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Original Research Article

MiR-128-3p inhibits the proliferation, migration and invasion of human nasopharyngeal carcinoma cells via EMT

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Abstract

Purpose: *To investigate the effect of overexpressing miR-128-3p on the migration, invasion, and proliferation of human nasopharyngeal cancer cells, and associated pathways.*
Methods: *Cell counting kit-8 (CCK-8) and clone creation tests were used to investigate the effect of miR-128-3p inhibitor on the growth of C666-1 cells. The inhibitor control and miR-128-3p inhibitor were transfected into human nasopharyngeal cancer cells (C666-1) and allowed to incubate for 48 h. Cell migration and invasion assays were conducted using Transwell and Scratch assays. Cell cycle was assessed using propidium iodide (PI) single-staining method, while expression of miR-128-3p was evaluated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Expression levels of vimentin, E-cadherin, and N-cadherin were determined by Western blot.*
Results: *MiR-128-3p inhibitor transfection in C666-1 cells significantly increased cell proliferation, cell viability, and clonal proliferation, and also significantly reduced miR-128-3p expression levels, and enhanced cell migration and invasion. In C666-1 cells, inhibition of miR-128-3p induced epithelial mesenchymal transition (EMT).*
Conclusion: *MiR-128-3p inhibits nasopharyngeal cancer cells from proliferating, migrating, and invading by regulating epithelial mesenchymal transition. MiR-128-3p may therefore be a viable therapeutic target for nasopharyngeal cancer. Future studies are required to yield pertinent data in support of this hypothesis.*

Keywords: *MiR-128-3p, Nasopharyngeal carcinoma, Epithelial mesenchymal transition (EMT), Cell migration, Cell invasion*

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INTRODUCTION

A malignant tumor that primarily impacts the region of the head and neck is known as nasopharyngeal carcinoma. The most prevalent histological subtype is squamous cell carcinoma, which arises from the mucosal epithelium of the nasopharynx. The incidence of nasopharyngeal carcinoma is relatively low in Western countries, but higher in Southeast Asian countries [1]. In China, Guangdong and Guangxi are the provinces with the highest incidence. In recent years, with the advancement of radiotherapy, targeted therapy and immunotherapy, prognosis

of nasopharyngeal carcinoma has improved, but is still unsatisfactory [2]. investigating the pathogenesis of nasopharyngeal carcinoma can help to find the causative genes of nasopharyngeal carcinoma and more effective treatments, which may be of great significance in providing patients with nasopharyngeal cancer a better prognosis. Numerous investigations have demonstrated the role of miRNAs in regulating the progression of drug resistance, invasion, metastasis, recurrence, and nasopharyngeal carcinogenesis. Although miR-128-3p prevents the growth of several malignancies, its function in nasopharyngeal carcinoma is unclear. This study investigated the function of miR-128-3p on the nasopharyngeal carcinoma cell line C666-1 and its mechanism, in order to shed light on the potential pathophysiology of the disease and offer a theoretical foundation for future research into relevant therapeutic targets.

EXPERIMENTAL

Cell culture

Saturated humidity was used to cultivate C666-1 cells (lot no. iCell-h378; Shanghai Saibakang Biotechnology Co., Shanghai, China) at 37 °C and 5 % CO_2 incubator with 1640 medium (lot no. SH30809.01; Hyclone, South Logan, UT, USA) + 10 % FBS (batch no. 141215; Hangzhou Tianhang Biotechnology Co., Hangzhou, China) + 1 % penicillin (double antibodies).

Cell transfection

Simultaneously a miR-128-3p inhibition group (miR-inhibitor) (Guangzhou RiboBio Co., Guangzhou, China) and a miR-128-3p inhibition negative control group (miR-NC-inhibitor) were set up. Following the instructions included with the Lipofectamine 2000 transfection kit (lot no. 11668019, Thermo Fisher Scientific, Waltham, MA, USA), C666-1 cells were transfected with both the miR-NC-inhibitor (Guangzhou RiboBio Co., Guangzhou, China) and the miR-inhibitor (Guangzhou RiboBio Co., Guangzhou, China) for 48 h prior to measurement.

Reverse transcription – quantitative polymerase chain reaction (RT-qPCR) was performed to investigate the effect of a miR-128-3p inhibitor on miR-128-3p expression in C666-1 cells. After transfected cells were collected, total cellular RNA was extracted with total RNA isolation reagent (TRIzol) (lot no. EP013, KeLu (Wuhan) Biotechnology Co., Wuhan, China). The miR-128-3p was determined using the EntiLinkTM 1st strand cDNA synthesis kit (lot no. EQ003, KeLu (Wuhan) Biotechnology Co., Wuhan, China), reverse transcription. Fluorescence quantitative PCR was performed using the EnTurboTM SYBR Green PCR Super Mix kit (lot no. EQ001; Kelu (Wuhan) Biotechnology Co., Wuhan, China), and the expression of miR-128-3p was assessed through the $2^{-\Delta\Delta CT}$ assay, with U6 serving as the internal reference. The primer sequences (Kingsray Biotechnology Co, Ltd., Nanjing, China) that were utilized are presented in Table 1.

Cell counting kit-8 (CCK-8) assay

After transfection, trypsin (lot no. GNM25200, Gino Biomedical Technology Co., (Hangzhou, China) digestion, and preparation into a cell suspension at a concentration of 1×10^5 cells/mL, 10,000 cells/well were inoculated onto 96-well plates. Thereafter, 100μL of the cell suspension was added to each well, and the plates were incubated for 2 h. After incubation, 10 μL of CCK-8 solution (batch no. C0038, Biyuntian Biotechnology Co., Shanghai, China) was added to each well, and incubated for 2 h. An enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the absorbance (A) value at 450 nm, and the proliferation inhibition rate (PI) was calculated using Eq 1.

$$PI = \{(A_{control} - A_{Experimental})/A_{control}\}100 \quad \ldots\ldots (1)$$

Cell scratch assay

A marker pen was used to draw horizontal lines across the six wells on the plate's back, with a spacing of 0.5 to 1.0 cm. After gathering and resuspending the transfected cells, 5×10^5 cells were introduced into the wells. On follow-up day, the cells were scribed using a 200 μL gun tip perpendicular to the horizontal line, rinsed three times with phosphate buffered saline (PBS) (batch no. GNM20012, Gino Biomedical Technology Co., Hangzhou, China), activated to serum-free mediums, and cultured at 37 °C in an incubator with 5 % CO_2. Finally, the scratch closure was observed at 0 and 24 h under an ordinary light microscope and images of the scratches were captured.

Table 1: Primer sequences

Primer	Upstream (5′-3′)	Downstream (5′-3′)
miR-128-3p	CCACAGTGAACCGGTCTCTTT	CTCAACTGGTGTCGTGGAGTC

Transwell assay

After diluting Matrigel (lot no. 354248, Corning, NY, USA) and 1640 medium in 1:3 respectively, 50 µL was introduced to the transwell and allowed to dry in the incubator. Following collection, the transfected cells remained suspended at 10^5 cells/mL, and 1 mL of the cell suspension was centrifuged at 1500 rpm for 5 mins, and the supernatant discarded. Thereafter, 1 mL of serum-free media was evenly spread inside the transwell chamber to create a transwell assay. The transwell chamber was filled with 200 µL of the cell suspension, after which 500 µL of complete media with 10 % FBS was added to the 24-well plate and the cells incubated for 48 h at 37 °C and 5 % CO_2. After incubation, the cells were removed from the chamber's inner membrane and fixed with paraformaldehyde for 20 mins, washed twice with PBS, and stained with 0.1 % crystalline violet for 10 mins. Thereafter, the non-inoculated side of the cells was photographed with an inverted microscope.

Clone formation in C666-1 cells

The cells were transfected, collected, resuspended with 1 mL of medium and diluted to appropriate concentration for counting. Each well of a 6-well plate was connected to 500 cells. After two weeks incubation, the 6-well plates were fixed in paraformaldehyde, stained with crystal violet, and captured on camera (cell number > 50). Clone formation rate (CF) was calculated using Eq 2.

$$CF = (NC/NI)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where NC is the number of clones formed while, NI is number of inoculated cells

Propidium iodide (PI) single-staining procedure

After transfecting and gathering the cells, ethanol was added to fix the cells at 4 °C for 24 h. Staining buffer (500 µL), propidium iodide (PI) working solution (10 µL), and RNase (10 µL) were added to the cells, incubated at 37 °C for 30 min, and then evaluated using flow cytometry.

Western blot (WB) assay

After transfecting and harvesting the cells, total cellular protein was extracted using radioimmunoprecipitation assay (RIPA) lysate (lot no. AS1004, ASPEN). A BCA protein quantification kit (lot no. AS1086, ASPEN) was employed to determine protein concentration. The protein samples were subsequently transferred to polyvinylidene difluoride (PVDF) membranes after been electrophoresed on sodium doceyl-sulfate polyacrylamide gel (SDS-PAGE). After that, the membranes were incubated for 1 h at room temperature with a blocking solution. Antibodies against Vimentin (lot no. #10366-1-AP; Wuhan Sanying Biotechnology Co., Wuhan, China), E-cadherin (lot no. #3195; CST, USA), N-cadherin (lot no. #13116; CST, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (lot no. ab181602; Abcam Cambridge, MA, USA) (1:10000 dilution) were applied to the membranes for specific protein determination, and the membranes were incubated at 4 °C for 12 h. After three TBST (Tris-buffered saline with Tween) washes, a HRP-labeled IgG antibody (lot no. AS1107; ASPEN) (1:10000 dilution) was then applied to the membranes. After incubation for 30 mins at room temperature, the membranes were soaked in TBST four times. Using an ECL mixture (lot no. AS1059; ASPEN), the exposure conditions were changed to reflect varying light intensity in order to visualize protein bands. Thereafter, the produced membranes were scrutinized and the optical density values of the target bands were identified using the AlphaEaseFC software processing system.

Statistical analysis

GraphPad Prism 9 was used for creating statistical plots and Statistical Packages for Social Sciences (SPSS version 26.0) was used to perform statistical analysis. Student t-test was used to comparison and $p < 0.05$ was considered statistically significant.

RESULTS

MiR-128-3p inhibitor decreases miR-128-3p expression in C666-1 cells

Transfection of miR-128-3p inhibitor into C666-1 cells for 48 h significantly decreased miR-128-3p expression ($p < 0.01$) in comparison to the negative control group (miR-NC-inhibitor group). This suggests that transfection was successful (Figure 1).

MiR-128-3p inhibitor promotes proliferation and clone formation of C666-1 cells

Using CCK-8 and clone formation assays, the effects of the miR-128-3p inhibitor on cell proliferation were evaluated and the results revealed that miR-128-3p inhibitor significantly accelerated the generation of clones and cell proliferation ($p < 0.01$) (Figure 2 A and B). When

paired with the findings of the cell proliferation experiment, the PI single-stain assay for cell cycle determination revealed an increase in S-phase cells (Figure 2 C), which raised the possibility that the miR-128-3p inhibitor promotes cell cycle in C666-1 cells.

MiR-128-3p inhibitor promotes migration and invasion of C666-1 cells

In contrast to the negative control group, the miR-128-3p inhibitor significantly enhanced cell migration ($p < 0.01$) (Figure 3 A). Additionally, a transwell assay was performed to examine the influence of the miR-128-3p inhibitor on the invasion of cells, and the findings showed that, in contrast with the control group; the miR-128-3p inhibitor significantly improved invasive capacity of C666-1 cells ($p < 0.01$) (Figure 3 B).

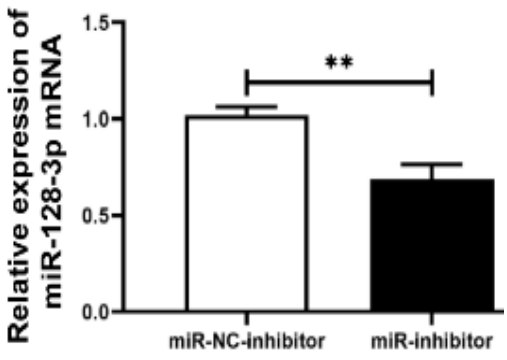

Figure 1: miR-128-3p inhibitor decreased C666-1 cell miR-128-3p expression. **$P < 0.01$

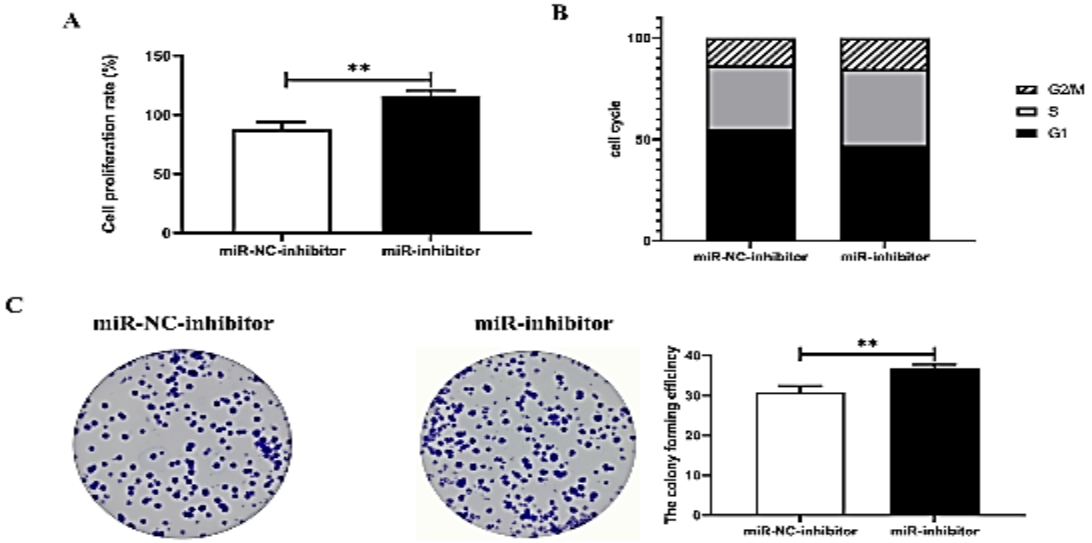

Figure 2: C666-1 cells proliferation (A) Cycle. (B) Clone formation ability (C) Cell cycle was promoted by miR-128-3p inhibitor. **$P < 0.01$

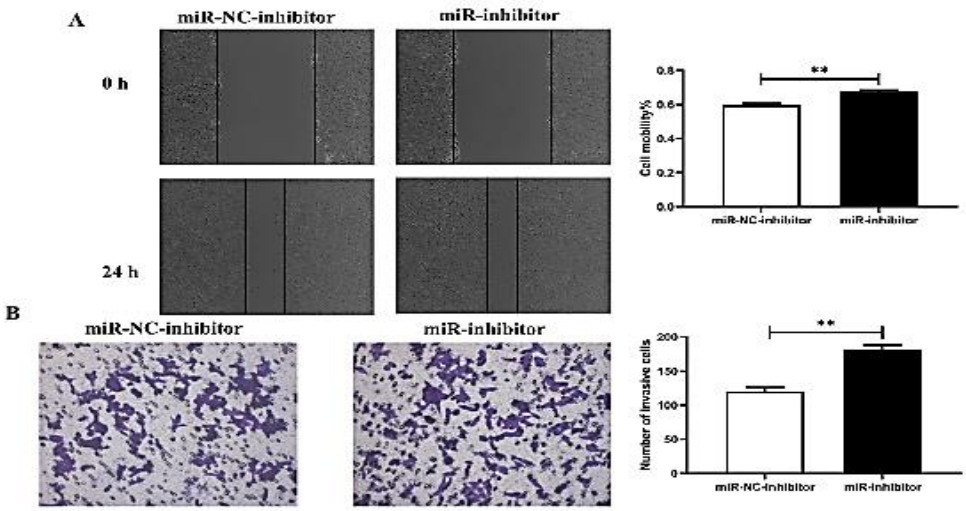

Figure 3: miR-128-3p inhibitor promoted migration (A) and invasion (B) of C666-1 cells. **$P < 0.01$

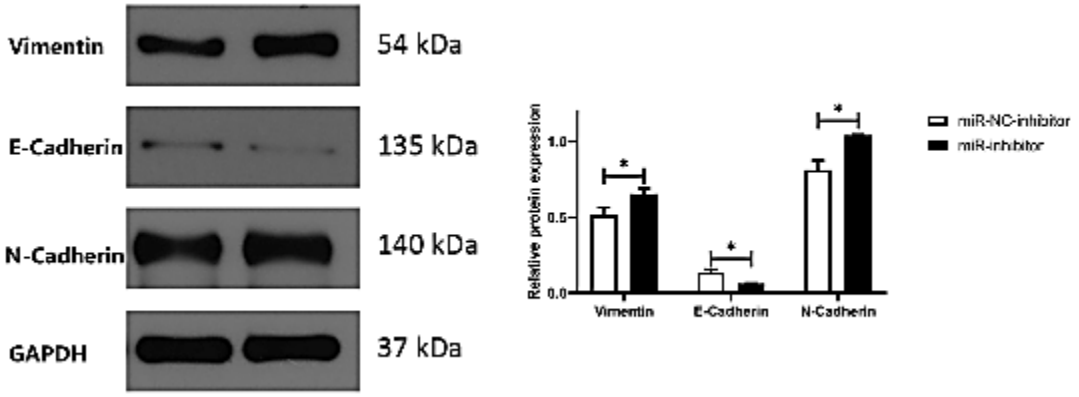

Figure 4: Effect of miR-128-3p inhibitor on proteins linked with epithelial mesenchymal transition in C666-1 cells

Vimentin, E-cadherin and N-cadherin proteins in C666-1 cells

The results of the western blot analysis indicated that inhibition of miR-128-3p significantly promoted the expression of vimentin and N-cadherin protein in C666-1 cells, and inhibited the expression of E-cadherin protein ($p < 0.05$) (Figure 4). This indicated that inhibition of miR-128-3p promotes epithelial mesenchymal transition.

DISCUSSION

High regional invasion and distant metastasis are characteristics of nasopharyngeal carcinoma, a rarely occurring malignant tumor that originates from the nasopharyngeal mucosa's epithelium. A large percentage of patients (60 – 85 %) are diagnosed with locally progressed or metastatic sickness as a result of atypical early symptoms, which has an adverse effect on prognosis and decreases the 5-year survival rate. The causative factors of nasopharyngeal carcinoma are currently believed to be associated with Epstein-Barr virus (EBV) infection [3]. Surgical treatment of nasopharyngeal cancer is challenging due to complexity of the anatomical structure of nasopharynx. Main approaches to treating nasopharyngeal cancer include radiotherapy, chemotherapy, and molecular targeted therapies.

In recent years, immunotherapy has been introduced and promoted with immune checkpoint inhibitors, improving the prognosis of some nasopharyngeal cancer patients. However, efficacy of immunotherapy remains unsatisfactory. It is therefore, important to look into the etiology and causative genes of nasopharyngeal cancer in order to improve prognosis of the disease. Thus, this study was aimed at investigating the role of miR-128-3p on nasopharyngeal carcinoma cells and its associated mechanism.

MiRNAs are a type of non-coding RNA molecules of 19–25 nucleotides that combine to the 3′-untranslated regions (3′-UTR) of target mRNAs to cause degradation and translation inhibition. These molecules have an essential role in malignancy, development, apoptosis, metastasis, and angiogenesis [4,5].

MiRNAs typically have oncogene or tumor suppressor properties. The proliferation, migration, invasion, differentiation, and recurrence of tumor cells, all of which are intimately linked to the development of tumors are impacted by dysregulation in their expression. MiRNAs have been shown in numerous studies to regulate expression of genes linked to treatment resistance, invasion, metastasis, recurrence, and nasopharyngeal carcinogenesis [2,4-6]. MiR-128-3p increases susceptibility of hepatocellular carcinoma cells to lenvatinib by regulating Akt, which mediates apoptosis; and extracellular-signal regulated kinase (ERK), which regulates cell cycle progression, inhibits tumor growth, and mediates EGFR-MAPK p38 signaling pathway to inhibit HCC cell metastasis in a nude mouse hepatocellular carcinoma cell model [7,8].

MiR-128-3p also inhibits the capacity of cells to proliferate and migrate, and its over-expression stops breast cancer cells in their G0/G1 phase by influencing expression of CDK2/CyclinE1 and CDK4/CDK6/CyclinD1 [9]. MiR-128 target ZEB1 to inhibit EMT, invasion and metastasis of pancreatic cancer cells [10], and overexpression induces glioblastoma cell GSC differentiation and enhances sensitivity of axitinib treatment [11,12]. Therefore, miR-128 is considered as an antitumor agent due to its inhibitory effects on a variety of tumors. Although the role of quite a few miRNAs in nasopharyngeal carcinoma (NPC) has been extensively studied [5,13,14], there is paucity of information on role of miR-128 in NPC.

The results of the RT-PCR experiment indicated a significant decrease in relative expression of miR-128-3p mRNA in transfected nasopharyngeal carcinoma cells. Also, the inhibitor of miR-128-3p stimulated the growth, clone formation, migration, invasion, and advancement of the cell cycle in nasopharyngeal cancer cells. Furthermore, this research has demonstrated that the process of epithelial-mesenchymal transition (EMT), which is essential for nasopharyngeal cancer metastasis, is exhibited by platinum-resistant cells which is in tandem with previous studies [1,15]. The control of EMT involves multiple signaling pathways, including but not limited to Notch, Wnt/β-catenin, TGF-ß1-FMNL3, PI3K-Akt, AKT-related miRNAs, and transcription factors.

It has been shown that ZEB1, MMP-2 and E-cadherin are key proteins involved in the metastasis of nasopharyngeal carcinoma [2,15]. By restoring E-cadherin expression and suppressing ZEB1 and MMP-2 expression, miR-429 over-expression prevented EMT and subsequently nasopharyngeal cancer cells' migration and invasion [5]. Previous research has demonstrated the involvement of miR-128-3p in the development of thyroid, non-small cell lung, ovarian, and oral cancers; nevertheless, the precise mechanism of action in nasopharyngeal carcinomas remains unknown [16,17]. In light of this current research, and the aforementioned findings, it is speculated that miR-128-3p decreases nasopharyngeal carcinoma cells' migration, invasion, and proliferation via a mechanism related to epithelial-mesenchymal transition (EMT).

Also, the results demonstrated that miR-128-3p inhibitor significantly increased expression of Vimentin, and N-Cadherin; inhibited the expression of E-Cadherin, and promoted the EMT process. These reactions led to an increase in the proliferation, invasion, and migration of nasopharyngeal carcinoma cells. Further *in vivo* and *ex vivo* studies are encouraged to validate these findings. It is suggested that miR-128-3p hinders epithelial mesenchymal transition, therefore decreasing the proliferation, migration, and invasion capabilities of nasopharyngeal cancer cells.

CONCLUSION

MiR-128-3p inhibitors are capable of improving the proliferation, migration, and invasion of nasopharyngeal carcinoma cells. It, therefore, follows that miR-128-3p suppresses cell expansion, migration, and invasion through the epithelial mesenchymal transition. As a result, miR-128-3p may be a viable therapeutic target for nasopharyngeal cancer, and future studies are required to yield pertinent data in support of this hypothesis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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