# MicroRNA-30a regulates cell proliferation, migration, invasion and apoptosis in human nasopharyngeal carcinoma via targeted regulation of ZEB2

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**Abstract.** MicroRNA-30a (miR-30a) was previously reported to serve as a tumor suppressor able to inhibit the development and progression of certain types of cancer. A number of previous studies demonstrated that zinc finger E-box binding homeobox 2 (ZEB2) may be regulated by miR-30a in clear cell renal cell carcinoma and breast cancer. However, the function of miR-30a in human nasopharyngeal carcinoma (NPC) remains unclear. The present study aimed to investigate the association between miR-30a and ZEB2 in NPC. Therefore, the expression levels of miR-30a and ZEB2 were measured in human NPC cells and tissues from patients with NPC, and the present results suggested that the expression level of miR-30a was significantly decreased in NPC tissues compared with paracancerous tissues. The direct interaction between miR-30a and the untranslated region of ZEB2 was examined using the dual-luciferase reporter assay, and ZEB2 was identified as a direct target of miR-30a. Additionally, the effects of miR-30a and ZEB2 overexpression on cell proliferation, migration, invasion and apoptosis were additionally investigated. Functional experiments identified that overexpression of miR-30a increased apoptosis and suppressed cell proliferation, cell migration and cell invasion by directly targeting ZEB2. Collectively, the present study suggested that miR-30a may serve an important role in the progression of NPC and may represent a novel target for the treatment of patients with NPC.

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## Introduction

Nasopharyngeal carcinoma (NPC) is a common type of epithelial squamous cell head and neck carcinoma, and is the most common type of nasopharyngeal tumor (1). Despite recent advances in the diagnosis and treatment of NPC, the 10-year survival rate of patients with NPS remains poor, and the occurrence rate in South-eastern Asia and North Africa increased over the past years (2). Therefore, the development of effective therapeutic strategies and novel prognostic molecular markers is necessary to improve the survival rate of patients with NPC.

A number of previous studies have demonstrated that multiple microRNAs (miRNAs) may act as oncogenes or tumor suppressor genes; therefore, the dysregulation of miRNAs was identified to be involved in the process of cancer development and progression (3,4). In addition, miRNAs may be used as molecular biomarkers for cancer prognosis and targeted therapies (5-8). miRNA-30a (miR-30a) was previously demonstrated to have an important role in the proliferative, metastatic and invasive potential of ovarian carcinoma (9), gallbladder cancer (10) and other types of cancer (11-13). However, the molecular mechanism underlying miR-30a function in human NPC remains unclear.

Epithelial-mesenchymal transition (EMT) is an important process for tumor cell invasion of epithelial and non-epithelial cancers, and zinc finger E-box binding homeobox 2 (ZEB2) was demonstrated to promote EMT (14). A previous study

demonstrated that ZEB2 served as a DNA-binding transcriptional repressor that may be able to interact with activated SMAD family member 1, thus regulating the bone morphogenetic protein signaling pathway (15). Previous studies investigating the role of ZEB2 in cancer identified that the expression of ZEB2 is important for the development of cancer (16), and the inhibition of ZEB2 may suppress cancer cell growth, migration and invasion (17). In addition, a previous studies demonstrated that the association between ZEB2 and Sp1 transcription factor was able to promote cancer cell survival and angiogenesis during metastasis via the upregulation of survivin and vascular endothelial growth factor (18). Certain miRNAs, including miR-335 and miR-200c, were identified to bind to the 3' untranslated region (3'-UTR) of ZEB2, inhibiting cancer progression (17,19); therefore, miRNAs may be used for the development of novel therapeutic strategies to treat cancer.

In the present study, ZEB2 was identified as a direct target gene of miR-30a in human NPC. miR-30a overexpression was identified to induce apoptosis and suppress proliferation, migration and invasion of NPC cells. The present data suggested that miR-30a may possess the potential to be used as a novel diagnostic marker and therapeutic target for the treatment of patients with NPC.

## Materials and methods

*NPC samples*. NPC and paracancerous tissues were collected from 4 patients at The People's Hospital of Longhua (Shenzhen, China) in August 2017. All volunteers [Patient 1 (a 46-year-old male), Patient 2 (a 59-year-old male), Patient 3 (a 53-year-old female) and Patient 4 (a 58-year-old male)] provided written informed consent. The consent procedure was approved by The Animal Care and Use Committee of People's Hospital of Longhua (Shenzhen, China). Inclusion criteria for the study were as follows: i) Having received a clinical or referral record in the electronic medical record of NPC during the period of the study; and ii) being ≥40 years of age at the time of diagnosis. Exclusion criteria for the study were as follows: i) Having a secondary cancer; ii) not having received primary care in the year prior to cancer diagnosis; and iii) not having any matched controls.

Following resection, the collected tissues were placed in a solution containing PBS, 0.1 g/ml streptomycin and 100 U/ml penicillin. The tissues were washed three times using PBS. Subsequently, NPC tissues and paracancerous tissues were lysed, and total RNA and protein were extracted for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot assay, respectively.

*Cell culture*. The human NPC cell line, C666-1, and 293 cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). The two cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 0.1 g/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified incubator at 37˚C with 5% $CO_2$. The culture medium was replaced every other day, and the C666-1 cells were passaged (dilution, 1:4) every 5 or 6 days.

*miR-30a preparation and transfection*. Potential target genes of miR-30a were first analyzed *in silico* using TargetScan 7.2 (http://www.targetscan.org). miR-30a (5'-UGUAAACAUCCU CGACUGGAAG-3') was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). A non-specific miRNA (5'-ACG UGACACGUUCGGAGAAUU-3') was used as the negative control (Ctrl miRNA). The reverse complementary sequence of miRNA-30a (5'-CUUCCAGUCGAGGAUGUUUACA-3') was used as the miR-30a inhibitor. Ctrl miRNA and miR-30a inhibitor was purchased from Sangon Biotech Co., Ltd. Cell transfection was performed using Lipofectamine® 3000 transfection reagent (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10 nM of miRNA, according to the manufacturer's protocol. Briefly, $1x10^5$ cells were transfected with miRNA molecules. Following transfection for 24 h, the cells in each group were harvested for subsequent experimentation.

*Overexpression of ZEB2 in NPC cells and grouping*. Total RNA was isolated from 293 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and used as a template to obtain genomic cDNA using a PrimeScript reverse transcription-polymerase chain reaction (RT-PCR) kit (cat. no. RR014B; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocols, and the coding sequence plus 3'-UTR of ZEB2 was amplified using PCR (Phusion® High-Fidelity DNA Polymerase, M0530L, New England BioLabs, Inc., Ipswich, MA, USA) and subsequently cloned into a pCI vector (Addgene, Inc., Cambridge, MA, USA) using *Nhe*I/*Sal*I restriction sites. The following primer sequences were used to amplify ZEB2: Forward (F), 5'-ATGAAGCAGCCGATCATG GCG-3' and reverse®, 5'-CACACATCTTGGAGCAAAAGC ATG-3'. PCR was performed under the following conditions: Initial denaturation at 95˚C for 5 min, 35 cycles of 95˚C for 35 sec, 60˚C for 35 sec and 72˚C for 2.5 min, followed by a final extension at 72˚C for 5 min.

To overexpress ZEB2 in NPC cells, C666-1 cells were transfected with the pCI-ZEB2 vector (final concentration, 1 $\mu$g/ml) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following transfection for 24 h, the cells in each group were harvested for the subsequent experimentation. The overexpression efficiency was examined by RT-qPCR. Cells transfected with Ctrl miRNA, pCI-empty vector, pCI-ZEB2 vector (ZEB2 vector), miR-30a, miR-30a inhibitor, pCI-empty vector + Ctrl miRNA (Ctrl), pCI-ZEB2 vector + Ctrl miRNA (ZEB2 overexpression), pCI-empty vector + miR-30a (miR-30a overexpression) and pCI-ZEB2 vector + miR-30a (ZEB2+miR-30a overexpression) were used for further analysis.

*RT-qPCR*. In total, 50 mg of homogenized NPC and paracancerous tissues were lysed using 1 ml Trizol® (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). A total of $5x10^6$ C666-1 cells from each group were lysed using 1 ml Trizol®. Total RNA was extracted using Trizol according to the manufacturer's protocol, and cDNA was synthesized using 1 $\mu$g RNA from each sample. RT was performed using a Transcriptor first-strand cDNA synthesis kit (Promega Corporation, Madison, WI, USA) under the following conditions: 20˚C for 10 min, 42˚C for 60 min and 95˚C for 5 min.

qPCR was conducted using an SYBR® Fast qPCR Mix kit (Takara Biotechnology Co., Ltd.). Following an initial polymerase activation and denaturation step at 50˚C for 2 min and 95˚C for 5 min, respectively, the samples in each group underwent 40 amplification cycles of 95˚C for 20 sec, 65˚C for 10 sec and 72˚C for 30 sec in the LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland). RT-qPCR results were quantified using the $2^{-\Delta\Delta Cq}$ method as previously described (20-22). In the present study, GAPDH and U6 were used as reference genes for the normalization of ZEB2 and miR-30a, respectively. The expression levels of the genes analyzed were normalized to their expression levels in the corresponding control group. The primer sequences used were the following: miR-30a F, 5'-ACACTCCAGCTGGGTTGC ATAGTCACAAAAGT-3 and R, 5'-ACACTCCAGCTGGGT GTAAACATCCTACACTCT-3'; U6 F, 5'-CTCGCTTCGGCA GCACA-3' and R, 5'-AACGCTTCACGAATTTGCGT-3'; ZEB2 F, 5'-CTCTTCCCACACGCTTAGTT-3' and R, 5'-GGC CTAAGCTTACAGTGTCATG-3'; GAPDH F, 5'-GGGAAA CTGTGGCGTGAT-3' and R, 5'-GAGTGGGTGTCGCTG TTGA-3'.

*Western blot analysis*. Radioimmunoprecipitation assay lysis buffer (RIPA; cat. no. 89900; Thermo Fisher Scientific, Inc.) was used to extract the total protein. The protein concentration was measured using a bicinchoninic acid assay kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Homogenized tissue (50 mg) was lysed using 0.3 ml RIPA lysis buffer. For the *in vitro* experiments, $1x10^6$ cells were lysed using 0.1 ml RIPA lysis buffer. Western blot assay was performed as previously described (23-25). In brief, protein (15 $\mu$g/lane) was separated via 10% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (Thermo Fisher Scientific, Inc.) for 2 h at room temperature, then incubated with primary antibodies overnight at 4˚C. In the present study, the primary antibodies used were: Anti-ZEB2 (1:1,000; Abcam, Cambridge, UK; cat. no. ab223688) and anti-GAPDH (1:5,000; Abcam; cat. no. ab8245). The secondary antibodies used were: Anti-mouse IgG [horseradish peroxidase (HRP)-conjugated; 1:5,000; Sigma-Aldrich; Merck KGaA; cat. no. A-9044] and anti-rabbit IgG (HRP-conjugated; 1:5,000; Sigma-Aldrich; Merck KGaA; cat. no. A-0545). Protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) and ChemiDoc Imagers (ChemiDoc™ XRS + System; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression was quantified using ImageJ 1.x software (National Institutes of Health, Bethesda, MD, USA).

*Dual-luciferase reporter assay*. The 3'-UTR sequence of human ZEB2 gene was amplified using PCR and cloned into a psiCHECK-1-based luciferase plasmid (Addgene, Inc., Cambridge, MA, USA) in which the *Renilla* luciferase sequence was replaced with a firefly luciferase sequence (restriction enzyme sites: *Nhe*I/*Sgf*I) from pGL-4.22 vector (Addgene, Inc.). pRL *Renilla* luciferase control reporter vectors (Promega Corporation) was co-transfected as an internal reference. The construction of the psiCHECK plasmid containing the mutated 3'-UTR of ZEB2 was performed as previously described (26-28). In brief, cell transfection was performed using Lipofectamine 3000 transfection reagent according to the manufacturer's protocol. Cells ($3x10^5$) were co-transfected with 1 $\mu$g plasmids, and miR-30a mimics, inhibitor or Ctrl miRNA for 24 h, then the dual luciferase assay was performed using a Dual Luciferase Assay Kit according to the manufacturer's instructions (Promega Corporation). In the present study, firefly and *Renilla* luciferase values were detected; for the evaluation of relative luciferase activity, the firefly luciferase activity was normalized to the *Renilla* luciferase value.

*Colony-formation assay, cell proliferation and cell cycle analysis*. To investigate the colony-forming ability of cancer cells, 100 cells were seeded into 12-well plates and incubated for 7 days in an incubator at 37˚C with 5% $CO_2$. The cells were subsequently fixed with 75% ethanol for 20 min at room temperature and stained using crystal violet (5 g/l) for 20 min at room temperature. Cell colonies in each groups were imaged using an Epson Perfection V600 scanner (Seiko Epson Corporation, Suwa, Japan) and the results were analyzed using BioSpot® version 5.0 software (Cellular Technology Limited, Cleveland, OH, USA).

To examine cell proliferation, the proliferation index of each group was assessed using a CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) as previously described (29). The proliferation index was calculated as the absorbance detected in the experimental group-the absorbance detected in the blank group.

To analyze the cell cycle, $5x10^6$ cells were fixed with 70% ethanol for 30 min at 4˚C. The cell samples were stained with 200 $\mu$l propidium iodide (PI; Beyotime Institute of Biotechnology, Haimen, China) in a solution containing ribonuclease A (Beyotime Institute of Biotechnology) for 10 min at room temperature. Subsequently, the samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

*Cell apoptosis assay*. Cell apoptosis assay was performed using a Cell Apoptosis Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. NPC cells in each group were dissociated into single cells with trypsin, washed with PBS, and incubated with a solution containing Annexin V-fluorescein isothiocyanate and PI. Cells were analyzed using a FACSCalibur flow cytometer and FlowJo 7.6.1.

*Cell migration and invasion assays*. The migration and invasion of cancer cells were measured using Transwell plates (pore filter size, 8 $\mu$m; Corning Inc., Corning, NY, USA). NPC cells were seeded in the upper chamber at a concentration of $1x10^5$ cells/well in serum-free DMEM. Inserts covered with Matrigel were used for invasion assays, whereas normal inserts were used for migration assays. High glucose DMEM (HyClone; GE Healthcare Life Sciences) containing 10% FBS was plated in the lower chamber. Subsequently, cells were incubated for 48 h at 37˚C. Cells on the upper chambers were removed, and migrating or invading cancer cells were fixed in 10% neutral buffered formalin for 15 min at room temperature, and stained with crystal violet (5 g/l) for 20 min at room temperature. The number of cancer cells in four randomly-selected fields of view were counted under a light microscrope (magnification, x100) for each group.

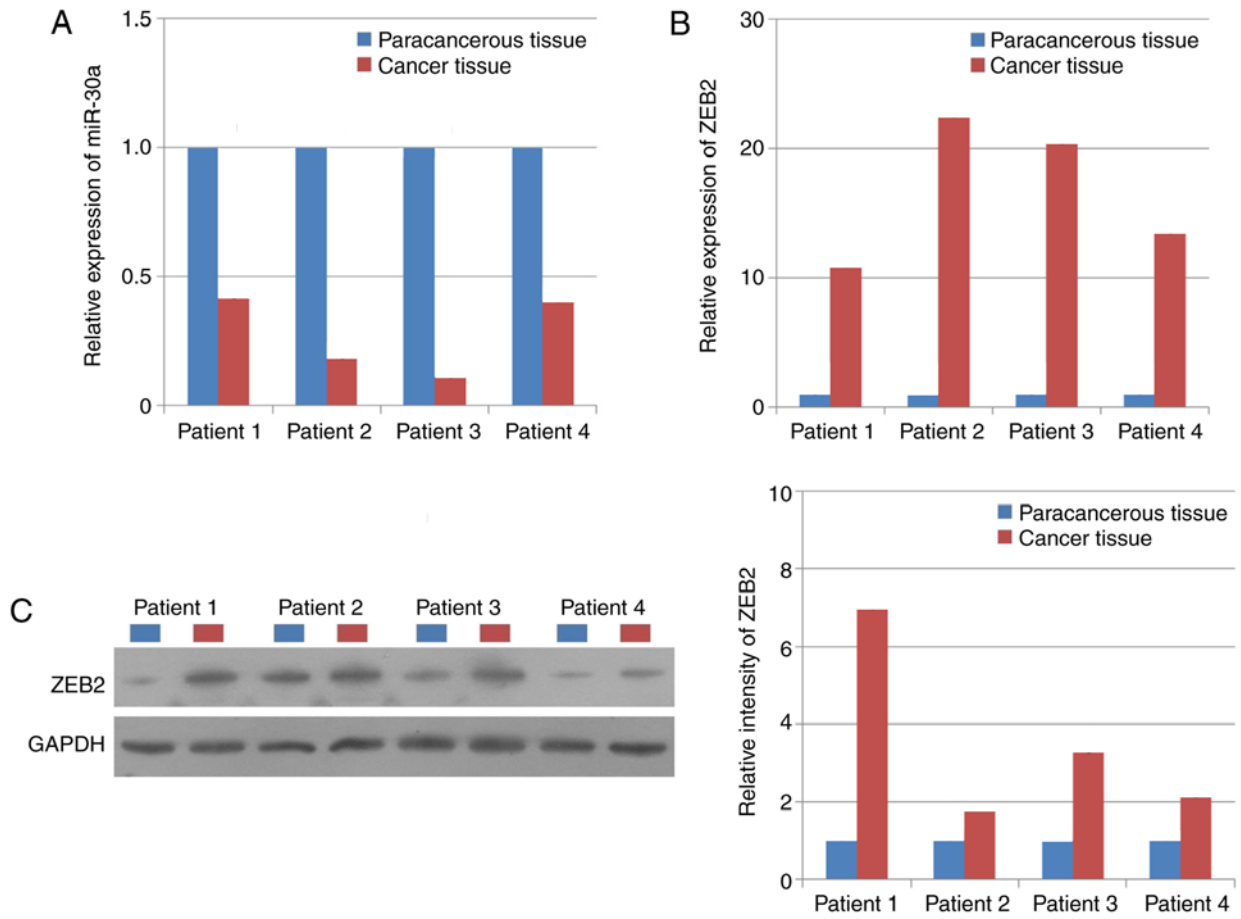


Figure 1. Expression levels of miR-30a and ZEB2 in human nasopharyngeal carcinoma tissues and paracancerous tissues. Expression level of (A) miR-30a and (B) ZEB2 was assessed using reverse transcription-quantitative polymerase chain reaction. (C) Protein expression levels of ZEB2 were measured using western blotting. miR-30a, microRNA-30a; ZEB2, zinc finger E-box binding homeobox 2.

*Tumor formation assay*. ZEB2 cDNA was obtained as described above. In addition, the miR-30a sequence was purchased from Sangon Biotech Co., Ltd., and the two sequences were separately cloned into pMXs-based retroviral plasmids (Addgene, Inc.). In total, 40,000 cancer cells were transduced in 6-well culture dishes using pMX-based retroviruses ($5x10^5$ PFU) as previously described (20). To obtain a stable overexpression, cells were infected using retroviral particles containing pMXs-ZEB2 (ZEB2 retrovirus), pMXs-empty vector (Ctrl retrovirus), pMXs-miR-30a (miR-30a retrovirus) and pMXs-ZEB2 + pMXs-miR-30a (ZEB2 retrovirus + miR-30a retrovirus).

Athymic nude mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). Male mice in each group (n=3/group, 12 mice in total; 22-25 g, 6-8 weeks old; n=3/group) were injected intrahepatically with infected cancer cells ($5x10^6$ cells/mouse). Mice were maintained in a fully controlled animal facility (12:12-h light:dark cycle at 22±2˚C with 50% humidity), and were housed in standard clear plastic cages with access to food and water *ad libitum*. Following 6 weeks, all animals were sacrificed using isoflurane gas, and tumor weight and volume were measured in each group. Tumor volume was calculated using the following formula: (length x width$^2$)/2. During the 6 weeks, animal health and behavior were monitored every other day. In case of body weight loss >20% within 3 weeks, mice were immediately sacrificed. The animal experiments were approved by The Animal Care and Use Committee of People's Hospital of Longhua (Shenzhen, China).

*Statistical analysis*. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The detection of different samples form each patients or different cell groups was repeated for 3 times. Unpaired Student's t-test was used to compare two groups. One-way analysis of variance followed by Bonferroni's post hoc test was used to compare three or more groups. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*ZEB2 is a direct target of miR-30a in human NPC cells*. Human NPC tissues and paracancerous tissues were collected from four patients to investigate the association between the expression levels of miR-30a and ZEB2. The result suggested that the expression level of miR-30a was significantly increased in human paracancerous tissues compared with NPC tissues (Fig. 1A); whereas the mRNA and protein expression levels of ZEB2 exhibited the opposite trend (Fig. 1B and C), suggesting that there was a negative association between the expression levels of miR-30a and ZEB2 in NPC tissues.

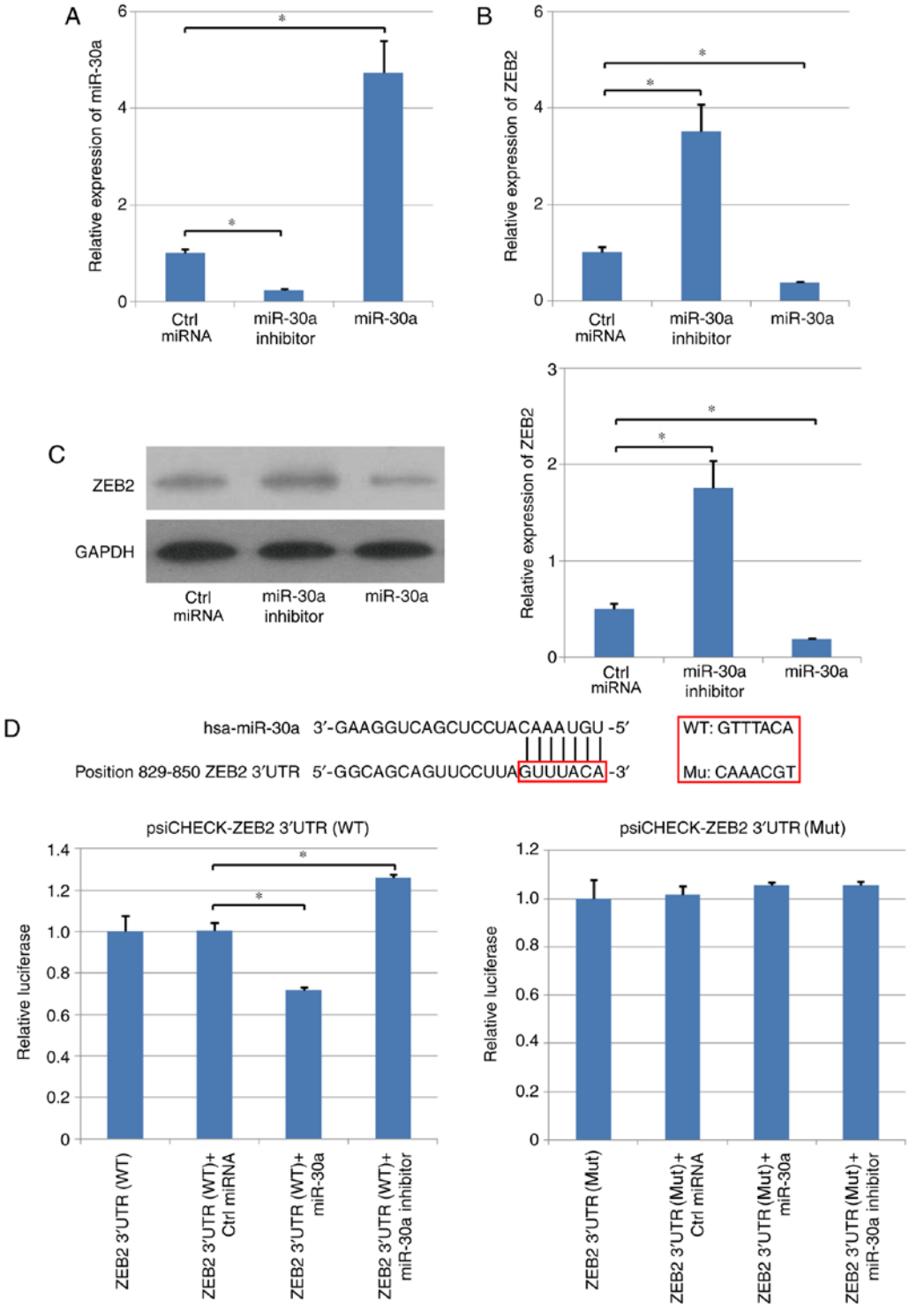


Figure 2. Expression level of ZEB2 is directly regulated by miR-30a. Effects of overexpression and inhibition of miR-30a on the expression level of ZEB2 in C666-1 human nasopharyngeal carcinoma cells. (A) Expression level of miR-30a in various experimental groups, as assessed by RT-qPCR. (B) mRNA and (C) protein expression levels of ZEB2 were determined using RT-qPCR and western blotting, respectively. (D) Direct interaction between miR-30a and ZEB2. Dual-luciferase reporter assay was used to examine the direct interaction between ZEB2 and miR-30a. *P<0.05. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-30a, microRNA-30a; Ctrl, control; miRNA, microRNA; ZEB2, zinc finger E-box binding homeobox 2; WT, wild-type; UTR, untranslated region; Mut, mutant.

In addition, a human NPC cell line, C666-1, was used to analyze the negative association between miR-30a and ZEB2 in NPC cells. miR-30a inhibitor or miR-30a were transfected in the C666-1 cells. RT-qPCR results suggested that miR-30a inhibitor decreased miR-30a expression level by >70%, and the expression level of miR-30a following overexpression increased by ~5-fold compared with the control group (Fig. 2A). In addition, the mRNA and protein expression levels of ZEB2 were decreased following overexpression of miR-30a in human NPC cells. Conversely, C666-1 cells transfected with miR-30a inhibitor exhibited an increased expression level of ZEB2 compared with cells transfected with the control miRNA (Fig. 2B and C).

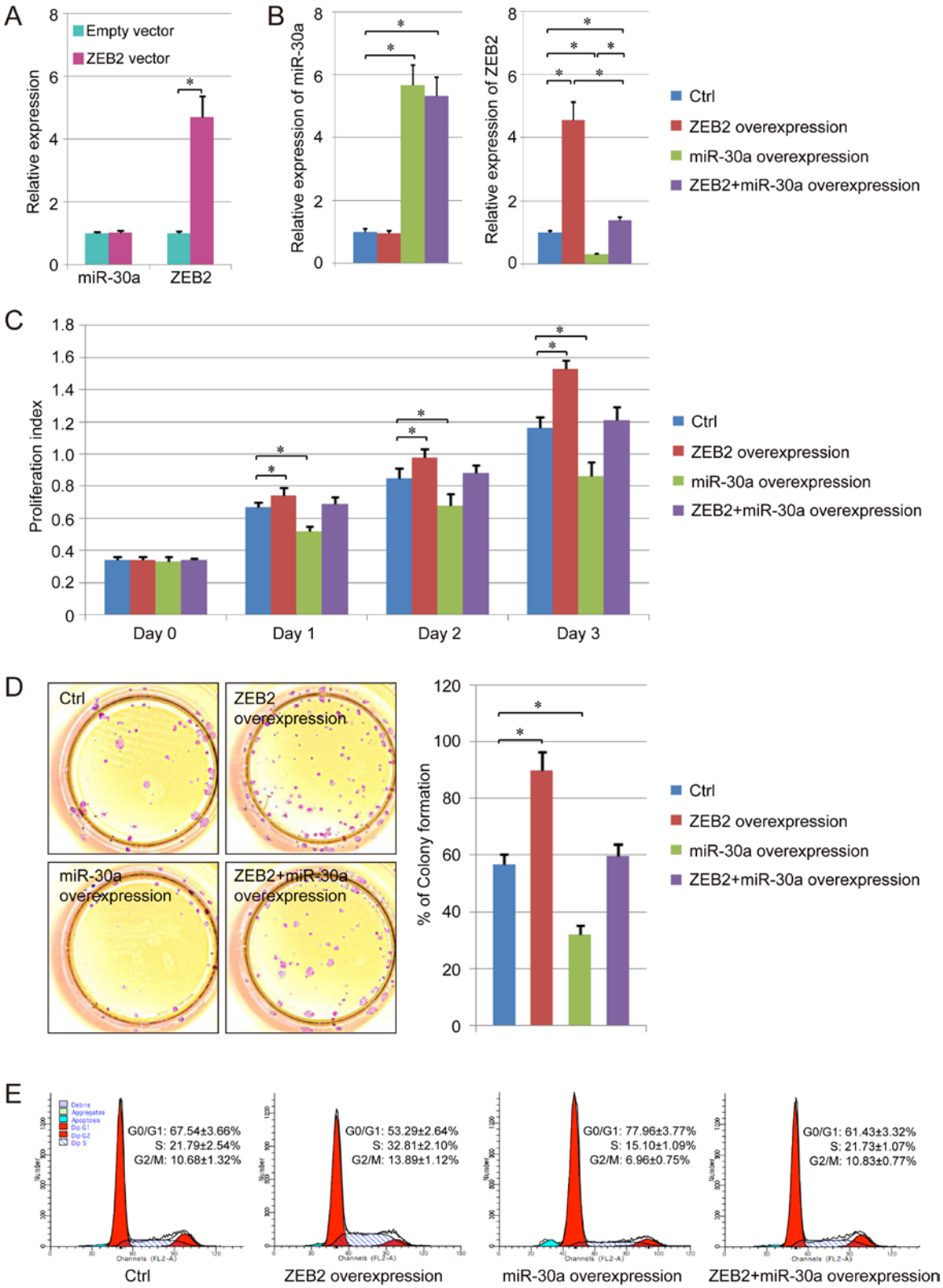


Figure 3. miR-30a overexpression inhibits the proliferative ability of human C666-1 cells by regulating ZEB2. (A) mRNA expression levels of miR-30a and ZEB2 following ZEB2 transfection. (B) mRNA expression levels of miR-30a and ZEB2 following ZEB2 and/or miR-30a transfection. (C) Effects of miR-30a and ZEB2 on cell proliferation, as assessed by Cell Cycle Kit-8 assay. (D) Colony formation assay and (E) cell cycle assay. *P<0.05 vs. corresponding control. ZEB2, zinc finger E-box binding homeobox; miR-30a, microRNA-30a; Ctrl, control.

The potential target genes of miR-30a were analyzed using TargetScan (Fig. 2D), suggesting that miR-30a may target the 3'-UTR of ZEB2 mRNA, regulating the expression level of this coding gene. Therefore, wild-type (WT) and mutant (Mut) 3'-UTRs of ZEB2 were cloned into psi-CHECK vectors, C666-1 cells were transfected, and dual-luciferase assay was performed. miR-30a repressed the luciferase activity of WT ZEB2-3'-UTR plasmid; however, transfection with miR-30a did not affect the

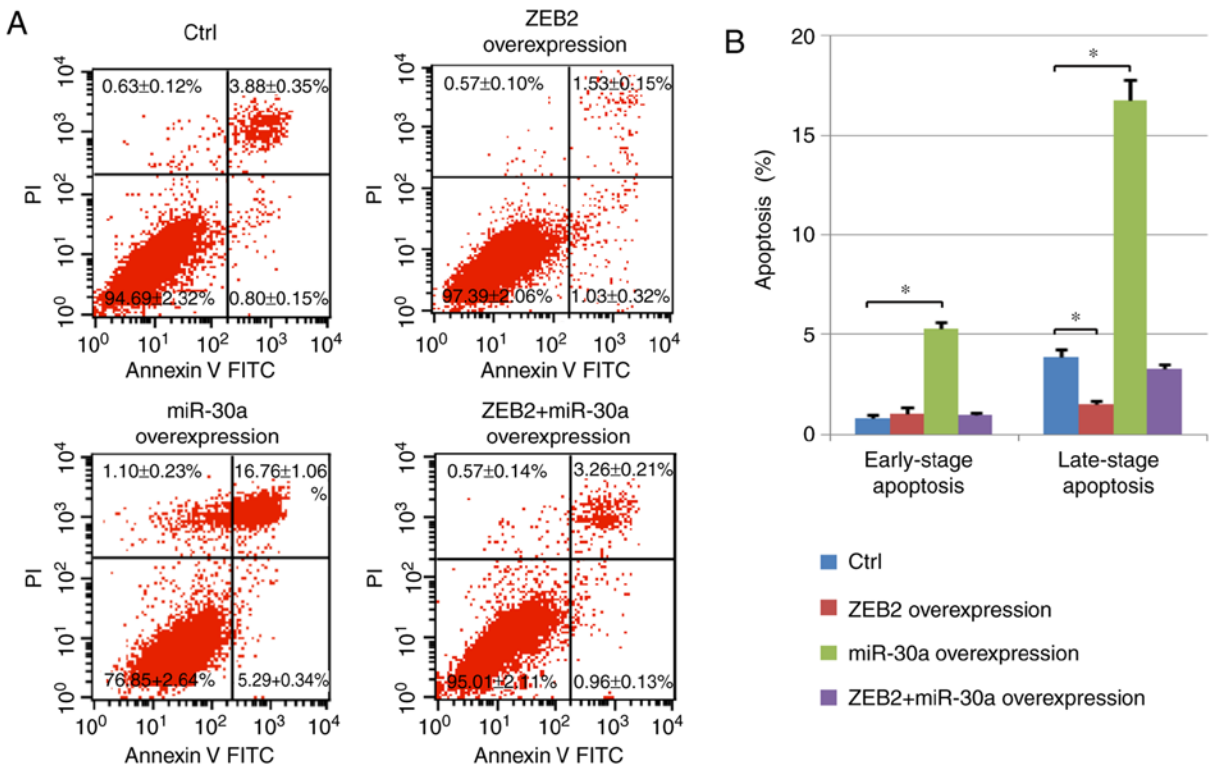


Figure 4. Effects of miR-30a and ZEB2 on cell apoptosis in C666-1 cells. (A) Cell apoptosis was analyzed using Annexin V-FITC/PI staining. Early- and late-stage apoptotic cells are presented in the lower right and in the upper right quadrants, respectively. (B) Early- and late-stage apoptotic cells. C666-1 cells transfected with empty vector and Ctrl miRNA were used as control group. *P<0.05. Ctrl, control; PI, propidium iodide; ZEB2, zinc finger E-box binding homeobox; miR-30a, microRNA-30a; FITC, fluorescein isothiocyanate.

luciferase activity of Mut ZEB2-3'-UTR plasmid (Fig. 2D). Furthermore, the inhibitory effects of miR-30a were suppressed by miR-30a inhibitor in the WT ZEB2-3'-UTR group; however, the luciferase activity in the Mut ZEB2-3'-UTR group was not altered (Fig. 2D). Collectively, the present results suggested that ZEB2 was a direct target of miR-30a.

*Effects of miR-30a on the proliferative ability of NPC cells.* ZEB2 overexpression did not affect the expression level of miR-30a; however, the expression level of ZEB2 increased. In addition, the RT-qPCR results suggested that the expression levels of miR-30a and ZEB2 increased following cotransfection of miR-30a and ZEB2 in human NPC cells (Fig. 3A and B).

Furthermore, the proliferative abilities of cells in various groups were assessed using a CCK-8 assay. The present results suggested that the number of proliferating cells was increased following ZEB2 overexpression. miR-30a overexpression decreased the proliferation index of normal NPC cells and cells overexpressing ZEB2 (Fig. 3C), consistently with the colony formation assay results (Fig. 3D).

The effects of miR-30a and ZEB2 on the cell cycle were further assessed using flow cytometry. miR-30a overexpression was identified to increase the percentage of cells in $G_0/G_1$ phase and decreased the percentage of cells in S phase and $G_2$/M phase in NPC cells. The opposite effect was observed in cells overexpressing ZEB2, and the cell cycle in ZEB2-overexpressing cells was partially reversed by overexpression of miR-30a (Fig. 3E). Collectively, miR-30a suppressed NPC cell proliferation by targeting ZEB2, downregulating its expression level.

*Effect of miR-30a on NPC cell apoptosis.* Cell apoptosis was measured using Annexin V/PI staining. The number of early-stage apoptotic cells (Annexin V-positive and PI-negative) and late-stage apoptotic cells (Annexin V-positive and PI-positive) was assessed by flow cytometry. The percentage of late-stage apoptotic cells increased following miR-30a overexpression. The increased apoptosis observed following miR30a overexpression was reversed by ZEB2 cotransfection. Conversely, ZEB2 transfection decreased the percentage of early-stage apoptotic cells in cells overexpressing miR-30a. Notably, ZEB2 overexpression decreased the number of late-stage apoptotic cells compared with the control group, and ZEB2 + miR-30a group exhibited an increased number of apoptotic cells compared with cells overexpressing ZEB2 alone (Fig. 4). Collectively, the present data suggested that the effect of miR-30a on cell apoptosis was dependent on ZEB2.

*miR-30a inhibits NPC cell migration and invasion.* The effects of ZEB2 and miR-30a on the migratory and invasive ability of cancer cells were analyzed using Transwell and Matrigel assays, respectively. The present results suggested that cell migration and invasion increased following ZEB2 overexpression. Furthermore, compared with the control group, human NPC cells exhibited decreased migration and invasion following miR-30a overexpression. In addition, miR-30a overexpression inhibited the effects of ZEB2 overexpression, suggesting a role for miR-30a in regulating the migration and invasion of human NPC cells via ZEB2 (Fig. 5).

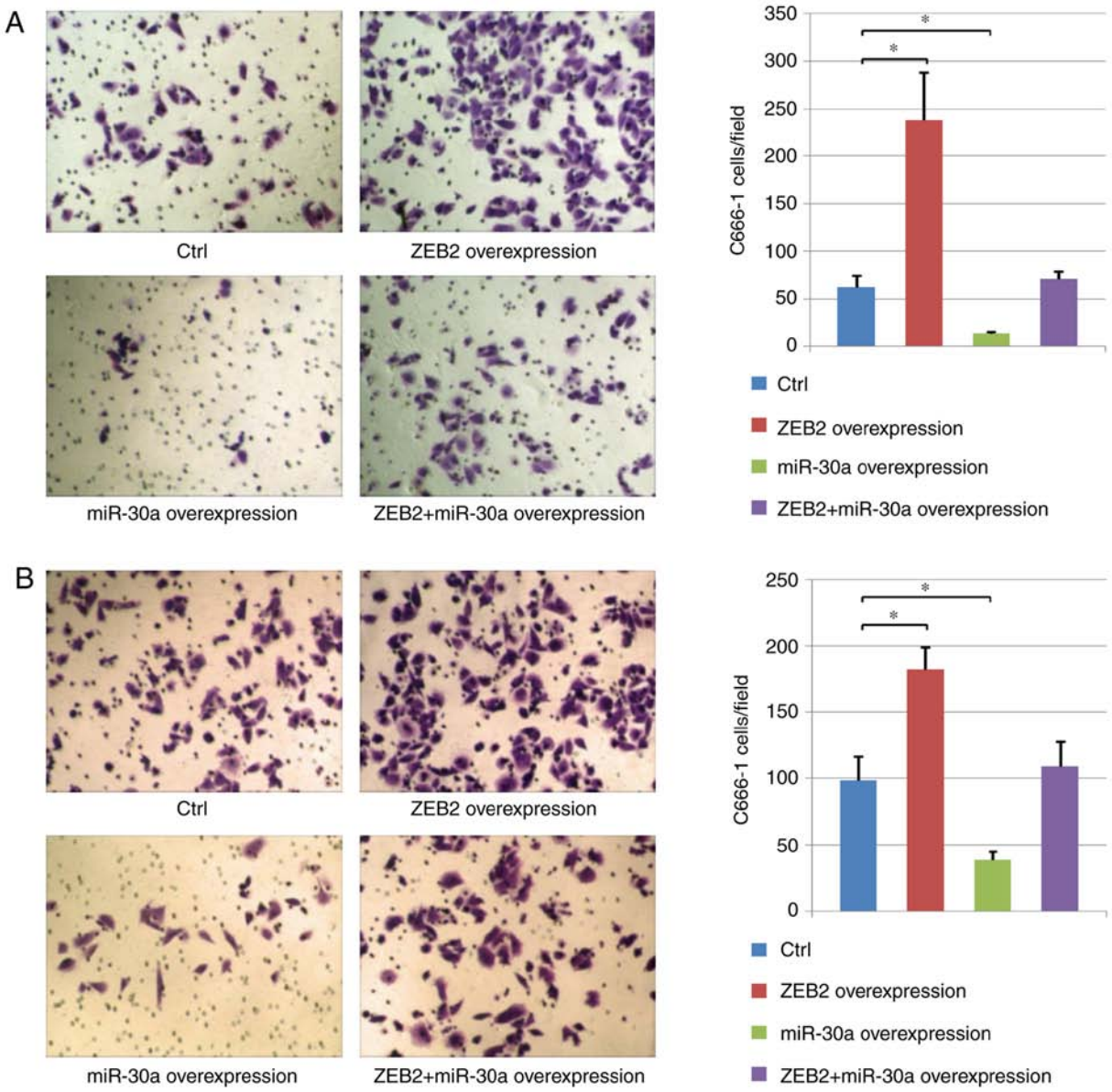


Figure 5. Cell migration and invasion assays. Effect of miR-30a and ZEB2 on the (A) migration and (B) invasion abilities of C666-1 human nasopharyngeal carcinoma cells. Cells transfected with empty vector and Ctrl miRNA were used as control group. *P<0.05. Ctrl, control; ZEB2, zinc finger E-box binding homeobox; miR-30a, microRNA-30a.

*Effects of miR-30a on NPC growth in vivo*. A ZEB2 overexpressing stable cell line, miR-30a overexpressing stable cell line and ZEB2 + miR-30a overexpressing stable cell line were established using retroviral infection. The expression levels of ZEB2 and miR-30a were assessed by RT-qPCR (Fig. 6A). The expression level of ZEB2 was suppressed by retroviral-mediated miR-30a overexpression compared with the control group. Following co-infection with miR-30a and ZEB2, the expression level of ZEB2 decreased significantly compared with cells infected with ZEB2 alone (Fig. 6A). In addition, the growth of cancer cells infected with various vectors was assessed *in vivo* using a xenograft model. The present results suggested that ZEB2 overexpression increased the weight and the volume of NPC and promoted tumor growth *in vivo*. Notably, tumor growth was suppressed by miR-30a overexpression, and growth of tumors overexpressing miR-30a was restored following concomitant overexpression of ZEB2 (Fig. 6B). Collectively, the present results suggested that miR-30a may exhibit the potential to suppress the growth of NPC *in vivo* by inhibiting the expression level of ZEB2.

## Discussion

Previous studies reported that miR-30a may have a role in cancer development and progression (9,13,30). A recent study suggested that miR-30a may modulate clear cell renal cell carcinoma (ccRCC) aggressiveness via repression of the expression level of ZEB2 (31). Chen *et al* (31), identified that, in ccRCC cells and tissues, the expression level of miR-30a was decreased, and decreased expression levels of miR-30a were associated with a poor prognosis in patients with ccRCC. In addition, overexpression of miR-30a in ccRCC cells was previously identified to suppress cellular proliferation, invasion and EMT by regulating ZEB2 *in vitro*

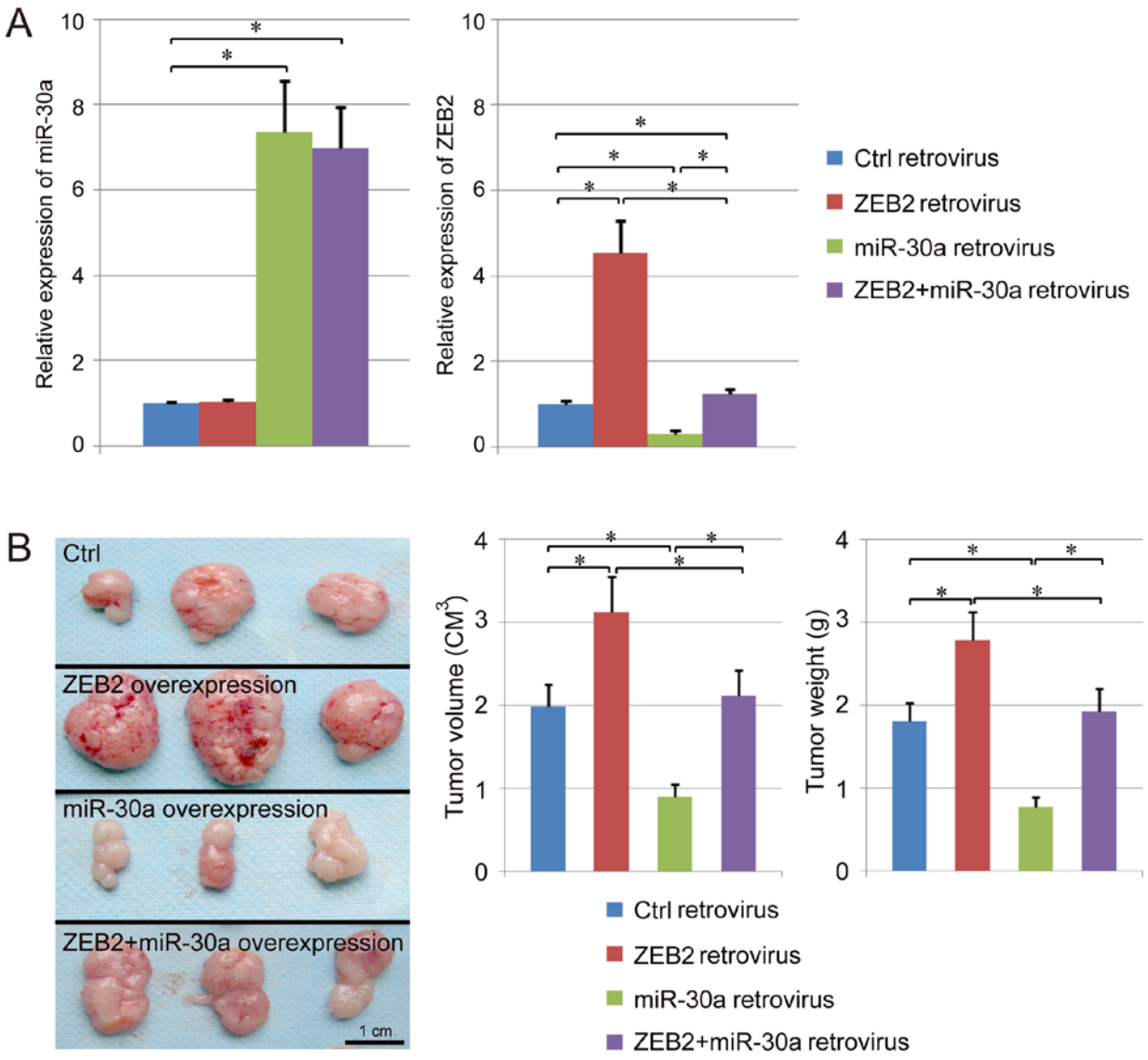


Figure 6. Effects of miR-30a and ZEB2 on tumor growth. (A) Expression levels of ZEB2 and miR-30a were measured using reverse transcription-quantitative polymerase chain reaction. (B) Quantification of tumor weight and volume (n=3 in each group). Scale bar, 1 cm. *P<0.05. miR-30a, microRNA-30a; Ctrl, control; ZEB2, zinc finger E-box binding homeobox.

and *in vivo*, indicating that the expression level of ZEB2 was negatively associated with miR-30a. The present results are in line with a previous study that demonstrated the direct association between miR-30a and ZEB2 in human breast cancer (32). Collectively, these previous studies suggested that miR-30a may serve a role in the regulation of cancer progression (31,32). Wang *et al* (33) identified an association between the expression level of miR-30a and the survival rate of patients with NPC. However, the results of Wang *et al* (33) were in contrast with previous studies (31,32). Wang *et al* (33) observed that the expression level of miR-30a in NPC primary tumors was decreased compared with metastatic tumors, and overexpression of miR-30a increased cell metastasis and invasion *in vitro* and *in vivo*. Mechanistically, this previous study identified that miR-30a interacted with the 3'-UTR of E-cadherin, decreasing its expression level and promoting epithelial-mesenchymal transition, thus decreasing the survival rate of patients with NPC (33). The present study aimed to investigate the functional association between miR-30a and ZEB2 in NPC. In the present study, miR-30a was identified to target the 3'-UTR of ZEB2, negatively regulating the expression level of ZEB2 in human NPC tissues and cells. miR-30a overexpression promoted cell apoptosis and inhibited the proliferative, migratory and invasive abilities of NPC cell, suggesting that miR-30a may serve a role in the development and progression of human NPC. Using the dual-luciferase reporter assay, the direct interaction between miR-30a and ZEB2 was observed, and the expression level of ZEB2 was identified to be suppressed by miR-30a. Furthermore, functional experiments were performed in the present study to investigate the effects of miR-30a on cell viability. Therefore, the effects of miR-30a, ZEB2 and miR-30a + ZEB2 overexpression on cell viability and proliferation were examined.

Although the role of miR-30a was previously investigated, the molecular mechanism underlying the function of miR-30a in human NPC remains unclear. In particular, the association between the downregulation of the expression level of miR-30a

and the development of NPC required further investigation. A previous study observed that the level of hypermethylation in the promoter of certain miRNAs increased in cancer cells, resulting in the downregulation of these miRNAs (34). Therefore, the hypermethylation of miRNA promoters may serve an important role in the regulation of miRNAs, thus modulating the development and progression of cancer. Further investigation is required to examine the association between hypermethylation and the regulation of miR-30a expression. Previous studies demonstrated that miR-30a may be downregulated by the long non-coding RNA deleted in lymphocytic leukemia 2, which exhibited an increased expression level in cancer cells, suggesting a possible mechanism underlying the regulation of miR-30a in NPC (31).

Previous studies observed that miR-30a was able to directly target multiple genes. For example, miR-30a was significantly downregulated in human gallbladder cancer, and E2F transcription factor 7 (E2F7) was identified to be a target of miR-30a (35). Overexpression of miR-30a inhibited the expression level of E2F7 and the re-establishment of the expression level of E2F7 reversed the inhibitory effects of miR-30a on cancer cell proliferation and metastasis (35). Notably, miR-30a may regulate the viability of cancer cells via multiple pathways, and the expression levels and roles of various target genes of miR-30a may be cancer type-specific. The present results suggested that, in human NPC, ZEB2 and ZEB2 downstream genes may serve an important role compared with other genes targeted by miR-30a. Nevertheless, further studies are required to investigate multiple signaling pathways associated with miR-30a. Understanding the important pathways and genes regulated by miR-30a in various types of cancers may aid the development of novel strategies to treat patients with cancer. Notably, in the present study, the number of patients was not sufficient to perform linear regression analysis of the expression levels of ZEB2 and miR-30a, and an increased number of patients is required in order to investigate the role of these two genes in further clinical studies.

The present results suggested that ZEB2 was a target of miR-30a in human NPC, and miR-30a negatively regulated the expression level of ZEB2 by directly binding to its 3'-UTR. miR-30a overexpression increased the level of apoptosis in human NPC cells and inhibited cell proliferation, migration and invasion in C666-1 cells by suppressing the expression level of ZEB2. Collectively, the present results suggested that miR-30a may have the potential to become a novel diagnostic biomarker in human NPC, and may facilitate the development of gene therapy strategies to treat NPC.

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**Availability of data and materials**

The analyzed datasets generated during the present study are available from the corresponding author on reasonable request.

**Authors' contributions**

WL, CX, MZ and SL conceived, designed and supervised the experiments. XC, JL, DS and SZ performed the experiments. XC, WL, WX, CX, MZ and SL analyzed the data. WX and DS contributed reagents, materials and analysis tools. MZ, SL, XC and JL drafted the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study (animal and human experiments) was approved by The Animal Care and Use Committee of People's Hospital of Longhua (Shenzhen, China). All patients provided written informed consent.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Yee-Lin V, Pooi-Fong W and Soo-Beng AK: Nutlin-3, A p53-Mdm2 antagonist for nasopharyngeal carcinoma treatment. Mini Rev Med Chem 18: 173-183, 2018.
2. Wu J and Hann SS: Functions and roles of long-non-coding RNAs in human nasopharyngeal carcinoma. Cell Physiol Biochem 45: 1191-1204, 2018.
3. Rupaimoole R and Slack FJ: MicroRNA therapeutics: Towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 16: 203-222, 2017.
4. Tutar L, Özgür A and Tutar Y: Involvement of miRNAs and pseudogenes in cancer. Methods Mol Biol 1699: 45-66, 2018.
5. Gabra MM and Salmena L: MicroRNAs and acute myeloid leukemia chemoresistance: A mechanistic overview. Front Oncol 7: 255, 2017.
6. Cheng L, Zhan B, Luo P and Wang B: miRNA375 regulates the cell survival and apoptosis of human nonsmall cell carcinoma by targeting HER2. Mol Med Rep 15: 1387-1392, 2017.
7. Poltronieri P: Editorial: Overview on microRNAs in cancer development and virus infection. Microrna 5: 80-82, 2016.
8. Tutar L, Tutar E and Tutar Y: MicroRNAs and cancer; an overview. Curr Pharm Biotechnol 15: 430-437, 2014.
9. Wang Y, Qiu C, Lu N, Liu Z, Jin C, Sun C, Bu H, Yu H, Dongol S and Kong B: FOXD1 is targeted by miR-30a-5p and miR-200a-5p and suppresses the proliferation of human ovarian carcinoma cells by promoting p21 expression in a p53-independent manner. Int J Oncol 52: 2130-2142, 2018.
10. Ye YY, Mei JW, Xiang SS, Li HF, Ma Q, Song XL, Wang Z, Zhang YC, Liu YC, Jin YP, *et al*: MicroRNA-30a-5p inhibits gallbladder cancer cell proliferation, migration and metastasis by targeting E2F7. Cell Death Dis 9: 410, 2018.
11. Guan Y, Rao Z and Chen C: miR-30a suppresses lung cancer progression by targeting SIRT1. Oncotarget 9: 4924-4934, 2018.
12. Zhang C, Ma X, Du J, Yao Z, Shi T, Ai Q, Chen X, Zhang Z, Zhang X and Yao X: MicroRNA-30a as a prognostic factor in urothelial carcinoma of bladder inhibits cellular malignancy by antagonising Notch1. BJU Int 118: 578-589, 2016.

13. Zhang S, Liu Q, Zhang Q and Liu L: MicroRNA-30a-5p suppresses proliferation, invasion and tumor growth of hepatocellular cancer cells via targeting FOXA1. Oncol Lett 14: 5018-5026, 2017.
14. Chen P, Liu H, Hou A, Sun X, Li B, Niu J and Hu L: Prognostic significance of zinc finger E-box-binding homeobox family in glioblastoma. Med Sci Monit 24: 1145-1151, 2018.
15. Verschueren K, Remacle JE, Collart C, Kraft H, Baker BS, Tylzanowski P, Nelles L, Wuytens G, Su MT, Bodmer R, *et al*: SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. J Biol Chem 274: 20489-20498, 1999.
16. He L, Yu K, Lu F, Wang J, Wu LN, Zhao C, Li Q, Zhou X, Liu H, Mu D, *et al*: Transcriptional regulator ZEB2 is essential for bergmann glia development. J Neurosci 38: 1575-1587, 2018.
17. Kan Q, Su Y and Yang H: MicroRNA-335 is downregulated in papillary thyroid cancer and suppresses cancer cell growth, migration and invasion by directly targeting ZEB2. Oncol Lett 14: 7622-7628, 2017.
18. Ko D and Kim S: Cooperation between ZEB2 and Sp1 promotes cancer cell survival and angiogenesis during metastasis through induction of survivin and VEGF. Oncotarget 9: 726-742, 2017.
19. Zhou X, Men X, Zhao R, Han J, Fan Z, Wang Y, Lv Y, Zuo J, Zhao L, Sang M, *et al*: miR-200c inhibits TGF-β-induced-EMT to restore trastuzumab sensitivity by targeting ZEB1 and ZEB2 in gastric cancer. Cancer Gene Ther 25: 68-76, 2018.
20. Liu P, Feng Y, Dong D, Liu X, Chen Y, Wang Y and Zhou Y: Enhanced renoprotective effect of IGF-1 modifed human umbilical cord-derived mesenchymal stem cells on gentamicin-induced acute kidney injury. Sci Rep 6: 20287, 2016.
21. Liu P, Cai J, Dong D, Chen Y, Liu X, Wang Y and Zhou Y: Effects of SOX2 on proliferation, migration and adhesion of human dental pulp stem cells. PLoS One 10: e0141346, 2015.
22. Liu P, Chen S, Li X, Qin L, Huang K, Wang L, Huang W, Li S, Jia B, Zhong M, *et al*: Low immunogenicity of neural progenitor cells differentiated from induced pluripotent stem cells derived from less immunogenic somatic cells. PLoS One 8: e69617, 2013.
23. Tao S, Liu P, Luo G, Rojo de la Vega M, Chen H, Wu T, Tillotson J, Chapman E and Zhang DD: p97 negatively regulates NRF2 by extracting ubiquitylated NRF2 from the KEAP1-CUL3 E3 complex. Mol Cell Biol 37: e00660-16, 2017.
24. Liu P, Feng Y, Dong C, Yang D, Li B, Chen X, Zhang Z, Wang Y, Zhou Y and Zhao L: Administration of BMSCs with muscone in rats with gentamicin-induced AKI improves their therapeutic efficacy. PLoS One 9: e97123, 2014.
25. Huang K, Liu P, Li X, Chen S, Wang L, Qin L, Su Z, Huang W, Liu J, Jia B, *et al*: Neural progenitor cells from human induced pluripotent stem cells generated less autogenous immune response. Sci China Life Sci 57: 162-170, 2014.
26. Yao Q, Pei Y, Zhang X and Xie B: microRNA-96 acts as a tumor suppressor gene in human osteosarcoma via target regulation of EZRIN. Life Sci 203: 1-11, 2018.
27. Liu Y, Wu T, Song J, Chen X, Zhang Y and Wan Y: A mutant screening method by critical annealing temperature-PCR for site-directed mutagenesis. BMC Biotechnol 13: 21, 2013.
28. Liu P, Zhang Y, Chen S, Cai J and Pei D: Application of iPS cells in dental bioengineering and beyond. Stem Cell Rev 10: 663-670, 2014.
29. Zhao L, Feng Y, Chen X, Yuan J, Liu X, Chen Y, Zhao Y, Liu P and Li Y: Effects of IGF-1 on neural differentiation of human umbilical cord derived mesenchymal stem cells. Life Sci 151: 93-101, 2016.
30. Li Y, Zhang J, Liu Y, Zhang B, Zhong F, Wang S and Fang Z: MiR-30a-5p confers cisplatin resistance by regulating IGF1R expression in melanoma cells. BMC Cancer 18: 404, 2018.
31. Chen Z, Zhang J, Zhang Z, Feng Z, Wei J, Lu J, Fang Y, Liang Y, Cen J, Pan Y, *et al*: The putative tumor suppressor microRNA-30a-5p modulates clear cell renal cell carcinoma aggressiveness through repression of ZEB2. Cell Death Dis 8: e2859, 2017.
32. di Gennaro A, Damiano V, Brisotto G, Armellin M, Perin T, Zucchetto A, Guardascione M, Spaink HP, Doglioni C, Snaar-Jagalska BE, *et al*: A p53/miR-30a/ZEB2 axis controls triple negative breast cancer aggressiveness. Cell Death Differ 25: 2165-2180, 2018.
33. Wang HY, Li YY, Fu S, Wang XP, Huang MY, Zhang X, Shao Q, Deng L, Zeng MS, Zeng YX and Shao JY: MicroRNA-30a promotes invasiveness and metastasis in vitro and in vivo through epithelial-mesenchymal transition and results in poor survival of nasopharyngeal carcinoma patients. Exp Biol Med (Maywood) 239: 891-898, 2014.
34. Lü L, Liu T, Gao J, Zeng H, Chen J, Gu X and Mei Z: Aberrant methylation of microRNA-193b in human Barrett's esophagus and esophageal adenocarcinoma. Mol Med Rep 14: 283-288, 2016.
35. Chen LL, Zhang ZJ, Yi ZB and Li JJ: MicroRNA-211-5p suppresses tumour cell proliferation, invasion, migration and metastasis in triple-negative breast cancer by directly targeting SETBP1. Br J Cancer 117: 78-88, 2017.