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MiR-221 affects the proliferation and apoptosis of laryngeal cancer cells through the PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: To investigate the effect of MiR-221 on proliferation and apoptosis of laryngeal carcinoma cells through the PI3K/AKT signaling pathway.

MATERIALS AND METHODS: LipofectamineTM 2000 liposomes were used to transfer MiR-221 analogue, MiR-221 NC into Hep-2 cells of laryngeal carcinoma. Real-time fluorescence quantitative polymerase chain reaction (PCR) method was used to detect the expression of MiR-221, MTT method was used to detect the proliferation of cells, flow cytometry was used to detect cell cycle, Western blotting was used to detect the expression of apoptosis proteinase-1 (Apaf-1) and cyclin-dependent kinase (CDK1, CDK2) protein and the activation of phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT).

RESULTS: Compared with MiR-221 NC group, the expression of MiR-221 in MiR-221 analogue group was up-regulated ($p<0.01$), the cell proliferation rate was decreased ($p<0.01$), the cell cycle was stagnated in the G1 phase ($p<0.01$), the expression levels of Cyclin A, CDK1, CDK2, PI3K, and p-AKT were significantly down-regulated ($p<0.01$), and the expression levels of Bax and Apaf-1 were significantly up-regulated ($p<0.01$).

CONCLUSIONS: MiR-221 analogues can significantly inhibit the proliferation and induce apoptosis of Hep-2 cells in laryngeal cancer, and this is achieved by blocking the PI3K/AKT signaling pathway, which also indicates that MiR-221 affects the proliferation and apoptosis of laryngeal cancer cells through the PI3K/AKT signaling pathway.

Key Words:
MiR-221, Hep-2 cells of laryngeal carcinoma, Cell proliferation, Cyclin, PI3K/AKT protein.

Introduction

Laryngeal cancer is one of the most common malignant tumors in the head and neck area[1]. It ranks second in the incidence of head and neck cancer and ranks 11th among all human cancers. Patients increased 5-year survival rate by receiving treatments such as chemotherapy, radiotherapy, and surgery[2]. However, recurrence after treatment is common. Therefore, it is important to identify new therapeutic targets for laryngeal cancer. MicroRNAs (miRNAs) are short, non-coding RNA molecules that, after transcription, regulate the expression of target genes and play a role in a variety of cellular, physiological, and pathophysiological processes[3,4]. MiR-221 is encoded from the X chromosome and acts as a carcinogenic MiRNA involved in various types of cancers[5]. It has been reported to be up-regulated in many types of tumors, including glioblastoma, bladder cancer, and papillary thyroid tumors[6,7]. MiR-221 has been shown to affect several cancer pathways by regulating multiple genes, such as estrogen receptor-αp27, p57, and receptor tyrosine kinases[8]. To the best of our knowledge, there have been no previous researches showing a link between MiR-221 and laryngeal squamous cell carcinoma (LSCC). This study aimed to elucidate the role of MiR-221 in laryngeal carcinoma cell line Hep-2. In addition, we focused on the effect of MiR-221 on PI3K/AKT signaling pathway-associated proteins, and initially explored the underlying mechanism of MiR-221 in Hep-2 cells.

Materials and Methods

Reagents and Instruments

Apoptotic protease activating factor-1 (Apaf-1), cyclin-dependent kinase (CDK) 1, CDK2, phosphatidylinositol 3-kinase (PI3K), GAPDH monoclonal antibody (CST; Danvers, MA,USA); Rabbit anti-human AKT and p-AKT polyclonal

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antibody (Abcam, Cambridge, MA, USA); methyl thiazolyl tetrazolium (MTT) and Dulbecco's Modified Eagle's Medium (DMEM) media were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA); protein concentration detection kit and cell cycle detection kit were purchased from Tiangen Biotechnology Co., Ltd. (Beijing, China); Total RNA extraction kit (QIAGEN, Germantown, USA), reverse transcription kit (Biotechnology Co., Ltd., Beijing, China), Lipofectamine™ 2000 (Thermo Fisher Sientific, Waltham, USA) flow cytometry (Beckman Coulter Biotechnology Co., Ltd., Atlanta, GA, USA); inverted fluorescence microscope (Nikon, Tokyo, Japan).

Cell Culture

The human Hep-2 laryngeal cancer cell line was purchased from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. Exponentially growing cells were used during the experiment. The specific culture method is referred to the literature[9].

MTT Assay for the Proliferation of Hep-2 Laryngeal Carcinoma Cells

1×10^4 Hep-2 laryngeal carcinoma cells were inoculated into 48-well plates, and after 24 h of culture, miR-221 analog and miR-221 NC were transfected into laryngeal carcinoma Hep-2 cells using Lipofectamine™ 2000 liposome. After continuing to culture for 48 h, MTT solution (final concentration 5 mg/ml) was added, and the cells were incubated in an incubator (37°C) for 4 h, and the supernatant was aspirated, followed by adding an appropriate amount of dimethyl sulphoxide (DMSO). The plate was gently shaken on a shaker for 10 min to dissolve the crystals, and the OD value was measured at a wavelength of 492 nm (detection of cell proliferation) using a microplate reader (Wellscan MKII type microplate reader, Labsystem, Shanghai, China), 3 multiple holes were set in each group. Cell proliferation was expressed as relative inhibition rate, and the relative inhibition rate was calculated.

Detection of the Cycle of Laryngeal Carcinoma Hep-2 Cells by Flow Cytometry

1.5×10^4 Hep-2 cells were seeded into 6-well plates, and after 24 h of culture, miR-221 analog and miR-221 NC were transfected into the laryngeal carcinoma Hep-2 cells, the culture was continued for 48 h, and were operated under aseptic conditions. When the cells were grown to log phase, 1×10^6/ml cells were collected in each group, appropriate amount of RNase (final concentration 10 mg/ml) was added, and gently pipetted with a pipette (non-contaminated pipette tip) to mix well, incubated for 1 h at room temperature. Then, PI staining solution was added, mixed by the same method as above, incubated at room temperature for 20 min in the dark, and cell cycle detection and analysis was performed by flow cytometry, each group repeated for 3 times.

Western Blotting Detection of Protein Expression in Hep-2 Cells

1.5×10^5 laryngeal carcinoma Hep-2 cells were seeded into 6-well plates, and after 24 h of culture, miR-221 analogues and miR-221 NC were transfected into laryngeal carcinoma Hep-2 cells, culture was continued for 48 h, and all were operated under aseptic conditions. When the cells grew to the log phase, the cells were collected, operated in a low temperature environment (ice bath), centrifuged, and finally the cell lysate was added to fully lyse the cells, and the supernatant was obtained by centrifugation (i.e., extraction of total protein), the protein concentration was determined using the purchased kit. After determining the protein concentration, proceed to the next step: the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was configured, sample processing was performed, and the extracted protein was subjected to high temperature denaturation (100°C), the loading buffer was added, mixed and load. Firstly, gel electrophoresis was performed for about 1-2 h, the film was transferred at low temperature for 45 min, then 5% skim milk powder was used for blocking for 1 h, and washed with Tris-Buffered Saline and Tween (TBST). Finally, the primary antibody solution was added, the antibody was diluted in a certain proportion (rabbit anti-human monoclonal antibody dilution 1:2000; rabbit anti-human polyclonal antibody dilution 1:500), incubated overnight at 4°C. On the next day, the primary antibody was recovered and washed, the secondary antibody solution was added (1:1000) and incubated for 1-2 h at room temperature. Finally, color exposure was performed, and each group was repeated 3 times.

Statistical Analysis

The data were processed using SPSS 20.0 statistical software (IBM, Armonk, NY, USA). The measurement data were expressed by $x \pm s$. The

difference between the two groups was statistically processed by the Student's *t*-test. *p*-value was considered statistically significant if $p<0.05$.

Results

Verification of MiR-221 Analogue Transfection

MiR-221 NC and MiR-221 analogs were transfected into Hep-2 cells, respectively. After 48 h of culture, the expression of MiR-221 was detected in the two groups. The results showed that the expression of MiR-221 in MiR-221 analogue group was significantly higher than that in MiR-221NC group ($p<0.01$), indicating that the MiR-221 analog was successfully transfected (Figure 1).

Effects of MiR-221 Analogues on Viability, Proliferation, and Cell Cycle of Hep-2 Cells

After transfection of Hep-2 cells with MiR-221 NC and MiR-221 analogs for 48 h, the relative inhibition rate of MiR-221 analogues was significantly lower than that of MiR-221 NC group ($p<0.01$), revealing that the proliferation of cells in MiR-221 analogue group was reduced, as shown in Figure 2. After transfection of Hep-2 cells with MiR-221 NC and MiR-221 analogs for 48 h, the cell cycle G1 of MiR-221 analogue group was significantly longer than that of MiR-221NC group, indicating that MiR-221 analogues make Hep-2 cell cycle blocked in the G1 phase (Table I).

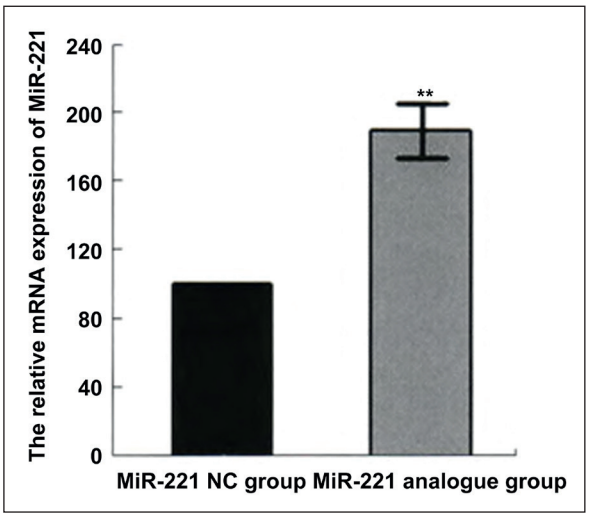

Figure 1. The expression of MiR-221 in cells transfected with MiR-221 NC and its analogues, **: $p< 0.01$.

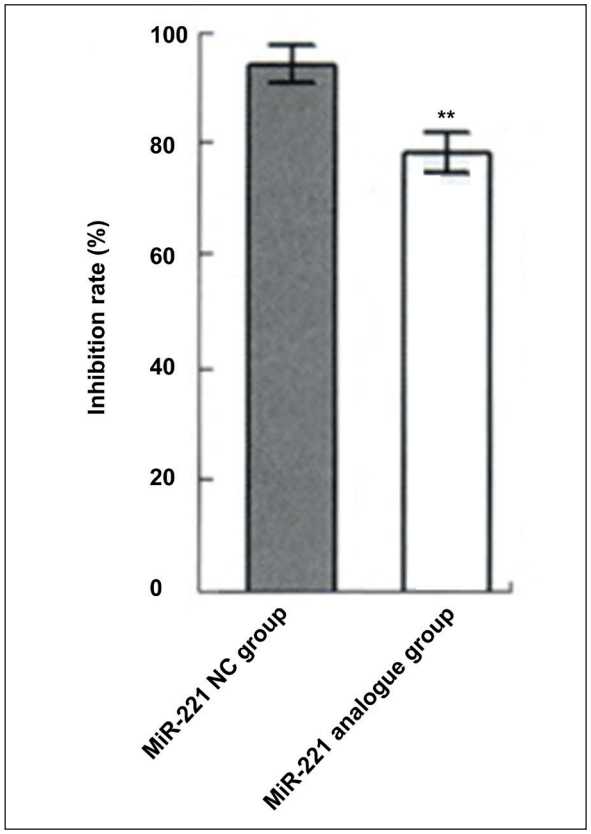

Figure 2. MTT assay of the effect of MiR-221 analogues on the proliferation of Hep-2 cells. **: $p < 0.01$.

Effects of MiR-221 Analogues on the Expression of Cyclin A, CDK1 and CDK2, and Other Related Proteins in Hep-2 Cells

MiR-221 NC and MiR-221 analogues were transfected into laryngeal carcinoma Hep-2 cells for 48 h, and the expression of related proteins was detected. Compared with MiR-221 NC group, the Cyclin A, CDK1 and CDK2 protein bands in MiR-221 analogue group were significantly shallower, indicating that the expression levels were significantly down-regulated ($p<0.01$). The protein bands of Bax and Apaf-1 were significantly deeper, showing that the expression of protein was significantly up-regulated ($p<0.01$), as shown in Table II.

Effect of MiR-221 on PI3K/AKT Signaling Pathway in Hep-2 Cells

After MiR-221 NC and MiR-221 analogues were transfected into laryngeal carcinoma Hep-2 cells for 48 h, the gray bands of PI3K and p-AKT in MiR-221 analogue group were significantly lower than those in MiR-221 NC group. Com-

Table I. The effect of MiR-221 analogues on Hep-2 cell cycle ($\bar{x} \pm s$, n=3, %).

Group	MiR-221 NC group	MiR-221 analogue group
G1	39.85 ± 3.58	53.79 ± 4.68#
S	34.61 ± 3.16	31.38 ± 2.93
G2	17.54 ± 1.60	13.83 ± 1.10

Note: Compared with the MiR-221 NC group: #p<0.01.

Table II. The effect of MiR-221 analogues on the expression level of marc-related proteins in Hep-2 cells of laryngeal carcinoma ($\bar{x} \pm s$, n = 3).

Group	Bax	Apaf-1	CyclinA	CDK1	CDK2
MiR-221 NC group	0.23 ± 0.02	0.16 ± 0.03	0.88 ± 0.08	0.89 ± 0.07	0.087 ± 0.06
MiR-221 analogue group	1.19 ± 0.12#	1.21 ± 0.09#	0.24 ± 0.02#	0.18 ± 0.02#	0.24 ± 0.01#

Note: Compared with the MiR-221 NC group: #p<0.01.

pared with MiR-221 NC group, the expression levels of PI3K and p-AKT protein in MiR-221 analogue group were significantly down-regulated, and the difference was statistically significant (p<0.01; Table III).

Discussion

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression by base-pair messenger RNA[10]. The current research results show that MiRNA plays an important role in key biological processes such as cell proliferation, apoptosis, development, and differentiation[11,12]. Among them, the association of MiRNA with diseases, especially the relationship between MiRNA and human cancer, has aroused great interest in both science and business. MiRNAs have been shown to play a crucial role in tumor formation, and many miRNAs have been identified as tumor suppressor genes or oncogenes[13]. Studies[14] have shown that miR-221 can induce cell apoptosis *in vitro* and *in vivo* in Hep-2 cells, which may be closely related to the development of laryngeal carcinoma and prognosis of patients. The results of this study referred that inhibition of miR-221 in Hep-2 cells induces apoptosis. MiR-221 is upregulated in a variety of malignancies. In addition, studies have shown that miR-221 can silence a variety of potential target genes to regulate cell life activities[15]. However, the specific mechanism of action of miR-221 in laryngeal cancer is not known[16,17]. The results of this study pointed that increased expression of miR-221 attenuated the proliferation of laryngeal carcinoma Hep-2 cells and arrested the cell cycle in the G1 phase, leading to cell replication failure, thereby inhibiting the malignant proliferation of laryngeal carcinoma Hep-2 cells. Apaf-1 is a constituent protein of apoptotic bodies, and apoptosis is caused by exogenous and endogenous factors[18]. In the intrinsic apoptotic pathway, cytochrome C, AP-1 interact with caspase-9 precursor, and caspase-9 precursor is activated. Studies have shown that Apaf-1 down-regulation is related with decreased apoptosis of colorectal cancer cells and poor prognosis, overexpression of Apaf-1 in U87 cells has the effect of inhibiting cell proliferation and promoting apoptosis[19,20].

Table III. The influence of MiR-221 analogues on expression levels of PI3K/AKT signaling pathway related proteins in Hep-2 cells of laryngeal carcinoma ($\bar{x} \pm s$, n = 3).

Group	PI3K	P-AKT/AKT
MiR-221 NC group	1.23 ± 0.10	1.09 ± 0.05
MiR-221 analogue group	0.18 ± 0.02#	0.19 ± 0.02#

Note: Compared with the MiR-221 NC group: #p<0.01.

The results of this study also reveal that Apaf-1 expression can induce apoptosis and cell cycle arrest in Hep-2 cells, which is consistent with previous studies. In addition, BAX is a pro-apoptotic protein, and Bax forms a heterodimer, which blocks the transmission of apoptotic signals and prolongs cell survival. In addition, the results of this study show that up-regulation of miR-221 leads to an increase in tyrosine kinase activity, thereby activating downstream related signals, promoting cell deterioration, and the PI3K/AKT signaling pathway is an important pathway. PI3K is a phosphokinase phosphorylated by phosphatidylinositol and similar compounds that produces important second messengers in the growth signaling pathway[21]. PI3K is a heterodimer of the regulatory unit p85 and the catalytic subunit p110, and P85 is activated (phosphorylated) by a protein tyrosine kinase that mediates the association of the p110 catalytic unit with the plasma membrane through its SH2 domain. PIK3R2 is a member of the PI3K p85 subunit family. PIK3R2 is an inhibitor of PI3K/Akt signaling pathway activation, which promotes phosphorylation of AKT protein. Phosphorylated AKT activates downstream target genes and is involved in cell life activities[22,23]. This study found that increased expression of miR-221 in laryngeal cancer cells significantly reduced the expression of PI3K and p-AKT.

Conclusions

Summarily, miR-221 inhibits the proliferation of laryngeal carcinoma Hep-2 cells and induces apoptosis, and it acts through the PI3K/AKT signaling pathway, but it is not ruled out that multiple signaling pathways also play a role at the same time, the occurrence of cancer involves a variety of signaling pathways, which can be followed for further research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Chen YF, Luo RZ, Li Y, Cui BK, Song M, Yang AK, Chen WK. High expression levels of COX-2 and P300 are associated with unfavorable survival in laryngeal squamous cell carcinoma. Eur Arch Otorhinolaryngol 2013; 270: 1009-1017.
2) Chu EA, Kim YJ. Laryngeal cancer: diagnosis and pre-operative work-up. Otolaryngol Clin North Am 2008; 41: 673-695.
3) Chang TC, Mendell JT. MicroRNAs in vertebrate physiology and human disease. Annu Rev Genomics Hum Genet 2007; 8: 215-239.
4) Latronico MV, Catalucci D, Condorelli G. Emerging role of microRNAs in cardiovascular biology. Circ Res 2007; 101: 1225-1236.
5) Ergun S, Arman K, Temiz E, Bozgeyik I, Yumrutas O, Safdar M, Dagli H, Arslan A, Oztuzcu S. Expression patterns of miR-221 and its target Caspase-3 in different cancer cell lines. Mol Biol Rep 2014; 41: 5877-5881.
6) Ciafre SA, Galardi S, Mangiola A, Ferracin M, Liu CG, Sabatino G, Negrini M, Maira G, Croce CM, Farace MG. Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun 2005; 334: 1351-1358.
7) Gottardo F, Liu CG, Ferracin M, Calin GA, Fassan M, Bassi P, Sevignani C, Byrne D, Negrini M, Pagano F, Gomella LG, Croce CM, Baffa R. Micro-RNA profiling in kidney and bladder cancers. Urol Oncol 2007; 25: 387-392.
8) He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, Calin GA, Liu CG, Franssila K, Suster S, Kloos RT, Croce CM, de la Chapelle A. The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A 2005; 102: 19075-19080.
9) Shen Z, Zhan G, Deng H, Kang C, Guo J. [Growth inhibitory effect of microRNA-519b-3p on larynx squamous Hep-2 cells]. Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 2014; 49: 151-156.
10) Li B, Lu Y, Wang H, Han X, Mao J, Li J, Yu L, Wang B, Fan S, Yu X, Song B. MiR-221/222 enhance the tumorigenicity of human breast cancer stem cells via modulation of PTEN/Akt pathway. Biomed Pharmacother 2016; 79: 93-101.
11) Mishra S, Yadav T, Rani V. Exploring miRNA based approaches in cancer diagnostics and therapeutics. Crit Rev Oncol Hematol 2016; 98: 12-23.
12) Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer 2006; 94: 776-780.
13) Jiang Q, Wang Y, Hao Y, Juan L, Teng M, Zhang X, Li M, Wang G, Liu Y. miR2Disease: a manually curated database for microRNA deregulation in human disease. Nucleic Acids Res 2009; 37: D98-104.
14) Xie B, Ding Q, Han H, Wu D. miRCancer: a microRNA-cancer association database constructed by text mining on literature. Bioinformatics 2013; 29: 638-644.
15) Sun X, Liu B, Zhao XD, Wang LY, Ji WY. MicroRNA-221 accelerates the proliferation of laryngeal cancer cell line Hep-2 by suppressing Apaf-1. Oncol Rep 2015; 33: 1221-1226.
16) Kan X, Sun Y, Lu J, Li M, Wang Y, Li Q, Liu Y, Liu M, Tian L. Coinhibition of miRNA21 and miRNA221 induces apoptosis by enhancing the p53 mediat-

ed expression of proapoptotic miRNAs in laryngeal squamous cell carcinoma. Mol Med Rep 2016; 13: 4315-4320.

17) Ricciardiello F, Capasso R, Kawasaki H, Abate T, Oliva F, Lombardi A, Misso G, Ingrosso D, Leone CA, Iengo M, Caraglia M. A MiRNA signature suggestive of nodal metastases from laryngeal carcinoma. Acta Otorhinolaryngol Ital 2017; 37: 467-474.

18) Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, Valtieri M, Calin GA, Liu CG, Sorrentino A, Croce CM, Peschle C. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci U S A 2005; 102: 18081-18086.

19) Lian S, Shi R, Bai T, Liu Y, Miao W, Wang H, Liu X, Fan Y. Anti-miRNA-23a oligonucleotide suppresses glioma cells growth by targeting apoptotic protease activating factor-1. Curr Pharm Des 2013; 19: 6382-6389.

20) Zang YS, Zhong YF, Fang Z, Li B, An J. MiR-155 inhibits the sensitivity of lung cancer cells to cisplatin via negative regulation of Apaf-1 expression. Cancer Gene Ther 2012; 19: 773-778.

21) Kern D, Regl G, Hofbauer SW, Altenhofer P, Achatz G, Dlugosz A, Schnidar H, Greil R, Hartmann TN, Aberger F. Hedgehog/GLI and PI3K signaling in the initiation and maintenance of chronic lymphocytic leukemia. Oncogene 2015; 34: 5341-5351.

22) Chen JJ, Zhou SH. Mesenchymal stem cells overexpressing miR-126 enhance ischemic angiogenesis via the AKT/ERK-related pathway. Cardiol J 2011; 18: 675-681.

23) Wang T, Gong X, Jiang R, Li H, Du W, Kuang G. Ferulic acid inhibits proliferation and promotes apoptosis via blockage of PI3K/Akt pathway in osteosarcoma cell. Am J Transl Res 2016; 8: 968-980.