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MicroRNA-29 targets FGF2 and inhibits the proliferation, migration and invasion of nasopharyngeal carcinoma cells *via* PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: Studies have indicated that miRNAs may prove essential therapeutic targets for the treatment of cancer. The study was designed to investigate the role and therapeutic potential of miR-29 in nasopharyngeal cancer.

MATERIALS AND MATERIALS: The quantitative Real-time polymerase chain reaction (qRT-PCR) was used for expression analysis. WST-1 assay was used for cell viability assessment. The 4′,6-diamidino-2-phenylindole (DAPI) staining and electron microscopic analysis was used for the detection of apoptosis and autophagy, respectively. Transwell assays were used for cell migration and invasion assay.

RESULTS: It was found that miR-29 is significantly downregulated in nasopharyngeal cell lines. Overexpression of miR-29 caused decrease in the viability of CNE2 nasopharyngeal cancer cells via induction of apoptosis and autophagy. Bioinformatics analysis revealed FGF2 to be the target of miR-29 in CNE2 cells, which was also confirmed by luciferase assay. The qRT-PCR results showed fibroblast growth factor 2 (FGF2) to be significantly upregulated in the nasopharyngeal cell lines. However, miR-29 overexpression in CNE2 cells resulted in post-transcriptional suppression of FGF2 expression. Nonetheless, silencing of FGF2 also caused inhibition of CNE2 cell proliferation via induction of apoptosis and autophagy. Overexpression of FGF2 could reverse the effects of miR-29 expression on the proliferation of CNE2 cells. Moreover, overexpression of miR-29 caused significant decline in the phosphorylation of PI3K and AKT expression cells and inhibits the migration and invasion of the CNE2 cells. Finally, miR-29 overexpression could also suppress the subcutaneous xenografted tumor growth.

CONCLUSIONS: The findings of the present study indicate the therapeutic implications of miR-29 in nasopharyngeal carcinoma.

Key Words:
MicroRNA, Apoptosis, Autophagy, MiR-29, Nasopharyngeal cancer.

Introduction

Nasopharyngeal cancer is one of the most prevalent malignant tumors in Southeast Asia and Southern China[1]. The early stage metastasis of nasopharyngeal carcinoma makes it one of lethal cancers[2]. The five-year survival rate under combined treatment with adjuvant cisplatin chemotherapy and radiotherapy is 50-60%[3]. The constant relapses and distant metastasis of nasopharyngeal cancer make it difficult to manage with the current treatment strategies[4]. Generally, surgical removal, systemic chemotherapy or radiotherapy is employed for nasopharyngeal carcinoma. However, owing to the severe adverse effects of available drug regimes, the patient's quality of life is drastically impaired[5]. Improvement of prevention through early detection and identification of the therapeutic targets may prove beneficial to curb nasopharyngeal cancer related mortalities[6]. Over the last few decades microRNAs (miRs) have been shown to exhibit therapeutic potential for treating numerous diseases[7]. Consisting of around 20 nucleotides, miRs nearly act in almost all mammalian biological pathways such as apoptosis, proliferation, reg-

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ulation of cell cycle and metabolism[8]. The miRs are aberrantly expressed under disease conditions such as cancer[9]. Each miR may modulate the expression of multiple mRNAs and may affect a wide array of processes, many of which are cancer related[10]. Among miRs, miR-29 has been shown to be dysregulated in several cancer types and has the potential to act as a therapeutic target for drugs[11]. MiR-29 has been shown to regulate the apoptosis, tumorigenicity, and prognosis of several types of cancers such as hepatocellular carcinoma[12]. The members of miR-29 family have also been shown to regulate the proliferation and invasion of gastric cancer cells[13]. In another study[14], miR-29 has been shown to negatively regulate the EMT regulator N-myc interactor in breast cancer. Furthermore, microRNA-29 has been reported to play a vital role in pathogenesis and progression of osteosarcoma[15]. In lung squamous cell carcinoma, miR-29 has been shown to act as a tumor suppressor by targeting LOX2[16]. However, the role and therapeutic potential of miR-29 are still unknown in nasopharyngeal cancer. Consistently, the present work was designed to elucidate the role of miR-29 in nasopharyngeal carcinoma and to explore its therapeutic po... We report that miR-29 is aberrantly down...ed in nasopharyngeal cancer and overexpres... miR-29 suppresses the proliferation of the naso...ryngeal cancer cells by targeting fibroblast grow... factor 2 (FGF2) via regulation of PI3K/AKT path...way. To sum up, the present stu...es that miR-29 may prove an essential th...euti...get for nasopharyngeal cancer.

Materials a... Metho...

Cell Lines and Cu...ditions

The normal nas...ryn... line (NP460) and nasopharyngeal cancer ... (CNE1, CNE2, SUNE1...K1) were pu...sed from American Ty... Cult...re Collection (ATCC, Manassas, V... USA)... cell lines were maintained in D...co'...odifie...agle's Medium supplemente... % feta...ine serum (FBS; Thermo Fisher ... ., Waltham, MA, USA)... tics (10... penicillin and 100 µg/m... ...ycin), and ... mM glutamine. The ce...were ... CO_2 incubator (Thermo F... Sci..., Waltham, MA, USA) at 37... % humidity and 5% CO_2. All transfection... ...rried out by Lipofectamine 2000 (Invitrogen... ...ad, CA, USA) as per the manufacturer's protocol.

qRT-PCR Analysis

The total RNA from th... ...mal a... na-sopharyngeal cancer cell... ...s was iso... by TRIzol Reagent (Invitro... ...Carlsbad, CA... ...A) following the manufact... ...structio... The cDNA was synthesi...ed usi... ...verse transcriptase (Prom..., Madison, ...A) and amplified with Pl...um S...R Green... qRT-PCR SuperMix-UDG ...agent... ...vitrogen Carlsbad, CA, USA) usi... ...6 seq...ce detection system (Bio-Rad, ... C... ...SA). The reaction mi...e consi... µl containing 1.5 mM ...$_2$, 2.5 units ...NA polymerase, 200 µM ... 0.2 µM of each primer and 0.5 µg D... Th... conditions were as follows: 95°C f... 20 s, f... y 40 cycles of 95°C for 15... ...d 58°C for 1 ... The expression was es-...ted by $2^{-\Delta\Delta Ct}$ method and actin was used as an ...ernal control. The qRT-PCR primers for miR-... were 5'-CGC... ATCCTGGATTTAGTAAGA ...GGGC-3'... ...ward) and 5'-CCGGAATTCA-... ...AGGTCAGTG- 3' (reverse) and for... ...primers were 5'-GGCTTCTTCCT-GCGCATCCA-3' (forward) and 5'-GCTCTTAG-GAGAC ATTGGAAGA-3' (reverse).

...ys of Cell Proliferation

The proliferation rate of CNE2 cells was monitored by WST-1 assay. In brief, the CNE2 cells were cultured in 96 well plates at the density of ...$\times 10^5$ cells/well. The cells were then transfected with miR-NC or miR-29 mimics and again incubated for 24 h at 37°C. This was followed by the incubation of the cells with WST-1 at 37°C for 4 h. The absorbance was then measured at 450 nm using a victor 3-microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals.

Cell Transfection

miR-29 mimics and NC were synthesized by RiboBio (Guangzhou, China). The transfection was carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. As the CNE2 cells reached 80%, the appropriate concentrations of miR-29 mimics or NC were transfected into these cells.

Analysis of Cell Death

After transfection with miR-NC or miR-29 mimics, the CNE2 cells were cultured in twenty-four well plates for 24 h at 37°C. The cells were then collected by centrifugation and washed with

PBS. After this, the cells were stained with DAPI for 25 min. The CNE2 cells were then washed with PBS and then observed both by fluorescence and phase contrast microscopy. For annexin V/PI assay, the miR-NC or miR-29 mimics transfected CNE2 cells (5×10^5 cells per well) were incubated for 24 h at 37°C. This was followed by the staining of these cells with annexin V-FITC or PI. The percentage of apoptotic CNE2 cells was determined by flow cytometry.

Electron Microscopy

Autophagy in transfected CNE2 cell was demonstrated by electron microscopy. In brief, the nasopharyngeal CNE2 cancer cells were treated transfected with appropriate constructs and incubated for 24 h. The cells were collected by trypsinization and subjected to washing which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by the treatment of the cells with ethanol and embedding in resin. Next, the thin section was cut with the help of an ultramicrotome and subjected to electron microscopy.

Transwell Assays

The effects of miR-29 overexpression on invasion ability of CNE2 cells were determined by transwell chambers with Matrigel. The CNE2 cells were transfected with miR-29 mimics and around 200 ml cell cultures were placed into the upper chambers and only medium was added in the bottom wells. After 24 h incubation, the cells were removed from the upper chamber and the cells that invaded via the chamber were subjected to fixation with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200× magnification. The same procedure was used for cell invasion; however, in case of migration assay the inserts were not coated with 50 µl extracellular matrix.

Target Identification and Dual-Luciferase Reporter Assay

The miR-29 target genes were identified by TargetScan software (http://www.targetscan.org). The miR-29 mimics or NC were co-transfected with plasmid pGL3-FGF2′-UTR-WT or pGL3-FGF2′-UTR-MUT into U87 cells. Dual-luciferase reporter assay (Promega, Madison, WI, USA) was carried out 48 h after transfection. Renilla luciferase was used for normalization.

Western Blotting

The normal and the glioma cell lines were cultured at 37°C for 24 and then centrifuged at high speed. The cell pellet was washed with PBS and then suspended again in RIPA buffer. Thereafter, the concentrations of the proteins were determined and equal concentrations of proteins were loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (15%). The samples were transferred to polyvinylidene difluoride (PVDF) membranes and blocking was done using skimmed milk powder. This was followed by membrane incubation with primary antibodies at 4°C for 24 h. Then, the membranes were incubated with horseradish peroxidase (HRP) labeled secondary biotinylated secondary antibodies for 1 h. The membranes were washed and immunoreactive bands observed by ECL-PLUS/Kit as per manufacturer's guidelines.

Statistical Analysis

All experiments were done in three biological replicates and the values represent the mean of three replicates ± standard deviation (SD). $p < 0.05$ was considered as significant difference. Student's t-test using Graph Pad prism 7 software (La Jolla, CA, USA) was used for the statistical analysis.

Results

miR-29 is Downregulated in Nasopharyngeal Cancer Cell Lines

Expression of miR-29 was examined in one normal NP460 and four nasopharyngeal carcinomas (CNE1, CNE2, SUNE1, and HK1) cell lines by qRT-PCR (Figure 1A). It was found that miR-29 is downregulated significantly ($p < 0.05$) in all the nasopharyngeal cancer cell lines. Furthermore, miR-29 was found to be downregulated in nasopharyngeal cancer lines by almost 10 fold relative to the normal NP460 cells. Lowest expression of miR-29 was reported in case of CNE2 cell line.

miR-29 Inhibits Proliferation of CNE2 Cells by Induction of Apoptosis and Autophagy

To find out the effects of miR-29 on the proliferation of the nasopharyngeal CNE2 cells, the CNE2 cells were transfected with NC or miR-29 mimics. The overexpression of miR-29 was confirmed by qRT-PCR, which showed about 4.6 fold upregulation of miR-29 in CNE2 cells (Figure 1B).

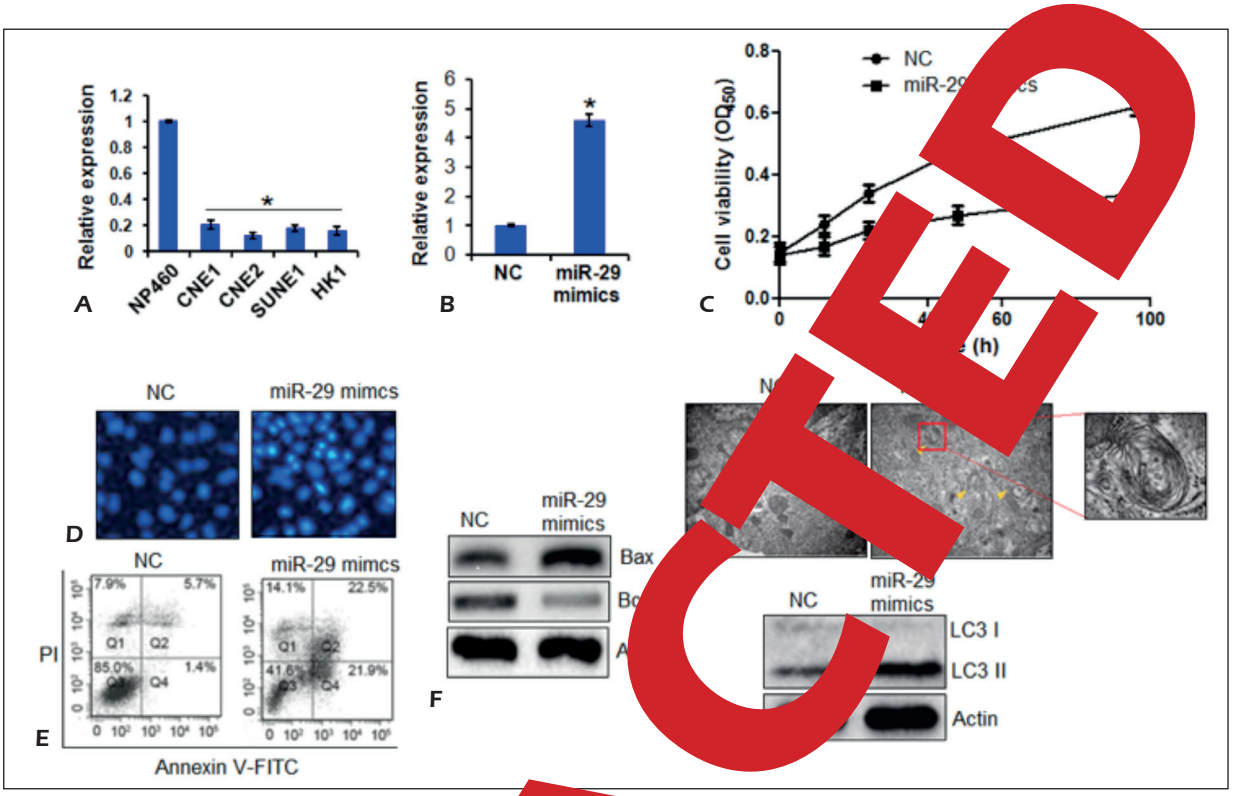


Figure 1. miR-29 inhibits the proliferation of naso… …lls via induction of apoptosis and autophagy. **A**, qRT-PCR showing the expression of miR-29 in normal … …sopharyngeal cancer cell lines (**B**) expression of miR-29 in NC and miR-29 mimics transfected CNE2 … **C**) CCK… …owing the viability of the. NC and miR-29 mimics transfected CNE2 cells (**D**) DAP staining of the NC and …29 m… …s transfected CNE2 cells (**E**) Annexin V/PI staining of the NC and miR-29 mimics transfected CNE2 cells (**F**) Expr… …ax and Bcl-2 in NC and miR-29 mimics transfected CNE2 cells (**G**) Electron microscopic images of NC and miR-29 … …ransfected CNE2 cells (Arrow heads depict autophagic vesicles) (**H**) Expression of LC3 I and LC3 II … …on in NC a… …-29 mimics transfected CNE2 cells. The experiments were performed in triplicate and the values re… … ± SD (*.<…

The proliferation rate of t… …miR-2… mimics' transfected CNE… cell… …nitored at different time interval… …WST-1 … …was found that transfectio… …miR-29 mim… …nto the CNE2 cells resu… …ignificant decline in the viability of C… …2 c… …re 1C). DAPI and annexin V/PI staining of t… …d miR-29 mimics transfe… …CNE2 cells we… …performed to observe if m… …29 in…uces apoptosis in CNE2 cells. The r… …ts sh… …d that miR-29 overexpression c… …the …uctio… apoptotic cell death of the … …lls. T… …CNE2 apoptotic cells increased f… …C to 22.5% in the miR-2… …s trans… …CNE2 cells (Figure 1D … …e induction of apoptosis in CNE2 cel… …was a… …nied by upregulation of … …n of BCl-2 (Figure 1F). Mo… …lectron microscopic analysis also showed… …rexpression of miR-29 in nasopharyngeal … cells resulted in autophagy as evident by the formation of the autophagic vesicles (Figure 1G). miR-29 overexpression that triggered autophagy was validated by examining the expression of LC3II. As expected, miR-29 overexpression enhanced the expression of LC3 II confirming the induction of autophagy in CNE2 cells (Figure 1H). Taken together, these results indicate that miR-29 overexpression inhibits the CNE2 cell proliferation by via induction of apoptosis and autophagy.

miR-29 Targets FGF2 in CNE2 Nasopharyngeal Cancer Cells

TargetScan analysis of miR-29 revealed FGF2 to be the target of miR-29 (Figure 2A), which was further confirmed by the dual luciferase assay (Figure 2B). Therefore, the expression levels of FGF2 were investigated in all the nasopharyngeal cancer cell lines as well as in the normal cell line. It was found that the expression of FGF2 was significantly upregulat-

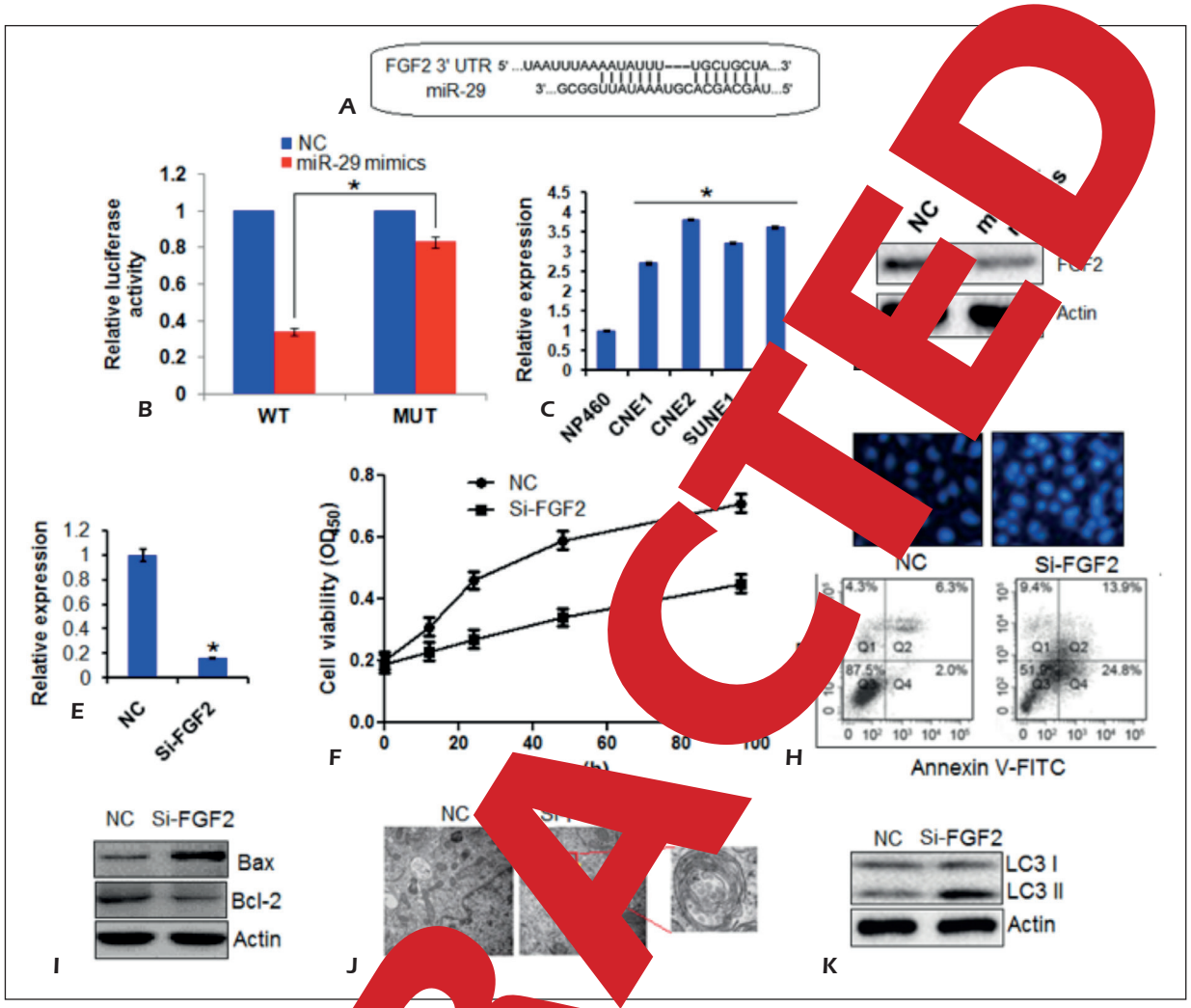


Figure 2. miR-29 exerts its effects ost-tra ression of FGF2. **A**, TargetScan analysis showing FGF2 as the target of mi-29 (**B**) Dual luciferase the expression of FGF2 in normal NP460 and four different nasopharyngeal cancer cell lines (**D**) We howing the expression of FGF2 in NC and miR-29 mimics transfected CNE2 cells (**E**) expression of F2F2 in and Si fected CNE2 cells (**F**) CCK-8 assay showing the viability of the NC and Si-FGF2 transfected CNE2 c) DAPI sta NC and Si-FGF2 transfected CNE2 cells (**H**) Annexin V/PI staining of the NC and Si-FGF2 tran CNE2 cells (**I**) ession of Bax and Bcl-2 in NC and Si-FGF2 transfected CNE2 cells (**J**) Electron microscopic ima Si-FGF2 transfected CNE2 cells (Arrow heads depict autophagic vesicles) (**K**) Expression of LC3 I and LC3 II in nsfected CNE2 cells. The experiments were performed in triplicate and the values represent mean ± SD (*p 05).

ed (3.6 fold) i e nasopharyngeal cancer cell lines (Figur). H ver, the expression of FGF2 in C de ased ificantly upon miR-29 over (Fig 2D). The effects of the FGF2 on f on rate of the nasopha cancer cells were also investi was found that the silencing of FG (Figu ed significant ($p < 0.05$) d in of the CNE2 nasopharyn er cells (Figure 2F). The decline is viab again found to be due to the induction tosis and autophagy (Figure 2G-K).

FGF2 Reverses Growth Inhibitory Effects of miR-29 on CNE2 Cells

miR-29 overexpression as well FGF2 silencing both inhibited the proliferation of the CNE2 cells through induction of apoptosis and autophagy. We sought to know if FGF2 overexpression could reverse the tumor suppressive effects of miR-29 on CNE2 cells. Interestingly, it was found that FGF2 overexpression in the miR-29 mimics transfected CNE2 cells promoted the proliferation of the CNE2 cells indicative of the inhibitory effects of the miR-29 overexpression are directly via suppression of FGF2 (Figure 3).

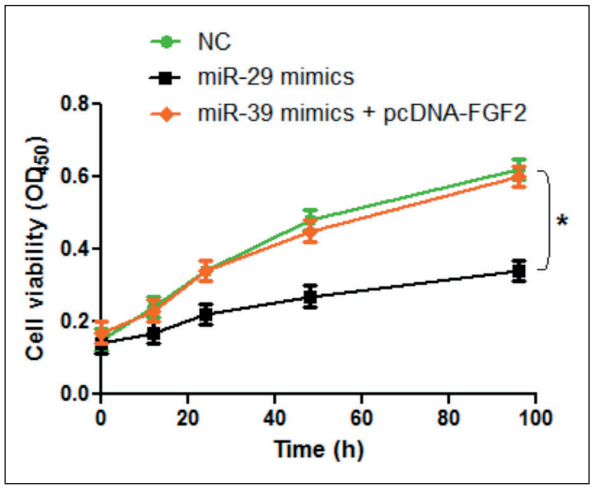
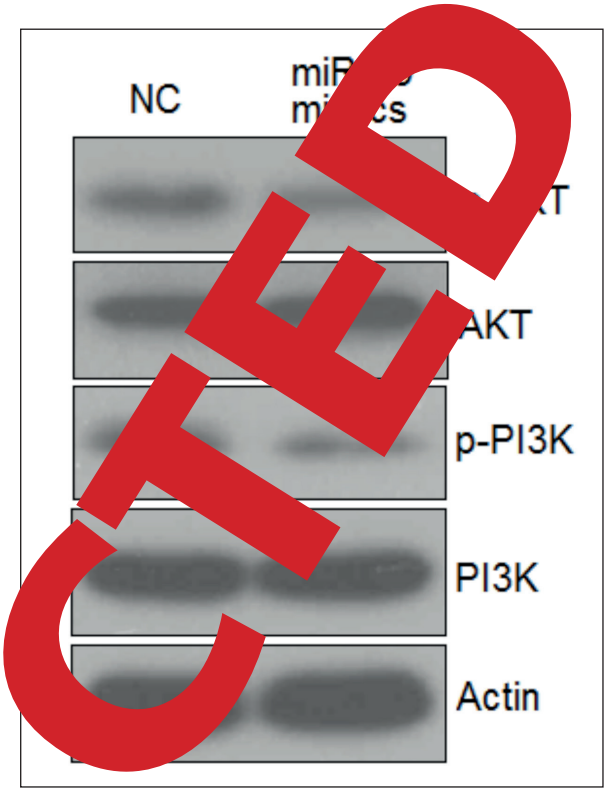


Figure 3. FGF2 overexpression reverses the effects of miR-29 overexpression on the viability of the CNE2 nasopharyngeal carcinoma cells. The experiments were performed in triplicate and the values represent mean ± SD (*p<0.05).



Effect of miR-29 overexpression on the PI3K/AKT pathway in CNE-2 cell lines. The experiments were in triplicate.

miR-29 Modulates PI3K/AKT Pathway in CNE2 Cells

This study explored the relation between 29 and FGF2 and FGF2 has been shown late the PI3K/AKT signaling pathway. The the effects of miR-29 overexpression were investigated on the PI3K/AKT signaling pathwa It was found that miR-29 overexpres inhibited the phosphorylation of both AK K (Figure 4).

miR-29 Inhibits the Mig and Invasion of the CNE2

The effects of miR- overexpr ere also investigated on t igration and ion of the CNE2 nasopha ncer cells. It was found that miR-29 exp sulted in the suppression of both migration a ion of the CNE2 cells (Fig 5A and B). The gration of CNE2 cells w hibited by 57% and the invasion was inh d by

Dis on

sopha inoma is one of the d neck malignancy[18]. The ea asis of nasopharyngeal carcinoma, late dia availability of therapeutic targets and the adv ects of the treatment strategies used for treatment are the main obstacles that limit its treatment[19]. It has been reported that miRNAs control the expression of around thirty percent of the human genes and are involved in a wide array of cellular processes[20]. Owing to the importance of the miRNAs in cellular and physiological processes, several studies[21] have revealed the potential of miRNAs as therapeutic targets. Herein, the role and therapeutic potential of the miR-29 were investigated in nasopharyngeal cancer. It was found that miR-29 was aberrantly downregulated in nasopharyngeal cancer cells. Kwon et al[22] carried out earlier have indicated that dysregulated expression of miR-29 may be associated with the development of pancreatic cancer. Moreover, miR-29 has been shown to be dysregulated in gastric cancer and in head and neck squamous cell carcinoma[23,24]. Overexpression of miR-29 in the CNE2 nasopharyngeal cancer cells caused significant reduction in the proliferation of the CNE2 nasopharyngeal cancer cells via induction of apoptosis and autophagy. These results are also supported by previous studies wherein miR-29 has been shown to inhibit the proliferation of gastric cancer and induces apoptosis in hepatocellular carcinoma cells[12,13].

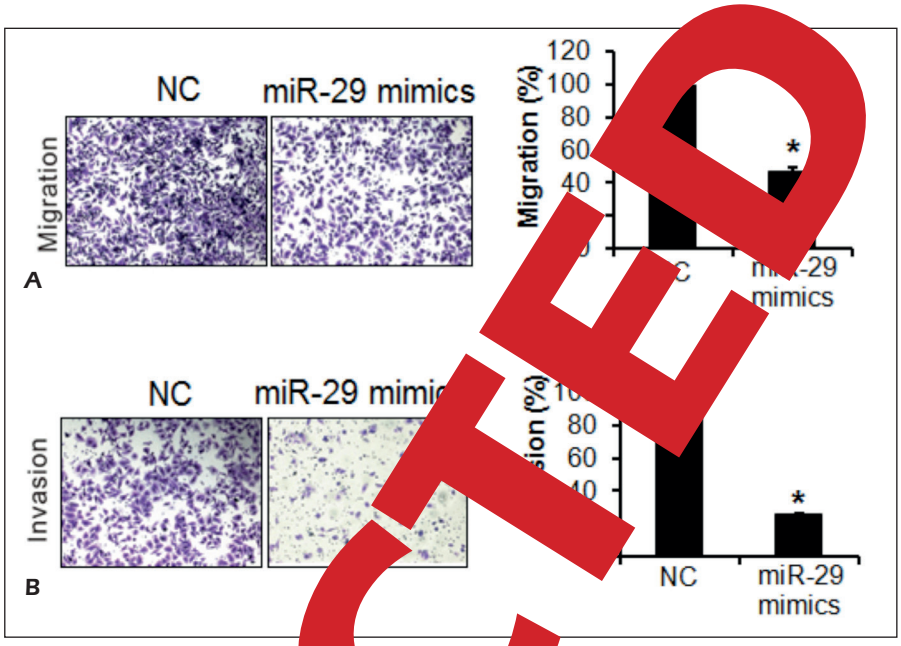


Figure 5. Effect of miR-29 overexpression on (**A**) migration (**B**) invasion of the CNE2 nasopharyngeal cancer cell lines. The experiments were performed in triplicate and the values represent mean ± SD (*p<0.05).

Bioinformatics analysis indicated FGF2 to be the potential target of miR-29. Herein, we observed that FGF2 is highly upregulated in nasopharyngeal cancer and miR-29 overexpression significant inhibition of the expression of Moreover, FGF2 silencing could also inhi growth of CNE2 nasopharyngeal cancer cell induction of apoptosis and autophagy similar that of miR-29 overexpression. F ther, FGF2 overexpression was found to re effects of miR-29 overexpression on t ion of the CNE2 cells. FGF2, is ge ly lo the nucleus and/or cytoplasm to be involved in the re ulatio igenesis and progression severa ncer ty ers such as lung and breast ers to name a [26]. Recently Zhu et al[27] at F2F2 regulates the PI3K/AKT pat y sopharyngeal carcinoma. Therefo the e of miR-29 overexpression e also invest d on the PI3K/AKT pa ay a d results showed that miR-29 over ressio d to the inhibition of the phosph ion he AK and PI3K. It is important to a n er of previously carried out studi inhibition of PI3K and A sphory with suppression of tum and progr ion of cancers[28]. The ov press 29 also resulted in the i on nd invasion of the CNE2 na al carcinoma suggesting that miR-29 may e a role in the metastasis of the nasopharyn rcinoma, which needs to be explored.

Conclusions

We showed that miR-29 is downregulated in the human nasopharyngeal cancer cells. Overexf miR-29 inhibits the proliferation of ynngeal cancer cells by inducing apoptos and autophagy via targeting FGF2 mediated PI3K/AKT pathway. Henceforth, miR-29 may rove to be an essential therapeutic target for the eatment of nasopharyngeal cancer.

Conflict of Interests

The authors declare that there are no conflicts of interest.

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