Downregulation of miR-429 and inhibition of cell migration and invasion in nasopharyngeal carcinoma

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Abstract. Viral, dietary and genetic factors have been implicated in nasopharyngeal carcinoma (NPC), however, the molecular mechanism underlying its pathogenesis remains to be fully elucidated. MicroRNAs (miRNAs) have been reported to be important in NPC tumorigenesis, with a previous miRNA microarray study showing the downregulation of miRNA (miR)-429 in NPC cells. However, the possible mechanisms of action of miR-429 have not been examined. In the present study, the expression profiles of miR-429 were detected using reverse transcription-quantitative polymerase chain reaction analysis in CNE-1 and CNE-2 cells, which are two generally used NPC cells with different degrees of differentiation. Subsequently, cell proliferation, invasion and migration were analyzed in miR-429-overexpressing CNE-2 cells, and the modulatory function of miR-429 was also investigated using two target genes, zinc finger E-Box-binding homeobox 1 (ZEB1) and CRK-like (CRKL), by transfection with miR-429 mimic or anti-miR-429. Significant changes in the expression of miR-429 were detected, particularly in low-differentiated CNE-2 cells, with higher levels of epidemicity and malignancy. Additional results revealed that miR-429 inhibited the invasion and migration of the CNE-2 cells, whereas no significant effect on cell growth was observed. In addition, the mRNA and protein expression levels of the two target genes, ZEB1 and CRKL, were negatively regulated by miR-429, demonstrated through gain-of-function and loss-of-function investigations, indicating that these two functional downstream targets may be involved in the inhibitory effects of miR-429 on NPC migration and invasion. miR-429 may act as a negative regulatory factor of NPC tumorigenesis, involving the functions of its downstream targets, ZEB1 and CRKL. The results suggested miR-429 as a potential candidate for miRNA-based prognosis or therapy against NPC.

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Introduction

MicroRNAs, a novel class of endogenous small non-coding RNAs, can modulate gene expression at the post-translational level by interacting with the 3' untranslated regions of target mRNAs, resulting in the inhibited translation or degradation of mRNAs (1,2). Substantial evidence has shown that the majority of the regulated genes of microRNAs are important in tumorigenesis, with aberrant microRNA expression reported in the development and progression of several types of tumor (3,4). The role of various microRNAs on invasion, migration and metastasis, which are essential steps during cancer progression, has been described (5-7).

Nasopharyngeal carcinoma (NPC) usually develops around the ostium of the eustachian tube in the lateral wall of the nasopharynx, and is widely prevalent in Southeast Asia, the Middle East and North Africa (8). NPC is also distinguished by its high rate of metastasis and poor prognosis among head and neck cancer (9). Epstein-Barr virus (EBV) infection, non-viral environmental risk factors and host genetics have been generally accepted as three predominant factors, which contribute to the development of NPC (10-12). However, the molecular mechanism underlying the pathogenesis of NPC remains to be fully elucidated.

Increasing evidence suggests that the distinct expression pattern of microRNAs can provide important insight into the molecular mechanism of tumorigenesis in NPC (13,14). Several dysregulated miRNAs have been shown to regulate cell growth, apoptosis and the metastasis of NPC (15-17). Previous systematic investigations of microRNA expression profiles in the stepwise development of NPC have also revealed 13 microRNAs, which may be the most important modulators during the development of NPC (18). MicroRNA-429 (miR-429), one of these microRNAs, has been reported to be important in certain types of cancer. miR-429 can induce the tumorigenesis of human non-small cell lung cancer and mesenchymal-to-epithelial transition in metastatic ovarian cancer cells (19,20). It can also inhibit cell invasion in

colorectal carcinoma, and the migration and invasion of breast cancer cells, in which the expression profiles of miR-429 are downregulated (21,22). However, the effects and possible mechanisms of action of miR-429 in the metastasis of NPC have not been examined.

In the present study, the expression levels of miR-429 were detected in CNE-1 and CNE-2, which are two generally used EBV-negative epithelial cells with different degrees of differentiation (23,24). To improve understanding of the regulatory mechanism of miR-429 in NPC, cell proliferation, invasion and migration were analyzed in miR-429-overexpressing CNE-2 cells. The modulatory function of miR-429 was also investigated through two representative target genes, zinc finger E-Box-binding homeobox 1 (ZEB1) and CRK-like (CRKL). The present study aimed to investigate the potential function of miR-429 in NPC tumorigenesis, using its target genes, including ZEB1 and CRKL, in order to determine the potential application of miR-429 in NPC treatment or prognosis determination.

Materials and methods

Cell lines and cell culture. NP69 cells, which are immortalized non-tumorigenic nasopharyngeal epithelial cells, were cultured in keratinocyte-serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with bovine pituitary extract, as described previously (25). The two tumorigenic NPC cell lines, comprising well-differentiated CNE-1 cells and poorly-differentiated CNE-2 cells, were maintained in our laboratory and cultured in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (BD Biosciences; Franklin Lakes, NJ, USA). All cells were grown in a humidified incubator at 37˚C with 5% CO_2. Following 24-h incubation to 100% confluence, the cells were fixed with absolute methanol (Sigma-Aldrich, St. Louis, MO, USA), stained with 0.4% (w/v) crystal violet (Sigma-Aldrich) in methanol and subsequently rinsed with water. Morphological images of three NPC cells were captured using a Nikon Eclipse TS100 inverted light microscope equipped with a Nikon Coolpix 4500 digital camera (Nikon Corporation, Tokyo, Japan).

Transfection with microRNA mimics. The miR-429 mimic (with a nonspecific miRNA control) and anti-miR429 (with a nonspecific anti-miRNA control) were all purchased from Dharmacon; Thermo Fisher Scientific, Inc.). RNAiMAX reagent (Invitrogen;Thermo Fisher Scientific, Inc.) was used to deliver the miRNA mimics (20 nM) into the cells, which were maintained in 6-well plates at a density of $1x10^6$ cells/well, and transfection was performed using Lipofectamine 2000 (Invitrogen;Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Transfected cells were incubated at 37˚C in complete medium, and the subsequent experiments were performed 48 h following transfection.

RNA extraction and reverse transcription. Total RNA was extracted from the cells using TRIzol (Invitrogen;Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, cells at ~80% confluence were seeded into 6-well plates and washed twice using ice-cold phosphate-buffered saline (PBS), following which 1 ml TRIzol was added to obtain the RNA. The quantity and quality of the extracted RNA were analyzed using a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Inc.). For mRNA analysis, cDNA was synthesized using Quant cDNA with random primers (Tiangen Biotech Co., Ltd., Beijing, China), and the miRNAs were reverse-transcribed, according to previously described method (26). Briefly, a specific reverse primer targeting individual miRNA was designed to complete the reverse transcription, and one miRNA-specific forward primer and one universal reverse primer were used in the subsequent quantitative polymerase chain reaction (qPCR) analysis.

qPCR. The qPCR detection was completed using SYBR Premix Ex Taq™ II (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. The qPCR procedure was performed in accordance with the protocol of Takara Bio, Inc. in a StepOne Plus Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The results were analyzed using the $2^{-\Delta\Delta Cq}$ method (27). U6 small nuclear RNA (U6-snRNA; Guangzhou RiboBio Co., Ltd., Guangzhou, China) and β-actin were used as internal controls for microRNA and mRNA, respectively. The primers used are listed in Table I.

Cell proliferation assays. Freshly sorted NP69, CNE-1 and CNE-2 cells were incubated at a density of 500 cells per well in a 96-well plate in triplicate to examine the growth rate. The cells transfected with miRNAs were reseeded at a density of $1.5x10^3$ cells/well in a final volume of 150 μl 48 h following incubation, and incubated at 37˚C overnight. The effects of miR-429 on cell growth and proliferation were determined using an MTT assay (Sigma-Aldrich), as described previously (28). During the subsequent 4 days, the absorbance of the cells stained with 50 μl MTT were measured at 570 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc.), and the measurements were used to construct a cell growth curve.

Cell invasion assays. Cell invasion was measured using Biocoat Matrigel Invasion Chambers (BD Biosciences), according to the manufacturer's protocol. In brief, the CNE-2 cells transfected with the miRNA mimics or inhibitors were plated 48 h post-transfection in serum-free medium ($2.5x10^4$ cells per Transwell) and allowed to migrate towards a 10% fetal bovine serum gradient for 12 h at 37˚C. Subsequently, the upper chambers were removed from the lower chambers and wiped using cotton swabs. The invaded cells were fixed using ≥99.9% methanol (Sigma-Aldrich), and visualized by 0.1% toluidine blue staining (Sigma-Aldrich) under a Nikon Eclipse TS100 light microscope, as described in a previous study (16). This experiment was independently repeated at least twice.

Western blot analysis. Western blot analysis was performed, as described previously (29). In brief, the cells were carefully collected with scrapers on ice and then subjected to lysis with radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Nanjing, China). Protein lysates

Table I. Primers used in quantitative polymerase chain reaction analysis.

Primer	Sequence (5'-3')
miR-429-R	CTCAACTGGTGTCGTGGAGTCGG CAATTCAGTTGAGACGGTTTT
miR-429-F	ACACTCCAGCTGGGTAATACTGTC TGGTAA
Universal-R	TGGTGTCGTGGAGTCG
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
ZEB1-F	GCACAACCAAGTGCAGAAGA
ZEB1-R	GCCTGGTTCAGGAGAAGATG
CRKL-F	CGCTCCGCCTGGTATATGG
CRKL-R	GGACACCGACAGCACATAGTC
β-actin-F	AGTGTGACGTGGACATCCGCA
β-actin-R	ATCCACATCTGCTGGAAGGTGGAC

miR, microRNA; ZEB1, zinc finger E-Box-binding homeobox 1; CRKL, CRK-like; R, reverse; F, forward.

were separated by 12% SDS-PAGE (Beyotime Institute of Biotechnology) and subsequently electrophoretically transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, ISA). Tris-buffered saline with Tween-20 (TBS-T) buffer, containing 10 mM Tris-HCl (pH 7.5; Beyotime), 150 mM NaCl (GR grade; Shanghai Hushi, China) and 0.1 % (v/v) Tween-20 (Sigma-Aldrich), was pre-prepared for use in the washing and blocking steps and was used as the antibody diluent. Following blocking with 5% (v/v) skimmed milk in TBS-T buffer for 1 h at room temperature, protein expression levels were analyzed following incubation with the following primary antibodies overnight at 4˚C: Polyclonal rabbit anti-ZEB1 (1:1,000; HPA027524; Sigma-Aldrich), monoclonal rabbit anti-CRKL (1:500; Y244; Abcam, Cambridge, UK) and monoclonal mouse anti-β-actin (1:5,000; A5441; Sigma-Aldrich). After rinsing three times with TBS-T, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-rabbit antibody (7074) or anti-mouse antibodies (both 1:2,000; 7076, both Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 60 min. Following washing in TBS-T, images of the immunoblots were acquired and analyzed using an ImageQuant LAS 4000 system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The primary antibodies used in the experiment were ZEB1, CRKL and β-actin.

Statistical analysis. The results of the quantitative data in the present study are expressed as the mean ± standard deviation. Two-tailed Student's *t*-test was used for comparisons of two independent groups, and Welch's corrected *t*-test was used for unequal variances. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-429 is decreased in human NPC cell lines. As an initial step in assessing the potential role of miR-429 in the development of NPC, the expression profiles were analyzed in two NPC-derived cell lines, CNE-1 and CNE-2, with different levels of differentiation. The immortalized NPC cells (NP69) were used as a negative control. The three carcinoma cell lines were incubated and observed under a Nikon Eclipse TS100 light microscope to confirm their morphologies (Fig. 1A). Further measurement of cell proliferation showed that CNE-1 and CNE-2 exhibited higher growth rates, compared with the NP69 cells. In addition, the CNE-2 cells exhibited higher proliferation rates, compared with the CNE-1 (Fig. 1B), indicating higher malignancy potential. Of note, the expression of miR-429 was suppressed more significantly in the CNE-2 cells, compared with the CNE-1 cells (Fig. 1C). CNE-2 is derived from poorly-differentiated NPC cells (24), and exhibits higher epidemicity and malignancy, compared with well-differentiated NPC cells, including CNE-1 cells (23). The results of the present study showed aberrant miR-429 expression in the low-differentiated NPC cells, indicating that miR-429 may be more important in the pathogenesis of NPC. The CNE-2 cells were selected to further investigate the functional role of miR-429 in NPC tumorigenesis.

Overexpression of miR-429 suppresses cell invasion and migration. In order to explore the functional roles of miR-429, miR-429 overexpression was induced in CNE-2 cells by transfection with miR-429 mimics, and the effects on cell proliferation, migration and invasion were investigated. Overexpression of miR-429 in CNE-2 cells was confirmed using RT-qPCR 48 h after transfection to ensure the induction had occurred (Fig. 2A), which demonstrated a significant 3,700-fold increase. The results indicated that the overexpression of miR-429 had minimal effect on cell proliferation over a 96-h period of detection (Fig. 2B). However, the miR-429-overexpressing cells demonstrated decreased invasion and migration, compared with the control group and the scrambled cells, which were transfected with nonspecific miRNA (Fig. 2C). Cell invasion was suppressed more markedly. Taken together, miR-429 may have regulated NPC tumorigenesis in a negative manner, indicating its potential in miRNA-based therapy against NPC. For further explanation of this negative regulation, target genes of miR-429 were also investigated.

miR-429 inhibits the expression levels of ZEB1 and CRKL. ZEB1, which is an important epithelial-to-mesenchymal transition (EMT) inducer (30), and CRKL, which has been identified as a candidate target of miR-429 (22), were selected in the present study as representatives to examine the regulator function of miR-429 in NPC cells.

The mRNA and protein expression levels of ZEB1 and CRKL were detected in response to the induced expression of miR-429 in CNE-2 cells by transfection with miR429 mimics. The results showed that ZEB1 and CRKL were suppressed significantly in the miR-429-overexpressing cells (Fig. 3). Compared with the scrambled cells, the expression levels of ZEB1 and CRKL were downregulated by ~3-fold and 6-fold,

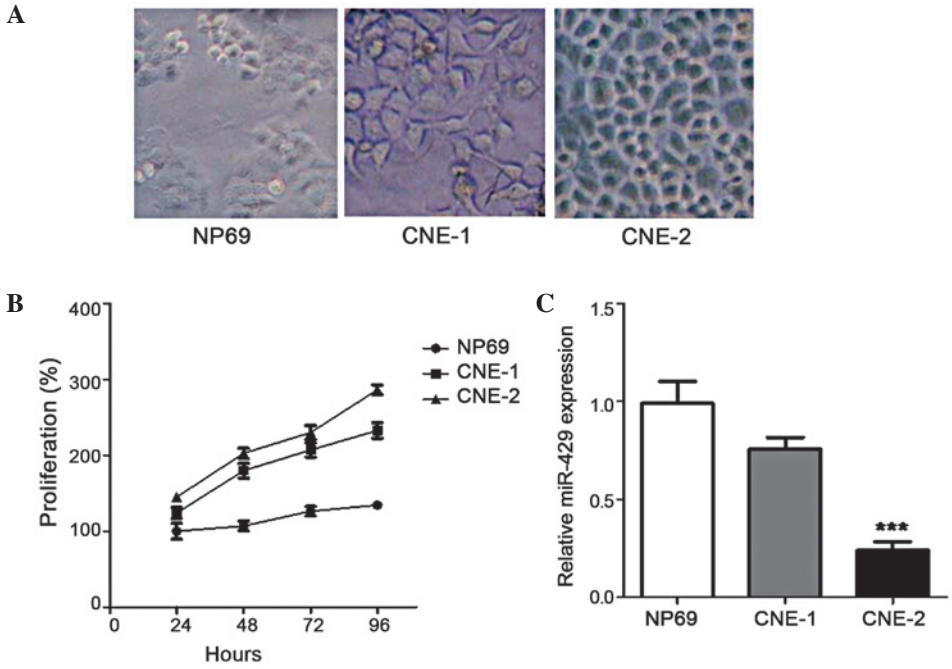

Figure 1. Detection of miR-429 expression profiles in human NPC cell lines. (A) Morphological images of three NPC cells following incubation for 24 h. The cells were stained with crystal violet and observed under a light microscope (magnification, x100). (B) Proliferation of NP49, CNE-1 and CNE-2 cells over 4 days. The proliferation rates of the two NPC cells were higher, compared with the NP69 cells. (C) Relative miR-429 expression levels in CNE-1 and CNE-2, compared with NP69 cells (***P<0.001). Expression of miR-l49 was significantly downregulated in the CNE-2 cells. NP69, immortalized non-tumorigenic nasopharyngeal epithelial cell; CNE-1: well-differentiated nasopharyngeal carcinoma cell; CNE-2: poorly differentiated nasopharyngeal carcinoma cell; miR, microRNA; NPC, nasopharyngeal cancer.

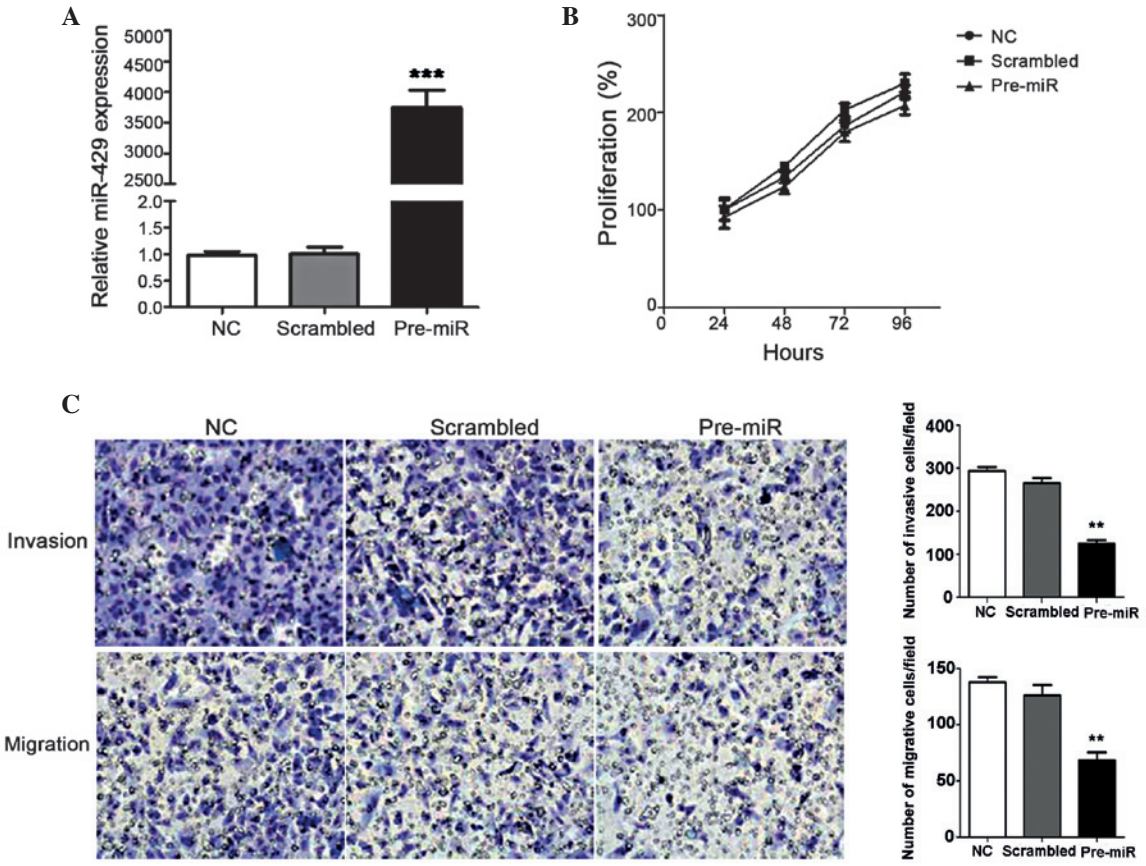

Figure 2. Effects of miR-429 overexpression on proliferation, invasion and migration of CNE-2 cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to confirm adequate induction of miR-429 overexpression in CNE-2 cells. (B) Overexpression of miR-429 had only a marginal effect on the proliferation of the CNE-2 cells. (C) Cell invasion and migration of CNE-2 cells were inhibited under miR-429 overexpression. Images were captured of tree fields of each chamber (magnification, x20) and the results were from duplicate chambers. Data are presented as the mean ± standard deviation. ***P<0.001 and **P<0.01 vs. scrambled. miR, microRNA; NC, normal control; Pre-miR, transfected with miR-429 mimic; Scrambled, transfected with nonspecific microRNA.

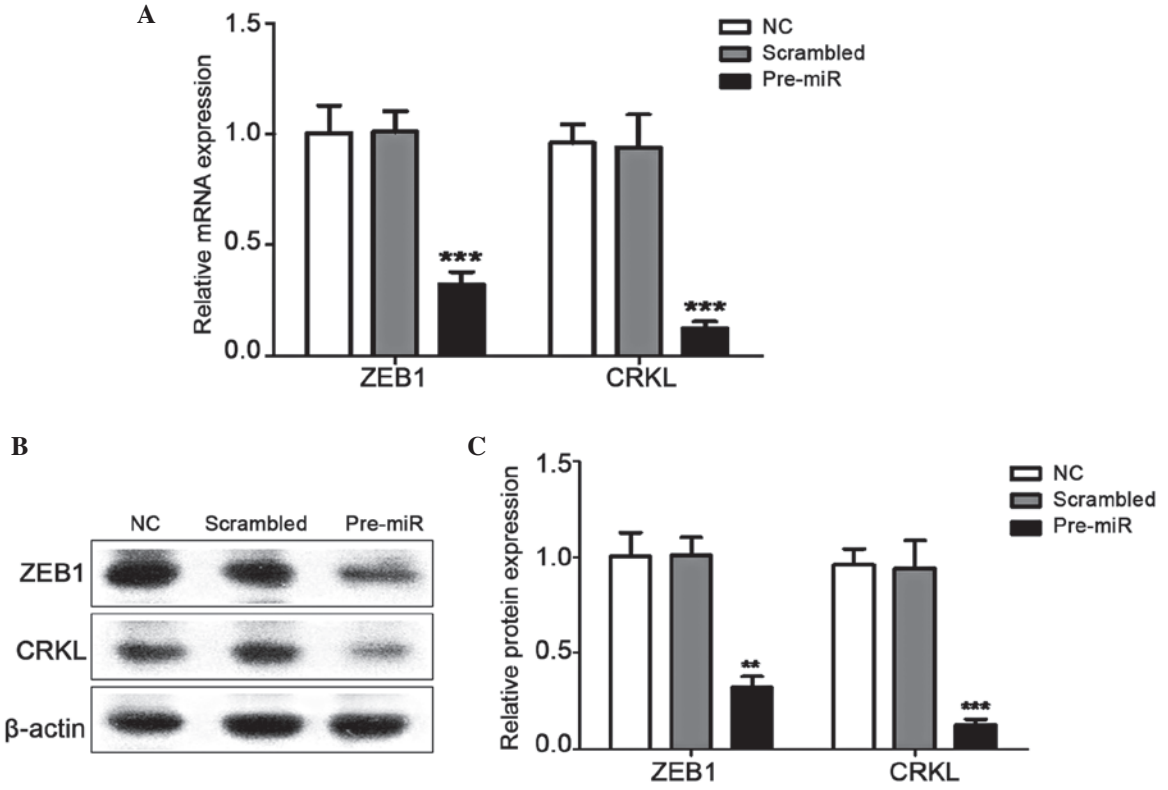

Figure 3. Downregulated expression profiles of ZEB1 and CRKL in cells overexpressing miR-429. Western blot analysis showed that the overexpression of miR-429 in CNE-2 cells resulted in reduced expression levels of ZEB1 and CRKL at the (A) mRNA and (B and C) protein levels. Data are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. scrambled. miR, microRNA; NC, normal control; Pre-miR, transfected with miR-429 mimic; ZEB1, zinc finger E-Box-binding homeobox 1; CRKL, CRK-like.

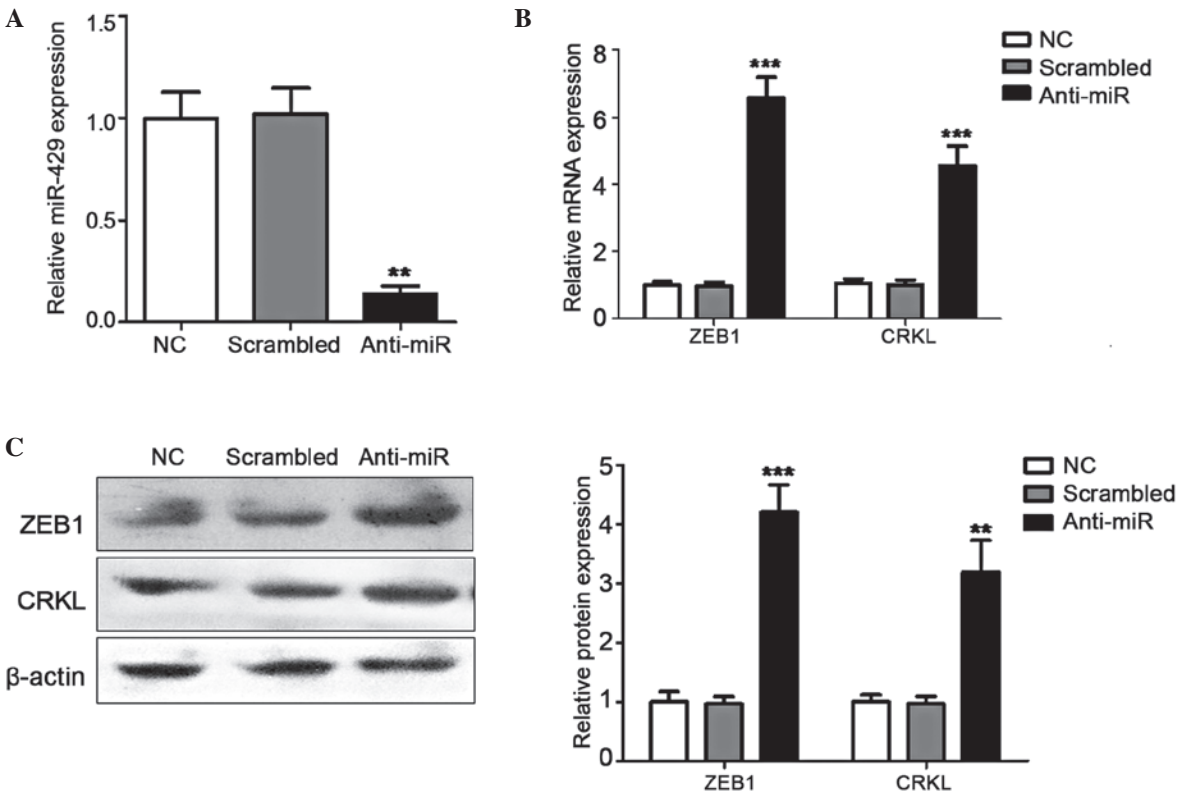

Figure 4. miR-429 silencing induced the expression of ZEB1 and CRKL. (A) Silencing of miR-429 in CNE-2 cells was quantified using reverse transcription-quantitative polymrerase chain reaction analysis to ensure adequate inhibition. Induced expression levels of ZEB1 and CRKL were detected in the miR-429-silenced cells at the (B) mRNA and (C) protein level. Data are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. scrambled. miR, microRNA; NC, normal control; anti-miR, transfection with anti-miR-429; scrambled, transfected with anti-miR as a negative control. ZEB1, zinc finger E-Box-binding homeobox 1; CRKL, CRK-like.

respectively in the pre-miR transfected cells at the mRNA and protein levels. These results indicated that miR-429 may regulate the development of NPC by inhibiting the function of its target genes.

miR-429 silencing induces the expression levels of ZEB1 and CRKL. The gain-of-function investigated described above indicated that ZEB1 and CRKL were suppressed by miR429. To verify this negative regulation, their relative expression levels were also investigated in miR-429-silenced cells through anti-miR429 transfection. miR-429 silencing was confirmed using RT-qPCR (Fig. 4A), and the expression levels of ZEB1 and CRKL were detected as described above. As expected, the expression levels of the two target genes were restored and even induced by the downregulation of miR-429 at the mRNA and protein levels (Fig. 4B and C). These results concluded that ZEB1 and CRKL were negatively regulated by miR429 in the NPC cells. Notably, miR-429 may suppress cell motility in NPC by negatively modulating its target genes, including ZEB1 and CRKL, indicating its potential as a candidate for miRNA-based prognosis or therapy against NPC.

Discussion

NPC has distinct ethnic and geographic distributions, and is particularly common in the southern Chinese population (31). The mechanism of NPC tumorigenesis is complex, involving aberrations in a variety of pathways and alterations in the expression levels of several proteins (32). Although high survival rates are reported for early stage NPC, the majority of NPC cases are diagnosed at an advanced stage. The prognosis for metastatic disease remains poor due to delays in seeking treatment following the onset of symptoms, although a thorough nasopharyngeal examination is difficult to complete (33). Therefore, identifying effective diagnostic biomarkers and targeted treatments for NPC is essential to improve the clinical outcomes. Differentially expressed miRNAs have been screened out for candidate biomarkers in NPC (34-36).

miR-429 is a member of the miR-200 family, and four members of this family have been found to be important in the regulation of EMT in various types of tumor (37). In the present study, miR-429 was markedly downregulated in poorly-differentiated CNE-2 cells (Fig. 1), in accordance with a previous microRNA microarray study (18). Further investigations indicated that the overexpression of miR-429 inhibited the cell migration and invasion of NPC cells *in vitro* (Fig. 2), which was also in accordance with previously reported results in other types of carcinoma (21,22,38). The upregulation of miR-429 inhibits invasion and promotes apoptosis in esophageal carcinoma cells by targeting B cell lymphoma-2 and SP1 (38). As described in breast cancer cells, miR-429 can suppress cell motility by negatively modulating several key invasion and metastasis inducers, including ZEB1 and CRKL. ZEB1, also known as δEF1, can repress the transcription of E-cadherin and regulate epithelial plasticity in breast cancer cells (39). CRKL, a tyrosine-phosphorylated protein, can transform fibroblasts and function in transformation via the BCR-ABL oncogene (40). In addition, CRKL is important in proliferation, migration and the evasion of apoptosis (22). Downregulation in the levels of ZEB1 and CRKL were detected in the miR-429-overexpressing CNE-2 cells (Fig. 3). In addition, the downregulation of miR-429 led to a reversal in the promoted expression profiles of ZEB1 and CRKL (Fig. 4). These results indicated that repression in the invasion and migration of miR-429-overexpressing NPC cells was closely associated with the functions of target genes, including ZEB1 and CRKL, regulated by miR-429.

In conclusion, significant changes in the expression of miR-429 were detected, particularly in low-differentiated CNE-2 cells. Further results showed that miR-429 inhibited the invasion and migration of CNE-2 cells. In addition, the mRNA and protein expression levels of the two target genes, ZEB1 and CRKL, were downregulated and upregulated by transfection with the miR429 mimic and anti-miR429, respectively. These results indicated that miR-429 may suppress cell motility in NPC by negatively modulating its target genes, including ZEB1 and CRKL. Therefore, miR-429 has potential for use as a biomarker of EMT in NPC, and also has potential therapeutic value in abating NPC metastasis, particularly in undifferentiated NPC cells. Further investigations may assist in clarifying the complex mechanisms of miR-429 regulation in NPC metastasis for improving prognosis and therapy.

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