MiR-205 promotes proliferation, migration and invasion of nasopharyngeal carcinoma cells by activation of AKT signalling

Yanjiao Mao, Shixiu Wu, Ruping Zhao and Qinghua Deng

Abstract

Objective: To examine the role of microRNA (miR)-205 in proliferation, migration and invasion of nasopharyngeal carcinoma (NPC).

Methods: The human NPC cell line CNE2 was transfected with miR-205 mimic, anti-miR-205 inhibitor or scrambled oligonucleotide (control). Cell proliferation was assessed via MTT assay. Cell migration and invasion were evaluated by transwell migration and Matrigel invasion assay, respectively. Radiation induced apoptosis was quantified via Caspase-Glo3/7 assay. Apoptotic proteins and epithelial–mesenchymal transition (EMT) proteins were semiquantified by Western blot analysis.

Results: Overexpression of miR-205 increased the proliferation, migration and invasion of CNE2 cells, and decreased radiation-induced apoptosis compared with control cells. MiR-205 overexpression downregulated E-cadherin and upregulated Snail expression via downregulation of PTEN and upregulation of AKT.

Conclusion: MiR-205 plays vital roles in tumourigenesis and tumour progression in NPC, and may be a potential treatment target.

Keywords
AKT, apoptosis, PTEN, metastasis, miRNA, miRNA-205, NPC, EMT

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Introduction

Nasopharyngeal carcinoma (NPC) is the most common primary malignancy of the nasopharynx, with a yearly incidence of 25 cases per 100 000 and a high prevalence in southeast Asia and southern China.¹

Department of Radiation Oncology, Hangzhou Cancer Hospital, Hangzhou First People’s Hospital, Hangzhou, China

Corresponding author:
Qinghua Deng, Department of Radiation Oncology, Hangzhou Cancer Hospital Hangzhou First People’s Hospital, Hangzhou 310000, China.
Email: yanjiaomao@gmail.com

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NPC has three subtypes based on degree of differentiation: keratinizing squamous cell carcinoma (SCC) (type 1); nonkeratinizing carcinoma (type 2); undifferentiated carcinoma (type 3). Types 2 and 3 NPC are strongly associated with Epstein–Barr virus (EBV). In patients with advanced type 3 NPC, the 5-year survival rate is <50%. Radiotherapy is the primary treatment for patients with NPC, but radioresistance and local recurrence are major limitations to its value. It is therefore important to investigate the molecular mechanisms and identify predictive markers in NPC.

MicroRNAs (miRNAs) are short non-coding RNA molecules that post-transcriptionally regulate gene expression by binding to the 3'-UTR of their target mRNAs. MiRNAs regulate many biological functions including tumour development, differentiation, proliferation and apoptosis. Expression profiling has shown that miRNA expression is different in NPC tissue compared with normal nasopharyngeal tissue, and aberrant miRNAs are correlated with clinical stage. In addition, some studies have demonstrated that EBV-encoded miRNAs play a critical role in the regulation of EBV infection and latency.

Growing evidence suggests that miRNAs affect NPC carcinogenesis and metastasis by activating various signalling pathways. For example, several miRNAs activate c-Myc, which then regulates tumour cell growth and carcinogenesis; in addition, MiR-200a regulation of ZEB2 and β-catenin promotes NPC cell migration and invasion.

The aim of the present study was to examine the roles of miR-205 in the proliferation, migration and invasion of NPC.

**Materials and methods**

**Cell culture**

The human NPC cell line CNE2 was obtained from the Cancer Centre of Hangzhou First People’s Hospital, Hangzhou, China. Cells were grown in RPMI 1640 medium (Invitrogen™, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified incubator in 5% carbon dioxide/95% air.

**Transfection**

MiR-205 mimic and anti-miR-205 inhibitor were purchased from GenePharma (Shanghai, China). When CNE2 cells reached 70% confluence, they were transfected with miR-205 mimic or anti-miR-205 inhibitor using Lipofectamine® 2000 (Invitrogen, USA) according to the manufacturer’s instructions. Scrambled oligonucleotide was used as negative control for the transfection.

**MiRNA quantification**

At 48 h after transfection, total RNA was extracted from 10⁶ cells using a mirVana® miRNA isolation kit (Ambion, Carlsbad, CA, USA) according to the manufacturer’s instructions, and cDNA was synthesized using a TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (qRT–PCR) was performed using the TaqMan® Universal PCR Master Mix. The cycling programme involved preliminary denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 65°C for 30s, and elongation at 70°C for 60s, followed by a final elongation step at 95°C for 5 min. Primer sequences were: GAPDH, sense 5'-GTCT CCTCTGACTTCAACAGCG-3' and anti-sense 5'-ACCACCTGTGGTGTAGGAA-3' (GAPDH); and miR-205, sense primer 5'-TTTTCAGACTCC-3' and anti-sense primer 5'-CTCTTGTCCTTCATT
CCACC-3’. MiR-205 was quantified by reference to GAPDH levels.

Cell proliferation
At 48 h after transfection, cell proliferation was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described. Briefly, 100 µl of transfected CNE2 cell suspension was inoculated to each well of a 96-well plate at 3 × 10⁴ cells/well. On alternate days, MTT solution (20 µl, 5 mg/ml) was added to each well, and the plates were incubated in the dark for 4 h at 37°C, followed by removal of the culture medium and addition of 100 µl dimethyl sulphoxide. Absorbance was measured at 492 nm, with 655 nm as the reference wavelength. Each group used six parallel wells and all experiments were carried out in triplicate.

Irradiation
At 48 h after transfection, cells were seeded in 24-well plates at density of 1 × 10⁵/well. After incubation for 24 h, cells were irradiated with 0, 2, 5 or 10 Gy. CNE2 cells were cultured for a further 36 h before apoptosis was quantified using a Caspase-Glo3/7 assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Luminescence was measured with a luminometer (Thermo Fisher Scientific, Waltham, MA, USA) using a 1 min lag time and 0.5 s/well read-time. Experiments were performed in triplicate.

Cell invasion assay
Cell invasion was quantified by Matrigel® invasion assay. At 48 h after transfection, CNE2 cells (transfected with either miR-205 mimic or anti-miR-205 inhibitor) were placed in the upper chamber of a BD Biocoat® Matrigel® Invasion Chamber (BD Bioscience, San Jose, CA, USA) in 0.5 ml DMEM with 0.1% bovine serum albumin. RPMI1640 containing 5% FBS was added to the lower chamber. Cells were incubated for 24 h then noninvading cells were removed with a cotton swab. Invasive cells were stained by Diff-Quik® (Thermo Fisher Scientific) stain and counted via light microscopy. The percentage of invasion was expressed as the ratio of invading cells over the cell number, normalized on day 2 of the growth curve.

Western blotting
At 48 h after transfection, 10⁶ transfected CNE2 cells were lysed with RIPA buffer (Beyotime, Jiangsu, China) then boiled for 5 min. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk in 1 × Tris buffered saline/0.1% Tween-20 (pH 7.4; TBST) at room temperature for 1 h, membranes were incubated overnight at 4°C with rabbit antihuman antibodies to E-cadherin, Snail, caspase 9, Bcl-2, Bax, AKT and PTEN (all 1:500 dilution; Cell Signaling Technology, Beverly, MA, USA), washed three times with TBST for 10 min at room temperature, then incubated with horseradish peroxidase-conjugated mouse antirabbit secondary antibody (1:1000 dilution; Cell Signaling Technology, USA) for 1 h at room temperature. Membranes were washed three times with TBST for 10 min at room temperature and immunoreactive signals were visualized using an EasySee® Western Blot Kit (TransGen Biotech, Beijing, China). Protein bands were quantified by densitometry (Tanon-1600 gel image system; BioTanon, Shanghai, China).

Statistical analyses
Data were presented as mean ± SD. Between-group comparisons were made using Student’s t-test. Statistical analyses
were performed using SPSS® version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows®. P-values < 0.05 were considered statistically significant.

Results
Transfection of CNE2 cells with miR-205 mimic resulted in significantly higher miR-205 expression (P = 0.0002, Figure 1a) and rate of cell proliferation at 7 days after transfection (P = 0.0081; Figure 1b) compared with cells transfected with control oligonucleotide. In addition, CNE2 cells transfected with miR-205 exhibited significantly lower levels of apoptosis after irradiation than control cells (2 Gy irradiation, P = 0.0387; 5 Gy, P = 0.0129; 10 Gy, P = 0.0102; Figure 1c). As shown in Figures 1d and 1e, the radiation-induced (2 Gy) increase in BAX and caspase-9 protein levels was reversed by miR-205 overexpression. In addition, levels of Bcl-2 protein were visibly increased in cells transfected with miR-205 compared with control cells.

Migration and invasion were significantly enhanced in CNE2 cells transfected with miR-205 mimic compared with control cells (migration, P = 0.0079; invasion, P = 0.0090; Figures 2a–d). In contrast, transfection with anti-miR-205 inhibitor significantly inhibited migration and invasion compared with control cells (migration, P = 0.0055; invasion, P = 0.0031; Figures 2e–h).

Western blot analysis of epithelial–mesenchymal transition (EMT) proteins is shown in Figure 3. E-cadherin levels were visibly decreased and N-cadherin and Snail levels were visibly increased in CNE2 cells transfected with miR-205 mimic, compared with control cells (Figures 3a and 3c) (P < 0.05). In contrast, levels of E-cadherin were increased, and N-cadherin and Snail were decreased, in CNE2 cells transfected with anti-miR-205 inhibitor, compared with control cells (Figures 3b and 3d) (P < 0.05).

Discussion
Studies have shown that miR-205 can act as either a tumour suppressor or an oncogene depending on the tumour type. Overexpression of miR-205 increased proliferation and inhibited radiation-induced apoptosis of CNE2 cells in the present study. Consistent with our findings, others have shown that miR-205 promotes growth, metastasis and chemoresistance in nonsmall cell lung cancer and is upregulated in NPC. There is evidence to suggest that miR-205 induces radioresistance in NPC cells via activation of PTEN. Taken together, these data suggest that miR-205 plays a critical role in NPC development and treatment.

Aggressive tumours are characterized by local invasion and distant metastasis. These processes include cell proliferation, changes in cell morphology and destruction of the extracellular matrix. EMT proteins play vital roles in tumour progression, invasion and metastasis, and the PTEN pathway is involved in EMT signalling in NPC. The tumour suppressor PTEN is crucial for multiple cellular processes including cell proliferation, apoptosis and cell migration, and negatively regulates AKT signalling. AKT controls multiple biological processes including cell proliferation, differentiation, apoptosis, glucose metabolism, and tumourigenesis, and promotes cell proliferation, survival and metastasis in NPC via inactivation of FKHR and
Figure 1. Micro RNA (miR)-205 promotes proliferation and reduces radiation-induced apoptosis in the human nasopharyngeal carcinoma cell line, CNE2. (a) Relative expression of miR-205 in control CNE2 cells and CNE2 cells transfected with miR-205 mimic. (b) Cell proliferation in transfected and control cells (c) Apoptosis (quantified via caspase 3/7 activity) in transfected and control cells treated with 0–10 Gy radiation. (d) Representative Western blot of apoptosis-associated proteins in transfected and control cells treated with 2 Gy radiation (e) Densitometric quantification of apoptosis-associated proteins in transfected and control cells treated with 2 Gy radiation (Black bars denote control and white bars denote miR-205 mimic). All experiments performed in triplicate.
Figure 2. Micro RNA (miR)-205 increases cell migration and invasion in the human nasopharyngeal carcinoma cell line, CNE2. (a, b) Representative light photomicrographs of Matrigel® invasion assay in (a) control cells and (b) cells transfected with miR-205 mimic. (c) Migration and (d) invasion of control cells and cells transfected with miR-205 mimic. (e, f) Representative light photomicrographs of Matrigel® invasion assay in (e) control cells and (f) cells transfected with anti-miR-205 inhibitor. (g) Migration and (h) invasion of control cells and cells transfected with anti-miR-205 inhibitor. All experiments performed in triplicate.
In accordance with the findings of others, overexpression of miR-205 downregulated PTEN and upregulated AKT levels, in the present study.

Migration and invasion of CNE2 cells were enhanced by overexpression of miR-205 in the present study. In addition, miR-205 overexpression resulted in downregulation of E-cadherin and upregulation of Snail proteins. E-cadherin, a member of the cadherin superfamily, is a calcium-dependent transmembrane glycoprotein involved in maintaining cell polarity and normal structure by strengthening intercellular adhesion. Downregulation of E-cadherin is a marker of EMT changes in epithelial cells. The zinc-finger transcription factor Snail is a prominent inducer of EMT that has been shown to inhibit E-cadherin expression and PTEN transcription. Snail is also associated with radioresistance, maintenance of a stem cell-like phenotype and poor prognosis. It is possible that elevated levels of Snail may

Figure 3. Micro RNA (miR)-205 regulates levels of epithelial-to-mesenchymal (EMT) markers in the human nasopharyngeal carcinoma cell line, CNE2. Representative Western blot of EMT markers in (a) control CNE2 cells and CNE2 cells transfected with miR-205 mimic, and (b) control CNE2 cells and CNE2 cells transfected with anti-miR-205 inhibitor. Densitometric quantification (relative to GAPDH levels) of EMT markers in (c) control CNE2 cells and CNE2 cells transfected with miR-205 mimic, and (d) control CNE2 cells and CNE2 cells transfected with anti-miR-205 inhibitor. Black bars denote control and white bars denote miR-205 mimic (c) and anti-miR-205 inhibitor (d). All experiments performed in triplicate.
contribute to PTEN downregulation in the present study.

In conclusion, our findings suggest that miR-205 promotes NPC cell proliferation, invasion and EMT, and inhibits radiation induced apoptosis. MiR-205 plays critical roles in the progression of NPC via regulation of PTEN and AKT signalling, and may represent a molecular treatment target for NPC.

Declaration of conflicting interest
The authors declare that there are no conflicts of interest.

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