miR-539 induces cell cycle arrest in nasopharyngeal carcinoma by targeting cyclin-dependent kinase 4

Ling-yan Lv1*, Yu-zhi Wang1, Qian Zhang2, Hong-rui Zang3 and Xing-jie Wang1

1Department of Otolaryngology, Affiliated Liaocheng Second People Hospital, Taishan Medical College, Liaocheng, China
2Department of Spinal Surgery, Affiliated Liaocheng Second People Hospital, Taishan Medical College, Liaocheng, China
3Department of Otolaryngology, Beijing Tongren Hospital, Beijing, China

Dysregulation of microRNAs has been demonstrated to contribute to malignant progression of cancers, including nasopharyngeal carcinoma (NPC). miR-539 was previously reported to be significantly downregulated in osteosarcoma. However, the potential role and mechanism of action of miR-539 in the initiation and progression of NPC remain largely unknown. Quantitative reverse transcription (RT)-PCR demonstrated that miR-539 was significantly downregulated in NPC tumour tissues compared with nontumour tissues. The cell viability, colony formation assay and tumourigenicity assays in nude mice showed that miR-539 could inhibit NPC cell growth in vitro and in vivo. The cyclin-dependent kinase 4 (CDK4) was verified as a miR-539 target gene using dual-luciferase reporter assays, quantitative RT-PCR and Western blotting and was involved in miR-539-regulated NPC cell growth. These results indicated that miR-539 plays an important role in the initiation and progression of NPC by targeting CDK4 and the miR-539/CDK4 pathway may contribute to the development of novel therapeutic strategies for NPC in the future. Copyright © 2015 John Wiley & Sons, Ltd.

KEY WORDS—nasopharyngeal carcinoma; miR-539; CDK4; cell cycle

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a nonlymphomatous squamous cell carcinoma arising from epithelial cells located in the nasopharynx, which is low globally but high in Southern China and Southeast Asia.1 The characteristics are highly malignant local invasion and early distant metastasis, and 30–40% of NPC patients will develop distant metastases with poor prognosis.2 NPC responds well to radiation therapy and adjuvant chemotherapy; however, the 5-year overall survival rate is still approximately 70%.3 Therefore, an improved understanding of the mechanisms of NPC tumourigenesis is urgently needed for the development of more effective therapies for NPC.

MicroRNAs (miRNAs) are endogenous noncoding 20- to 22-nucleotide RNAs that have been identified as post-transcriptional regulators of gene expression.4 The miRNAs mainly bind to the 3′ untranslated regions (UTRs) of target mRNAs, resulting in mRNA degradation or the blockade of mRNA translation. Increasing evidence showed that miRNAs have significant roles in diverse biological processes.5 Meanwhile, deregulation of miRNAs has been observed in a wide range of human diseases, including cancer.6 In human cancer, miRNAs can function as oncogenes or tumour suppressor genes during tumour development and progression.7

In this study, miR-539 was found frequently downregulated miRNAs in human NPC compared with adjacent nontumour tissues. Further investigation revealed that miR-539 could induce cell cycle arrest in NPC cell lines. Moreover, cyclin-dependent kinase 4 (CDK4) was characterized as a direct target of miR-539.

MATERIALS AND METHODS

Tissue specimens and cell lines

Human NPC and adjacent nontumour tissues were obtained from surgical specimens immediately after resection from patients undergoing primary surgical treatment of NPC in Affiliated Liaocheng Second People Hospital, Taishan Medical College, China. The samples were frozen in liquid nitrogen and stored at −80 °C until use. NPC and adjacent nontumour tissues were obtained from 56 patients, and their profiles are shown in Table 1. Written consent for tissue donation (for research purposes) was obtained from the patients before tissue collection, and the protocol was approved by the Institutional Review Board of Taishan Medical College.

Human embryonic kidney cells (HEK293T) and human NPC cell lines SUNE-1 and CNE-1 were maintained in Dulbecco’s modified Eagle’s medium (DMEM, HyClone,
Table 1. The data of patients (n = 56)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.6 ± 12.1</td>
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<tr>
<td>Gender (male)</td>
<td>41 (73.2%)</td>
</tr>
<tr>
<td>Tumour size (cm)</td>
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<tr>
<td>Tumour grade</td>
<td></td>
</tr>
<tr>
<td>Well-differentiated (G1–2)</td>
<td>31</td>
</tr>
<tr>
<td>Moderately differentiated (G3)</td>
<td>22</td>
</tr>
<tr>
<td>Poorly differentiated (G4)</td>
<td>3</td>
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<tr>
<td>TNM stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>24</td>
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<td>II</td>
<td>19</td>
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<td>III</td>
<td>11</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
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</tbody>
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TNM, tumour-node-metastasis

*aDiameter of the biggest nodule

Quantitative real-time PCR

Synthesis of cDNA and quantitative real-time (qRT)-PCR analysis of miRNA expression was carried out with TaqMan microRNA assay kits (Applied Biosystems) according to the manufacturer’s protocol. Briefly, total RNA of clinical samples or NPC cell lines was extracted using TRIzol Reagent (Invitrogen). Reverse transcriptase reactions were incubated for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at 4 °C. The cDNA product was used for the following qRT-PCR analysis directly. The PCR reaction were incubated at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. PCR reactions were run on a StepOne Plus real-time PCR machine (Applied Biosystems), and the data were analyzed by SDS v2.3 software. The fold change was calculated using the 2^(-ΔΔCt) method and presented as the fold expression change in tumours relative to their corresponding normal tissues after normalization to the endogenous control.

Transfection

The transfection was carried out using FuGene HD transfection reagent (Roche, Indianapolis, IN, USA) following the manufacturer’s protocol. In brief, 2 × 10^4 SUNE-1 and CNE-1 cells were transfected with indicated plasmid DNA, miRNA duplex (GenePharma, Shanghai, China) and siRNA (GenePharma) and collected 24–48 h after transfection.

The construction of luciferase reporter plasmids and dual-luciferase reporter assay

The fragment of 3′-UTR of CDK4 (1204–2020 nt, GenBank accession no. NM_000075) containing the putative miR-539 binding sequences (1378–1385 nt) was amplified with the primers 5′-GCAATGGAGTGGCTGCCATGG-3′ (forward) and 5′-TGCCATTTAAAAATCTATATTGC-3′ (reverse). The PCR product was cloned into Firefly luciferase reporter vector pGL3 (Promega Corporation, Madison, WI, USA), termed as pGL3-CDK4-3′-UTR. The plasmid that carried the mutated sequence in the two complementary sites for the seed region of miR-539 was generated based on pGL3-CDK4-3′-UTR plasmid by MutanBEST Kit (Takara Bio Inc., Shiga, JP), termed as pGL3-CDK4-3′-UTR-mut. The plasmids were confirmed by sequencing analyses.

HEK293T cells seeded in a 24-well plate in triplicate were cotransfected with pGL3-CDK4-3′-UTR or pGL3-CDK4-3′-UTR-mut and miR-539 mimic or nonrelative control RNA duplex (NC duplex, GenePharma) by using FuGene HD transfection reagent. The pRL-TK (Promega Corporation, Madison, WI, USA) was also transfected as a normalization control. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation) and recorded by multi-plate reader (Synergy 2, BioTek).

Cell viability and colony formation assay

Twenty-four hours after transfection, 1000 transfected SUNE-1 and CNE-1 cells were placed in a fresh 96-well plate in triplicate and maintained in DMEM containing 10% FBS for 5 days. Cells were tested for proliferation per 24 h using Cell Titer-Blue cell viability assay (Promega Corporation, Madison, WI, USA) according to the manufacturer’s instructions, and the fluorescence value was recorded by multi-plate reader (Synergy 2, BioTek, Winooski, VT, USA).

Twenty-four hours after transfection, 2000 transfected SUNE-1 and CNE-1 cells were placed in a fresh 6-well plate in triplicate and maintained in DMEM containing 10% FBS for 2 weeks. Cell colonies were fixed with 20% methanol and stained with 0.1% Coomassie brilliant blue R250 at room temperature for 15 min. The colonies were counted by ELISPOT Bioreader 5000 (BIO-SYS, Karben, GE).

Cell cycle and cell apoptosis assay

Forty-eight hours after transfection, 1 × 10^5 transfected SUNE-1 and CNE-1 cells were harvested, washed once in phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4 °C overnight. Staining for DNA content was performed with 50 mg/mL propidium iodide and 1 mg/mL RNase A at room temperature for 30 min. Populations in G0–G1, S and G2–M phase were measured by Cell Lab Quanta SC flow cytometry (Beckman Coulter, Fullerton, CA, USA), and the data were analyzed by FlowJo v7.6 software. For apoptosis assay, PBS washed cells were incubated with FITC-Annexin V (Promega Corporation) for 15 min at 4 °C in the dark, according to the manufacturer’s instructions. After staining, the cells were incubated with propidium iodide for 5 min at 4 °C in the dark and then analyzed by Cell Lab Quanta SC flow cytometry, and the data were analyzed by FlowJo v7.6 software.
**Tumourigenicity assays in nude mice**

Male BALB/c nude mice (5 to 6 weeks of age) were obtained from Shanghai Experimental Animal Center (Shanghai, China). After adjacent nontumourous tissues were electrophoresed in 10% NC transfected or nontransfected SUNE-1 cells (1 × 10^5) were suspended in 100 μL PBS and then injected s.c. into left side of the posterior flank of six BALB/c nude mice, respectively. NC transfected or nontransfected SUNE-1 cells (1 × 10^5) were injected subcutaneously into right side of same 12 mice. Tumour growth was examined daily, and the tumour volumes were calculated every week using the formula for hemi-ellipsoids: \( V = \text{length (cm)} \times \text{width (cm)} \times \text{height (cm)} \times 0.5236. \) After 5 weeks, the mice were sacrificed and the tumours were dissected and photographed.

**Western blot**

Protein extracts from NPC tissues or SUNE-1 cells were prepared by a modified radioimmunoprecipitation assay buffer with 0.5% sodium dodecyl sulphate (SDS) in the presence of proteinase inhibitor cocktail (Complete Mini, Roche). Twenty-five micrograms of protein of NPC tissues and their adjacent nontumourous tissues were electrophoresed in 10% SDS-PAGE mini gels and transferred onto PVDF membranes (Immobilon P-SQ, Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk, the membranes were incubated with rabbit anti-CDK4 antibody (1:1000 dilution, Epitomics, Inc., Burlingame, CA, USA) or mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:5000 dilution, Epitomics, Inc.) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (1:10,000 dilution, KPL, Gaithersburg, MA, USA) for 1 h at room temperature. Finally, signals were developed with Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA), visualized by the Gene Gnome HR Image Capture System (Syngene, Frederick, MD, USA) and analyzed by GeneTools (Syngene).

**Prediction of miRNA targets**

MiRanda (http://www.microrna.org/microrna/home.do) was used to predict the target genes and the conserved sites bound by the seed region of miR-539.

**Statistical analysis**

Data are presented as mean ± SD. Comparisons were made by using a two-tailed t test or one-way ANOVA for experiments with more than two subgroups. Correlation analysis was made by using Spearman correlation coefficient. \( P < 0.01 \) was considered statistically significant.

**RESULTS**

**miR-539 correlates negatively with CDK4**

To investigate the role of miR-539 in NPC tumourigenesis, firstly, the expression of miR-539 was measured in 56 NPC tumour tissues and nontumour tissues by qRT-PCR. miR-539 showed significantly downregulated in NPC tissues compared with nontumour tissues (Figure 1A). However, CDK4 was found to be upregulated in NPC tumour tissues compared with adjacent nontumour tissues (Figure 1B, upper panel). A negative correlation was found between the upregulated CDK4 protein and downregulated miR-539 (\( r = -0.5511, P < 0.001 \)) (Figure 1B, lower panel).

**miR-539 inhibits cell growth**

The significant reduction of miR-539 expression in NPC tissues indicated possible biological significance in tumourigenesis. At first, the effect of miR-539 on cell growth was evaluated in SUNE-1 and CNE-1 cells transfected with or not, miR-539 mimic, miR-539 inhibitor or NC duplex. The expression of miR-539 was increased 25-fold (SUNE-1) and 22-fold (CNE-1) in cells transfected with 20 nM miR-539 mimic but decreased 30-fold (SUNE-1) and 43-fold (CNE-1) in cells transfected with 20 nM miR-539 inhibitor (Figure 2A).

From 2 days (SUNE-1) or 3 days (CNE-1) after the transfection, the viability of cells transfected with miR-539 mimic significantly decreased compared with that of NC duplex transfected or nontransfected cells, but the viability of cells transfected with miR-539 inhibitor significantly increased (Figure 2B). These results indicate that miR-539 could inhibit cell growth. To validate the inhibitory effect of miR-539 on cell growth, the colony formation assay was performed in SUNE-1 and CNE-1 transfected with or not, miR-539 mimic, miR-539 inhibitor or NC duplex. As showed in Figure 2C, SUNE-1 and CNE-1 cells transfected with 20 nM miR-539 mimic displayed much fewer and smaller colonies (218 or 161 colonies) compared with NC duplex transfected (783 or 729 colonies) and nontransfected cells (756 or 692 colonies), but cells transfected with 20 nM miR-539 inhibitor displayed much more and larger colonies (1238 or 1349 colonies).

To further confirm the previous findings, an in vivo mouse model was used. For the duration of the treatment with miR-539 mimic or miR-539 inhibitor for 5 weeks, tumour volume curves revealed a significant decrease in growth rates at the third, fourth and fifth weeks after treatment with miR-539 mimic and a significant increase in growth rates at the fourth and fifth weeks after treatment with miR-539 inhibitor, whereas no significant differences in tumour growth rates were observed between the NC group and the control group (Figure 2D). These results indicate that introduction of miR-539 significantly inhibits tumourigenicity of SUNE-1 cells in xenograft nude mouse model.

**miR-539 induces cell cycle arrest**

Cell cycle analysis showed, but the percentages of miR-539 mimic transfected SUNE-1 cells in the S phase were 8.73% less than that of NC mimic transfected or nontransfected...
cells, which paralleled with a 7.82% increase in the G0–G1 phase (Figure 3A). In miR-539 inhibitor transfected cells, the percentages of cells in the S phase were 6.32% more than that of NC inhibitor transfected or nontransfected cells, which paralleled with a 6.61% decrease in the G0–G1 phase (Figure 3A). The similar results were obtained in CNE-1 cells. In addition, in miR-539 mimic or miR-539 inhibitor transfected cells, the percentages of apoptotic cells were much same to that of control group (Figure 3B). Together, these results indicate that miR-539 could suppress NPC cell proliferation by inducing cell cycle arrest but not inducing cell apoptosis.

**CDK4 is a direct target of miR-539**

It is generally accepted that miRNAs exert their function through regulating the expression of their downstream target genes. CDK4 was predicted as a potential target of miR-539 by MiRanda (http://www.microrna.org/microrna/home.do). The 3'-UTR of CDK4 mRNA contained a complementary site for the seed region of miR-539 (Figure 4A). To validate whether CDK4 is a direct target of miR-539, a human CDK4 3'-UTR fragment containing wild-type or mutant miR-539 binding sequence was cloned downstream of the firefly luciferase reporter gene in pGL3. In HEK293 cells cotransfected with the reporter plasmids and miR-539 mimic or NC duplex, the luciferase activity of the reporter that contained wild-type 3'-UTR was significantly suppressed by miR-539 mimic, but the luciferase activity of mutant reporter was unaffected (Figure 4B), indicating that miR-539 may suppress gene expression through miR-539 binding sequence at the 3'-UTR of CDK4. Furthermore, transfection of miR-539 mimic decreased CDK4 expression and transfection of miR-539 inhibitor increased CDK4 expression in SUNE-1 cells at protein (Figure 4C) but not mRNA level (data not shown), suggesting that CDK4 expression could be inhibited by miR-539 at post-transcriptional level. Together, the results show that miR-539 could regulate the expression of endogenous human CDK4 by directly targeting the 3'-UTR of CDK4 mRNA, and human CDK4 is a new target of miR-539.

**CDK4 knockdown could induce cell cycle arrest**

To identify whether inhibition of CDK4, just like miR-539 restoration, also resulted in NPC repression, the effects of knockdown of CDK4 on cell growth were examined. Firstly, SUNE-1 cells were transfected with or not, CDK4 siRNA or control siRNA. Forty-eight hours after transfection, a dose-dependent knockdown of CDK4 was observed in SUNE-1 cells (Figure 5A). In cell viability assay and cell cycle analysis, in vitro knockdown of CDK4 repressed cell viability (Figure 5B) and induced cell cycle arrest (Figure 5C). The similar data were obtained in CNE-1 cells transfected with CDK4 siRNA (data not shown). These results indicate that CDK4 is most likely involved in the induction of cell cycle arrest by miR-539.

**DISCUSSION**

miRNAs are key regulators of numerous cellular processes, and abnormal expression of miRNAs has been closely related to the initiation and progression of malignant tumours. Recently, several miRNAs have been found to be dysregulated in NPC, such as miR-663, miR-144, miR-26a, miR-451 and miR-9, which function as oncogenes or tumour suppressors depending on their targets. In a recent study, the dysregulation of miR-539 in a small cohort of Duchenne muscular dystrophy patients, miR-539 may represent a novel therapeutic target in the treatment of heart failure by targeting O-GlcNAcase (OGA), which modulates the function of many nuclear and cytoplasmic proteins; miR-539 was downregulated in osteosarcoma, miR-539 was upregulated in alcoholic steatohepatitis. However, to date, studies of the biological function and molecular mechanism of miR-539 in NPC remain limited. Therefore, miR-539 was selected for further investigation in the present study.

In this study, we observed that miR-539 is frequently downregulated in NPC tumour tissues. Functional analyses
Figure 2. miR-539 inhibits cell growth. (A) The expression of miR-539 in SUNE-1 and CNE-1 cells with transfection of miR-539 mimic, miR-539 inhibitor or NC duplex. (B) The effect of miR-539 on cell viability of NSCLC cell lines. (C) The effect of miR-539 on colony formation of NPC cell lines. (D) The effect of miR-539 on the tumour growth in xenograft nude mice. Representative results (B–D) in SUNE-1 and CNE-1 cells transfected with miR-539 mimic, miR-539 inhibitor, NC mimic or NC inhibitor. Column, mean of three independent experiments; bars, SD; *P < 0.01; **P < 0.001
revealed that depletion of miR-539 suppressed NPC cell growth \textit{in vitro} and inhibited NPC tumour growth \textit{in vivo}. Based on MiRanda search, CDK4 was predicted as the target of miR-539. CDK4 had been reported to be regulated by several miRNAs, including miR-206,18 miR-506,19 miR-138,20 miR-124,21 miR-54522 and miR-302.23 We identified CDK4 as a target of miR-539 in NPC, which may provide new insights into the mechanisms underlying tumourigenesis. In addition, the cell cycle is regulated by a family of the CDKs and their activating partners (cyclins). The G1/S phase transition is regulated primarily by D-type cyclins (D1, D2 or D3) in complex with CDK4/CDK6 and E-type cyclins (E1 or E2) in complex with CDK2. These complexes cooperate in phosphorylating and preventing Rb binding to E2F, thus activating E2F-mediated transcription and driving cells from G1 into S phase.24 Whether the CDK/pRb/E2F pathway is involved in the miR-539 induction of cell cycle arrest needs further study.

In conclusion, we here provide evidence that low expression of miR-539 contributes to the cell viability and cell cycle in NPC by directly binding CDK4 3′-UTR. Therefore, miR-539 may function as a tumour suppressor in NPC and serve as a useful therapeutic agent for miRNA-based NPC therapy.

Figure 3. miR-539 induces cell cycle arrest. (A) The effect of miR-539 on cell cycle of NPC cell lines. (B) The effect of miR-539 on cell apoptosis of NSCLC cell lines. Column, mean of three independent experiments; bars, SD; *P < 0.01

Figure 4. CDK4 is a direct target of miR-539. (A) The putative miR-539 binding sequence in the 3′-UTR of CDK4 mRNA. Mutation was generated on the CDK4 3′-UTR sequence in the complementary site for the seed region of miR-539. (B) Suppressed luciferase activity of wild-type 3′-UTR of CDK4 by miR-539 mimic. HEK293T cells were cotransfected pGL3-CDK4-3′-UTR or pGL3-CDK4-3′-UTR-mut, and miR-539 mimic or NC duplex. Firefly luciferase activity of each sample was measured 48 h after transfection and normalized to Renilla luciferase activity. Column, mean of three independent experiments; bars, SD; *P < 0.01. (C) The expression of endogenous CDK4 regulated by miR-539. The expression level of endogenous CDK4 in SUNE-1 cells was analyzed 48 h after transfection with miR-539 mimic, miR-539 inhibitor or NC duplex by Western blotting. GAPDH was used as an internal control.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES