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ORIGINAL ARTICLE

MicroRNA-299 targets VEGFA and inhibits the growth, chemosensitivity and invasion of human nasopharyngeal carcinoma cells

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Summary

Purpose: *Nasopharyngeal cancer (NC) causes significant mortality and is one of the common types of head and neck cancers (HNC). The incidence of NC is increasing at an alarming rate and therapeutic targets are lacking. This study was performed to investigate the role and therapeutic implications of miR-299 in NC.*

Methods: *The normal nasopharyngeal cell line NP460 and the nasopharyngeal cancer cell lines CNE1, CNE2, SUNE1 and HK1 were used in the present study. qRT-PCR was used for expression analysis. MTT assay was used for cell viability assessment.*
Acridine orange (AO)/ethidium bromide (EB) DAPI and Annexin V/propidium iodide (PI) staining assays were used for the detection of apoptosis. Transwell assay was used for cell invasion assay. Western blot analysis was used to determine protein expression.

Results: *The expression of miR-299 was significantly (up to 9-fold) decreased in NC cells. Ectopic expression of miR-299 suppressed the proliferation by promoting the apoptosis of HK1 NC cells. The percentage of apoptotic HK1 cells was 6.3% in NC and 28.3% in miR-299 mimic-transfected cells. The apoptosis promoted by miR-299 overexpression was also associated with enhancement of Bax, depletion of Bcl-2, and activation of caspases 3 and 9 in HK1 cells. The TargetScan analysis showed vascular endothelial growth factor A (VEGFA) to be the target of miR-299. Additionally, the expression of VEGFA was enhanced in all NC cells and miR-299 ectopic expression could cause suppression of the VEGFA expression in HK1 cells. Suppression of VEGFA also inhibited the proliferation of the HK1 cells, similar to that of miR-299 overexpression. The miR-299 overexpression enhanced the chemosensitivity of the HK1 cells to 5-FU and also caused a decrease in their invasion ability.*

Conclusion: *miR-299 may exhibit a therapeutic implication in NC and may prove useful in NC treatment.*

Key words: *nasopharyngeal cancer, microRNA, apoptosis, VEGFA, invasion*

Introduction

Nasopharyngeal cancer (NC) is one of the most common types of head and neck tumors in Southeast Asia and Southern China [1]. Metastasis of NC at early stage makes it one of the lethal cancers [2]. The 5-year survival rate under combined treatment with adjuvant cisplatin chemotherapy (CT) and radiotherapy (RT) is about 50-60% [3]. The frequent relapses and distant metastasis make its treatment very complicated with the current treatment strategies [3]. Generally, surgical removal, systemic CT or RT are employed for NC; however, owing to the severe adverse effects, quality of life is severely impaired [4]. Improvement of prevention through early detection and identification of the therapeutic

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targets may prove beneficial to decrease NC-related mortality [4]. Worldwide researches carried out have shown that microRNAs (miRs) may exhibit therapeutic properties in treating human diseases including cancer [5]. The miRs regulate the expression of target genes via post transcriptional regulation and are around 19-23 nucleotides in length [6]. The miR-299 has been shown to control a diverse array of molecular processes [7]. In lung cancer, the expression of miR-299 is dysregulated and has been shown to regulate the doxorubicin sensitivity [8]. In laryngeal carcinoma, miR-299 inhibits cell proliferation [9]. In another study, miR-299 has been shown to suppress the growth of colon cancer cells [10]. In another study, miR-299 has been shown to regulate the invasive behavior of breast cancer cells [11]. However, there is no report on the role and therapeutic implication of miR-299in NC.

Methods

Cell lines and culture conditions

The normal nasopharyngeal cell line (NP460) and NC cell lines (CNE1, CNE2, SUNE1, HK1) were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The cells were cultured in an incubator (Thermo Fisher Scientific, Inc.) at 37°C, 98% humidity and 5% CO_2. All transfections were carried out by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as per the manufacturer's protocol.

Expression analysis

The TRIzol reagent (Invitrogen) was used to extract RNA from the tissues and cell lines. This was followed by purification of the RNA by RNeasy Mini Kit (Qiagen). The complementary DNA (cDNA) was then synthesized with the help of miScript Reverse Transcription Kit (Qiagen). Afterwards the cDNA was amplified by using SYBR Premix Ex *Taq*™ (TaKaRa, Otsu, Shiga, Japan).

Cell transfection

The miR-299 mimics and negative control (NC) were synthesized by RiboBio (Guangzhou, China). The transfection was then carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instruc¬tions. As the HK1 cells reached 80% confluence, the appropriate concentrations of miR-299 mimics or NC were transfected into these cells.

Cell viability

The HK1 cells were transfected and then cultured for 24 h. Subsequently, the cells were treated with MTT (500 µg/mL) for 4 h. The MTT, a yellow tetrazole, is reduced to blue formazan in living cells. DMSO (10%) was then added to dissolve the blue formazan crystals formed from MTT. Finally, the optical density (OD) was taken at 570 nm to monitor the cell viability at 0, 12, 24, 48, 72, and 96 h time intervals.

AO/EB staining assay

The HK1 cells (0.6×10^6) were cultured in 6-well plates for 24 h at 37°C. Subsequently, 25 µl of cell culture were put onto glass slides and stained with a 1 µL solution of AO/EB or DAPI. The slides were then covered with cover slips and examined under fluorescence microscope.

Annexin V/PI staining assay

ApoScan kit was used to determine the apoptotic HK1 cell percentage. In brief, transfected HK1 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining these cells with annexin V-FITC or PI. The percentage of apoptotic HK1 cells at each concentration was then determined by flow cytometry.

Target identification and dual-luciferase reporter assay

The miR-299 target was identified by TargetScan online software (http://www.targetscan.org). The miR-299 mimics or negative control were co-transfected with plasmid pGL3-VEGFA´-UTR-WT or pGL3-VEGFA´-UTR-MUT into HK1 cells. Dual-luciferase reporter assay (Promega) was carried out 48 h after transfection. *Renilla* luciferase was used for normalization.

Transwell assay

The effects of miR-299 overexpression on the invasion ability of HK1 cells was determined by transwell chambers (8 mm pore size, Corning, NY, USA) with Matrigel (Millipore, Billerica, USA) The HK1 cells were transfected with miR-299 mimics and negative control and around 200 ml cell culture were placed onto the upper chambers and only medium was placed in the bottom wells. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers 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 200x magnification.

Western blotting

The transfected HK1 cells were harvested and lysed in radioimmunoprecipitation lysis buffer (20mM HEPES, 350 mM Nacl, 20% glycerol, 1% Nonider P40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethane sulfonyl fluoride, protease inhibitor cocktail and phosphatase inhibitor cocktail). A total of 45 µg protein/lane was separated on 10% SDS-PAGE gel. Proteins were then transferred to nitrocellulose membranes, blocked with bovine serum albumin for 45 min at room temperature and probed with primary antibodies (Santa Cruz Biotechnology Inc, Dallas, TX, USA) overnight at 4°C. Proteins were then incubated with horse radish peroxidase-conjucat-

ed anti-rabbit secondary antibody (SC-2357-CM) for 1 h overnight at 4°C. WEST-SAVE UpTM luminal-based enhanced chemiluminescent reagent was then used to visualize bands (AB Frontier, Co, Ltd, Seoul, Korea).

Statistics

The experiments were performed in triplicate and the values are presented as the mean±SD of three independent experiments. Student's t-test (for comparison between two samples) and one-way analysis of variance (ANOVA) followed by Tukey's test (for comparison between more than two samples) were used for statistical analyses using GraphPad Prism software (version 7; Graph Pas Software Inc, La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-299 is downregulated in nasopharyngeal cancer cells

The expression of miR-299 was examined in different NC cells by qRT-PCR and the results showed that it was significantly decreased in the NC cells compared to the normal cells (Figure 1A). The expression of miR-299 was found to be up to 7.14 fold lower in the NC cells. The HK1 showed the lowest expression of miR-299 across all the cell lines.

Suppression of HK1 nasopharyngeal cancer cell proliferation by miR-299

In order to determine the effects of miR-299 overexpression on the growth of the HK1 NC cells, the miR-299 mimics-transfected HK1 cells were subjected to MTT assay. The overexpression of miR-299 was confirmed by qRT-PCR which showed 6.3-fold increase in miR-299 expression (Figure 1B). Thereafter, the cell viability was assessed at different time intervals. It was found that miR-299 overexpression caused significant decline in the viability of the HK1 cells (Figure 1C). The miR-299 overexpression also caused decrease in the colony formation potential of the HK1 cells (Figure 1D).

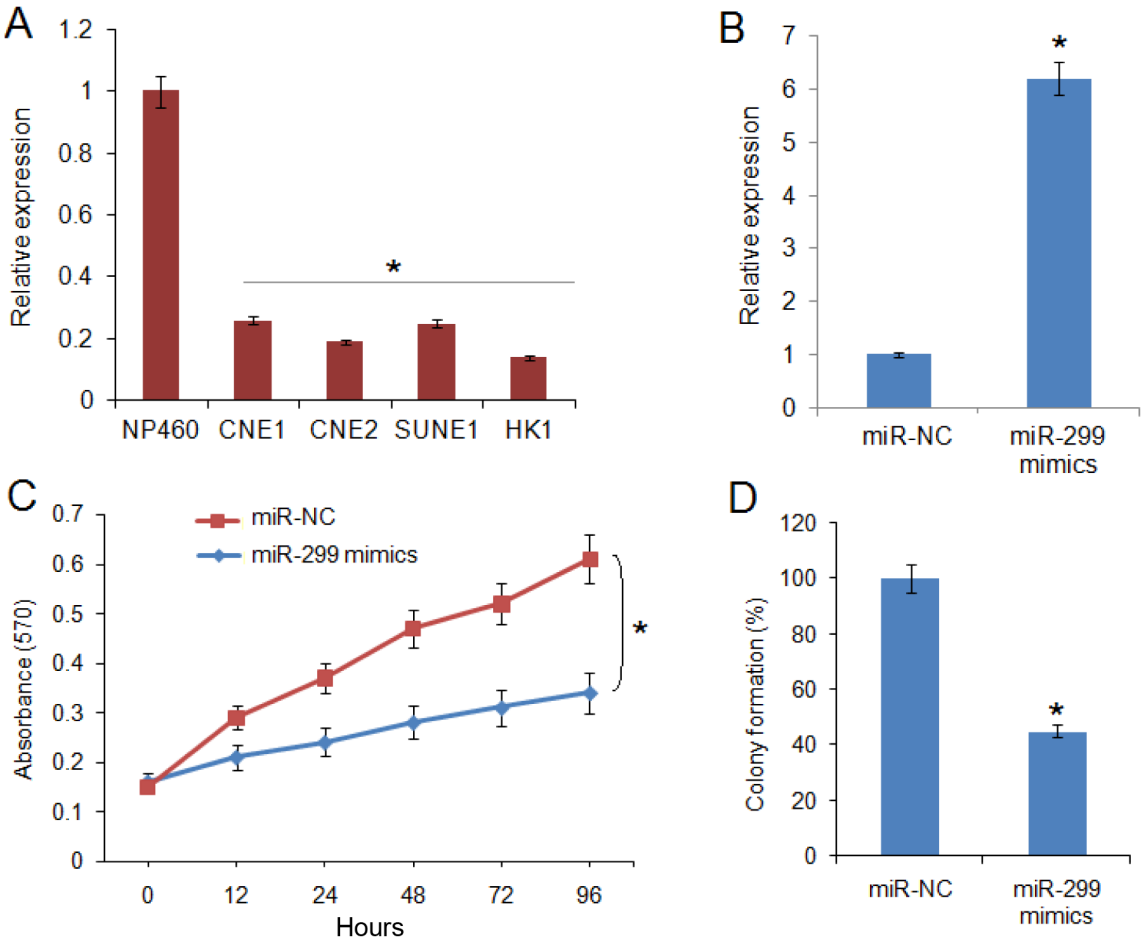

Figure 1. A: Expression of miR-299 in normal NP460 and nasopharyngeal carcinoma cells. **B:** Expression of miR-299 in miR-NC and miR-299 mimic-transfected HK1 nasopharyngeal cancer cells. **C:** Cell viability of miR-NC and miR-299 mimic-transfected HK1 nasopharyngeal cancer cells. **D:** Colony formation of miR-NC and miR-299 mimic-transfected HK1 nasopharyngeal cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

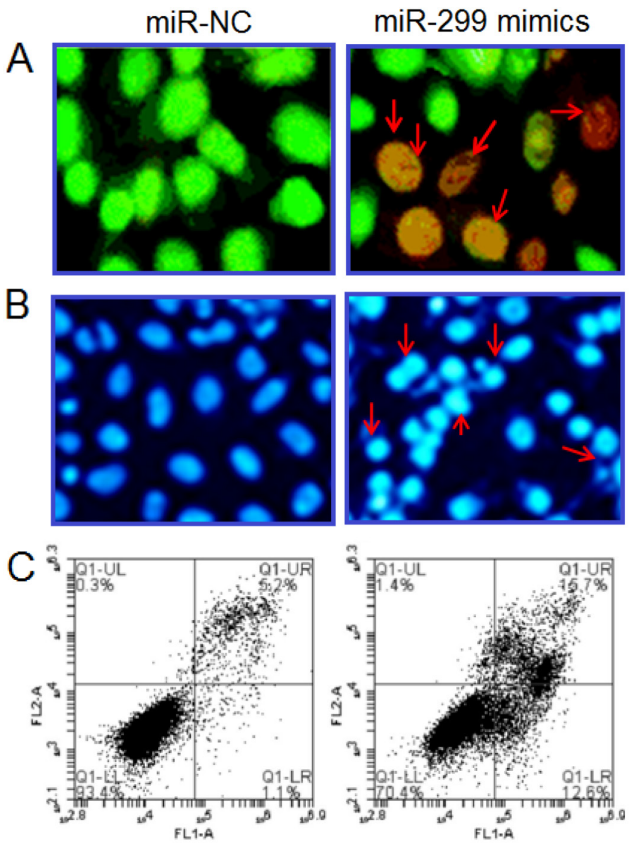

Figure 2. A: AO/EB staining, **B:** DAPI staining, **C:** Annexin V/PI staining of the miR-NC and miR-299 mimic-transfected HK1 nasopharyngeal cancer cells. The Figure shows that miR-299 overexpression induces apoptosis in HK1 cells. Green color depicts normal cells and orange color apoptotic cells. Arrows also show apoptotic cells. The experiments were performed in triplicate.

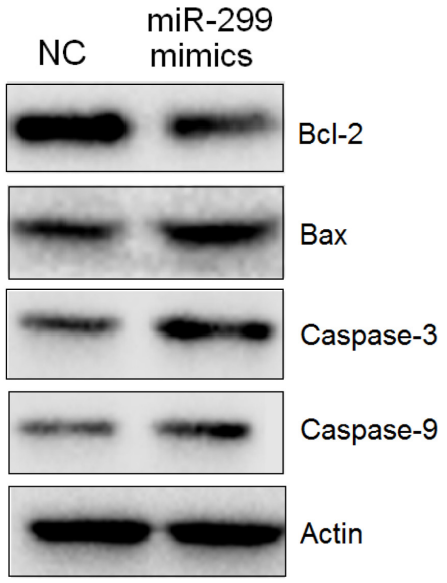

Figure 3. Expression of Bax, Bcl-2, caspase-3 and 9 in miR-NC and miR-299 mimic-transfected HK1 nasopharyngeal cancer cells. The Figure shows that miR-299 overexpression enhances the expression of Bax, Caspase-3 and Caspase-9 and decreases the expression of Bcl-2. The experiments were performed in triplicate and expressed as mean ± SD.

Induction of apoptosis in HK1 cells by miR-299 overexpression

To determine the reasons behind the inhibition of proliferation triggered by miR-299 overexpression, the miR-299 mimics-transfected HK1 cells were subjected to AO/EB, DAPI, and Annexin V/PI staining. The results of AO/EB and DAPI staining revealed that overexpression of miR-299 triggered apoptosis of the HK1 cells (Figure 2A and 2B). Annexin V/PI staining showed that the percentage of the apoptosis in miR-299 mimic-transfected HK1 cells was 28.3% compared to the 6.3% in NC-transfected cells (Figure 2C). Overexpression of miR-299 also resulted in enhancement of Bax expression and depletion of Bcl-2 expression as well as the activation of caspases 3 and 9 (Figure 3).

miR-299 targets VEGFA in HK1 nasopharyngeal cancer cells

The TargetScan analysis showed that miR-299 targets VEGFA in HK1 NC cells (Figure 4A). Dual luciferase also confirmed VEGFA as the target of miR-299 (Figure 4B). Therefore, the expression of VEGFA was also determined across all NC cells. The results showed that the expression of VEGFA was significantly increased in all NC cells (Figure 4C). The fold upregulation of VEGFA ranged between 2.9 to 4.4 in NC cells. Nonetheless, miR-299 overexpression caused suppression of VEGFA in HK1 cells (Figure 4D). The impact of VEGFA on HK1 cell proliferation: VEGFA was silenced in HK1 cells (Figure 4E) and it was found that VEGFA silencing also resulted in inhibition of the HK1 cell viability and colony formation (Figure 4F and 4G). Nonetheless, rescue assays showed that restoration of VEGF2A expression in HK1 cells overexpressing miR-299 could rescue the inhibitory effects of miR-29 overexpression on cell proliferation (Figure 5).

miR-299 enhances the drug sensitivity of HK-1 cells

To assess whether miR-299 has any effect on the 5-flourouracil (5-FU) sensitivity of the HK1 cells, the cells were subjected to 2 μM treatment or miR-299 mimics transfection or 5-FU plus miR-299 mimics transfection. The viability of all these cell groups was monitored by MTT assay at 0, 12, 24, 48, 72, and 96 h time intervals. It was found that cell viability of miR-299 mimics or 5-FU-treated cells was significantly higher compared to HK-1 cells treated with 5-FU plus miR-299 mimics transfection (Figure 6). This suggests that miR-299 enhances the sensitivity of the miR-299 mimics cells to 5-FU.

miR-299 inhibits THE invasion of HK1 cells

The transwell assay was used to determine the effects of miR-299 overexpression and showed that miR-299 significantly suppressed the invasion of the HK1 cells. The migration of the HK1 cells was suppressed up to 63% by miR-299 overexpression (Figure 7).

Discussion

NC is one of the common types of head and neck malignancies [12]. Early metastasis of NC, late diagnosis, unavailability of therapeutic targets, and the adverse effects of therapies are the main obstacles that limit its treatment [13]. The diverse role that miRs play in humans by controlling the expression of approximately 30% of the human genes indicates that miRs may prove useful therapeutic targets for treating human diseases including cancer [14]. Herein, we investigated the role of miR-299 in NC and the results indicated that the expression of miR-299 is significantly increased in NC cells. In malignant mesothelioma cells, miR-299 has also been shown to be dysregulated, validating

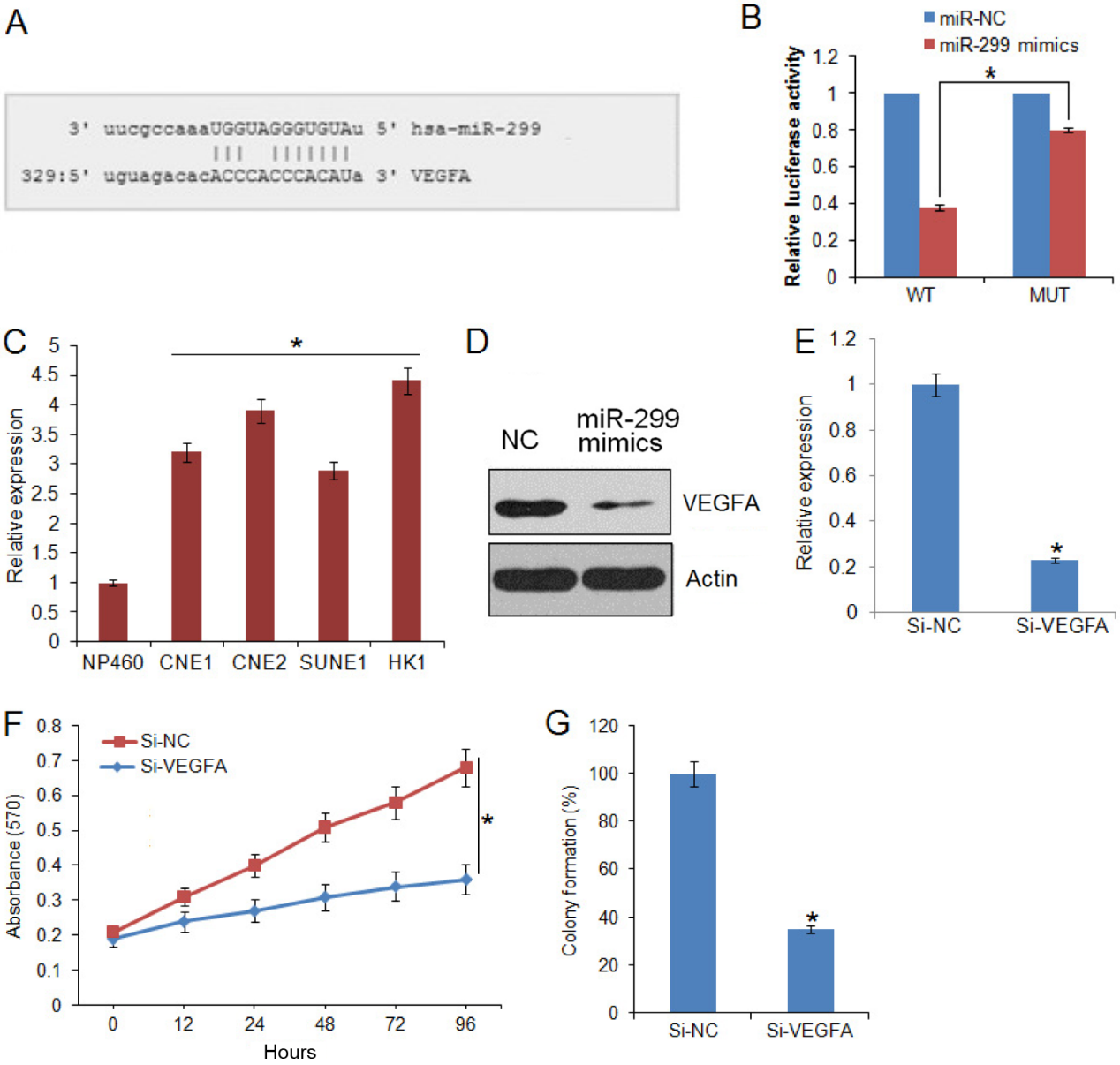

Figure 4. A: TargetScan analysis showing VEGFA as the target of miR-299. **B:** Dual luciferase assay. **C:** Expression of VEGFA in normal NP460 and other nasopharyngeal cancer cells. **D:** Western blot analysis showing the expression of VEGFA in miR-NC or miR-299 mimic-transfected HK1 nasopharyngeal cancer cells. **E:** Expression of VEGFA in si-NC or Si-VEGFA-transfected HK1 cells. **F:** Cell viability of Si-NC and Si-VEGFA-transfected HK1 nasopharyngeal cancer cells. **G:** Colony formation of the Si-NC and Si-VEGFA-transfected HK1 nasopharyngeal cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

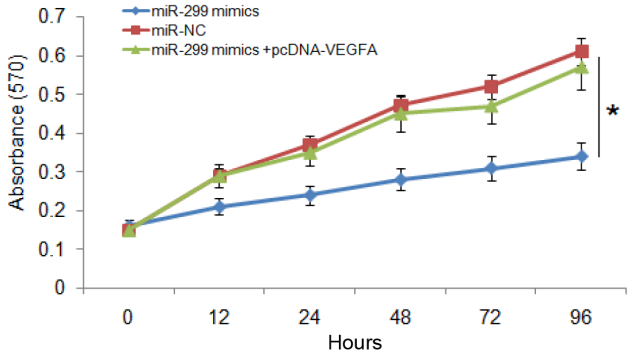

Figure 5. Cell viability of miR-299 mimics, miR-NC, miR-299 mimics + pcDNA-VEGFA-transfected HK1 cells showing rescue effects of VEGFA overexpression. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

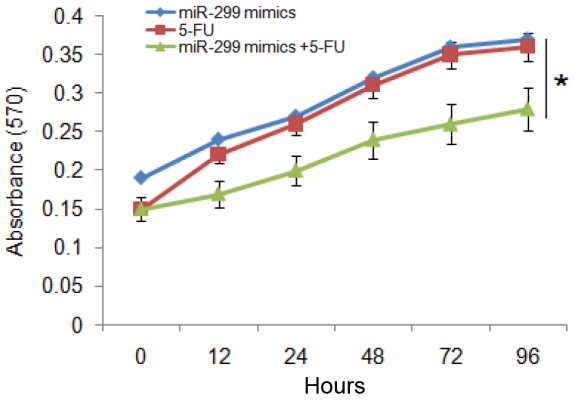

Figure 6. miR-299 enhances the chemosensitivity of HK1 nasopharyngeal cancer cells to 5-FU. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

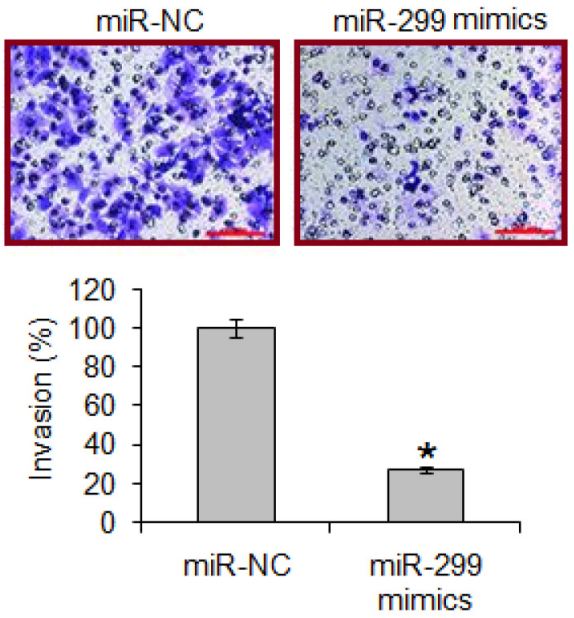

Figure 7. Transwell assay showing the invasion of the miR-NC and miR-299 mimic-transfected HK1 nasopharyngeal cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

the findings of the present study [15]. Nonetheless, overexpression of miR-299 inhibits the growth of HK1 cells. These findings are all confirming previous observations wherein miR-299 has been shown to regulate the growth of diverse cancer cells. In prostate cancer cells, miR-299 has been shown to regulate the cell proliferation via activation of apoptotic cell death [16]. Consistently, we performed the DAPI and AO/EB of the miR-299 mimics-transfected NC cells to ascertain whether miR-299 overexpression induces apoptosis. Intriguingly, it was found that ectopic expression of miR-299 promotes the apoptotic cell death of the HK1 NC cells. Moreover, miR-299 mimics-promoted apoptosis in the HK1 NC cells was also associated with enhancement of Bax, and depletion of the Bcl-2 expression, and activation of caspase-3 and caspase-9. miRs have been shown to perform their functions by modulating the expression of their target genes such as ABCE1, telomerase reverse transcriptase, VEGFA, AND OCT4 [8-11]. Herein, we found that miR-299 targeted VEGFA in NC cells and overexpression of miR-299 suppressed the VGEFA expression. To ascertain the impact of miR-299 on the invasion of the HK1 NC cells, transwell assay was performed and was found that miR-299 overexpression suppressed the invasion of the HK1 cells. This observation is also in concordance with a previous research wherein miR-299 regulates the migration and invasion of human ovarian cancer cells [17].

Conclusion

In conclusion, the findings of the present study suggest that miR-299 regulates the proliferation of nasopharyngeal cancer cells by inhibiting the expression of VEGFA. Furthermore, miR-299 also regulates the chemosensitivity and invasion of the nasopharyngeal cancer cells. Therefore, miR-299 may exhibit therapeutic implications in nasopharyngeal cancer and may prove useful in the treatment of this disease.

Conflict of interests

The authors declare no conflict of interests.

References

1. Zhang XX, Chang ZET, Liu Z et al. Medical History, Medication Use, and Risk of Nasopharyngeal Carcinoma. Am J Epidemiol 2018;187:2117-25.
2. Adham M, Kurniawan AN, Muhtadi AI et al. Nasopharyngeal carcinoma in Indonesia: epidemiology, incidence, signs, and symptoms at presentation. Chin J Cancer 2012;31:185-96.
3. Chang ET, Adami HO. The enigmatic epidemiology of nasopharyngeal carcinoma. Cancer Epidemiol Biomarkers Prev 2006;15:1765-77.
4. Haleshappa RA, Thanky AH, Kuntegowdanahalli L, Kanakasetty GB, Dasappa LJ. Epidemiology and outcomes of nasopharyngeal carcinoma: Experience from a regional cancer center in Southern India. South Asian J Cancer 2017;6:122-4.
5. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. Annu Rev Med 2009;60:167-79.
6. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. Nat Rev Drug Discov 2010;9:775.
7. Fateh A, Feizi MA, Safaralizadeh R, Azarbarzin S. Importance of miR-299-5p in colorectal cancer. Ann Gastroenterol 2017;30:322.
8. Zheng D, Dai Y, Wang S, Xing X. MicroRNA-299-3p promotes the sensibility of lung cancer to doxorubicin through directly targeting ABCE1. Int J Clin Exp Pathol 2015;8:10072.
9. Li M, Chen SM, Chen C et al. microRNA 299 3p inhibits laryngeal cancer cell growth by targeting human telomerase reverse transcriptase mRNA. Molec Med Rep 2015;11:4645-9.
10. Wang JY, Jiang JB, Li Y, Wang YL, Dai Y. MicroRNA-299-3p suppresses proliferation and invasion by targeting VEGFA in human colon carcinoma. Biomed Pharmacother 2017;93:1047-54.
11. Göhring AR, Reuter S, Clement JH et al. Human microRNA-299-3p decreases invasive behavior of cancer cells by downregulation of Oct4 expression and causes apoptosis. PLoS One 2017;12:e0174912.
12. Wei WI, Sham JS. Nasopharyngeal carcinoma. Lancet 2005;365:2041- 54.
13. Crooker K, Aliani R, Ananth M, Arnold L, Anant S, Thomas SM. A Review of Promising Natural Chemopreventive Agents for Head and Neck Cancer. Cancer Prev Res 2018;11:441-50.
14. Carthew W, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2018;136:642-55.
15. Guled M, Lahti L, Lindholm PM et al. CDKN2A, NF2, and JUN are dysregulated among other genes by miRNAs in malignant mesothelioma-a miRNA microarray analysis. Genes Chromosomes Cancer 2009;48:615-23.
16. Formosa A, Markert EK, Lena AM et al. MicroRNAs, miR-154, miR-299-5p, miR-376a, miR-376c, miR-377, miR-381, miR-487b, miR-485-3p, miR-495 and miR-654-3p, mapped to the 14q32. 31 locus, regulate proliferation, apoptosis, migration and invasion in metastatic prostate cancer cells. Oncogene 2014;33:5173.
17. Zhao R, Liu Q, Lou C. MicroRNA-299-3p regulates proliferation, migration and invasion of human ovarian cancer cells by modulating the expression of OCT4, Arch Biochem Biophys 2018;6:5-9.