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Effect of Silencing miRNA-221 on Proliferation of Tongue Squamous Cell Carcinoma Cells

Shuqi He, Xian Li, Renfa Lai *

Department of Stomatology, The First AffiliatedHospital of Jinan University, Stomatology Medical School of Jinan University, Guangzhou 510632,China.

*****Corresponding author: Ren-fa Lai, Department of Stomatology, The First Affiliated Hospital of Jinan University, Stomatology Medical School of Jinan University, Guangzhou 510632, China.Tel: +86 13318818388, E-mail:tlrf@ jnu.edu.cn

ABSTRACT

Objective: To investigate the effect of microRNA(miR)‑ 221 in proliferation of tongue squamous cell carcinoma(TSCC) cells in vitro and in vivo.

Methods: The UM1 cell line was transfected with miR‑ 221 inhibitor, and qRT‑ PCR analysis was used to detect the expression level of miR‑ 221 in UM1 cell line after transfection. MTS assay was performed to analyze the effect of miR-221 on proliferation after transfection. Western blot analysis was used to demonstrate the metastasis associated proteins after transfection. Tumorigenesis model in nude mice was performed to analyze the effect of miR-221 on proliferation of UM1 cell line after transfection in vivo. Data were analyzed using the Statistical Package for the Social Science (SPSS), Version 19.0. For all statistical analyses, $P<0.05$ was considered statistically significant.

Results: Ectopic transfection of the miR-221 inhibitor to the UM1 cell led to decrease inmiR-221 expression ($P<0.01$); after transfection, the proliferation rate of UM1 cell was reduced ($P<0.05$); the expression level of E-Cadherin in cells was increased, while that of the MMP2, MMP9, Vimentin were decreased. The results of the subcutaneous xenograft model in nude mice showed that low-expression of miR-221 led to decrease in tumorigenesis ability ($P< 0.01$). Immunohistochemical staining showed that E-Cadherin was increased, and MMP2, MMP9, Vimentin were decreased.

Conclusion: miR-221 gene silencing could suppress the proliferation of TSCC in vitro and in vivo.

Keywords: MicroRNA-221; tongue squamous cell carcinoma; proliferation; in vitro; in vivo

1. INTRODUCTION

Tongue squamous cell carcinoma (TSCC) is the most common oral cancer, accounting for about 1/3 to 1/2 of the prevalence rate of oral cancer. Due to abundant blood supply and lymphatic reflux of tongue, TSCC has the characteristics of high malignancy, infiltration and growth, postoperative recurrence and distant metastasis (Zanoni DK, et al. 2017). As the mechanism of TSCC is unknown, the five-year survival rate of patients still lingers at 45% to 50%, regardless of certain progress made in surgery, radiotherapy, chemotherapy, immunotherapy and multidisciplinary combination therapy (Siegel RL, et

al.2018).Therefore, it is increasingly important to improve the survival rate of TSCC patients and clarify the molecular mechanism of its occurrence and development, so as to facilitate early detection, early diagnosis and early treatment.

MicroRNA (MicroRNA) is a kind of endogenous non-coding small RNA with 18~25 bases, which widely exists in various eukaryotes and participates in regulating the proliferation, invasion and migration of tumor cells. More and more studies have shown that it can be used as a new target for tumor treatment (Esquela-KerscherA, SLACK FJ.2006). There have been reports of miRNA-221 in other malignant tumors at home and abroad, but few reports have been made on TSCC research, and the mechanism of action is still unclear.

In this study, a highly invasive TSCC cell line UM1 with high expression of miR-221 was selected as the research object, and a low expression vector of miR-221 was constructed. Through in vitro and in vivo experiments, the relationship between miR-221 expression level and tongue squamous cell carcinoma proliferation was discussed, and the related mechanism was preliminarily explored.

MATERIALS AND METHODS

1 Celllines and culture

TheTSCC cell line UM1 was cultured in RPMI 1640 supplemented with 10% fetal bovineserum (Gibco, Newyork, USA), penicillin (100U/mL), and streptomycin (100 μg/mL). Cells were maintained at 37◦C in a humidified incubator with 5% CO_2, and were passaged on reaching 80–85% confluence.

2 miRNA inhibitor and transfection

A negative control (miR-NC) and miR-221 inhibitor were purchased from Ribo Biotech (Guangzhou, China). The sequences for transfection are shown in Table 1.miR-221inhibitor is chemically modified antisense oligonucleotide, which can compete against endogenous miRNAs in RNA-induced silencing complex incorporation. Cells were plated at 50% confluence and transfected with 100 nM miR-221 inhibitor using Lipofectamine^{TM}RNAiMAX Transfection Reagent (Invitrogen, CA, USA), according to the manufacturer's protocol. Cells were harvested at 24h or 48 h after transfection.

Table-1: miR-221 inhibitor sequences for transfection

Name	Sequence
miR-221 inhibitor	GAAACCCAGCAGACAAUGUAGCU
miR-221 NC	UCUACUCUUUCUAGGAGGUUGUGA

3 Quantitative Real-Time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, CA, USA). To analyze miR-221 expression, reverse transcription PCR was performed using specific stem-loop reverse transcription primers, miRNA first strand synthesis was performed using a First Strand Synthesis Kit (Takara, Dalian, China), and qRT-PCR was performed using a Mir-X miRNA qRT-PCR SYBRKiton an Applied Biosystems 7500system. U6 was used as an internal control. The primer sequences used in qRT-PCR are shown in Table 2. Gene expression was measured in triplicate, quantified using the$2^{-\Delta\Delta CT}$ method, and normalized to a control.

Table-2: Primers for qRT-PCR

Primer name	Sequence(5'-3')
miR-221	AGCTACATTGTCTGCTGGGTTTC
miR-221 F	ACACTCCAGCTGGGAGCTACATTGTCTGCTGG
miR-221 RT	CTCAACTGGTGTCGTGGA
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT

F: forward primer, R: reverse primer, and RT: reverse transcription primer.

4 MTS experiment

At 24h, 48h and 72h after transfection, cellTiter96AQ single solution cell proliferation detection reagent was added to each well, and 10ul detection solution was added to every 100ul culture medium. After culturing in an incubator with saturated humidity at 37℃and 5% CO_2 for 4h, the 96-well plate was taken out and put in a microplate reader to detect the absorbance at the wavelength of 490 nm. For the transfected miR-221inhibitorgroup and NC group, three multiple holes were set for each group in each experiment. The mean and standard deviation of absorbance values of each group were calculated, and a histogram was drawn.

5 Western blot

48h after transfection, the cells were washed with phosphate buffer (PBS) for 3 times and lysed using RIPA buffer (Beyotime Biotechnology, Nantong, China). The total protein was determined using a BCA Protein Assay kit (Beyotime Biotechnology, Nantong, China). Equal amounts of total protein were loaded in sample path, separated and then transferred to PVDF membranes (Millipore, Massachusetts, USA). Membranes were blocked for 1h with 5% milk in TBST, incubated with rabbit anti-human E-Cadherin monoclonal antibody (1:1000, CST, Massachusetts, with 0.1% entoiodine, the cell suspension was injected subcutaneously into the right USA), or rabbit anti-human Vimentin monoclonal antibody (1:1000, Abcam, Cambridge, England), rabbit anti-human MMP-2 monoclonal antibody (1:1000, Abcam, Cambridge, England), rabbit anti-human MMP-9 monoclonal antibody (1:1000, Abcam, Cambridge, England). Then washed three times with TBST. The HRP coupled secondary antibody (1: 500, Zhongshan Jiqiao, Beijing, China) was incubated at room temperature for 1 h, TBST was used to wash the membrane, and proteins were visualized using ECL.

6 Nude mice experiment

The experimental animals were BALB/C nude mice aged 4 to 5 weeks. The twelve mice were male, weighing 16g to 20g. They were provided by Experimental Animal Center of Southern Medical University (animal license number: 4400210010608).

24h after transfection, the cells of miR-221 inhibitor group and miR-NC group were centrifuged for 5 min at 1000 r/min with 0.25% EDTA suspension. After blowing with PBS, the cells were centrifuged again, which was repeated for 3 times. Then, the cells were resuspended in serum-free RPMI 1640 culture medium containing 1% antibiotics, and the concentration was adjusted to 5×10^7 cells/ml and placed on ice for later use. After disinfection of local skin armpit with a 1 ml injector, and each one was inoculated with 0.2 ml(1×10^7 cells). At

the end of the third week, the nude mice were killed by cutting off their necks and the tumor mass was peeled off. The tumor volume was calculated according to the following formula: tumor volume (mm^3)= 0.5× long diameter (mm)× short $diameter^2$(mm^2). The tumor was preserved in 4% formaldehyde solution for later use. Conventional paraffin sections, hematoxylin-eosin staining and immunohistochemistry were performed. Finally, histopathological examination was performed.

7 STATISTICAL ANALYSES

SPSS 19.0 software was used for statistical analysis of the data, and Results were represented as mean±standard deviation (SD). Two groups were analyzed by independent sample t-test, and multiple groups were analyzed by one-way ANOVA. *P*values <0.05 were regarded as statistically significant.

RESULTS

The qRT-PCR results after transfection of miR-221 inhibitor/miR-NC in UM1 cells are shown in Figure 1. The relative expression of miR-221 in miR-221 inhibitor group was lower than that in miR-NC group, and the difference was statistically significant (*P*<0.01).

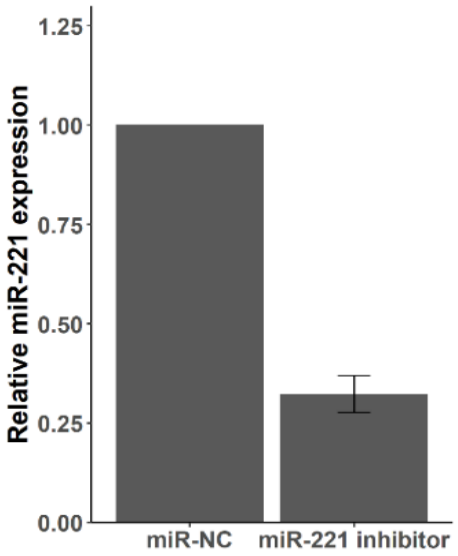

Figure 1. miR-221 was successfully low-expressed in UM1 cells. qRT-PCRwas used to detect the low-expression efficiency of miR-221 in UM1 cell after transfection. Means ± SD. ***P*< 0.01 vs miR-NC group.

The effect of low expression of miR-221 on the proliferation of UM1 cells was detected by MTS method (Figure 2). The results showed that the proliferation level of miR-221 inhibitor group was significantly lower than that of miR-NC group since 48h, and the difference was statistically significant (*P*<0.05). That is, down-regulation of miR-221 expression for 48h could significantly inhibit the proliferation of UM1 cells in vitro.

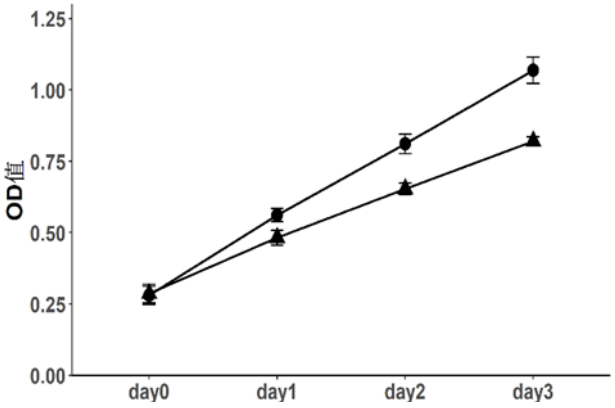

Figure 2. The effect of miR-221 low-expression on the proliferation of UM1 cell. Means ± SD. ∗*P*< 0.05 vs miR-NC group.

At 48h after transfection of miR-221 inhibitor in UM1 cells, Western blot results showed that the expression level of E-Cadherin increased and the expression levels of Vimentin, MMP2 and MMP9 decreased in UM1 cells after down-regulating miR‑ 221 (*P*<0.05).

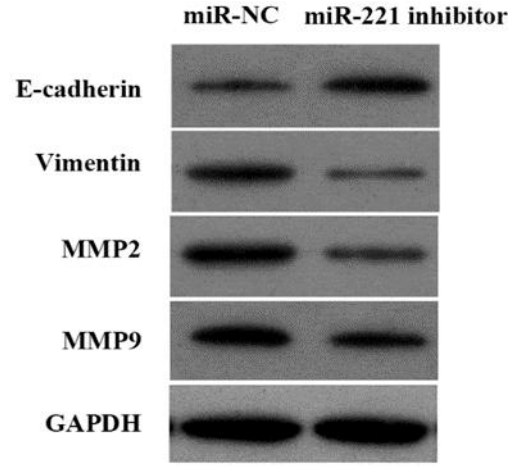

Figure 3. The effects of miR-221 low-expression on tumor metastasis-related protein expression of UM1 cell. Means ± SD. ∗*P*< 0.05 vs miR-NC group.

The model of subcutaneous tumor transplantation in nude mice was established by subcutaneous injection of miR-221 inhibitor group and miR-NC group cells. The effect of inhibiting miR-221 expression on subcutaneous tumor formation of UM1 cells was observed and analyzed, and the difference of transplanted tumor volume was compared. After 3 weeks, the nude mice were killed by cutting off their necks. The tumors were fixed with 4% formaldehyde solution, and then stained with hematoxylin-eosin (HE). Under 400× light microscope, the cancer cells in the two groups were distributed in clumps, with abundant cytoplasm and large nucleus, which accorded with the structural characteristics of squamous cell carcinoma. Compared with the experimental group, most cancer cells in miR-NC group showed focal necrosis (Figure 4A). The statistical results showed that the transplanted tumor volume of miR-221 inhibitor group was (0.5072±0.1179)cm^3, which was significantly smaller than that of miR-NC group (0.8433±0.1511) cm^3 (*P*<0.01; Figure 4B).

Immunohistochemistry (IHC) was used to detect the expression of tumor metastasis-related proteins. As shown in Figure 4C, the positive sites of Vimentin protein expression were mainly located in cytoplasm and nucleus, and the positive sites of E-cadherin protein expression were mainly located in cell membrane. MMP2 and MMP9 were mainly located in cytoplasm and nucleus. In miR-NC group, Vimentin, MMP2 and MMP9 were obviously stained with brown color, but the expression of brown color in miR-221 inhibitor group decreased. On the contrary, the expression of E-cadherin protein was brownish yellow in miR-221 inhibitor group, but decreased in miR-NC group. The results indicated that silencing miR-221 could down-regulate the expression of Vimentin, MMP2 and MMP9, and up-regulate the expression of E-cadherin. This suggested that silencing miR-221 could inhibit the process of tumor

metastasis in transplanted tumor tissues of nude mice.

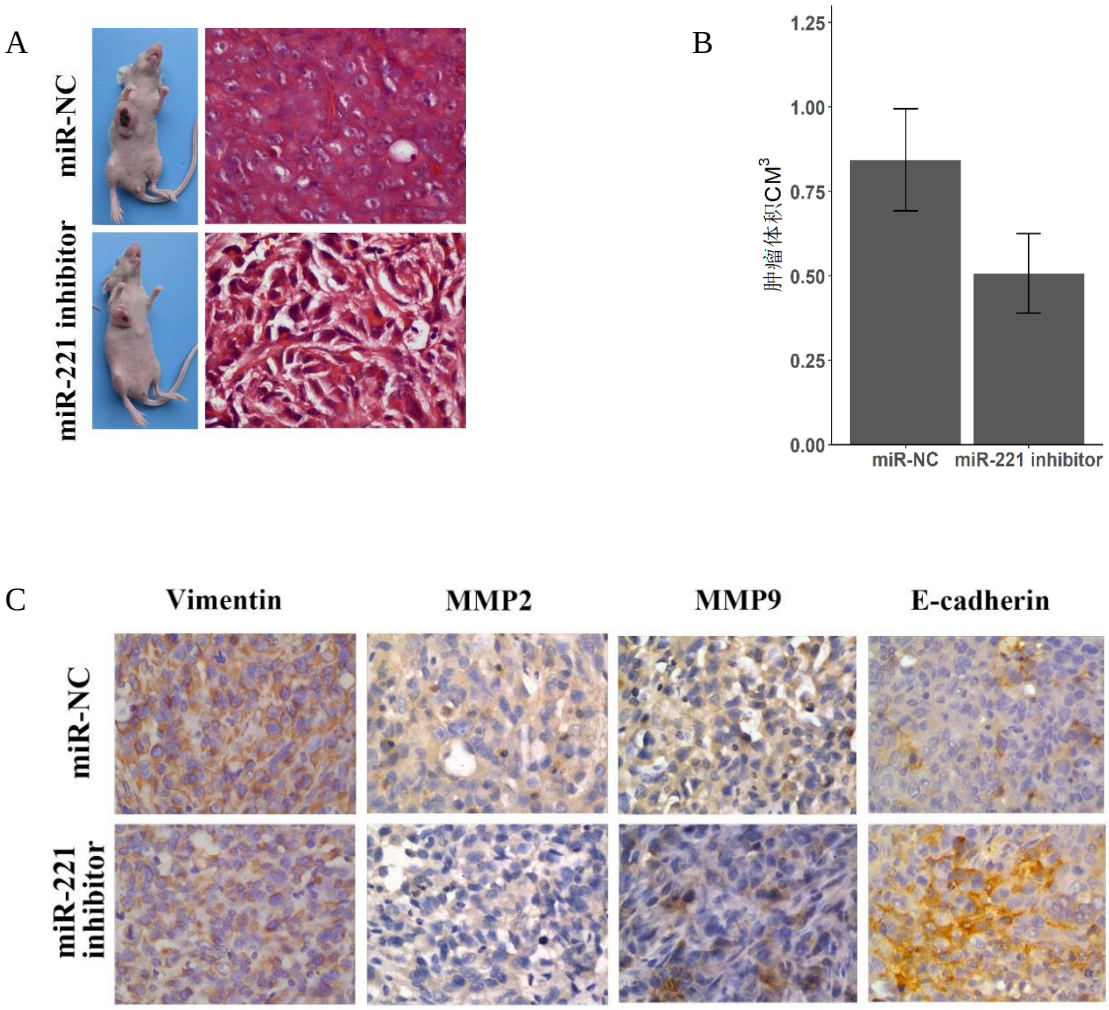

Figure 4. The effects of miR-221 low-expression on tumorigenesis of UM1 cell. A. Light microscopy showed that the cancer cells were consistent with the structural characteristics of TSCC, while most of the cancer cells in the miR-NC group presented focal necrosis (×400). B. The tumor volume in group miR-221 inhibitor was significantly smaller than that in groupmiR-NC. Means ± SD. ∗P< 0.05 vs miR-NC group. C. The expression of the metastasis associated proteins (Vimentin, MMP2, MMP9, E-Cadherin) in two groups.

DISCUSSION

miRNA is a kind of endogenous non-protein encoded small RNA with a length of 18-25 nucleotides. It participates in the early development of organisms, cell proliferation, invasion, apoptosis, metabolism, differentiation and other physiological processes (Garzon R, et al. 2010). With the development of bioinformatics technology, more than 1000 miRNAs have been found in human genome and recorded in miRbase database. At present, it is found that miRNA regulates about 30% of human genes, participates in the occurrence and development of tumors by regulating target genes and plays the role of oncogene and

tumor suppressor gene with significant influence on the invasion and metastasis of cancer cells (Berezikov E, et al. 2005; Baranwal S, et al. 2010).

miR-221/222 is located in chromatin Xp11.3, and has a common gene sequence and the same target gene. miR-221/222 can complement and pair with the specific sequence of 3'UTR region of target gene mRNA to form a miRNA complex (Leva GD, et al. 2012).miR-221 has been studied mostly, it can inhibit the growth and proliferation of human gastric cancer cells, induce apoptosis and reduce tumor invasion and metastasis. In patients with poor prognosis of gastric cancer, the expression level of miR-221 is increased, and its expression level is closely related to the decline of survival rate and the increase of recurrence rate (Liu K, et al. 2012). Yang et al.verified by qRT-PCR that the expression levels of miR-221 and miR-222 in oral squamous cell carcinoma tissues are higher than those in normal tissues, and the over expression of miR-221/222can promote cell proliferation, colony formation and cell migration (Yang CJ, et al. 2011). Western blot show that the expression of miR-221/222 is up-regulated and the protein levels of P27 and P57 are decreased. Therefore, miR-221/222 may promote the occurrence and development of oral squamous cell carcinoma by inhibiting the expression of tumor suppressor genes P27 and P57. Chen et al. discussed the effect of miR-221 on the chemosensitivity of TSCC (Chen D, et al. 2016). When miR-221 expression is inhibited, Tca811 and UM2 increase the chemosensitivity of TSCC to adriamycin and promote apoptosis. It is confirmed that silencing miR-221 increases the sensitivity of tongue squamous cell carcinoma to adriamycin by up-regulating the expression of tumor suppressor gene TIMP3.

To further study the effect of miR-221 on the proliferation of tongue squamous cell carcinoma cells, our group first detected the expression of miR-221 in four types of tongue squamous cell carcinoma cells (Tca8113/CAL27/UM1/UM2), and finally selected UM1 cells with high expression as experimental research subject(He SQ, et al. 2015). The activity of miR-221 in UM1 cell line was down-regulated, and the changes of cell proliferation activity after transfection were detected by MTS method. The tumor-forming ability of transfected cells was detected by subcutaneous transplantation model in nude mice. MTS results showed that the proliferation activity of cells in experimental group decreased after transfection for 24h. The experimental results of subcutaneous tumor transplantation model in nude mice confirmed that silencing of miR-221 and then inoculated subcutaneously in nude mice to delay tumor formation. As a result, the tumor growth was slowed down and the tumor volume was reduced, proving that inhibition ofmiR-221 could inhibit the growth of UM1 cells.

Epithelial-mesenchymal transition (EMT) can change the phenotype of tumor cells, so as to obtain high invasiveness. It plays a vital role in the occurrence, development and metastasis of various malignant tumors (Pastushenko I, et al. 2018). Vimentin is a marker of epithelial-mesenchymal transition, which is not expressed in normal epithelial cells, but expressed in epithelial cells when epithelial-mesenchymal transition occurs in tissues (Franke WW, et al. 1982). E-cadherin is involved in regulating epithelial formation and maintaining internal environment stability (Bhatt T, et al. 2013). The decrease or increase of its expression in tumor tissue has a good clinical guiding significance for judging the prognosis of tumor patients. Matrix metalloproteinases (MMPs) are a family of endogenous zinc-dependent proteases, whose main function is to degrade

extracellular matrix components. MMP-2 is one of the most important matrix metalloproteinases, which can degrade type IV collagen, promote angiogenesis, regulate intercellular adhesion and promote tumor invasion and metastasis (Chant Rain CF, et al.2006). MMP-9 is also considered as an important molecular marker of tumor invasion and metastasis. It can regulate cell adhesion by degrading the molecular substances of extracellular matrix (Yamada K, 2008). In this study, it was confirmed from the cellular and animal levels that the expression levels of Vimentin, MMP2 and MMP9 protein were down-regulated after low expression of miR-221. While the expression level of E-cadherin protein was up-regulated. To sum up, it can be inferred that miR-221 can regulate the proliferation level of TSCC by affecting the expression of tumor metastasis-related proteins in cells.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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