

Original Article

Downregulation of miR-204 is associated with poor prognosis and promotes cell proliferation in hypopharyngeal squamous cell carcinoma (HSCC)

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Abstract: MicroRNAs (miRNAs) were identified to be involved in tumor progression and prognosis. However, the clinical significance and biological function of miR-204 in hypopharyngeal squamous cell carcinoma (HSCC) are not well appreciated. Therefore, the aim of this study is to explore the role of miR-204 in HSCC. The miR-204 expression was determined in 56 pairs of HSCC tumor and adjacent non-tumor tissues by quantitative real-time reverse transcriptase-PCR (qRT-PCR). Kaplan-Meier survival curve analysis and log-rank test were used to analyze the association between miR-204 expression and clinicopathological parameters and the over survival (OS) time in HSCC patients. Univariate and multivariate Cox analysis was applied to investigate the predicted risk factors for OS. Moreover, CCK8 cell proliferation assays, flow cytometry analysis and western-blot analysis were performed to examine the cell growth and cell cycle related protein expression in FaDu cells. In the study, our results reported that miR-204 was down-regulated in HSCC tissues. The patients with lower miR-204 expression had significantly poor OS time. Multivariable Cox analysis demonstrated that lower miR-204 expression was an independent risk factor in HSCC patients. Furthermore, CCK8 cells assays and cell cycle analysis showed that over-expression of miR-204 significantly inhibited cell proliferation, S phase cell number and inhibited the cell cycle related protein expression of CyclinD1, CDK4 and CDK6, but up-regulated the p21 expression in FaDu cells. Thus, our study demonstrated that miR-204 was downregulated in HSCC and upregulation of miR-204 suppressed cell proliferation, which highlighted that miR-204 could have potential therapeutic applications in HSCC.

Keywords: Hypopharyngeal squamous cell carcinoma, miR-204, cell proliferation, prognosis

Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) accounts for about ~5% in all head and neck malignant tumors and is an aggressive malignancy presenting an incidence of about 10 cases per million people-years in the world [1]. Advances in surgical techniques and peri-operative management have enhanced the over survival rates in HSCC patients. Due to high rate of local-regional recurrence, distant invasion or second primary tumors, the HSCC remains unsatisfactory prognosis and the 5-year survival rates about 35% [2, 3]. Thus, to investigate and identify novel therapeutic targets in HSCC could significantly improve the treatment of HSCC.

MicroRNAs (miRNAs) are a conserved class of about 22 nucleotide RNAs that acting as key

roles in various biological processes including tumor progression [4, 5]. Recent findings have demonstrated that miRNAs were association with hypopharyngeal squamous cell carcinoma progression. Such as, microRNA-504 inhibited cancer cell proliferation via targeting CDK6 in hypopharyngeal squamous cell carcinoma cells [6]. MiR-489 acted as a tumour-suppressive miRNA and targeted PTPN11 to inhibit cell proliferation [7]. MiR-21 was up-regulated in HSCC and upregulated expression of mir-21 was associated with clinical stage, T classification, pathologic differentiation, and lymph node positivity [8]. In addition, MiR-15a induced HSCC cell apoptosis by targeting BCL2L2 and BCL2 in HPV-positive HSCC [9]. MiR-140-5p suppressed tumor migration and invasion by inhibiting ADAM10-mediated Notch1 signaling pathway in HSCC [10].

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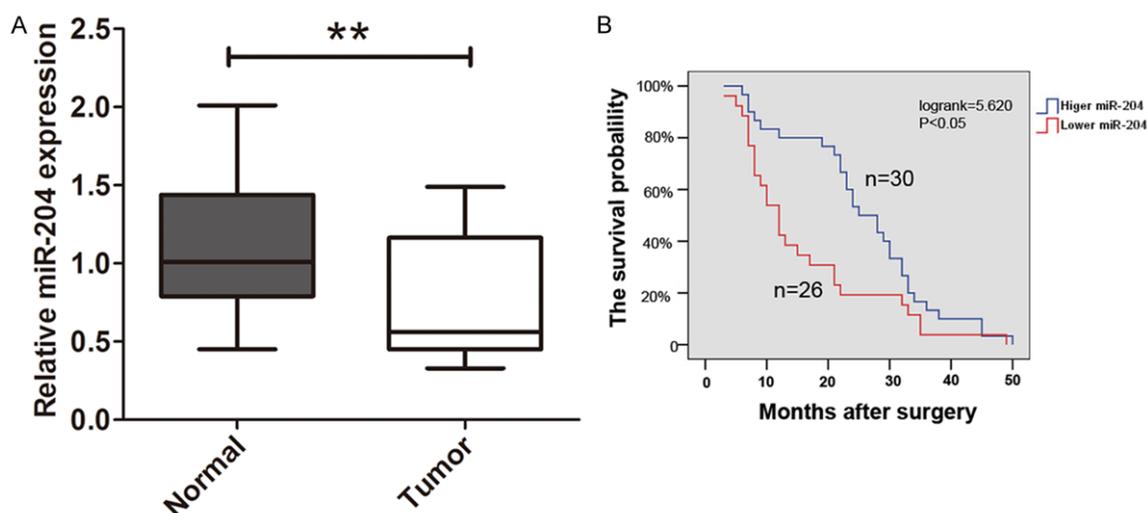


Figure 1. Expression of miR-204 in HSCC tissues was down-regulated. A. Relative expression levels of miR-204 in HSCC tissues and adjacent normal tissues were evaluated by qRT-PCR assays (n=56). U6 was used the control. B. The association between miR-204 expression and the over survival time was analyzed by Kaplan-Meier survival analysis and log-rank test, U6 was used the control, **P<0.05.

Decreased miR-204 expression was identified in many human solid tumors, including non-small cell lung cancer [11], bladder cancer [12], gastric cancer [13], and endometrioid endometrial cancer (EEC) and so on [14]. Flores-Pérez et al found that miR-204 targeted ANGPT1 and TGFBR2 genes and regulated cell angiogenesis in breast cancer [15]. Lin et al reported miRNA-204-5p functioned as tumor suppressor to promote cell apoptosis by targeting BCL2 in prostate cancer [16]. However, the biological role of miR-204 has not been studied in HSCC so far.

In present study, our results revealed that miR-204 was down-regulated in HSCC tissues compared adjacent normal tissues. Lower miR-204 expression had significantly poor OS and acted as an independent factor in predicting poor prognosis of HSCC patients. Moreover, over-expression of miR-204 inhibited the cell proliferation in FaDu cells. Thus, our study suggested that miR-204 could act as a potential therapeutic target of HSCC.

Materials and methods

Patient specimens

The 56 cases of tissue specimens of HSCC and matched adjacent non-tumor tissue were collected from patients undergoing surgical resection between March 2008 and Feb 2013 at

Department of Otorhinolaryngology, The Second Clinical Medical College of Jinan University. Patients had no received chemotherapy or radiation therapy before operation in this study. HSCC tissues were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. The over survival is defined from the surgery time to death or tumor recurrence. Written informed consents were obtained from all subjects, and this consent procedure was approved by the ethics boards of The Second Clinical Medical College of Jinan University.

Cell culture

The HSCC cell lines FaDu were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Invitrogen, Carlsbad, CA, USA), and supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere containing 5% CO_2 at 37°C

Cell proliferation assay

FaDu cells were transfected using miR-204 mimic or miR-NC and seeded in 96-wellplates at 4000 cells per well. After transfection at 1, 2, 3, 4 and 5 days, cell proliferation was determined by CCK-8 assay (Dojindo, Japan) according to the manufacturer's instructions.

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Table 1. Correlation between miR-204 expression and clinicopathologic data in HSCC patients was evaluated

Characteristics	Patients number	MiR-204 expression		Chi-squared <i>P</i> -value
		Lower (n=26)	Higher (N=30)	
Age (years)				0.666
≤60	22	11	11	
>60	34	15	19	
Gender				0.536
Male	32	16	16	
Female	24	10	14	
Primary location				0.317
Sinus piriformis	23	12	11	
Posterior pharyngeal wall	23	8	15	
Postcricoid area	10	6	4	
Smoking				0.666
Yes	34	15	19	
No	22	11	11	
Differentiation degree				0.016**
Well differentiation	12	5	7	
Moderate differentiation	23	6	17	
Poor differentiation	21	15	6	
Lymph node metastasis				0.009**
NO	21	5	16	
N1-N3	35	21	14	
T category				0.303
T1, T2	19	7	12	
T3, T4	37	19	18	
Clinical stage				0.025**
I-II	24	7	17	
III-IV	32	19	13	

**P<0.05.

Real-time quantitative RT-PCR

Total RNA from tissues and cells were isolated with Trizol reagent (TAKAKA, Dalian, China), the experiment was carried out according previous description [17]. The first-strand cDNA was generated with a miRNA cDNA Kit First Strand Synthesis Kit according to the manufacturer's instructions (TAKALA, Dalian, China) and the QRT-PCR was performed by miRNA Real-Time PCR Assay Kit (TAKALA, China). The expression of miR-204 was quantified by measuring cycle threshold (Ct) values and normalized using the $2^{-\Delta\Delta Ct}$ method. The U6 were used for normalization. The primers sequence for miR-204, forward, 5'-CAUCUUCAGUACAGUGUUGGA-3' and for U6, forward, 5'-CTCGCTTCGGCAGCACA-3'.

Flow cytometric analysis for cell cycle

Cells were obtained and washed in PBS for two times, followed by permeabilization with cold 70% methanol for overnight at 4°C. Cell cycle for G1, G2, S phase were detected using Cell Cycle and Apoptosis Analysis Kit (Beyotime, Shanghai, China) according to the manufacturer's instruction and was detected at 488 nm using a flow cytometer (BD Biosciences, San Jose, CA) and analyzed with ModFit Lt (Verity Software House).

Western-blot assays

The FaDu cells were lysed with RIPA (Sigma) and supplemented with protease inhibitors (Sigma) according to the manufactures protocol. Equivalent amounts of cell protein (40 µg) were separated electrophoretically in 8-10% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was blocked with 5% nonfat milk, the membranes were incubated with primary antibodies with CyclinD1 (1:1000, Santa Cruz Biotechnology, Inc., USA), CDK4 (1:500, Santa Cruz Biotechnology, Inc., USA), CDK6 (1:500, Santa Cruz Biotechnology, Inc., USA), p21 (1:1000, CST, USA) and GAPDH (1:1,000, Abcam, USA), at

4°C overnight. Then, the membranes were incubated for 2 hours at room temperature using horseradish peroxidase-conjugated secondary antibody. GAPDH was used as a protein-loading control. The immune complexes were detected by chemiluminescence (ECL).

Statistical analysis

Statistical analysis were evaluated by using the SPSS software (17.0: IBM SPSS, IL, USA), the P<0.05 as the statistical significance. All statistical tests were two-sided. The Cox proportional hazards regression analysis were performed to evaluate the association of clinicopathological factors and miR-204 to the OS. The Kaplan-Meier curves were also performed.

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Table 2. The results of univariate and multivariate Cox regression analysis were showed in 56 cases HSCC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
Gender	1.081	0.632-1.689	0.783			
Age	1.212	0.733-2.063	0.538			
Primary location	1.159	0.694-1.968	0.567			
Smoking	0.946	0.344-1.762	0.932			
Differentiation degree	1.943	1.244-3.109	0.004**	1.668	1.044-2.788	0.034**
Lymph node metastasis	2.122	1.441-3.321	0.001**	1.699	1.198-2.988	0.029**
T category	1.233	0.879-2.155	0.422			
Clinical stage	2.238	1.335-3.359	0.001**	1.967	1.122-2.987	0.003**
miR-204	0.268	0.139-0.662	0.001**	0.319	0.176-0.790	0.001**

Abbreviations: HR, hazard ratio, CI, confidence interval, **P<0.05.

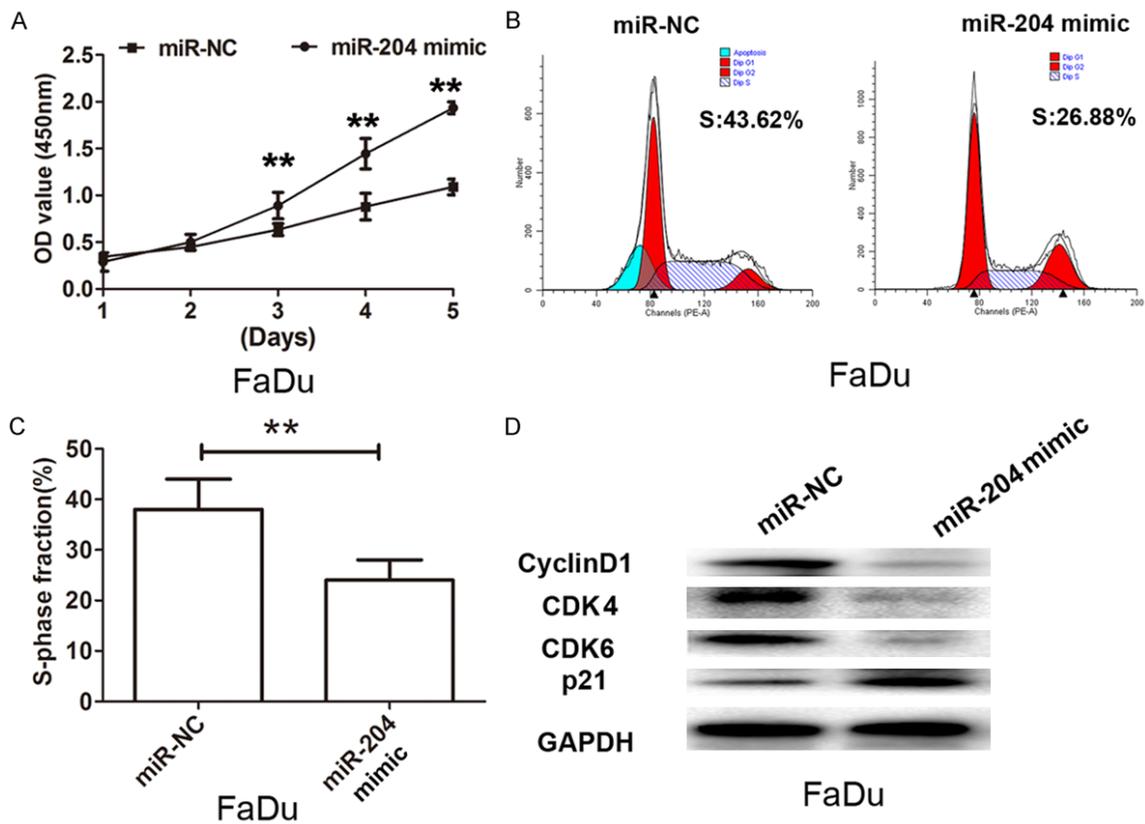


Figure 2. MiR-204 inhibited the FaDu cell proliferation and S phase cell proportion in HSCC. A. CCK8 cell proliferation assays were performed to detect the cell proliferation ability when miR-204 mimic was transfected into FaDu cell. B, C. Cell cycle assays and cell cycle analysis were performed to detect the cell cycle progression when miR-204 mimic was transfected into FaDu cell. D. The relative expression levels of Cyclin D1, CDK4, CDK6 and p21 were detected by Western-blot assays when miR-204 mimic was transfected into FaDu cell. The data are presented as the mean \pm SD for three independent experiments, **P<0.05.

Results

The expression of miR-204 in HSCC tissues

The 56 cases human HSCC tissues and adjacent normal tissues were detected to investi-
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gate the relative expression levels of miR-204 using qRT-PCR assays. The data showed that the expression levels of miR-204 were significantly down-regulated in 38 out of 56 cases of HSCC tissues, compared with adjacent normal

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tissues (**Figure 1A**, $P < 0.05$). To further assess the association between clinicopathological features and miR-204 expression levels in HSCC patients, the median fold value of miR-204 expression (0.532) in HSCC tissues was used as the cut-off point to classify HSCC patients into two groups: low-level expression of miR-204 and high-level expression of miR-204. The results of Pearson's correlation χ^2 test showed that miR-204 expression was significantly associated with tumor differentiation degree ($P = 0.016$), lymph node metastasis ($P = 0.009$) and clinical stage ($P = 0.025$) (**Table 1**, $P < 0.05$). MiR-204 expression was not associated with other clinicopathological factor such as, age, gender, smoking, and so on.

Expression of miR-204 was correlated with patients' overall survival (OS) time

Furthermore, we explored that whether miR-204 correlated with HSCC patients' OS. Kaplan-Meier survival curves and log-rank test was used to analyze the association between miR-204 expression and the over survival (OS) time. The results showed that HSCC patients with lower expression of miR-204 exhibited a poor the over survival time, compared with HSCC patients with higher expression levels of miR-204 (**Figure 1B**, Log-rank = 5.620, $P < 0.05$). Moreover, univariate and multivariate Cox analysis showed that tumor differentiation degree (HR=1.668, 95% CI: 1.044-2.788, $P = 0.034$), lymph node metastasis (HR=1.699, 95% CI: 1.198-2.988, $P = 0.029$), clinical stage (HR=1.967, 95% CI: 1.122-2.987, $P = 0.003$) and lower miR-204 expression (HR=0.319, 95% CI: 0.176-0.790, $P = 0.001$) were independent risk factor for predicting the prognosis of HSCC patients (**Table 2**, $P < 0.05$).

MiR-204 inhibits the HSCC cell proliferation, S phase cell number and cell cycle related protein expression

To determine whether the miR-204 expression levels affected cell proliferation in HSCC cell, we performed the CCK8 cell proliferation and cell cycle assays to detect the cell proliferation ability in FaDu cell. The results exhibited that when miR-204 was up-regulated by transfected by miR-204 mimic, the cell proliferation was inhibited in FaDu cell by CCK8 assays (**Figure 2A**). Furthermore, compared the control group, we also found that S phase cell number was

reduced when miR-204 was up-regulated in FaDu cells by cell cycle analysis (**Figure 2B, 2C**). More, we found that the cell cycle related protein expression levels of Cyclin D1, CDK4 and CDK6 were down-regulated, but p21 expression was up-regulated when miR-204 was up-regulated in FaDu cells (**Figure 2D**). Thus, these findings revealed that miR-204 acted as a tumor suppressor in HSCC cells by inhibiting the HSCC cell proliferation, S phase cell number and cell cycle related protein.

Discussion

Hypopharyngeal squamous cell carcinoma (HSCC) represents a poor prognosis compared to other head and neck squamous cell carcinomas [17]. Recent reports have suggested that microRNAs affect process of cell formation and development, cell proliferation and cell apoptosis in normal or malignant cells in human body [18, 19]. It had been identified that miR-204 functions as a tumor-suppressor via promoting cell apoptosis, conferring the resistance of cancer cells to chemotherapy, and suppressing the self-renewal of cancer stem cells (CSCs) and the epithelial to mesenchymal transition (EMT) [19, 21-23]. In the study, significantly down-regulated miR-204 was found in HSCC tumor tissues, compared to non-tumor tissues. Decreased miR-204 expression was significantly association with the over survival (OS) time and was also an independent predicted risk factor in HSCC by multivariate Cox analysis. These data showed that miR-204 could function as a biomarker for predicting the prognosis in HSCC patients.

MiR-204 had been reported as a tumor suppressor in some cancers. Such as, decreased expression of miR-204 in plasma was associated with a poor prognosis in patients with non-small cell lung cancer [24]. MiR-204 expression was also been found to be correlated with chemotherapeutic resistance of breast cancer patients and decreased expression of miR-204 is associated with poor prognosis in patients with breast cancer [25]. MiR-204 inhibited EBV positive C666-1 cell invasion and metastasis partly through targeting cdc42 [26]. Low expression of microRNA-204 (miR-204) is associated with poor clinical outcome of acute myeloid leukemia (AML) patients [27]. In the study, our data demonstrated that cell prolifer-

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ation was significantly inhibited after over-expression of miR-204 compared the control group by CCK8 cell proliferation in FaDu cells. Furthermore, we also found that the S phase cell number was decreased when miR-204 was up-regulated in FaDu cells. After up-regulation of miR-204, western-blot analysis showed that cell cycle related protein CyclinD1, CDK4 and CDK6 expression levels were decreased, but increased expression of p21. Thus, these results revealed that miR-204 acted a tumor suppressor in HSCC cell.

In summary, our study demonstrated that miR-204 expression was down-regulated in HSCC and low miR-204 expression was also an independent predicted risk factor in HSCC. Function analysis demonstrated that miR-204 acting as a tumor suppressor in HSCC cells. Thus, our results suggested that miR-204 could act as a predicted biomarker and potential therapeutic target in HSCC.

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Disclosure of conflict of interest

None.

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