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MicroRNA-326 inhibits cell proliferative capacity and invasiveness through inhibiting the expression of ETS1 in nasopharyngeal carcinoma

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Abstract. – OBJECTIVE: This study aims to detect microRNA-326 expression in nasopharyngeal carcinoma (NPC) tissues and cell lines and to explore the potential mechanism of microRNA-326 inhibiting the proliferative capacity and invasiveness of NPC cells.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine microRNA-326 expression in 40 cases of NPC tissue samples and cell lines. Meanwhile, microRNA-326 mimics were transfected into NPC cells to up-regulate microRNA-326 level. Next, the influence of microRNA-326 mimics on the proliferation and invasiveness of NPC cells was observed by Cell Counting Kit-8 (CCK-8) assay and transwell assay, respectively. Bioinformatics analysis was applied to search for target genes which may have direct effects on microRNA-326. An EST1 luciferase reporter vector containing microRNA-326 binding site was constructed and the binding relation between ETS1 and microRNA-326 was detected by Dual-Luciferase reporting assay. Lastly, the underlying mechanism of microRNA-326 and ETS1 in NPC was further verified via cell reverse experiments.

RESULTS: Compared with control group, microRNA-326 expression was found remarkably decreased both in NPC tissue specimens and cell lines. The low expression of microRNA-326 could predict poor prognosis of patients with NPC. *In vitro* cell experiments revealed that overexpression of microRNA-326 remarkably inhibited the proliferative capacity and invasiveness of NPC cells. Bioinformatics analysis and Dual-Luciferase assay results suggested that microRNA-326 may bind to ETS1. Cell reverse assay indicated that inhibiting ETS1 expression partially reversed the changes in cell biological behavior induced by the down-regulation of microRNA-326 in CNE1 and 5-8F cell lines.

CONCLUSIONS: Overexpression of microRNA-326 in NPC cells could remarkably inhibit the proliferative capacity and invasiveness of NPC cells. In addition, microRNA-326 may participate in the development of NPC by inhibiting ETS1 expression.

Key Words:
Nasopharyngeal carcinoma (NPC), MicroRNA-326, Cell proliferation, Cell invasion, ETS1.

Introduction

Nasopharyngeal carcinoma (NPC) is a common tumor of the head and neck and it is highly prevalent in Southeast Asia. Its incidence is closely related to Epstein Barr virus infection[1]. NPC is characterized by deep location, strong invasiveness, rapid progression, and easy recurrence[2]. Radiotherapy treatment is the first choice for NPC at present. With the progress of radiation technology and the extensive application of intensity-modulated radiation therapy (IMRT), the limitation of the NPC lesions control has improved significantly. However, due to the deep location of the disease and the difficulty in early diagnosis, some patients have distant metastases at the time of treatment. Despite that the radiation and chemotherapy combined with targeted drug therapy and surgery are optional, the overall effect is still poor[3,4]. Therefore, the innovation of therapeutic methods and the exploration of the molecular mechanism of nasopharyngeal cancer have become the hot spots in the field of nasopharyngeal cancer research.

At present, the etiology and pathogenesis of NPC are not completely understood, while in recent years, the exploration of molecular medicine in the field of NPC mainly focuses on the

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apoptosis mechanism of tumor cells and the regulation of mRNA and protein of related genes[5]. Compared with other types of tumors, the exploration of miRNAs in the field of NPC has not been widely analyzed. MiRNA is a highly conserved non-coding single-stranded small RNA molecule with a length of about 18-25 nucleotides, which inhibits the expression of target genes by binding to the 3'UTR sequence of target genes[6,7]. In recent years, reports on miRNAs in NPC have been increasing, indicating that miRNAs received more and more attention by many researchers in the field of NPC. However, the specific role of miRNA in the development and pathological course of NPC remains to be further explored.

Among them, microRNA-326 has been found to be dysregulated in a variety of tumors and acts as a tumor suppressor gene, such as cervical cancer, prostate cancer, non-small cell lung cancer, melanoma, etc.[8-11]. In addition, the existing literature has shown that microRNA-326 expression is decreased in NPC[12], suggesting that it may play a tumor-inhibiting role. Nevertheless, the role of microRNA-326 in NPC remains unclear and needs further validation. ETS1, a member of the ETS transcription factor family, is an important transcription factor that mediates extracellular matrix degradation, cell migration, angiogenesis, and drug resistance. Increasing attention has been given to the regulatory role of ETS1 in tumors[13]. Scholars[14,15] have reported that the poor prognosis of some tumors, such as prostate cancer and breast cancer, is correlated with the high expression of ETS1. Therefore, ETS1 gene may be a potential target for the treatment of NPC. The purpose of this work was to investigate the role of microRNA-326 in regulating proliferative capacity and invasiveness in NPC and the mechanism of its interaction with ETS1.

Patients and Methods

Paients and Sample Collection

Tumor tissue samples and normal control ones of 40 patients diagnosed with NPC were collected and stored in a liquid nitrogen tank for use. The research was approved by the hospital Ethics Committee and patients volunteered to participate in the study and signed written informed consent.

Cell Culture

Normal control cells NP69 and human NPC cell lines (CNE1, CNE2, 6-10B, and 5-8F) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The above cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and cultured in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

1x10^5 NPC cells were plated into a 6-well plate and transfection was performed when the cell density reached about 80%; transfection was carried out according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). MicroRNA-326 mimics, microRNA-326 inhibitor and ETS1 siRNA, and corresponding negative references were all designed and synthesized by GenePharma (Shanghai, China). After the cells were transfected for 6 h, the complete medium was added and the culture was continued for another 48 h.

RNA Extraction

The tissue was lysed with 1 mL TRIzol (Invitrogen, Carlsbad, CA, USA) and extracted by adding chloroform and pre-cooled isopropanol. After centrifugation, the precipitate was gently washed with 75% ethanol and resuspended in 50 μL of diethylpyrocarbonate (DEPC) water (Beyotime, Shanghai, China). The total RNA was quantified by a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) instrument, and the extracted RNA was then stored in a –80°C for later use.

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

The cDNA was reverse transcribed according to the instructions of the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). MiRNA quantitative PCR procedures were performed according to the miScript SYBR Green PCR Kit instructions. QRT-PCR was performed on the ABI7500 instrument according to the SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan) instructions. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are used as internal parameters, respectively. The relative concentration of the sample was calculated by the $2^{-\Delta\Delta CT}$ method.

Cell Proliferation Assay

The treated NPC cells were seeded in a 96-well plate at 4×10^5/mL per well and cultured for 0, 24, 48, and 72 h, and 5 replicate wells were set in

each group. Subsequently, 10 uL of Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well and cultured for further 2 h at 37°C in the dark. Then, the optical density (OD) value was measured at 450 nm. The experiment was repeated three times separately.

Cell Invasion Assay

1×10^5 NPC cells suspended in serum-free medium were seeded into the upper Matrigel invasion chamber and the complete medium was added to the lower chamber. After being cultured for 24 h in an incubator, the cells remaining in the upper chamber membrane were carefully discarded, while the cells adhering to the membrane were fixed with 4% paraformaldehyde, followed by staining with crystal violet. Cells were counted and observed under a fluorescence microscope.

Luciferase Reporting Assay

3×10^4 treated NPC cells were seeded in 24-well plates. After 48 h of co-transfection with the corresponding plasmid and microRNA-326 mimics or negative control, luciferase activity was measured and recorded using a Promega kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Western Blot Assay

The protein in the cells was extracted using a cell lysate containing protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China), and the protein concentration was determined according to the bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China). The total protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), which were incubated with primary antibodies overnight and subsequently with secondary antibodies at room temperature for 1 h. Finally, the protein bands were detected by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. Two-sample *t*-test statistical method was used for comparison between the groups. $p<0.05$ was considered statistically significant.

Results

MicroRNA-326 is Lowly Expressed in NPC

We detected the relative expression of microRNA-326 in NPC tissues and normal control tissues by qRT-PCR. Results showed that microRNA-326 was remarkably downregulated in NPC tissues compared with the normal control group (Figure 1A). Besides, we found that microRNA-326 was remarkably under expressed in NPC cell lines (Figure 1B). According to the expression level of microRNA-326 in NPC, we divided the patients into microRNA-326 high expression group and microRNA-326 low expression group. By analyzing the clinical information, we found that compared with microRNA-326 high expression group, the overall survival was remarkably reduced in the miR-326 low-expression group (Figure 1C), suggesting that low-expression microRNA-326 predicted poor prognosis in patients. These results suggested that microRNA-326 may play an important role in the progression of NPC.

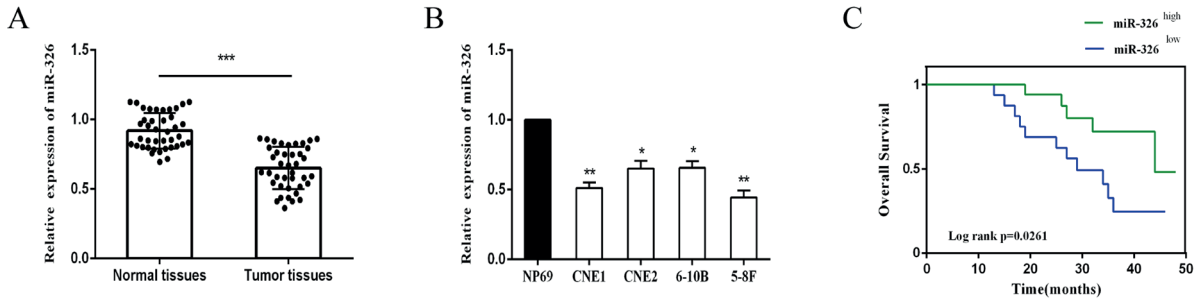

Figure 1. MiR-326 is under expressed in NPC. **A,** Expression of miR-326 in NPC tissues. **B,** Expression of miR-326 in NPC cell lines. **C,** Expression level of miR-326 is associated with the prognosis of patients with NPC. *$p<0.05$; **$p<0.01$; ***$p<0.001$

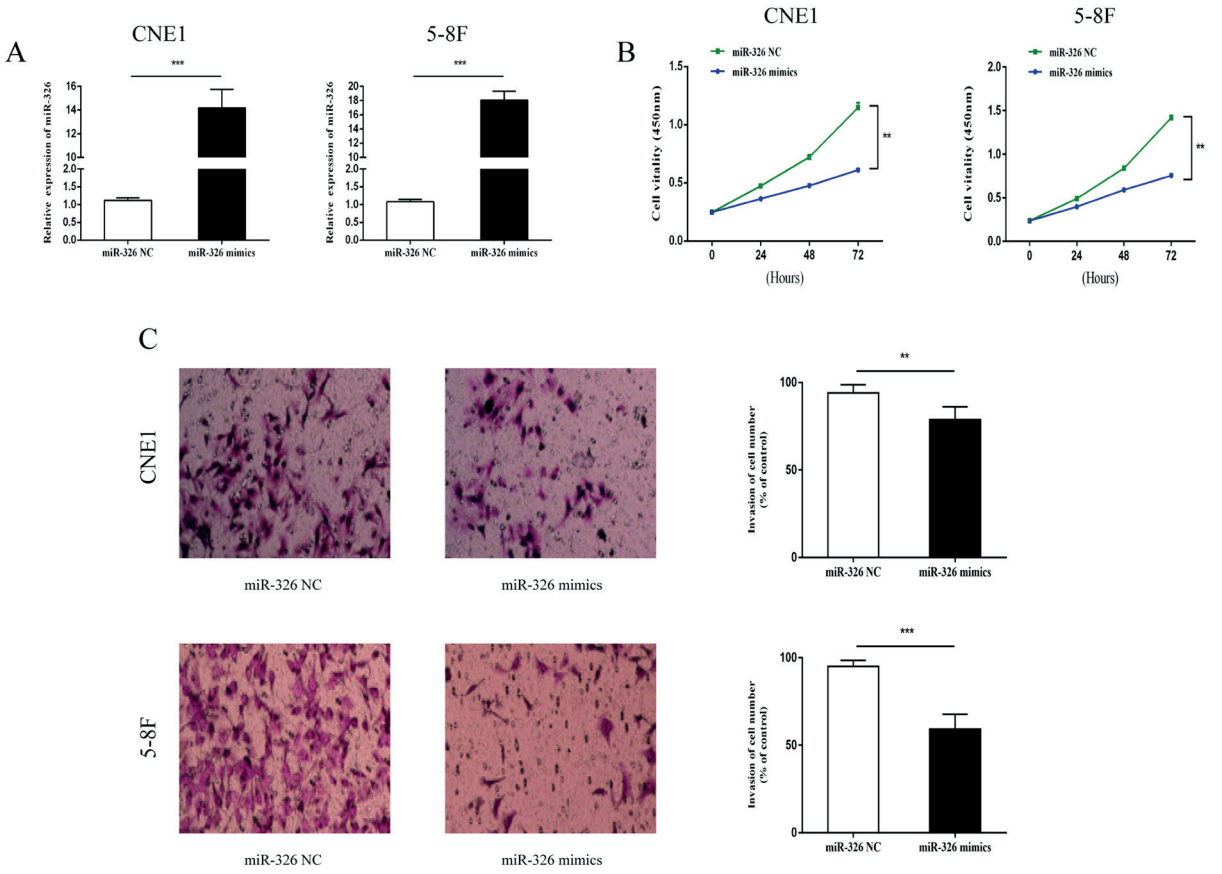

Figure 2. Overexpression of miR-326 inhibits proliferation and invasion of NPC cells. **A,** Expression of miR-326 after transfection of miR-326 mimics in CNE1 and 5-8F cell lines. **B,** CCK8 assay detects changes in cell proliferation capacity after overexpression of miR-326 in CNE1 and 5-8F cell lines. **C,** Transwell invasion assay detects changes in cell invasive ability after overexpression of miR-326 in CNE1 and 5-8F cell lines. (Magnification: 20×). *$p<0.05$; **$p<0.01$; ***$p<0.001$

Overexpression of MicroRNA-326 Inhibits Proliferative Capacity and Invasiveness of NPC Cells

We transfected microRNA-326 mimics in CNE1 and 5-8F cells and verified transfection efficiency by qRT-PCR (Figure 2A). We then tested the proliferation of cells after overexpression of microRNA-326 in CNE1 and 5-8F cell lines. Results of CCK8 assay showed that microRNA-326 overexpression remarkably inhibited cell proliferation in NPC cells CNE1 and 5-8F (Figure 2B). Next, we examined the effect of microRNA-326 on the invasion of NPC cells by the transwell invasion assay. Results show that microRNA-326 overexpression attenuated the invasive ability of CNE1 and 5-8F cells (Figure 2C). These findings indicated that microRNA-326 can remarkably inhibit the growth and metastasis of NPC cells.

MicroRNA-326 can Combine with ETS1

To explore the biological mechanism of microRNA-326, we predicted the target gene that could bind to microRNA-326 by bioinformatics and finally selected ETS1 through a functional analysis. To verify the binding of microRNA-326 to ETS1, we designed and constructed the ETS1 wild type plasmid (ETS1-WT) and the ETS1 mutant plasmid (ETS1-MUT) (Figure 3A). Subsequently, the binding relation between the two was detected by a dual luciferase reporter gene assay and the results showed that after transfection of miR-326 mimics in CNE1 and 5-8F cells, the luciferase activity of the ETS1-WT 3'UTR group was significantly decreased, while ETS1-MUT group showed no significant change, indicating that ETS1 binds to miR-326 (Figure 3B). Then, we detected the relative expression of ETS1 in NPC by qRT-PCR. Results

showed that ETS1 was remarkably expressed in NPC compared with the control group (Figure 3C). To verify the regulation of ETS1 by microRNA-326, we overexpressed or inhibited microRNA-326 in CNE1 and 5-8F cells and detected the expression of ETS1 by qRT-PCR and Western blot. Results indicated that mRNA and protein expression of ETS1 were remarkably decreased after overexpression of microRNA-326 in CNE1 and 5-8F cell lines, while the opposite result was observed after microRNA-326 was down-regulated (Figure 3D-3G), which demonstrated that microRNA-326 was able to bind to ETS1 and inhibit its expression in NPC cells.

MicroRNA-326 Promotes Proliferative Capacity and Invasiveness of NPC Cells by Binding ETS1

We examined the effects of microRNA-326/ETS1 on cell proliferative capacity and invasiveness by *in vitro* cell experiments. The results showed that knockdown of ETS1 partially reversed the promoting effect of microRNA-326 on cell proliferation (Figure 4A). At the same time, we found that interfering with ETS1 partially attenuated the promoted cell invasion ability after inhibition of microRNA-326 (Figure 4B). Results indicated that microRNA-326 may inhibit the proliferative capacity and invasiveness of cells by

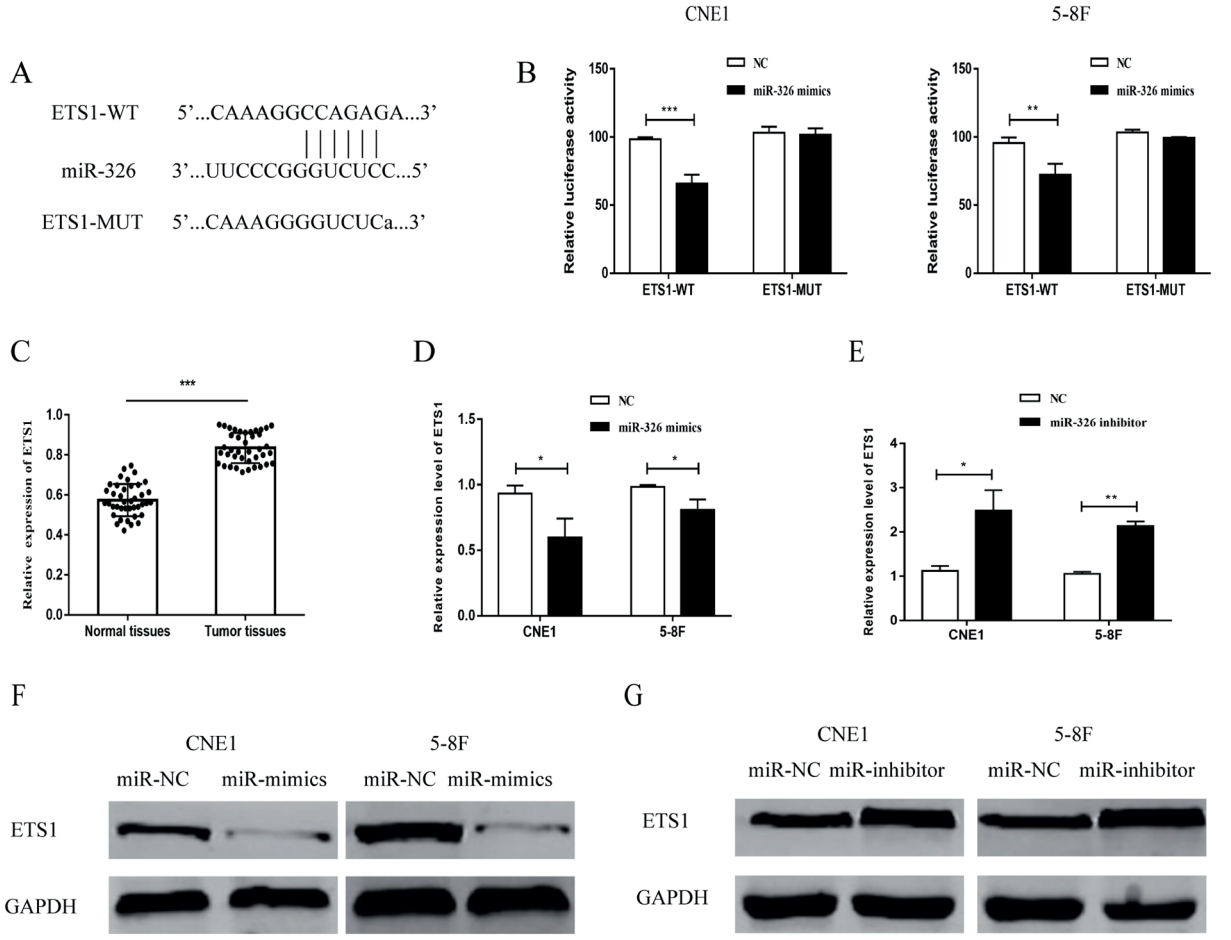

Figure 3. MiR-326 is able to bind ETS1. **A,** Bioinformatics predicted the binding site of miR-326 and ETS1 and constructed ETS1 wild type plasmid (ETS1-WT) and ETS1 mutant plasmid (ETS1-MUT). **B,** Dual luciferase reporter assay for binding of ETS1 to miR-326 in CNE1 and 5-8F cell lines **C,** QRT-PCR was used to detect the expression of ETS1 in NPC. **D,** ETS1 mRNA expression was decreased after overexpression of miR-326 in CNE1 and 5-8F cell lines. **E,** ETS1 mRNA expression was increased after interference with miR-326 in CNE1 and 5-8F cell lines. **F,** After overexpression of miR-326 in CNE1 and 5-8F cell lines, ETS1 protein expression was reduced. **G,** After interference with miR-326 in the CNE1 and 5-8F cell lines, ETS1 protein expression was increased. *p<0.05; **p<0.01; ***p<0.001

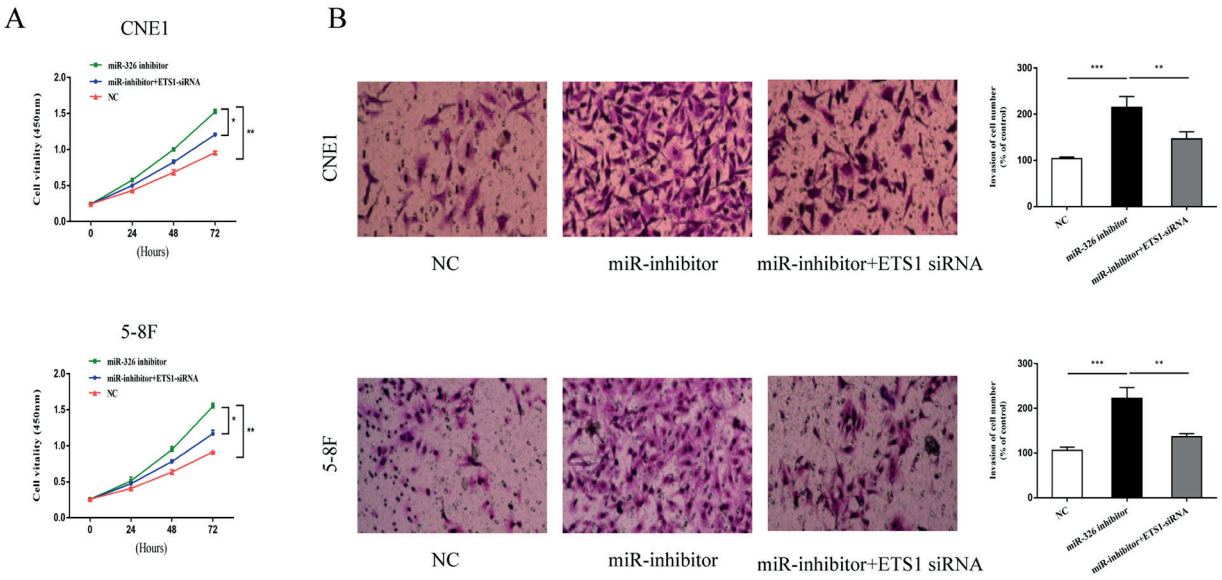

Figure 4. MiR-326 inhibits the expression of ETS1 and thereby inhibits cell proliferation and invasion. **A,** CCK8 assay showed that interference with miR-326 in CNE1 and 5-8F cells promoted cell proliferation, and while interfering with ETS1, the promoting effect was reversed. **B,** After interfering with miR-326 in CNE1 and 5-8F cells, the cell invasion ability was enhanced, and the cell invasion ability was relatively weakened after interfering with ETS1. (Magnification: 20×) **p*<0.05; ***p*<0.01; ****p*<0.001.

inhibiting the expression of ETS1, and thus participated in the progression of NPC.

Discussion

Due to the development of radiotherapy technology and treatment mode, local control and overall survival rate of patients with nasopharyngeal carcinoma have been effectively improved[16]. However, the prognosis of 10-20% patients with nasopharyngeal carcinoma is still poor due to local recurrence and distant metastasis[17]. Therefore, exploring the molecular mechanism of invasion and metastasis of nasopharyngeal carcinoma may provide new effective strategies for the prevention and treatment of nasopharyngeal carcinoma.

Specific miRNAs play a pivotal role in disease progression. Based on their role in cancer, miRNAs can be divided into two groups with opposite effects i.e., carcinogenic and anticancer[18]. MiRNA functions mainly by binding to the 3'UTR region of the target gene. Previous studies[19] have shown that many miRNAs with abnormal expression play a critical role in the development of NPC. For example, mir-144-3p promotes NPC through its interaction with PTEN[20]. MiRNA-506 inactivates the Wnt/beta-catenin signaling pathway by downregulating LHX2, thereby inhibiting tumor growth and metastasis of NPC[21]. MiRNA-342-3p inhibits the invasive phenotype of NPC cells by targeting FOXQ1[22]. MiRNA-372 enhances radio sensitivity and inhibits invasion and metastasis of NPC cells by activating the p53 signaling pathway[23]. Also, mir-449b targets TGFBI to regulate the TGF beta pathway to induce cisplatin resistance in NPC[24].

In this research, we found that microRNA-326 expression in NPC tissues and cell lines was both decreased and the low expression of microRNA-326 could be used to predict the poor prognosis of patients with NPC. Subsequently, through *in vitro* experiments, we demonstrated that overexpression of microRNA-326 in NPC cells remarkably weakened cell proliferative capacity and invasiveness ability, suggesting that microRNA-326 may play a role as an oncogene in NPC. MiRNAs can perform their biological functions by regulating the expression of target genes. Therefore, we suggested that microRNA-326 may be involved in the progression of NPC by regulating the expression of downstream target genes. With the help of bioinformatics prediction tools, we found that several target genes have potential binding sites with microRNA-326. Previous studies have shown that ETS1 is closely related to the

growth and metastasis of tumor cells. Therefore, we chose ETS1 as a possible binding target.

ETS (Erythroblastosis virus E26 oncogene homolog) was discovered in the research on avian retrovirus E26, possessing a highly conserved DNA binding domain, the ETS domain[25], which can bind to specific sequences to regulate the expression of target genes. ETS1 is an important member of the ETS transcription factor family. ETS transcription factor families plays a pivotal role in regulating cell proliferation, differentiation, apoptosis, angiogenesis, tissue reconstruction, migration, malignant transformation, and distant metastasis[25-26].

Subsequently, we found that microRNA-326 could bind to ETS1 and the knockdown of microRNA-326 in cells was capable of enhancing ETS1 expression. Results of cell function experiments revealed that microRNA-326 was able to inhibit the proliferative capacity and invasiveness of NPC cells, while ETS1 promoted the growth and metastasis of cells. MicroRNA-326 may regulate the proliferative capacity and invasiveness of NPC cells by inhibiting the expression of ETS1, thus playing a critical role in the development of NPC.

Conclusions

The results of this report revealed that microRNA-326 was downregulated in NPC tissues, which could inhibit cell proliferative capacity and invasiveness. The potential mechanism might be that microRNA-326 exerted its biological functions by binding and down-regulating ETS1, which provide a potential therapeutic target for nasopharyngeal cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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