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Original Article

Bcl-2 regulation by miR-429 in human nasopharyngeal carcinoma in vivo

Wenmei Su[1*], Yanming Lin[1*], Fenping Wu[2], Hongsheng Guo[3], Lixia Li[1], Suwen Zhu[1], Zhennan Lai[1], Rong Liang[1], Zhixiong Yang[1]

[1]Department of Oncology in Affiliated Hospital of Guangdong Medical College, Zhanjiang, Guangdong, China; [2]Department of Radiotherapy, The Tumor Hospital of Chengdu, Chengdu, Sichuan, China; [3]Department of Histology and Embryology, Guangdong Medical College, Dongguan, Guangdong, China. [*]Equal contributors.

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Abstract: Radioresistance and chemoresistance severely restrict the clinical treatment of nasopharyngeal carcinoma (NPC). A considerable number of evidence indicates that the abnormal expression of microRNAs (miRNAs) contributes to cancer progression and sensitivity to chemotherapy and radiation. Bcl-2 plays an important role in the pathogenesis of NPC by regulating apoptosis. This study aimed to explore Bcl-2 regulation by miR-429 in human NPC in vivo. The aberrant miR-429 expression in CNE-2 and 5-8F cells was compared with that in NPC cells, and tumor and normal tissues were screened through quantitative reverse transcription polymerase chain reaction (qRT-PCR). Bioinformatic analyses, quantitative real-time PCR assays, and Western blot were performed to explore the regulation of miR-429 targeting Bcl-2. miR-429 expression was significantly decreased in CNE-2 and 5-8F cells compared with that in NP-69 cells. Bcl-2 was predicted to be the downstream target of miR-429, which downregulated Bcl-2 expression in transfected CNE-2 and 5-8F cells according to Luciferase reporter assay. QRT-PCR identified reduced expression of Bcl-2 targeted by miR-429. Western blot analysis consistently showed that the expression level of Bcl-2 was significantly decreased in both CNE-2 and 5-8F cell lines infected with miR-429 compared with those infected with miR-NC. miR-429 contributes to the chemoresistance and radioresistance of NPC. This finding establishes Bcl-2 as a novel regulator of miR-429 and maybe a potential therapeutic target for NPC.

Keywords: Apoptosis, nasopharyngeal carcinoma, miR-429, Bcl-2

Introduction

Nasopharyngeal carcinoma (NPC) is a highly prevalent malignant cancer in Southern China and Southeastern countries, with a distinct ethnic and geographic distribution [1, 2]. However, NPC cells can easily invade local tissues and even metastasize to remote organs, resulting in relapse and metastasis that lead to poor prognosis [3]. Therefore, the pathogenesis of NPC must be further elucidated to discover new therapeutic approaches.

Chemotherapy resistance is one of the key causal factors in cancer death. Increasing evidence has revealed that microRNAs (miRNAs) are involved in chemoresistance of different kinds of human cancers. miRNAs are a kind of small non-coding RNAs that negatively regulate gene expression at the posttranscriptional level [4]. miRNAs are important regulators in various biological and pathological processes, as well as in tumorigenesis, progression, invasion, and metastasis of NPC [5-18]. Recent studies showed that miRNAs are involved in regulating resistance to tumor cell concurrent chemotherapy [19, 20].

MiR-429 is a member of the miR-200 family, which plays a key role in tumorigenesis, migration, and chemotherapy tolerance [21, 22]. Some studies found that miR-429 may inhibit the proliferation of tumor cells and promote apoptosis as a tumor suppressor miRNA [23, 24]. A recent research pointed out that the expression level of miR-429 in NPC tissue was decreased compared with that in the epithelial tissue [25]. However, the function of miR-429 on NPC chemoresistance and radioresistance is largely unknown.

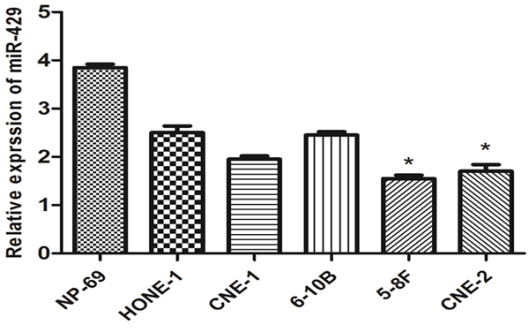

Figure 1. Histograms of the average relative expression of miR-429 in normal nasopharyngeal epithelial cell line (NP-69) and NPC cell lines (CNE-1, CNE-2, 5-8F, 6-10B, and HONE-1) determined using qRT-PCR. The miR-429 expression is significantly lower in the NPC cell lines than in the NP-69 ($^{*}P < 0.05$).

Apoptosis is a key pathologic feature of NPC, which is a severe clinical condition with high morbidity and mortality. Bcl-2 plays an important role in the pathogenesis of NPC by regulating apoptosis. However, the Bcl-2 regulation in NPC, particularly through miRNAs remains unclear. This study intended to find out the molecular mechanism of miR-429 in NPC cells and sensitization effect of chemotherapy and radiotherapy, as well as to find out whether miR-429 plays deleterious or protective roles in NPC by regulating Bcl-2. The results can provide new clinical treatment strategies to solve the high tolerance for chemotherapy and radiotherapy in NPC.

Materials and methods

Cell culture and transfection

Nasopharyngeal cancer cell lines (CNE-1, CNE-2, 5-8F, 6-10B, and HONE-1) and non-malignant nasopharyngeal epithelial cell (NP-69) were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin in humidified 5% CO_2 atmosphere at 37°C as previously described [26]. For transfection, cells were cultured up to 70% confluency and transfected with miR-429 mimics or its vector control, miR-NC, using Lipofectamine 2000 (Life Technologies, USA) by incubating with OptiMem-I media for 4 h. The cells were then cultured in fresh DMEM with 10% fetal bovine serum. The relative level of miR-429 in transfected cells were examined using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Cell viability assay

Cell viability was quantitatively assessed using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan). Viability assay was briefly conducted in 96-well plates, and absorbance was assessed using a microplate reader at 450 nm according to the manufacturer's instructions.

MTT cell viability assay

The CNE-2 and 5-8F cells were seeded in 96-well culture plates with 1×10^4 cells/well and incubated at 37°C with 5% CO_2. After treatment with different piceatannol concentrations, MTT assay (Amresco, Solon, USA) was performed. Twenty μl of MTT solution (5 mg/ml) was briefly added to each well, and the cells were continuously incubated for 4 h. Formazan crystals were then dissolved in 150 μl of DMSO. The optical density of the wells was measured with using a microplate reader (BioTek, Richmond, USA) at 490 nm.

Western blot

Equal amounts of cellular protein extracts (401 g) were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and transferred into polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Each membrane was incubated overnight at 4°C with a primary antibody against Bcl-2 orb-actin (Abcam, Cambridge, UK) and subsequently incubated with appropriate secondary antibodies at room temperature for 2 h. The membranes were then exposed to enhanced chemiluminescent plus reagents (Thermo Fisher Scientific, Dartford, UK). Protein quantification was performed using the Biospectrum-510 imaging system (UVP, Upland, CA, USA).

qRT-PCR

Total RNA, including miRNA, was extracted using a mirVana miRNA isolation kit (Ambion, Carlsbad, CA, USA). miR-429 was reverse transcribed using a specific stem-loop primer (Applied Biosystems, Carlsbad, CA, USA) and

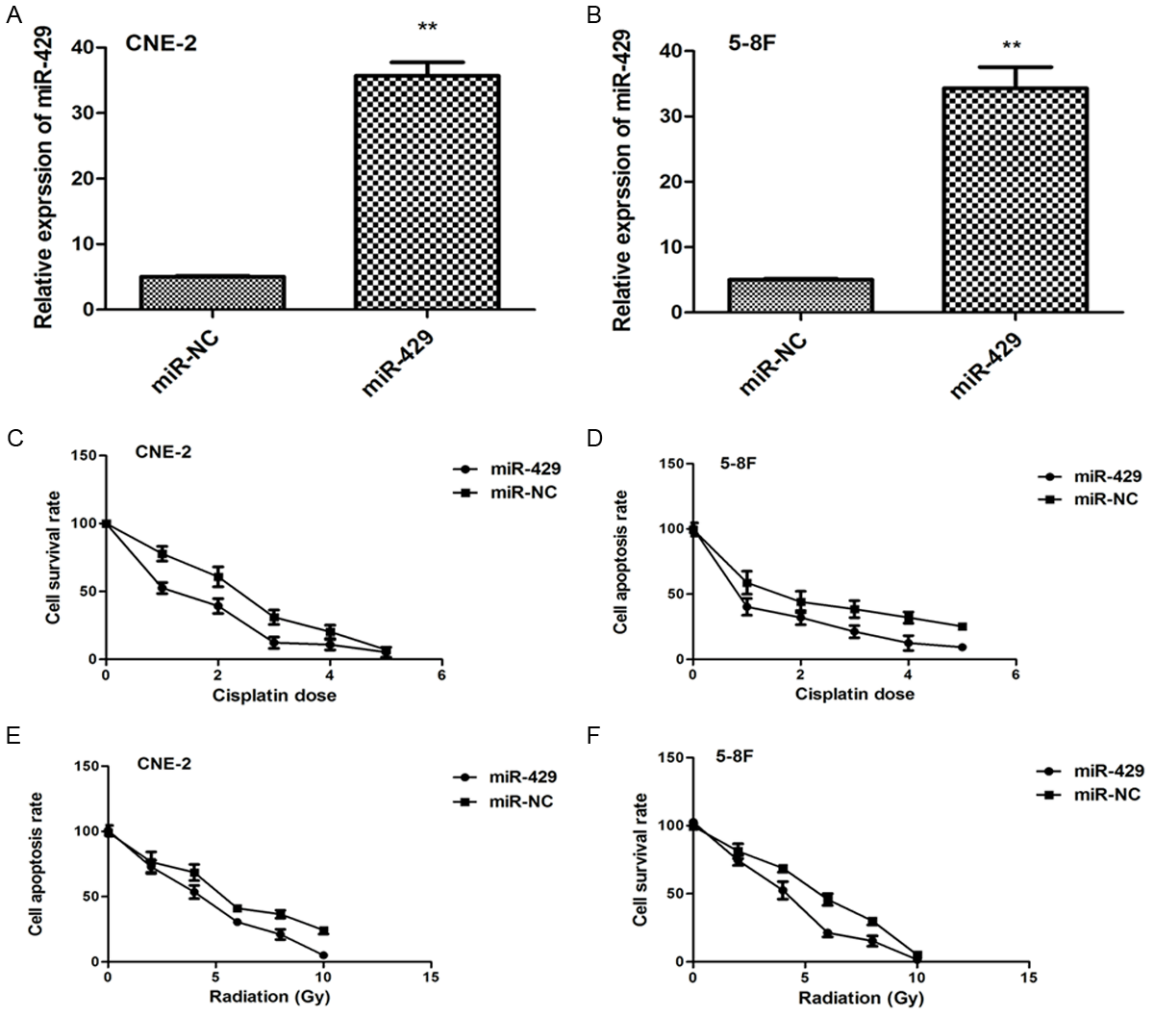

Figure 2. A, B. Using miR-429 mimic transient transfection the CNE-2 and 5-8F cell lines. The miR-429 mimics can increase the expression levels of miR-429 in NPC cell lines compared with blank control group (miR-NC). C, D. The survival rates of different cell groups determined using MTT assay in chemotherapy; the results are the average of three independent experiments ± standard deviation ($^{*}P < 0.05$; $^{**}P < 0.01$). E, F. The survival rates of different cell groups determined using MTT assay in radiotherapy; the results are the average of three independent experiments ± standard deviation ($^{*}P < 0.05$; $^{**}P < 0.01$).

quantified using real-time PCR with the TaqMan MicroRNA assay kit (Applied Biosystems). U6 was used to normalize miR-429 expression. Bcl-2 mRNA expression was quantified using real-time PCR with an RNA PCR kit (TaKaRa, Dalian, China) using primers synthesized by TaKaRa. GAPDH was used as an internal control for the mRNA expression analysis of Bcl-2.

Bioinformatic analysis

Human Bcl-2 3'-UTR sequences were retrieved from the Entrez Nucleotide database (www.ncbi.nlm.nih.gov/nuccore). The potential miRNA binding site in the Bcl-23'-UTR was predicted using TargetScan (www.targetscan.org) and miRanda (www.micorrna.org).

Luciferase reporter assay

The luciferase reporter gene plasmid containing putative miR-429 binding site at the 3'-UTR of Bcl-2 mRNA was purchased from GenePharma. For the luciferase reporter experiment, 5-8F and CNE-2 cells were seeded in 12-well plates and cotransfected with luciferase reporter vectors and the miR-429 mimic or corresponding negative control. Luciferase activity was measured using the Dual-Light Chemiluminescent Reporter Gene Assay Sys-

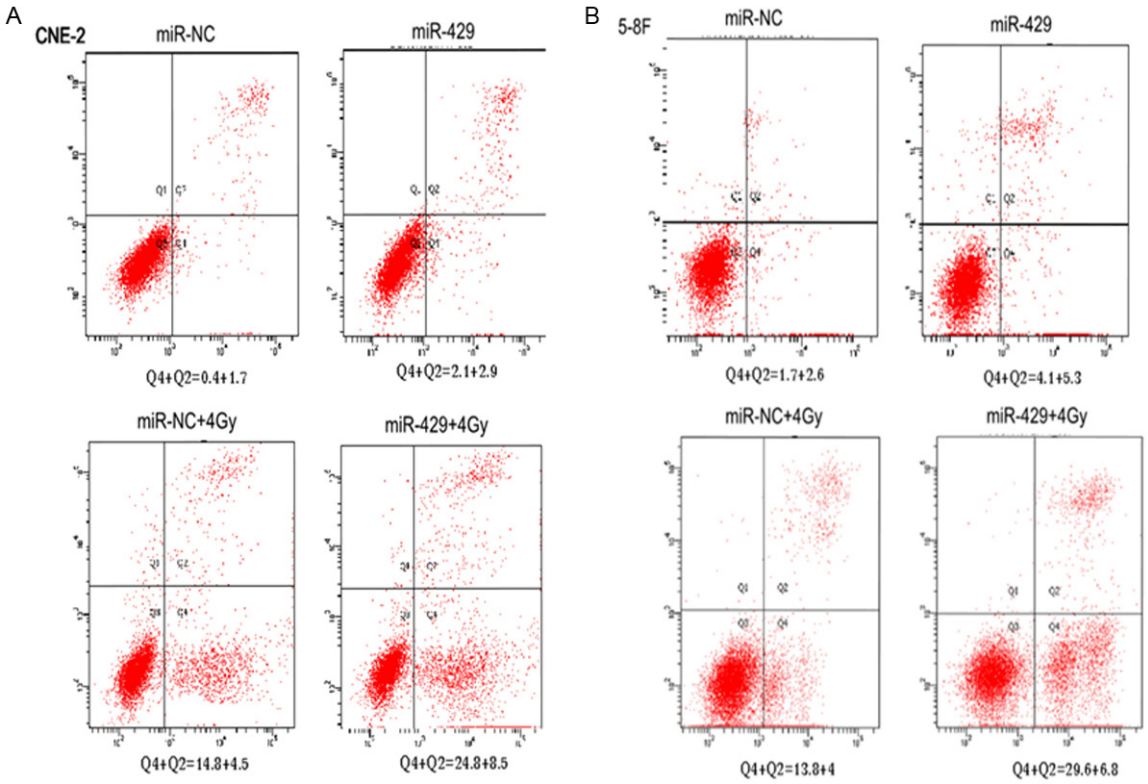

Figure 3. MiR-429 can cause NPC cell apoptosis compared with the blank control group (miR-NC).

tem (Applied Biosystems) and normalized to the b-galactosidase activity.

Establishing a radioresistant NPC CNE-2 and 5-8F cell lines

CNE-2 and 5-8F cells were cultured in a T75 flask (Corning Incorporated, Corning, New York, NY, USA) and subjected to 2 Gy irradiation (IR) using a RS 2000 biological irradiator (Rad Source Technologies, Inc., Suwanee, GA, USA). Following the first radiation exposure, the cells were cultured and passaged twice. The surviving cells were subsequently treated with the same assay as previously described; however, the IR dose was increased to 4, 6, 8 and 10 Gy after each dose had been administered twice. Following the complete radiation treatment, the surviving cells were cultured and defined as the radioresistant NPC cell line.

Statistical analysis

All data were presented as the mean ± standard deviation. Statistical analysis was performed using the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). Statistical comparisons between pairs of groups were analyzed using the two-tailed Student's t-test. Statistical comparisons among multiple groups were analyzed using the Kruskal-Wallis test followed by the Wilcoxon Rank Sum test with Bonferroni adjustments (for non-normal distributions) or a one-way analysis of variance followed by the Student-Newman-Keuls test (for normal distributions). A *P* value of less than 0.05 was considered to be statistically significant.

Results

Expression of miR-429 in nasopharyngeal cancer cell lines

Real time qRT-PCR was used to measure miR-429 mRNA expression levels in five nasopharyngeal cancer cell lines (CNE-1, CNE-2, 5-8F, 6-10B, and HONE-1) and a non-malignant nasopharyngeal epithelial cell (NP-69). The miR-429 expression was obviously reduced in CNE-2 and 5-8F cells compared with that in NP-69 cell (**Figure 1**). These data indicated that miR-429

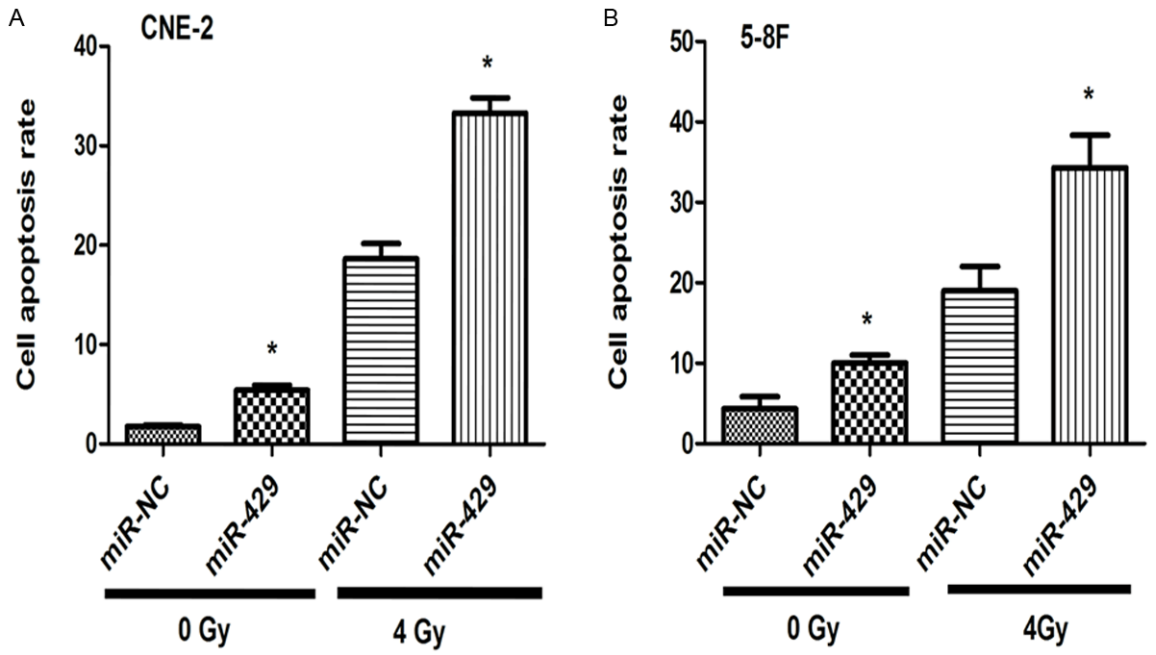

Figure 4. The apoptosis rate of NPC cell by flow cytometry analysis is higher in the miR-429 + 4 Gy group than in the miR-429 or 4 Gy single role group (*P < 0.05).

was low expressed in cell lines from NPC patients (P < 0.05). Therefore, the most sensitive 5-8F and CNE-2 two cell lines were chosen for the following experiment.

MiR-429 sensitizes chemotherapy and radiotherapy of NPC cells

To further confirm the involvement of miR-429 in regulating cisplatin sensitivity to 5-8F, CNE-2, and NP-69 cells, we exogenously upregulated miR-429 expression in CNE-2/cisplatin and 5-8F/cisplatin cells and observed its impact on cisplatin sensitivity using MTT assay. As shown in **Figure 2A** and **2B**, stable miR-429-infected CNE-2/cisplatin and 5-8F/cisplatin cells were established with significantly upregulated miR-429 expression level compared with the controls (P < 0.01). Furthermore, drug sensitivity was determined using MTT assay at 72 h with different cisplatin doses (0, 1, 2, 3, 4, and 5 μm/ml). These findings suggest that miR-429 may modulate sensitivity to cisplatin in NPC cells (**Figure 2C** and **2D**).

We continued to investigate the role of miR-429 in radiotherapy sensitization. Using different doses of CNE-2 and 5-8F cell lines undergoing radiation, we found that compared with the light treatment group, miR-429-expressed cells were more sensitive to radiation-induced cell death, especially in the 4 Gy group (**Figure 2E** and **2F**). Therefore, the most sensitive 4 Gy is chosen for the apoptotic rate of the experiment in 5-8F and CNE-2 two cell lines. The results showed that miR-429 expression can lead to NPC cell apoptosis. When miR-429 expression and radiation ray (4 Gy) affect the cells at the same time, NPC cell apoptosis rate becomes significantly higher than in pure miR-429 or ray action group (P < 0.01) (**Figures 3** and **4**). Therefore, miR-429 can increase the sensitivity of NPC cells to chemotherapy and radiotherapy.

Relationship between Bcl-2 and miR-429

Bcl-2 can cause insensitivity to chemotherapy and radiotherapy treatments [27, 28]. To explore the effect of miR-429 regulation on the sensitivity of NPC cells to chemotherapy, two computational algorithms, TargetScan and miRanda, were used to determine whether Bcl-2 3'-UTR can target miR-429. **Figure 5A** shows that Bcl-2 is theoretically a potential target gene of miR-429, and the predicted binding site between miR-429 and Bcl-2 3'-UTR is also illustrated.

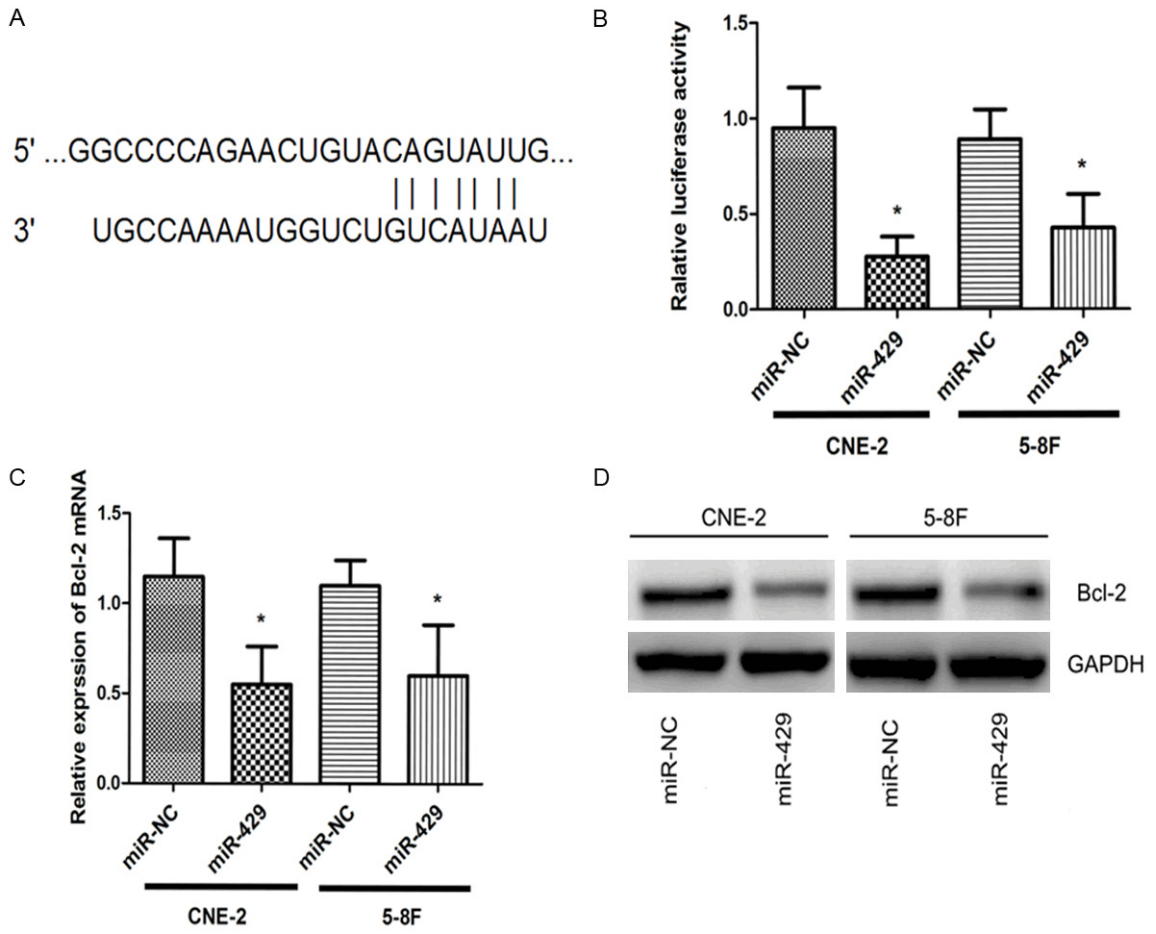

Figure 5. A. Sequence alignment of putative miR-429 binding site in the 3'-UTR of Bcl-2 mRNA. B. MiR-429 down-regulated Bcl-2 expression in transfected CNE-2 and 5-8F cells according to Luciferase reporter assay. C. qRT-PCR was used to detect the transfection efficiency of miR-429 mimics in CNE-2 and 5-8F cells. D. Western blot was conducted to detect the protein expression of Bcl-2 and miR-429 in CNE-2 and 5-8F cells.

We subcloned the full-length Bcl-2 3'-UTR into a luciferase reporter vector to confirm Bcl-2 3'-UTR targeting miR-429. **Figure 5B** shows that adding miR-429 mimics dramatically suppressed the luciferase activity of Bcl-2 3'-UTR upon co-transfection with the luciferase vector. As shown in **Figure 5C** and **5D**, Bcl-2 mRNA levels and protein were reduced by miR-429 mimics but increased by miR-429 transfection. QRT-PCR identified reduced Bcl-2 expression when targeted by miR-429 (**Figure 5C**). Western blot analysis consistently showed that the expression level of Bcl-2 was significantly decreased in both CNE-2 and 5-8F cell lines infected with miR-429 compared with those infected with miR-NC (**Figure 5D**). These results confirmed that miR-429 maybe affect the 3'-UTR of Bcl-2 mRNA, resulting in Bcl-2 degradation and inhibition of its expression.

Discussion

NPC is a common type of head and neck cancer in Southeast Asia, especially in Southern China [29]. Radiotherapy and chemotherapy are routine NPC treatments, but patients with NPC are relatively sensitive to chemotherapy treatment, which is also one of the reasons why the 5-year survival rate in patients with NPC is not high [30]. Therefore, new NPC treatment strategies are being explored. Oncogenes and tumor-suppressing genes may form a very complicated network, and their interaction that leads to cancer remains unclear [31]. Few reports have indicated that miRNAs were abnormally expressed in different cancers, and more researchers have paid close attention to the molecular mechanisms of miRNAs in tumorigenesis [32]. Several studies have suggested that the abnor-

mal expression of miRNAs with tumor cells is highly related to radiation and chemotherapy tolerance [33, 34]. Recent studies have indicated the role of miR-429 as a suppressor in tumor cell growth in several cancers [35, 36]. The present study found that miR-429 is relatively lower in patients with NPC cell lines than those with NP-69. QRT-PCR also revealed that miR-429 was one of the most down regulated miRNAs in induced radioresistant and chemoresistant NPC cells, which suggests that miR-429 may modulate the sensitivity of NPC cells to chemotherapy and radiotherapy. In our study showed that miR-429 inhibited tumor growth and enhanced the sensitivity of NPC to chemotherapy and radiotherapy based on MTT assay and colony formation assay. Then, we restored miR-429 in the radioresistant and chemoresistant cells by joining miR-429 mimics together, thereby improving the sensitivity to radiotherapy and chemotherapy.

Furthermore, the miR-429 in NPC cells was found to inhibit the expression level of Bcl-2. Excessive activation of Bcl-2 may lead cancer patients undergoing radiotherapy and chemotherapy to become insensitive. miR-429 showed obvious inhibiting effect on the luciferase intensity of Bcl-2 3'-UTR reporter, which indicates that Bcl-2 may be a factor affecting miR-429 by directly inhibiting its expression, thereby playing an important role in chemotherapy sensitization. Given that miR-429 expression is decreased in patients with NPC, highly activating Bcl-2 resulted in chemotherapy tolerance for patients with NPC. MiR-429 was down-regulated in nasopharyngeal cancer cell lines. Through bioinformatic analysis and 3'-UTR luciferase reporter assay, we determined that Bcl-2 can be a factor affecting miR-429, which can down-regulate endogenous Bcl-2 mRNA and protein levels. The expression of miR-429 mimics led to inhibited Bcl-2 expression. Finally, we confirmed that miR-429 down-regulation in nasopharyngeal cancer is inversely associated with Bcl-2 expression level. In conclusion, this study proves that during NPC development, loss of some tumor suppressor miRNAs, such as miR-429, led to relative sensitivity to chemotherapy, radiotherapy, and other treatments. These findings suggest that Bcl-2 may serve as major factor affecting miR-429 and paves the way for a better understanding of the mechanism underlying NPC pathogenesis and development of novel targeted therapies. Although new therapeutic ways for miRNA targets still need further improvement, this study provides a new theoretical basis and prompts the feasibility of NPC treatment based on miRNAs.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhannan Lai, Rong Liang and Zhixiong Yang, Department of Oncology in Affiliated Hospital of Guangdong Medical College, Zhanjiang 524023, Guangdong, China. Tel: +86-759-2387458; Fax: +86-759-2388305; E-mail: laizhenn@163.com (ZNL); 455822394@qq.com (RL); yangzhixiong1963@hotmail.com (ZXY)

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