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miR-206 inhibits human laryngeal squamous cell carcinoma cell growth by regulation of cyclinD2

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Abstract. – OBJECTIVE: Accumulating evidence has shown that microRNAs (miRNAs) are aberrantly expressd in many malignancies and crucial to tumorigenesis. Herein, we identified the role and mechanism of miR-206 in laryngeal squamous cell carcinoma (LSCC) growth.

PATIENTS AND METHODS: Quantitative real-time PCR was performed to detect the relative expression level of miR-206 in LSCC tissues. Crystal violet and flow cytometry were conducted to explore the effects of miR-206 on the proliferation and cell cycle of human LSCC cell line, respectively. The impact of miR-206 overexpression on putative target cyclinD2 were subsequently verified via Western blot. Tumor growth assay was performed to testify the effect of miR-206 on the tumor growth *in vivo*.

RESULTS: MiR-206 expression was frequently ($p < 0.05$) down-regulated in LSCC specimens. Overexpression of miR-206 in Hep-2 cell inhibited the proliferation by blocking the G1/S transition as well as suppressed the growth of xenograft tumors in mice, implying that miR-206 functions as a tumour suppressor in the progression of LSCC. Overexpression of miR-206 significantly decreased ($p < 0.05$) the protein level of cyclinD2, which has previously been identified as a direct targets of miR-206.

CONCLUSIONS: Altogether, our results identify a crucial tumour suppressive role of miR-206 in LSCC growth, at least partly via up-regulation of cyclinD2 protein levels, and suggest that miR-206 might be a candidate prognostic predictor or an anticancer therapeutic target for LSCC patients.

Key Words:
miR-206, Laryngeal squamous cell carcinoma, Proliferation, CyclinD2.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide[1]. Laryngeal carcinoma is one of the most frequently occurring tumors of HNSCC, and more than 95% of laryngeal cancers are laryngeal squamous cell carcinomas (LSCC)[2,3]. Although diagnosis and treatment of LSCC have been improved, some cases of LSCC are still not diagnosed until the disease is at an advanced stage[4,5]. Therefore, it is urgently necessary to explore the molecular mechanisms of LSCC, which will greatly contribute to increase survival rates and improve prognosis.

Recently aberrant expression of microRNAs (miRNAs) is reported in various types of cancers[6]. MiRNAs are a class of 22 nucleotides single-stranded RNAs that negatively regulate gene expression post-transcriptionally[7]. Since miRNA can potentially regulate hundreds of targets genes, miRNAs play important regulatory roles in a wide range of biological functions such as cell growth, proliferation, differentiation and cell death[8,9]. Growing evidence has indicated that miRNAs were deregulated in many types of cancers and these unique miRNA expression profiles would be a useful biomarker for each cancer type[10]. In terms of miR-206, it has been reported to be downregulated in several types of cancer such as breast cancer[11], gastric cancer[12], endometrioid adenocarcinoma[13] and hepatocellular carcinoma[14]. However, the role of miR-206 and its direct functional targets in human LSCC are still poorly understood.

In the present study, we investigated expression levels of miR-206 in primary LSCC and adjacent normal tissues. Overexpression of miR-206 in Hep-2 cells inhibited the proliferation by blocking the G1/S transition as well as suppressed the growth of xenograft tumors in mice. In addition, a reverse correlation between miR-206 and cyclinD2 expression was noted in Hep-2 cells. Based on these results, we postulate that miR-206 functions as a tumour suppressor in the progression of LSCC by regulation of cyclinD2.

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Patients and Methods

Tissue Samples

Fresh tissues of human laryngeal carcinoma and adjacent non-tumor tissues were obtained from fifteen patients who underwent surgery at First Affiliated Hospital of Xinxiang Medical University (Xinxiang, Henan, CHN). Written consent of tissue donation for research purposes was obtained from each patient. The study protocol was approved by the Scientific and Ethical Committee of Xinxiang Medical University. None of patients had received chemotherapy, radiotherapy and immunotherapy before surgery. Both tumor and adjacent non-tumor tissues were sampled, as confirmed by pathological examination. The specimens were snap frozen in liquid nitrogen and stored at -80ºC.

Cell Culture

LSCC cell line Hep-2 cell was purchased from the Cell Resource Center of Shanghai Academy of Life Science of Chinese Academy of Science (Shanghai, CHN). Cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in a humidified atmosphere containing 5% CO_2 at 37°C. The growth medium was renewed every other day.

Cell Transfection

The miR-206 mimics and non-specific miR control (NC) were synthesized and purified by Genepharma Biotech (Shanghai, China). Cells cultured in 6-well culture plate were transfected with miR-206 mimics or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, respectively. After 48 hours transfection, cells were harvested for further experiments.

Quantitative Real-Time PCR (qPCR)

Total RNA from human laryngeal tissues and cells was prepared using Trizol reagent (Invitrogen). The cDNA was generated from 3 g of total RNA with oligo dT primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. For analysis of miR-206 expression, quantitative real-time PCR (qPCR) was performed using Platinum SYBR Green qPCR SuperMix-UDG system (Invitrogen) on an ABI7900HT System. U6 small nuclear RNA was used as an internal control to normalize RNA input. Gene expression was normalized to internal controls and fold changes were calculated using relative quantification (2-ΔΔCt).

Cell Proliferation

Cells after transfected with miR-206 mimics or NC were cultured in 96-well plates (3,000 cells/well), After 48 and 72 hours of culture, the number of cells was determined using crystal violet assay. Wells containing known cell numbers (0, 1,000, 2,000, 5,000, 10,000, 20,000, or 40,000 cells/well; six wells/cell density) were treated in the similar fashion to establish standard curves. Cell proliferation studies were run in four independent experiments.

Cell Cycle Analysis

To detect the cell cycle distribution, cells after transfected with miR-206 mimics or NC for 48 hours were collected and washed twice with ice-cold phosphate buffered saline (PBS), and then fixed them in ice-cold 70% ethanol at 4°C overnight. Following the cells were stained with propidium oxide using the Cycle Test Plus DNA Reagent Kit (BD Biosciences, San Jose, CA, USA) following the protocol and analysed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA), and the results were interpreted using Modifit and CellQuest software. The percentages of the cells in G0-G1, S, and G2-M phases were counted and compared. All of the samples were assayed in triplicate.

Western Blot

Hep-2 cells transfected with miR-206 mimics or NC were prepared, 20 g of proteins were fractionated by SDS-PAGE, and transferred to nitrocellulose membrane. After SDS-PAGE and blotting, the membrane was probed with cyclinD2 antibody (1:2000; Sigma-Aldrich, St. Louis, MO, USA) and anti-GAPDH (1:5000; Kangcheng, Shanghai, CHN). Protein bands were visualized by enhanced chemiluminescence. Western blotting of GAPDH on the same membrane was used as a loading control.

Tumor Growth Assay

Twelve BALB/c nude mice aged 5 to 6 weeks were used in this tumor growth assay. Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Xinxi-

ang Medical University. Six mice were injected subcutaneously with 100 l suspension of (1×10^6) miR-206 transfected Hep-2 cells. The other six mice were injected subcutaneously with NC transfected Hep-2 cells as control. After 30 days, the experiment was terminated and the tumors were then excised. The weights of tumors were measured.

Statistical Analysis

All values were expressed as means ± SD. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc analysis. Statistical significance was defined as $p < 0.05$.

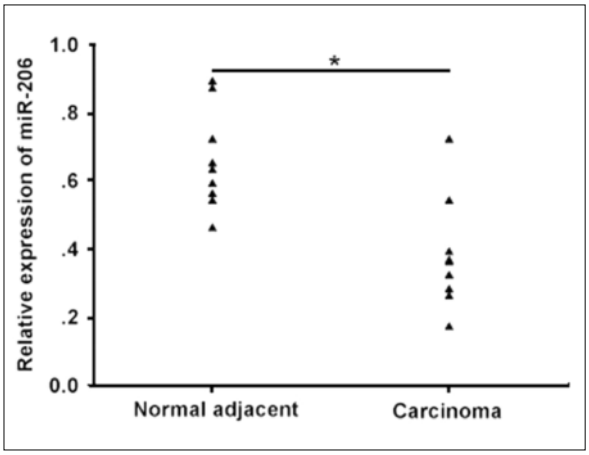

Figure 1. Decreased expression of miR-206 in patient's LSCC tissues. MiR-206 expression was examined in primary tumor (n = 15) or in adjacent normal tissues (n = 15) of LSCC patients by quantitative real time PCR. *Different ($p < 0.05$) from normal controls.

Results

MiR-206 Was Frequently Down-Regulated in Clinical Specimens

We firstly examined miR-206 expression levels in 15 pairs of frozen samples from LSCC patients by TaqMan real time RT-PCR. Our results showed that miR-206 expression was significantly ($p < 0.05$) decreased in LSCC tissues in comparison with adjacent normal tissue (Figure 1).

Effect of miR-206 on LSCC Cell Proliferation and cell cycle

To validate if miR-206 regulates LSCC cell growth, we performed a proliferation assay by transfecting miR-206 mimics or NC into Hep-2 cells. After transfection, the miR-206 is highly increased when compared to NC (Figure 2A). As expected, the overexpression of miR-206 induced significant inhibition ($p < 0.05$) on cell proliferation in Hep-2 cells (Figure 2B). Meanwhile, compared to NC, the cell ratio of G0/G1-phase robustly increased ($p < 0.05$) and the cell ratio of S-phase significantly decreased ($p < 0.05$) correspondingly after transfected with miR-206 mimics (Figure 3).

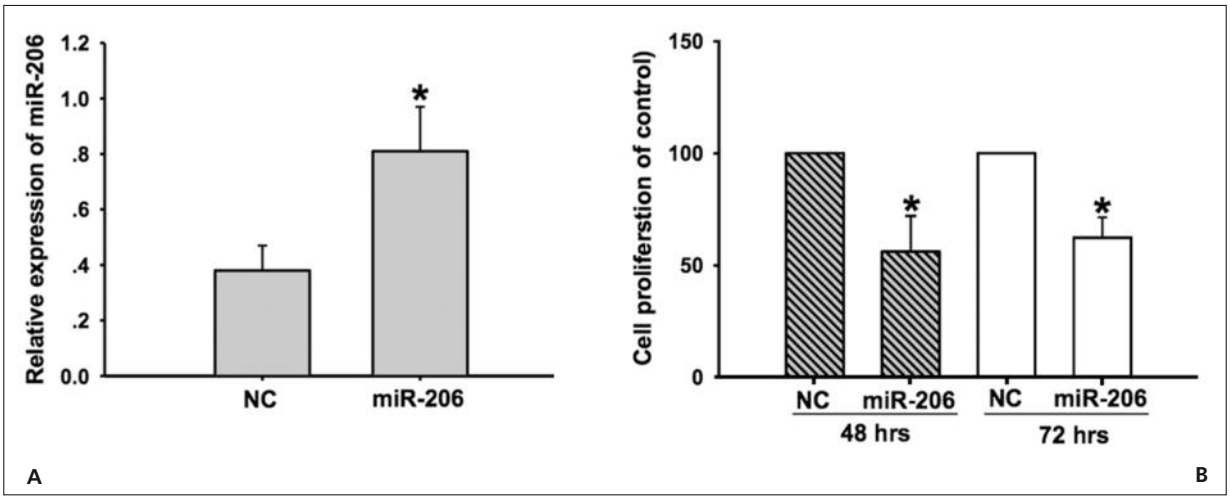

Figure 2. Ectopic overexpression of miR-206 inhibits LSCC cell proliferation. ***A,*** LSCC cells (Hep-2) were transfected with miR-206 or NC, and miR-206 expression was examined by quantitative real time PCR. ***B,*** Cell proliferation was measured by crystal violet assay at different time points. *Different ($p < 0.05$) from NC control.

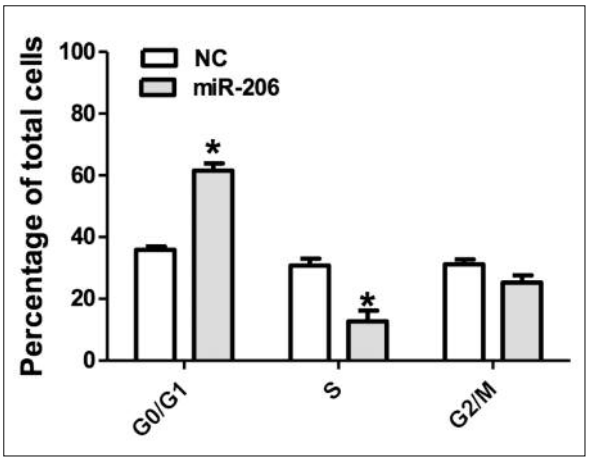

Figure 3. Effects of miR-206 overexpression on Hep-2 cell cycle. Cell cycle were determined by flow cytometric analysis. The results are represented as a percentage of cell number relative to the control cells (n = 4). *Different ($p < 0.05$) from NC control.

Overexpression of miR-206 Silenced CyclinD2

To explore the mechanism of growth inhibition induced by miR-206, we investigated whether miR-206 could regulate cyclinD2 expression in LSCC cells. After transfected with various concentration of miR-206 (0, 10, 20, 30 nM) and then examined cyclinD2 expression levels. As shown in Figure 4, overexpression of miR-206 caused a dose-dependent ($p < 0.05$) decrease in cyclinD2 protein levels.

MiR-206 Suppresses Tumorigenicity in vivo

To confirm the above findings, an *in vivo* tumor model was used. MiR-206 mimics-transfected Hep-2 cells and NC-transfected Hep-2 (NC) were injected separately into two groups of nude mice (n = 16). 30 days after injection, the average tumor weight was significantly reduced in the miR-206 group (Figure 5), but no difference in body weight was found between the miR-206 and the NC treated mice (data not shown).

Discussion

Dysregulation of miRs has been observed to be correlated with tumorigenesis and progression in different types of tumor. In the present study, we demonstrated that miR-206 was frequently downregulated in priamry LSCC specimens. Transfection of miR-206 mimics in Hep-2 cells was able to reduce cell proliferation and arrest cell cycle. Moreover, miR-206 significantly inhibited tumor growth *in vivo*. Finally, cyclinD2 was identified as a direct target of miR-206. Therefore, these results highlight the significance of miR-206 as a tumor suppressor in LSCC growth in part by targeting cyclinD2.

Previous studies[11-14] indicated decreased miR-206 expression and its tumor suppressive function in many human malignancies. In agreement with these reports, our current work also found that miR-206 was frequently downregulated in LSCC clinical specimens. To reveal the roles of miR-206 in LSCC growth, we ectopically increased the miR-206 levels in Hep-2 cell and observed significant inhibitory effects on cell proliferation and xenograft tumor growth both *in vitro* and *in vivo*, revealing its potential tumor suppressor role in LSCC growth and development. These findings were similar to the investigations in ovarian cancer[15], hepatocellular carcinoma[16]

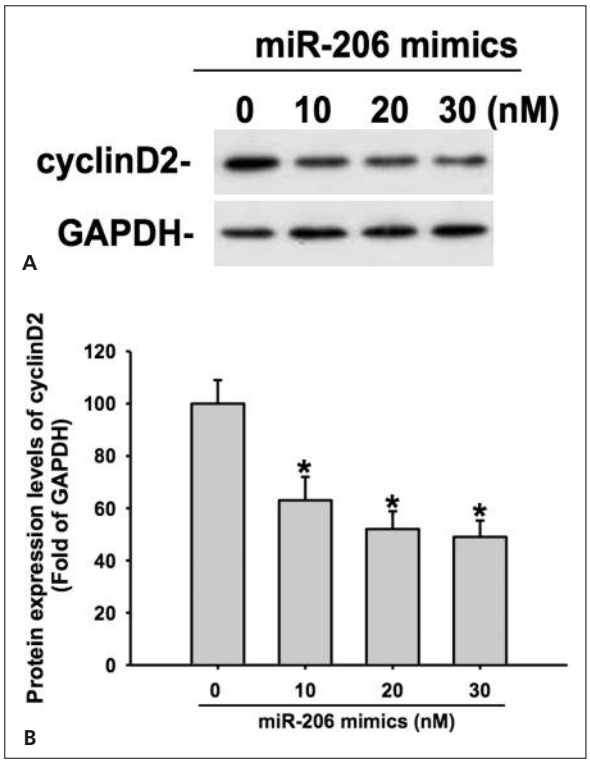

Figure 4. ***A, B,*** Overexpression of miR-206 reduced the protein levels of cyclin D2. The protein levels of cyclin D2 were detected by Western blot after transfected with miR-206 and NC. The relative expression of cyclin D2 was normalized to the endogenous control GAPDH. *Different ($p < 0.05$) from the NC control.

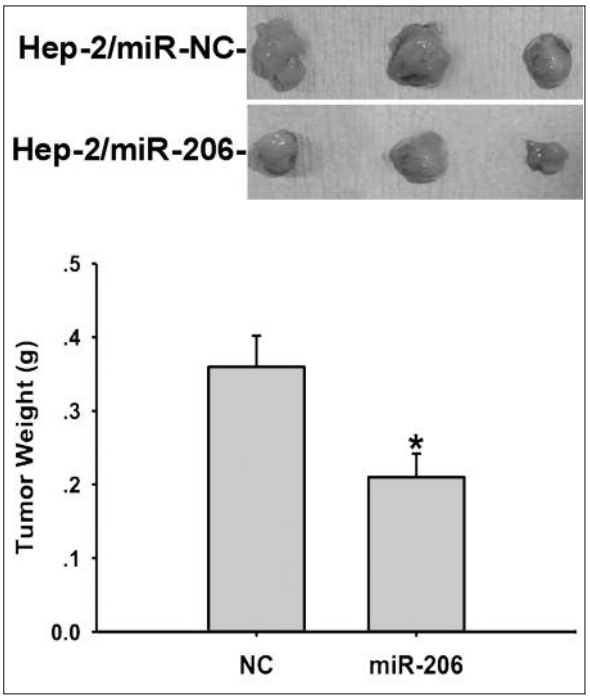

Figure 5. miR-206 inhibits tumor growth *in vivo*. Hep-2 ells transfected with miR-206 or NC were injected subcutaneously in the flanks of nude mice, respectively (n =12). After 30 days, the experiment was terminated and the tumors were collected. ***A,*** Representative photographs of subcutaneous tumor regeneration from Hep-2/miR-NC and Hep-2/miR-206 cells. ***B,*** Average of xenograft tumor weight. *Different ($p < 0.05$) from NC control.

and melanoma[17] that restoration of miR-206 expression suppresses cell proliferation in these tumor cell lines.

It is now clear that miRNAs execute their oncogenic or tumor suppressor functions by regulating the expression of target genes[18,19]. With regard to miR-206, several targets have been demonstrated in recent reports including cyclinD2[20-22]. Cyclin D2 is a protein belonging to the highly conserved cyclin family, which characterized as the regulator of the cell cycle at the point of transition from G1 to S phase during the cell cycle[23-25]. Furthermore, ectopic overexpression of cyclinD2 was frequently observed in various cancers including B-cell malignancies[26], gastric cancer[27], kidney cancer[28]. Our results demonstrated that increase expression of miR-206 dose-dependently silenced cyclinD2 expression, which partially involved in the impaction on LSCC cell proliferation and cell cycle. All these findings documented that miR-206 repressed cyclinD2 expression, which in turn inhibited the growth and tumorigenicity of LSCC cells.

Conclusions

We proved that miR-206 was markedly down-regulated in human LSCC tissue. Increasing the expression of miR-206 may lead to LSCC cell proliferation suppression and cause cell cycle arrest. Though there is still much to learn about the role of miR-206 in LSCC tumorigenesis, miR-206 provides us with a new potential target for LSCC treatment.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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