ORIGINAL RESEARCH

Labeling and Diagnostic Value of miRNA-28-3p in Patients with Nasopharyngeal Carcinoma

Gang Huang, BM; Na Zhao, BM; Jianwei Wu, BM; Xiaojuan Zhou, BM; Wan Liu, BM

ABSTRACT

Objective • To explore how miRNA-28-3p targets BIN1 and affects the biological behavior of nasopharyngeal carcinoma cells, and to evaluate its potential as a predictive biomarker for NPC.

Methods • We studied 100 nasopharyngeal carcinoma patients who underwent surgery between January 2021 and January 2022. Tissues from tumors and adjacent normal areas were analyzed. Participants were categorized into two groups: one with nasopharyngeal carcinoma and another with adjacent normal tissue. Additionally, the nasopharyngeal carcinoma cell line CNE-2 was divided into three groups: an untreated control, a negative control with NC plasmid, and a test group treated with a miRNA-28-3p inhibitor. Key techniques included PCR, Western blotting, CCK-8, flow cytometry, focusing on the interactions between miRNA-28-3p and BIN1 and their predictive significance for NPC.

Results • miRNA-28-3p levels were significantly higher in nasopharyngeal carcinoma tissues compared to adjacent normal tissues ($P < .05$). The predictive performance of miRNA-28-3p for NPC featured an AUC > 0.75 with sensitivity and specificity both exceeding 70% ($P < .001$). In nasopharyngeal carcinoma cells, miRNA-28-3p levels were significantly elevated compared to normal cells ($P < .05$). Transfection with the miRNA-28-3p inhibitor increased apoptosis and BIN1 protein levels while reducing cell proliferation, invasion, and migration significantly ($P < .05$).

Conclusion • miRNA-28-3p is overexpressed in nasopharyngeal carcinoma and inhibits tumor cell proliferation, migration, and invasion, while promoting apoptosis by targeting BIN1. The level of miRNA-28-3p expression serves as a sensitive indicator for predicting nasopharyngeal carcinoma, affirming its potential as a diagnostic biomarker and underscoring the significance of these findings in enhancing understanding and clinical management of NPC. (*Altern Ther Health Med.* 2024;30(7):84-89).

Gang Huang, BM, Associate Chief Technician; **Xiaojuan Zhou**, BM, Associate Chief Technician; **Wan Liu**, BM, Technician-In-Charge, Department of Laboratory; Ganzhou Cancer Hospital; Ganzhou; China. **Na Zhao**, BM, Associate Chief Technician, Blood Transfusion Research Laboratory; Ganzhou City Center Blood Bank; Ganzhou; China. **Jianwei Wu**, BM, Associate Professor, Department of Medical Technology; Gannan Healthcare Vocational College; Ganzhou; China.

Corresponding author: Gang Huang, BM
E-mail: huang567gang@163.com

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a significant global health concern due to its high metastatic potential and prevalence in specific regions. This malignancy originates from the epithelial cells of the head and neck and is characterized by its aggressive invasion and metastasis. Traditional treatment primarily involves radiotherapy, which has seen enhancements from targeted therapies and chemotherapy, significantly improving overall treatment outcomes. Despite these advances, the prognosis for NPC has not yet met patient expectations.[1] Micro RNAs (miRNAs) play critical roles in gene regulation by binding to non-coding regions of target genes, inducing degradation or inhibiting translation.[2,3] MiRNA-28-3p, in particular, has been shown to promote cancer in various malignancies such as colon, esophageal, and breast cancer. It modulates apoptosis resistance through targeting apoptosis inhibitor 5, influencing cancer cell proliferation and metastasis, which has been well-documented in numerous studies. However, reports on miRNA-28-3p's expression and mechanisms in NPC are relatively sparse. [4] This study aims to elucidate the role of miRNA-28-3p in the development and progression of NPC, establishing a theoretical foundation for subsequent clinical research. By investigating miRNA-28-3p, this research seeks to identify new therapeutic targets to reduce metastasis and recurrence and improve clinical outcomes in NPC patients. The objectives of this study are to explore miRNA-28-3p's biological functions and its potential as a biomarker and therapeutic target in NPC. This research could significantly impact clinical strategies, offering new avenues for treatment and enhancing patient prognosis. Proper citation and clarity in presentation will ensure the accessibility and utility of this study's findings to the broader scientific community.

PATIENTS AND METHODS

General Information

A total of 100 NPC patients treated with surgical resection from January 2021 to January 2022 were selected. The collected samples included tumor excision tissue and 2 cm of adjacent normal tissue, grouped for study as nasopharyngeal carcinoma and paracancer tissue groups. The age range of the patients was 45-65 years, with a mean age of 50.24±4.13 years, reflecting the peak incidence ages for NPC. There were 54 males and 46 females, representing the gender distribution typically observed in NPC, which aligns with global epidemiological data.

Inclusion criteria: (1) Diagnosed and confirmed to meet the nasopharyngeal carcinoma diagnostic criteria; (2) Comprehensive pathological data without missing details; (3) Signed informed consent; (4) No concurrent malignant diseases or metastasis.

Exclusion criteria: (1) Patients with defined severe organic diseases (e.g., chronic heart failure, advanced liver cirrhosis); (2) Autoimmune diseases; (3) Serious organ failure (e.g., renal failure requiring dialysis, respiratory failure requiring mechanical ventilation); (4) Prior history of chemoradiotherapy or immunotherapy.

Cell lines and instrumental reagents

The nasopharyngeal epithelial NP69 cell line and the nasopharyngeal carcinoma CNE-2 cell line, both used in this study, were donated by the Chinese Academy of Medical Sciences. These cell lines have been validated for NPC research, demonstrating consistent phenotypic and genotypic characteristics that are crucial for the reliability of experimental outcomes. Further details on the experimental instruments and reagents are provided in Table 1.

Methods

Cell culture, transfection and grouping. The NPC cell CNE-2 strains were randomly divided into 3 groups. Group A was not transfected, group B was transfected with the NC plasmid, and group C was transfected with the miRNA-28-3p inhibitors. This grouping helps delineate the specific effects of miRNA-28-3p inhibition compared to baseline and control conditions.

Determination of miRNA-28-3p expression level. Total RNA was extracted using TRIzol, followed by measuring RNA concentration and purity. cDNA synthesis was performed through reverse transcription. Quantitative real-time PCR (qRT-PCR) was set up with GAPDH as the internal reference, employing SYBR Green to quantify miRNA-28-3p levels, with specific cycling conditions designed to optimize amplification and detection.

Expression method of Western blottingBIN1 protein. Sample preparation involved thorough grinding, addition of lysis buffer, and subsequent centrifugation. Protein concentration was measured using a BCA assay. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and detected using specific antibodies. The process included blocking, antibody incubations, and extensive washing, followed by chemiluminescent detection and analysis using Gel-Pro32 software to quantify BIN1 expression.

Cell proliferation capacity was determined by C C K-8 assay. Cell proliferation was quantified using a CCK-8 assay at 96 hours post-transfection, measuring optical density at 450 nm. Apoptosis was assessed by flow cytometry using Annexin V-FITC and PI staining. Cell invasion and migration were evaluated using a Matrigel-coated Transwell setup, with results quantified after 48 hours. Specific assay conditions, such as reagent volumes and incubation times, were optimized based on preliminary experiments to ensure reproducibility and reliability of results.

Apoptosis was measured by flow cytometry. CNE-2 cells were cultured and collected for 48 h. PBS and 500 μL of binding buffer were added for cell resuspension. Annexin V-FITC and PI were 10 μL and 5 μL, respectively, according to the temperature requirements. The apoptosis rate was measured after 15 min.

Cell invasion and migration capacity were assessed by Transwell assay. The Matrigel matrix glue was diluted at the ratio of 1:8 and coated in the upper chamber of Matrigel cells, and 100μl bloodless medium was added to each well and cultured for 30 minutes. The basement membrane was hydrated, and the excess liquid in the upper chamber was sucked out for use after air drying for 3h. CNE-2 cells at logarithmic growth stage were taken, digested with 0.25% pancreatic enzyme for 3min, centrifuged at 2000rpm for 3min, the supernatant was discarded, the cells were washed twice with PBS, the cells were suspended in serum-free medium, and the cell density was adjusted to $1×10^5$ cells /ml. 200ul cell suspension was added to the upper chamber of the Transwell chamber, 600μL serum medium was added to the lower chamber, and the mixture was placed in an incubator. After 48 h, the culture medium was discarded, and the concentration of 4% paraformaldehyde was placed and fixed. The staining solution was 1% crystal violet, and the dosage was 400 μL. The invaded cells were observed and recorded by microscope. The cell migration capacity manipulation step was consistent with the invasion manipulation.

Verification of BIN1 targeting by miRNA-28-3p. According to the instructions of Lipofectamine 2000, the mi R-28-3p mutant (3'-UTR-MUT) and wild type (3'-UTR-MT) on the 3'-UTR carrying BIN1 were constructed and transfected into CNE-2 cells, which were mutant group and wild type group, respectively. NC plasmid and miRNA-28-3p inhibitor were transfected, respectively. After transfection for 48 h, the old cell culture medium was discarded, 100μl reporter cell lysate was added to each well for cell lysis, and the cells were transferred to an EP tube for centrifugation and mixing. The luciferase assay working liquid was prepared by adding the luciferase assay substrate to the luciferase assay buffer at the ratio of 1:100. Each cell sample was added with 100μl firefly luciferase detection reagent. According to the luciferase kit instructions, luciferase activity was determined by enzymoleter. Relative luciferase activity = reporter enzyme activity/sea kidney luciferase activity.

Observation Indicators

(1) The expression levels of miRNA-28-3p were compared between the nasopharyngeal carcinoma group and the adjacent carcinoma group; (2) ROC curve was drawn to analyze the clinical efficacy of miRNA-28-3p in predicting nasopharyngeal carcinoma, including sensitivity, specificity, and area under the ROC curve (AUC). (3) The expression levels of miRNA-28-3p were compared between the normal cell group and nasopharyngeal carcinoma cell group; (4) The results of BIN1 luciferase assay were compared between the mutant group and wild-type group; (5) The BIN1 protein levels of A group, B group, and miRNA-28-3p inhibitor group were compared; (6) The differences in cell proliferation, apoptosis, invasion and migration ability between A group, B group and miRNA-28-3p inhibitor group were compared.

Statistical analysis

Statistical analysis was conducted using SPSS 26.0. Descriptive statistics were calculated as mean ± SD. Group comparisons were performed using *t* tests for two groups or F-tests for multiple groups. Specific tests, such as Pearson correlation for continuous variables, were applied where relevant. A $P < .05$ was considered statistically significant, guiding the interpretation of data towards clinical relevance.

RESULTS

Comparison of miRNA-28-3p expression between nasopharyngeal carcinoma group and para-carcinoma group

The expression level of miRNA-28-3p had higher values and was statistically different in the NPC group ($P < .05$). Table 2.

Clinical efficacy of miRNA-28-3p in predicting NPC

The AUC of miRNA-28-3p in predicting nasopharyngeal carcinoma was 0.752, and the sensitivity and specificity were 70.00% and 72.50%, respectively, $P < .001$. Attached Table 3, Figure 1.

Comparison of miRNA-28-3p expression between normal cell group and nasopharyngeal carcinoma cell group

The miRNA-28-3p expression level had higher values and was statistically different in the NPC cell group ($P < .05$). Table 4.

Table 1. Reagents and instrument information

Instruments and reagents	**manufacturing enterprise**
Total RNA extraction reagent, TRIzol	American Invitrogen, Inc
qPCR primer	Shanghai Yingjun Biotechnology Company
Rabbit anti-human bridging integration factor 1 (bridging integrator1, BIN1)	Abcam Corporation, USA
The Luciferase Reporter Gene Kit p RL-CMV	American Promega, Inc
The mi R-28-3p inhibitor, the mi R-NC plasmid	American Thermo Fisher, Inc

Table 2. Compares the miRNA-28-3p expression differences between the NPC and adjacent groups ($\bar{x} \pm s$, n=100)

group	**miRNA-28-3p**
NPC group	7.56±2.14
Next to the cancer group	5.21±1.43
t	9.130
P value	<.001

Table 3. Clinical efficacy of mirNA-28-3P in predicting nasopharyngeal carcinoma

index	**sensitivity (%)**	**specificity (%)**	**cutoff value**	**AUC**	**standard error**	***P* value**	**95%*CI***	
							Lower limit	**Top limit**
miRNA-28-3p	70.00	72.50	2.105	0.752	0.048	.000	0.658	0.846

Figure 1. ROC curves for predicting NPC by miRNA-28-3p

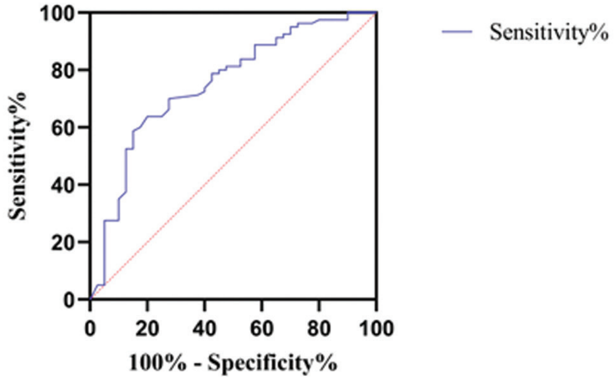


Table 4. Comparison of miRNA-28-3p expression levels between normal cell group and nasopharyngeal carcinoma cell group ($\bar{x} \pm s$)

group	**miRNA-28-3p**
The NPC cell group	4.43±1.02
Normal cell group	1.03±0.67
t	27.860
P value	<.001

Table 5. Comparison of BIN1 luciferase experimental results between mutant group and wild-type group ($\bar{x} \pm s$)

group	**transfection miRNA-28-3p**	**transfection NC plasmid**	***t***	***P* value**
Mutant group	1.11±0.37	1.17±0.35	-1.178	.240
Wild type group	0.67±0.12	1.13±0.31	-13.838	<.001

Compare the experimental results of BIN1 luciferase between mutant group and wild-type group.

There was no significant difference in luciferase activity between transfected NC plasmid and miRNA-28-3p inhibitor in the mutant group ($P > .05$). The luciferase activity of the wild-type group transfected with miRNA-28-3p inhibitor was significantly higher than that of the NC plasmid transfected with mirNA-28-3P inhibitor ($P < .05$). Table 5.

Table 6. Comparison of BIN1 protein levels among A group, B group and miRNA-28-3p inhibitor group ($\overline{x} \pm s$)

group	BIN1alb
A group	1.09±0.22
B group	1.07±0.24
C group	3.42±1.21^a
F	5.343
P value	<.001

[a]indicate the alignment with groups A and B, with $P < .05$.

Figure 2. Figure of BIN1 protein bands

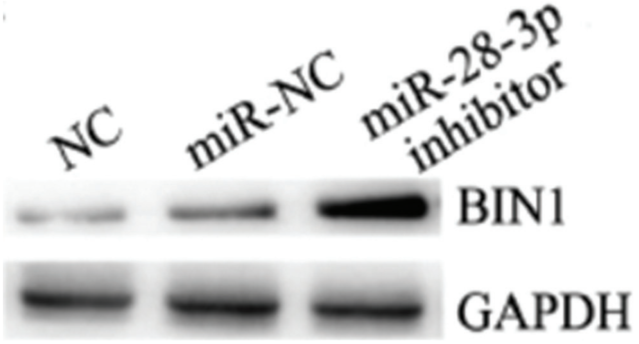
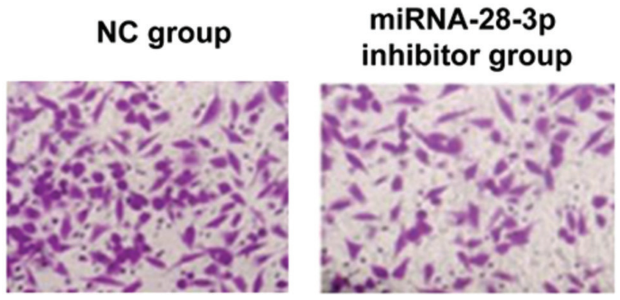


Table 7. contrasts the differences in cell proliferation, apoptosis, invasion, and migration capacity in the NC, miR-NC, and miRNA-28-3p inhibitor groups ($\overline{x} \pm s$)

group	cell multiplication	apoptosis	Cell invasion	cell migration
A group	0.89±0.07	10.89±0.52	112.02±4.22	56.43±3.22
B group	0.87±0.04	11.33±0.45	107.05±2.24	52.33±3.24
C group	0.43±0.02^a	32.44±1.43^a	42.54±1.43^a	26.45±2.09^a
F	7.354	6.433	5.874	5.613
P value	<.001	<.001	<.001	<.001

[a]indicate the alignment with groups A and B, with $P < .05$.

Figure 3. Cell invasion.



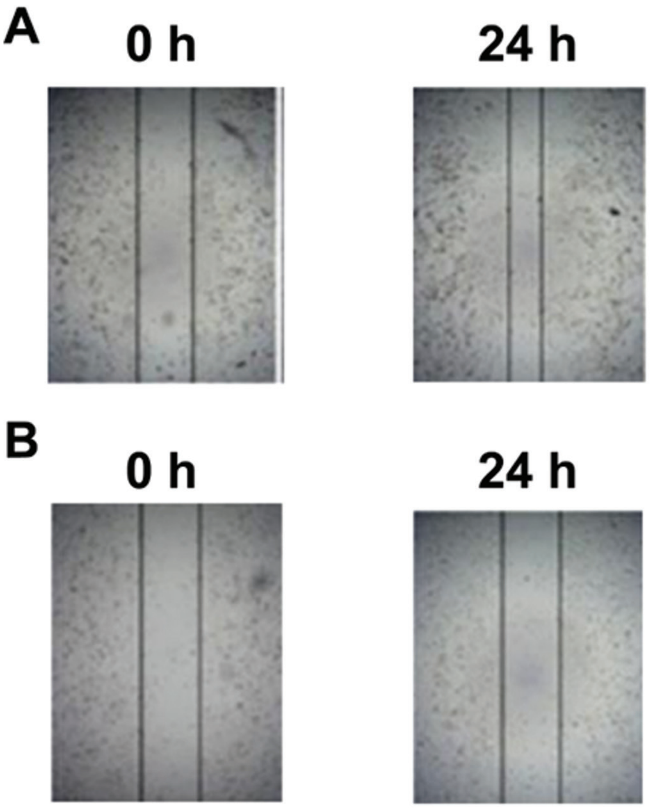
Compare the differences in BIN1 protein levels among the A group, B group, and miRNA-28-3p inhibitor group.

The expression level of BIN1 protein in group C was higher than that in group A and group B, and it was statistically different ($P < .05$). Attached Table 6, Figure 2.

Differences in cell proliferation, apoptosis, invasion, and migration capacity were compared between the A group, miR-NC, and miRNA-28-3p inhibitor groups.

The values of cell proliferation, cell invasion, and cell migration were lower than those of groups A and B, and the level of apoptosis was higher, and the data were statistically different ($P < .05$). Attached Table 7, Figure 3-4.

Figure 4. Cell migration at different time points.



Note: A is NC group and B is miRNA-28-3p inhibitor group.

DISCUSSION

Nasopharyngeal carcinoma (NPC) is a malignant tumor with high incidence worldwide. Although there are various precise treatment schemes, such as chemotherapy, radiotherapy, and targeted therapy, its prognosis is relatively poor.[6,7] Abnormal expression of oncogenes can promote tumor cell proliferation, metastasis, and other malignant biological behaviors, which play an important role in the occurrence and development of malignant tumors. Therefore, exploring the expression and mechanism of oncogenes closely related to nasopharyngeal carcinoma is of great significance for the research and update of potential therapeutic targets of nasopharyngeal carcinoma.[8]

Studies[9] have confirmed that the vast majority of human gene transcripts do not contain non-coding RNAs with effective open reading frames, among which miRNA is an important component, and there are certain differences in the target proteins and functions that different miRNAs can regulate. The miRNAs are mainly paired with the bases of the untranslated regions at the 3 ‘end of the target mRNA in a complete or incomplete complementary manner to degrade or inhibit the target mRNA at the post-transcriptional level. MiRNAs also participate in gene regulation at the post-transcriptional level and play a variety of biological functions. Previous studies have pointed out that miRNA-28-3p can play a significant role in the occurrence and development of various malignant tumors.[10]

In this study, the expression level of miRNA-28-3p was analyzed from two aspects of nasopharyngeal carcinoma tissues and cell lines, and the results showed that miRNA-28-3p was abnormally high in nasopharyngeal carcinoma tissues and cell lines, which was basically consistent with the above results. It is further suggested that the abnormally high expression of miRNA-28-3p plays a certain role in promoting the occurrence and development of nasopharyngeal carcinoma. Some studies have analyzed the expression differences of miRNA-28-3p in different pathological characteristics of malignant tumor tissues and concluded that the expression level of miRNA-28-3p is closely related to the diameter and TNM stage of malignant tumors.[11] This study did not enter into an in-depth analysis from this aspect, which can be further extended as a new idea in subsequent research. This study conducted an in-depth analysis from the perspective of miRNA-28-3p expression and nasopharyngeal carcinoma biological function. The results showed that nasopharyngeal carcinoma cells transfected with miRNA-28-3p inhibitor significantly reduced cell proliferation, invasion, and migration, and enhanced cell apoptosis ability. It further indicated that the abnormal increase of miRNA-28-3p expression level could enhance the tumor suppressive effect on nasopharyngeal carcinoma tissues.

BIN1 is a ligand-protein that shows abnormally low expression in various malignancies, which has a significant tumor suppressor effect.[12] Some studies[13] believe that the reduced expression level of BIN1 in malignant tumor cells will induce oxaliplatin resistance, and the abnormal shear of the BIN1 gene will cause the loss of its tumor suppressor function in the process of tumor development. Some studies have suggested that BIN1 is negatively regulated by miRNA-28-3p in malignant tumors, and miRNA-28-3p overexpression can inhibit BIN1 expression, inhibit the proliferation of malignant tumor cells through this way, and induce oxaliplatin resistance.[14] To further clarify the mechanism of the anti-tumor effect of abnormally high expression of miRNA-28-3p on nasopharyngeal carcinoma, a luciferase activity reporter assay was used to analyze the targeting effect between miRNA-28-3p and BIN1. The results showed that BIN1 fluorescence activity was significantly enhanced after transfection with miRNA-28-3p inhibitor. Combining with the expression results of BIN1 protein in the A group, B group, and miRNA-28-3p inhibitor group, the expression level of BIN1 protein in the miRNA-28-3p inhibitor group was significantly higher, which indicated that miRNA-28-3p had a certain targeted regulation effect on BIN1. In addition, it can inhibit the proliferation, migration, and invasion of nasopharyngeal carcinoma cells and promote cell apoptosis through this pathway. From the perspective of the BIN1 expression its mechanism may be BIN1 can weaken by combining the body protein or reverse tumor cells' malignant transformation process, thereby inhibiting tumor cell proliferation, and can suppress the inflammatory factor regulating the inflammatory pathways through expression, at the same time can reduce tumor lymphocyte infiltration degree, to weaken the tumor immune escape response. Furthermore, it can promote cell apoptosis and inhibit cell proliferation, migration and invasion.[15] The data obtained in this study cannot explain the targeting mechanism of miRNA-28-3p on BIN1, so the targeting mechanism between the two still needs to be further verified in subsequent studies. In addition to regulating BIN1, mirNA-28-3p can also participate in the occurrence and progression of nasopharyngeal carcinoma through various ways. For example, miRNA can affect the mitosis of cancer cells by regulating the expression of Plk1, a key regulator of mitotic G2/M phase transformation. miRNA-28-3p can also affect the metastasis of cancer cells by regulating the expression of epithelial-specific protein E-cadherin, matrix metalloproteinases, vascular endothelial growth factor, epidermal growth factor receptor, and other transfer-related genes.

There are still some shortcomings in this study, such as a small sample size included in the study and no in-depth analysis of miRNA-28-3p expression differences on different pathological features, which may affect the deviation and integrity of the study data. In addition, as a preliminary study, this study only verified that miRNA-28-3p affects the occurrence and progression of nasopharyngeal carcinoma through targeted regulation of the BIN1 gene. Still, the signaling pathways involved and potential mechanisms of action are not yet clear, so it is necessary to improve the research ideas and conduct in-depth analysis on large samples in the future to provide references for clinical treatment targets of nasopharyngeal carcinoma and improve the clinical prognosis of patients.

In conclusion, miRNA-28-3p is highly expressed in nasopharyngeal carcinoma tissues and cell lines. miRNA-28-3p has a targeted regulatory effect on BIN1 and can inhibit cell proliferation, migration, and invasion and promote cell apoptosis through this pathway. The expression level of miRNA-28-3p can be used as a sensitive index to predict nasopharyngeal carcinoma.

DATA AVAILABILITY

The simulation experiment data used to support the findings of this study are available from the corresponding author upon request.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to report that are relevant to this article.

FUNDING

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AUTHOR CONTRIBUTIONS

GH and NZ designed the study and performed the experiments, NZ and JW collected the data, XZ and WL analyzed the data, and GH and NZ prepared the manuscript. All authors read and approved the final manuscript.

ETHICAL COMPLIANCE

The ethics committee of Ganzhou Cancer Hospital approved this study. Signed written informed consent were obtained from the patients and/or guardians.

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