some genes have been validated as miR-19b target genes, including *TNFAIP3*, *BCL3*, *SMAD4*, *PTPRG*, and *PITX1*.[9-13] However, the underlying molecular mechanism of miR-19b-regulated NPC tumorigenesis is still to be further investigated.

The rat sarcoma (RAS) family has three members: HRAS, NRAS, and KRAS, which encode a 21-kDa guanosine triphosphate (GTP)-binding proteins.[14] Among RAS proteins, KRAS in the Harvey and Kirsten strains of the RAS/mouse sarcoma virus was first identified as the transforming factor.[15] In complex cell signaling networks, KRAS functions as a critical "on–off" switch, which relays extracellular signals to the nucleus and connects upstream signals with the downstream signaling pathways.[16] These signaling pathways are referred to cell differentiation, cell proliferation, migration, invasion, and cell cycle.[17,18] In this study, we suggested that miR-19b-targeted KRAS, a key downstream effector of the phosphoinositide 3-kinase/ serine/threonine kinase 1 (PI3K/AKT) and Mitogen Activated Protein Kinase/extracellular regulated kinase (MAPK/ERK) signaling pathway, has long been identified to play an important role to monitor cancer cell growth.[19]

Recently studies have reported that miR-19b plays important roles in cancer *via* affecting different cell signaling pathways. In our study, we aimed to further identify the roles of miR-19b and its molecular as well as cellular mechanisms in cancer. Overexpression of miR-19b inhibited cell proliferation, cell migration, and tumor growth by suppressing a key target KRAS. We further defined miR-19b-induced chemosensitivity of cancer cells to cisplatin (CDDP) through KRAS suppression. In our study, these results revealed a new molecular mechanism of miR-19b in cancer, and it possessed a potential to be used as a novel strategy to develop miR-19b-based therapeutics.

Materials and Methods

Clinical Tissues and Cell Culture

The NPC specimens and normal specimens were collected from clinical patients undergoing NPC resection. All these specimens were conserved in liquid nitrogen after surgery. Informed consents were obtained from all patients participating in this study. This study was approved by the Research Ethics Committee of Zhengzhou University. NP69 cell lines, as human immortalized nasopharyngeal epithelial cells, were cultured in keratinocyte/serum-free medium, then supplemented with various growth factors; human NPC cell line CNE1, C666, SUNE1, and HNE1 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, while HEK293T cells was cultured in Dulbecco's modified Eagle medium. All cells were incubated at 37°C incubators.

Establishment of Stable Cell Lines

According to the manufacturer's manual, lentivirus carrying miR-19b or miR-NC was packaged in HEK293T cells to stably overexpress miR-19b in cancer cells (Thermo Fisher Scientific, Rockford, Illinois). Indicated cells were infected with lentivirus carrying miR-19b or miR-NC and selected by puromycin (Sigma-Aldrich, China) for 2 weeks.

Oligonucleotides and Cell Transfection

MiR-19b mimics and miR-NC were chemically synthesized (GenePharma, Shanghai, China). Cells at 50% to 70% confluence were transfected with miR-19b or miR-NC using Lipofectamine reagent (Invitrogen, China). After 24 or 48 hours transfection, cells were harvested.

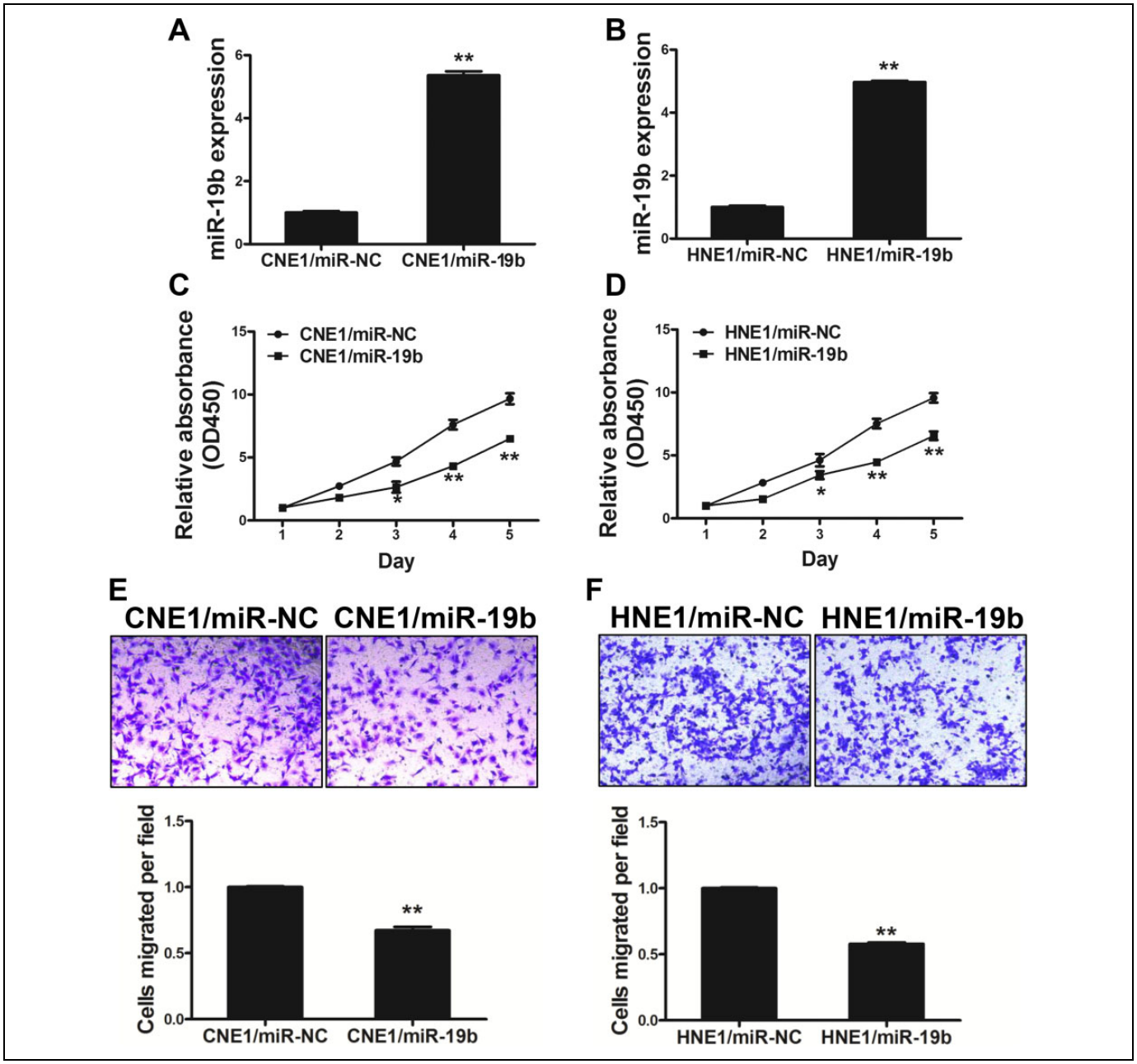

Figure 2. Overexpression of miR-19b inhibits the ability of cell proliferation and cell migration in cancer cells. A and B, Relative expression levels of miR-19b in CNE1/miR-19b, CNE1/miR-NC, HNE1/miR-19b, and HNE1/miR-NC stable cell lines were confirmed by RT-qPCR. C and D, Overexpression of miR-19b arrested cell proliferation in CNE1 and HNE1 cells. E and F, MiR-19b overexpression reduced cell migration in CNE1 and HNE1 cells. Data represent mean (SD) of 3 replicates. *Significant difference at $P < .05$. **Significant difference at $P < .01$. miR-19b indicates microRNA-19b-5p; NC, negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation.

Quantitative Reverse Transcription Polymerase Chain Reaction

Purified RNA was extracted and stored at −80°C. According to the manufacturer's instructions, qRT-PCR analysis for miR-19b was carried out using the RT reagent kit (Takara, China). Furthermore, 500 ng total RNA was reversely transcribed into complementary DNA, and qRT-PCR was performed using SYBR Green Master Mix (Takara, China) on a 7900HT Applied Biosystem. The miR-19b expression was determined relative to internal U6, and relative fold changes were calculated by $2^{-\Delta\Delta Ct}$.

Cell Proliferation Assay

Cell Counting Kit-8 (CCK8 kit; Dojindo Laboratories, Japan) assay was used to determine viability of cells. Cells were seeded at a density of 2000 per well. After 24, 48, 72, and 96 hours incubation, CCK8 was added into each well, followed by

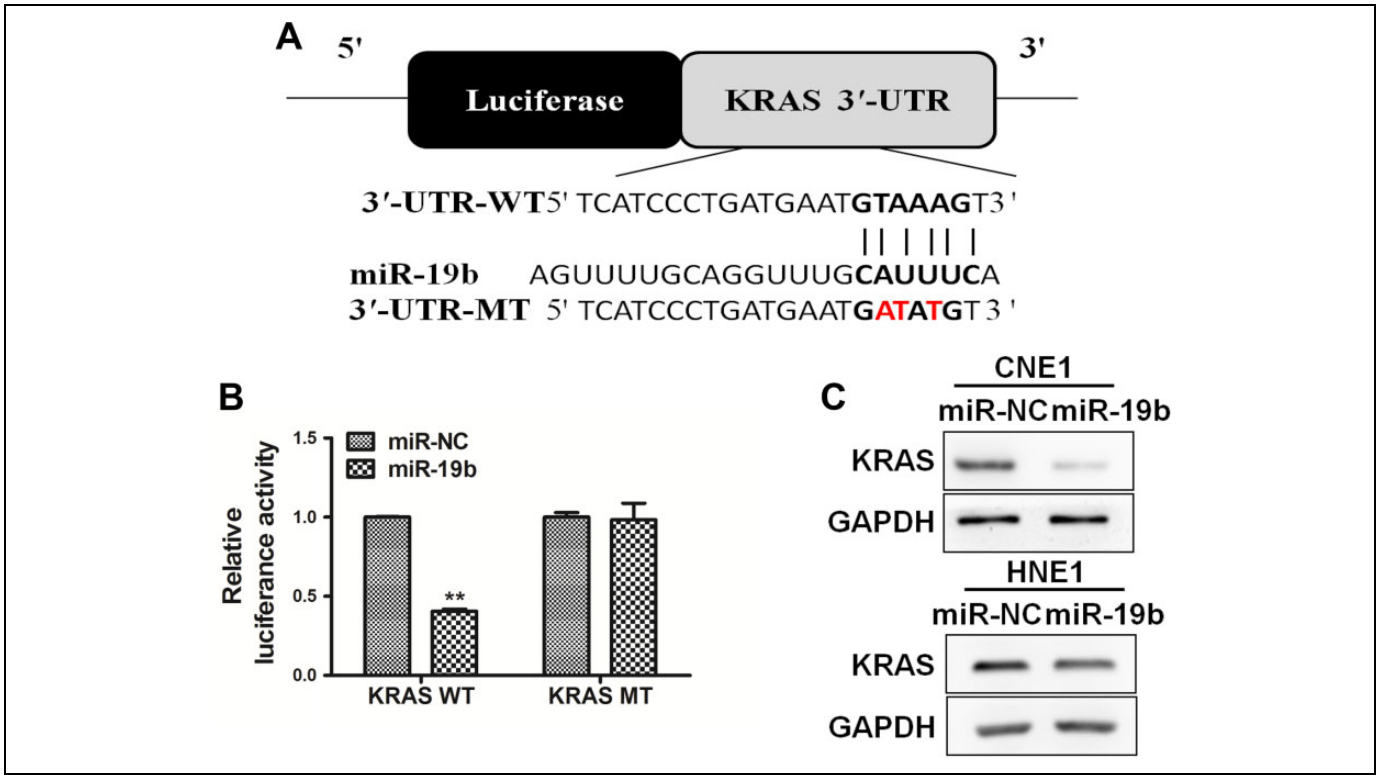

Figure 3. KRAS is a target of miR-19b. A, Sequence of the miR-19b-binding site within the human KRAS 3′-UTR and a schematic diagram of the reporter construct showing the entire KRAS 3′-UTR sequence and the mutant KRAS 3′-UTR sequence. The mutant nucleotides of the KRAS 3′-UTR are labeled in red. B, Luciferase assay on CNE1 cells, which were cotransfected with miR-NC or miR-19b and a luciferase reporter containing the full length of KRAS 3′-UTR (WT) or a mutant (MT) harboring 4 mutant nucleotides of the miR-19b-binding site. Luciferase activities were measured 24 hours posttransfection. MiR-19b markedly suppressed luciferase activity in KRAS 3′-UTR (WT) reporter constructs. The data represent means (SD) for separate transfections (n = 4). C, The immunoblotting showed that expression levels of KRAS were decreased in cells with miR-19b overexpression. Data represent mean (SD) of 3 replicates. **Significant difference at $P < .01$. miR-19b indicates microRNA-19b-5p; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; UTR, untranslated region.

1-hour incubation. Absorbance was then determined at a wavelength of 450 nm.

Cell Migration Assay

The different effects of miR-NC or miR-19b on cell migration were investigated with migration chambers. The transfected cells (5×10^4) were seeded in the upper well containing serum-free RPMI-1640, and RPMI-1640 containing 10% fetal bovine serum (FBS) was applied to the lower. After 18 to 24 hours, cells were fixed with 3% paraformaldehyde and stained with 0.1% crystal violet, and the absorbance was recorded at 570 nm.

Western Blot

Cells and tissues were treated according to previous study, and cells were harvested after 24 hours and lysed in Radio Immunoprecipitation Assay (RIPA) buffer supplemented with indicated protease inhibitors on ice for 30 minutes. After 15-minute centrifugation, concentrations of protein were determined and then separated by 8% to 10% sodium lauryl sulfate polyacrylamide gels. The proteins were detected with KRAS antibody (1:1000; Cell Signaling Technology, Danvers, Massachusetts) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Bioworld Technology, Atlanta, Georgia).

Dual-Luciferase Reporter Assay

TargetScan software was used to predict miR-19b-binding sites of targeted gene. The 3′-UTR of KRAS containing the miR-19b-binding site was amplified by PCR. To generate a construct containing the mutant miR-19b-binding site, 3 nucleotides were substituted. Constructs were transfected into cells in 24-well plates and cotransfected with miR-19b or miR-NC. After 24 hours, assays were performed using the dual-luciferase reporter assay system (Promega, Wisconsin, USA).

In Vitro *Chemosensitivity Assay*

Cancer cells were cultured in a 96-well plate overnight at a density of 4000 cells per well. Freshly prepared CDDP solution (Sigma-Aldrich) was added into medium to obtain final indicated concentrations (1.25, 2.5, 5, 10, 20, 40, and 80 μM), then detected by CCK8 kit 48 hours later.

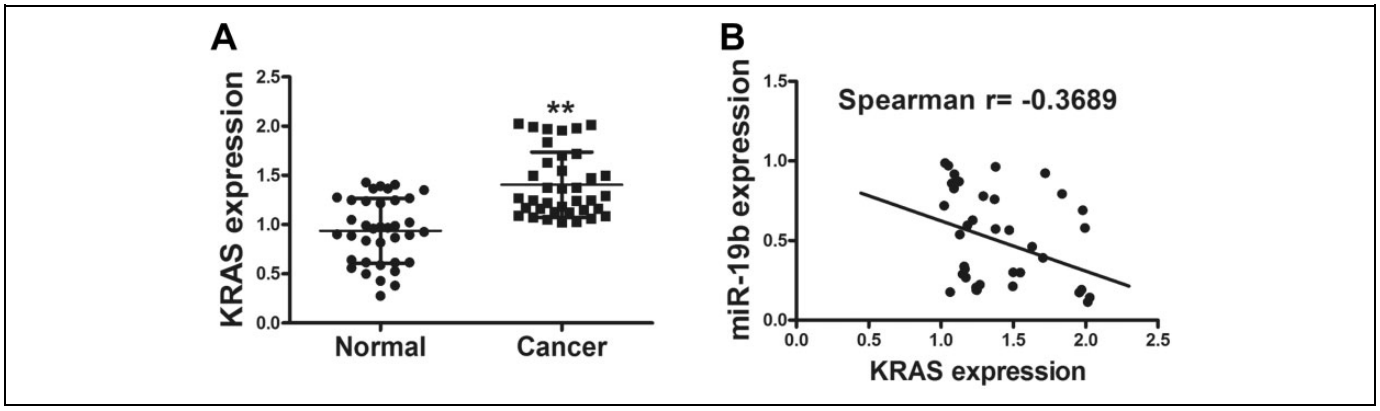

Figure 4. The expression of KRAS was inversely correlated with miR-19b expression in human clinical specimens. A, The expression of KRAS in adjacent normal tissues and human cancer specimens was determined by qRT-PCR analysis, and fold changes were obtained from the ratio of KRAS and GAPDH levels. B, Spearman analysis was conducted between expression of miR-19b and KRAS. Data represent mean (SD) of 3 replicates. **Significant difference at $P < .01$. miR-19b indicates microRNA-19b-5p; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; UTR, untranslated region.

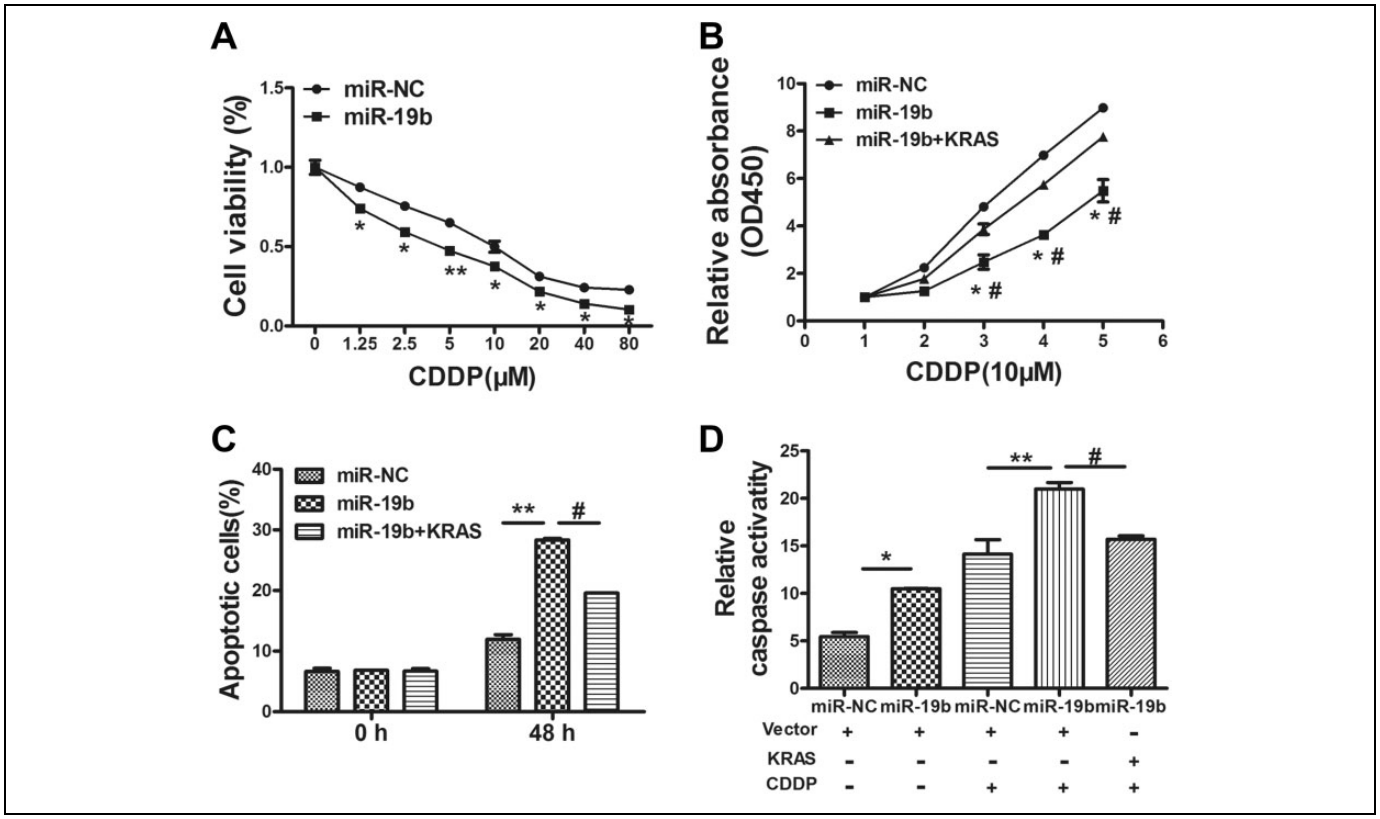

Figure 5. MiR-19b regulates cisplatin (CDDP) chemosensitivity by targeting KRAS in nasopharyngeal carcinoma cells. A, CNE1 cells stably expressing miR-NC or miR-19b were treated with different concentrations of CDDP for 48 hours and analyzed by CCK8 assay. B, CNE1 cells stably expressing miR-NC, miR-19b, or miR-19b in combination with KRAS overexpression were treated with 10 μM of CDDP for indicated time points. Cell proliferation was analyzed by CCK8 assay. C, D, Cell apoptosis assay by flow cytometry and caspase analysis were analyzed in CNE1 cells with 10 μM CDDP treatment. Data represent mean (SD) from 3 replicates. *Significant difference at $P < .05$. **Significant difference at $P < .01$. *Significant difference at $P < .05$ compared to miR-NC control. #Significant difference at $P < .05$ compared to miR-19b and KRAS overexpression. miR-19b indicates microRNA-19b-5p; NC, negative control; SD, standard deviation.

Apoptosis Assay

Apoptosis assay was measured by flow cytometry. For Annexin V staining, indicated reagents and buffer were added to the cell samples and incubated for 15 minutes. Samples were analyzed by flow cytometry (BD Biosciences, Bedford, Massachusetts) within 1 hour. Three experiments were performed in triplicate.

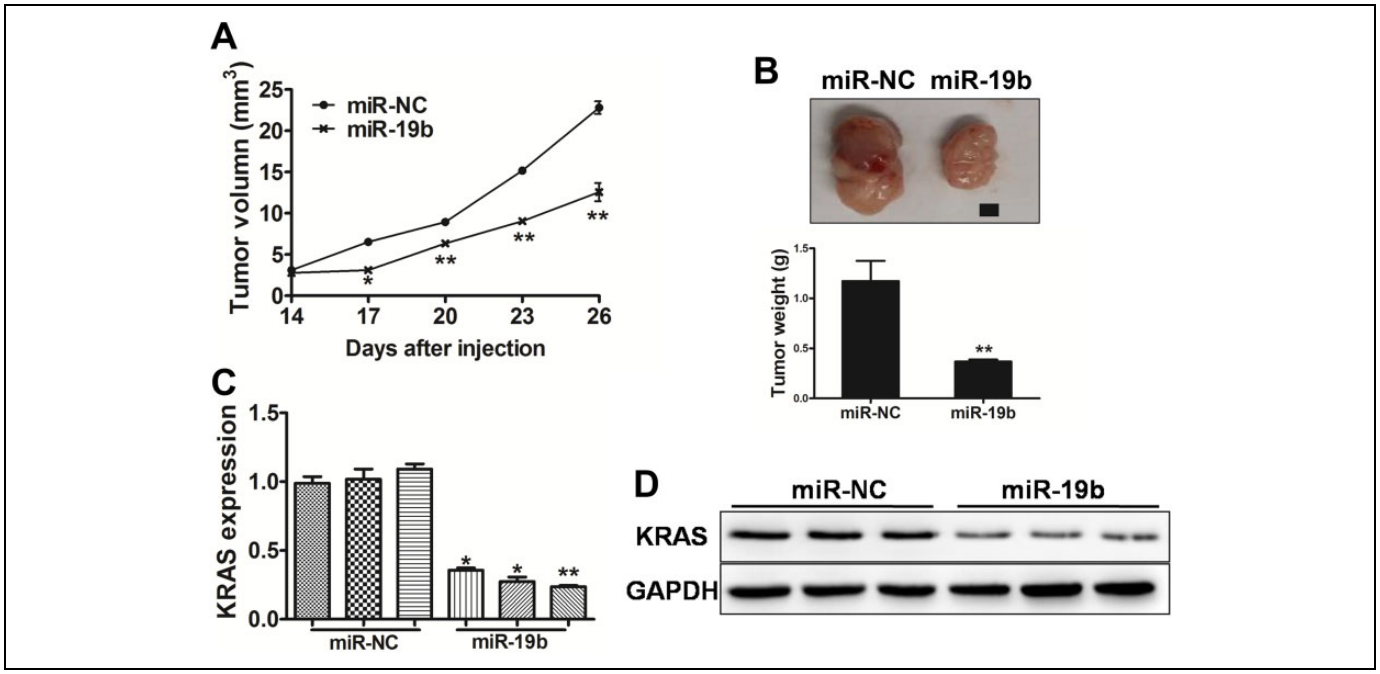

Figure 6. MiR-19b inhibits tumorigenesis *in vivo*. A, B, Tumor growth assay in nude mice. Representative pictures of xenograft tumors. Bar: 2 mm. Tumor growth curve (A) and average of xenograft tumors (B) between the groups of miR-NC and miR-19b. C, The expression levels of KRAS were analyzed in tumor tissues by qRT-PCR. D, Protein levels of KRAS in xenograft tumors. Data were presented by mean (SD). $*P < .05$, $**P < .01$. miR-19b indicates microRNA-19b-5p; NC, negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation.

Caspase-3 Activity Assay

The activity of caspase-3 was evaluated by kit (Beyotime, Jiangsu, China). According to manufacturer's instructions, caspase-3 activity is based on detection of the chromophore *p*-nitroanilide, which was quantified by determining the absorbance at a wavelength of 405 nm.

Xenograft Studies

For tumor growth assay, female nude mice (6-week-old) were purchased from Beijing Vital River Laboratory Animal Technology and maintained in special pathogen-free conditions. Animal protocols were approved by the Animal Welfare Committee of Zhengzhou University. Aliquots of cells (5×10^6) were suspended in 150 mL of FBS-free medium and injected into mice. Tumor size was measured using Vernier caliper every 2 days when they were visible, and tumor volume was calculated according to the formula: volume $= 0.5 \times$ length $\times$ width^2.

Statistical Analysis

All experiments in this study were performed in triplicate, and data were analyzed with GraphPad Prism 5 software (La Jolla, California). Statistical evaluation for data analysis was determined by *t* test, and $P < .05$ was considered statistically significant. The correlation between miR-19b and KRAS levels in cancer specimens was analyzed by Spearman rank test.

Results

MiR-19b Is Significantly Downregulated in Cancer Tissues

In our study, we assessed the miR-19b expression levels in 37 pairs of clinical specimens, and Figure 1A suggested that the miR-19b expression in tumor tissues was significantly reduced compared with those controls. In addition, all human cancer samples, according to World Health Organization (WHO) classification, were divided into grade I, grade II, and grade III–IV, and miR-19b expression in high-grade tumors (WHO grade III-IV) was distinctly reduced than that in low-grade tumors (WHO grades I and II; Figure 1B). Our results indicated that the progressive loss of miR-19b might be associated with the cancer disease progression.

Overexpression of miR-19b Inhibits Activity of Cell Proliferation and Cell Migration of Cancer Cells

In this study, we established stable cell lines with lentiviral constructs harboring miR-19b or miRNA-negative control (miR-NC) in CNE1 and HNE1 cells (Figure 2A and B). Cell viability assay indicated that the miR-19b overexpression significantly reduced the rate of cell proliferation at 48 hours after the cell seeding in CNE1 and HNE1 cells (Figure 2C and D). Since cell migration is key specialty of malignant tumor, we also investigated the effects of miR-19b on migration, showing that overexpression of miR-19b also markedly decreased the migration ability of cancer cells (Figure 2E and F). Thus, these

results showed that overexpression of miR-19b inhibits activity of cell proliferation and cell migration of cancer cells.

MiR-19b-Targeted KRAS and KRAS Levels Were Inversely Correlated With miR-19b Levels in Human Clinical Specimens

In our study, targetScan and miRanda software were used to suspect potential targets of miR-19b in cancer, which then analyze the underlying molecular mechanism of miR-19b in cancer. Figure 3A reveals the 3′-UTR fragment of KRAS contained the binding site of miR-19b. CNE1 cells were transfected with reporter plasmids, plus either miR-19b or miR-NC mimics. Our study showed that miR-19b significantly reduced the luciferase activity of the wild-type reporter, while the mutant reporter insignificantly affected (Figure 3B). Western blotting analysis was conducted to determine the KRAS expression. We found that KRAS expression was downregulated in miR-19b-treated cells at the protein level (Figure 3C). These results showed that miR-19b directly targeted KRAS. Then, we investigated the KRAS expression at the mRNA level in human specimens, which showed that KRAS levels were significantly higher in tumor tissues than normal tissues (Figure 4A). Next, we testified the relationship between KRAS and miR-19b levels in NPC specimens. Spearman rank correlation analysis showed that KRAS and miR-19b were inversely correlated in human NPC specimens (Figure 4B; $r = -0.3689$). Thus, these results suggested that miR-19b directly targeted KRAS, and KRAS levels were inversely correlated with miR-19b levels in human clinical specimens.

MiR-19b Renders Cancer Cells Sensitive to CDDP Treatment by Targeting KRAS

Resistance to CDDP treatment is one of the major causes for the failure of chemotherapy in clinical treatment of various cancers. Therefore, it is urgent to discover novel strategies to promote drug effect. In this study, we suggested that miR-19b significantly promoted chemosensitivity to CDDP treatment in CNE1 cells (Figure 5A). Then, cell growth rate was assayed in the presence of CDDP at indicated time points, which found that reversed expression of KRAS resulted in more resistance to CDDP treatment in miR-19b-overexpressed cells (Figure 5B). To investigate whether miR-19b and KRAS play a role in cell apoptosis after treatment of CDDP, fluorescence-activated cell sorting and caspase-3 analysis were performed. That is, the combination of miR-19b and CDDP significantly induced cell apoptosis and caspase-3 activity; however, reversed expression of KRAS partially abolished the apoptotic activity induced by miR-19b and CDDP (Figure 5C and D). These results indicated that miR-19b renders cancer cells more sensitive to CDDP treatment for inducing cell apoptosis by targeting KRAS.

MiR-19b Inhibits Tumor Growth In Vivo

To test the effect of miR-19b on tumor growth, CNE1 cells overexpressing miR-19b or miR-NC were subcutaneously injected into both posterior flanks of nude mice ($n = 5$). Xenograft tumor volumes were measured every 2 days when they were visible. On day 17 after postimplantation, tumor growth of miR-19b overexpression group was found significantly smaller than the control group. Then, 28 days later, nude mice were killed, and xenografts were collected. Tumor growth rate was reduced in miR-19b group compared with miR-NC (Figure 6A). When compared with controls, the average tumor weight of miR-19b overexpression group was markedly reduced by 60% (Figure 6B). Total proteins and RNAs from representative tumor samples were analyzed by Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR), which determined that miR-19b suppressed its target KRAS expression *in vivo* (Figure 6C and D).

Discussion

Recently, studies have suggested that miR-19b is downregulated in several cancer types, including NPC. Here, in this study, we showed that miR-19b was downregulated in cancer tissues compared with normal controls, and the miR-19b was decreased in high-grade tumors compared to that in low-grade tumors, indicating that miR-19b was correlated with cancer progression. More interestingly, we further found overexpression of miR-19b inhibited cell proliferation, cell migration, and tumor growth. Thus, we demonstrated that miR-19b regulated cancer growth, which provides novel therapeutic strategies for cancer diagnosis, prevention, and treatment.

KRAS, a 21-kDa GTP-binding proteins, belonging to the RAS family, has previously been demonstrated to be associated with various biological processes in cancers.[20,21] Abnormality of RAS signal pathway is often occurred in various human cancers. Wang *et al* have reported that miR-143 acts as a tumor suppressor in glioma by targeting N-RAS and enhances temozolomide effect.[22] Jiang *et al* reported that miR-124 enhances chemosensitivity by targeting R-Ras and N-Ras.[23] However, a more effective therapeutic approach directly targeting RAS remains to be developed. In this study, KRAS was experimentally validated as direct target of miR-19b. First, miR-19b directly recognized the 3′-UTR of KRAS by luciferase reporter assay. Second, KRAS expression was significantly decreased at the protein level in cancer cells stably expressing miR-19b. Third, KRAS were upregulated in cancer tissues and inversely correlated with miR-19b levels. These results show that miR-19b is a tumor suppressor through targeting KRAS.

Recently, miRNAs have been proposed to play essential roles in regulation of drug resistance. Resistance to clinical chemotherapy is induced by a lot of factors, including individual differences in patients and epigenetic or genetic changes in tumors.[24] In our study, we suggested that miR-19b promoted the inhibition effects of CDDP. Flow cytometer and caspase-3 assay demonstrated that cancer cells with miR-19b have higher

CDDP sensitiveness to cell apoptosis. Therefore, it is feasible that restoration approach of miR-19b may offer a regulatory strategy to overcome chemoresistance to CDDP in NPC.

In this study, we demonstrated that miR-19b played a vital role in suppressing cancer growth through inhibition of KRAS. Although we ensured that miR-19b could inhibit the most phenotype of cancer by directly targeting KRAS, there might be many other targets of miR-19b, which also could affect progression of cancer. Nonetheless, we showed that such effect was exerted through the suppression of KRAS. Thus, future more studies are required to signify various targets and regulatory pathways of miR-19b.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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