Int J Clin Exp Pathol 2015;8(5):4773-4781
www.ijcep.com /ISSN:1936-2625/IJCEP0006289

Original Article

Targeting miR-21 with AS-miR-21 suppresses aggressive growth of human tongue squamous cell carcinoma *in vivo*

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Received January 23, 2015; Accepted March 22, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: MicroRNAs (miRNAs) are involved in many human malignant tumors. Notably, miR-21 was identified to contribute to tumorigenicity. To investigate the repressive effect of targeting miR-21 with AS-miR-21 on proliferation of tongue squamous cell carcinoma (TSCC). We established the Tca8113-luc cell line with stable luciferase expression using pGL6-luciferase (pGL6-luc) plasmid transfection. TSCC xenograft models were characterized by high tumorigenicity rate and stable growth. Intratumor injection of Oligofectamine™-mediated AS-miR-21 significantly inhibited TSCC growth. The suppression of malignant phenotype was also accompanied by decreased photon signals, rare necrosis foci, smaller nucleuses, weakly stained nucleuses, atypia reversal and tumor angiogenesis reduction. Additionally, miR-21 expression was markedly decreased in TSCC xenografts and the apoptotic index was increased. Intratumor injection of AS-miR-21 into TSCC xenografts could reduce expression of miR-21, promote apoptosis of TSCC cells and inhibit TSCC proliferation.

Keywords: Tongue squamous cell carcinoma, miR-21, AS-miR-21, gene therapy, *in vivo* imaging system

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in the world [1]. More than 500,000 new cases are diagnosed each year and the trend is rising. Over the past three decades, OSCC treatments did not sufficiently improved long-term survival rates, with a 5-year survival rate of only 27% in advanced OSCC patients [2]. The development of safer and more effective treatments is a key for improving long-term survival and quality of life in OSCC patients. It is thus vital to understand the molecular events leading to OSCC and the mechanisms involved in initiation and progression of OSCC.

It is well known that microRNAs (miRNAs) are differentially expressed in a large proportion of human malignant tumors [3]. Of these miRNAs, miR-21 deserves a special attention. MiR-21 was found to be up-regulated in almost all solid tumors and it may down-regulate tumor suppressor, cell differentiation and/or apoptosis-associated genes in order to promote tumorigenicity [4]. It was noted in tongue squamous cell carcinoma (TSCC) *in vitro* that targeting miR-21 using antisense oligonucleotides remarkably inhibited proliferation, promoted apoptosis and suppressed migration and invasion [5]. Hence, specifically targeting miR-21 by antisense oligonucleotides could provide novel insights into OSCC gene therapy.

The objectives of the present study were: first, to screen stable transfected Tca8113 cells, derived from TSCC, with pGL6-luc (Tca8113-luc) plasmid; then to establish a TSCC xenograft model by subcutaneous inoculation of Tca8113-luc cells into nude mice; and finally to assess the effects of intratumor injection of Oligofectamine™-mediated AS-miR-21 on TSCC growth using *in vivo* imaging and TUNEL assays.

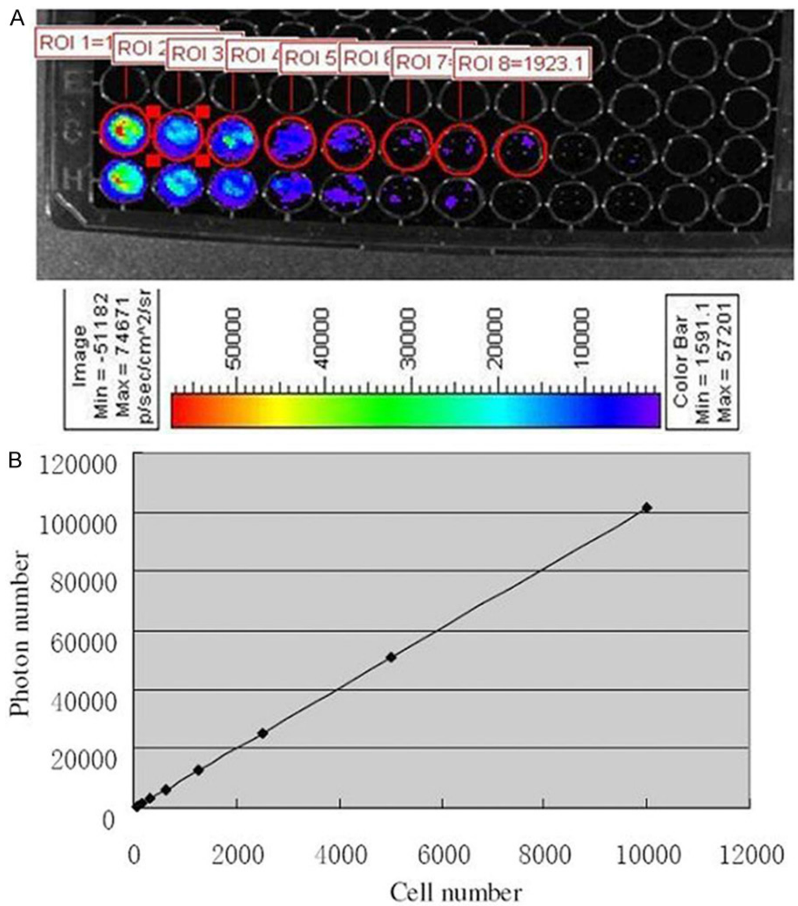


Figure 1. Detection of Luciferase activity in Tca8113-luc cells. A. Identification of luciferase activity in Tca8113-luc cells; B. Correlation between Tca8113-luc cell number and photon emission.

Materials and methods

Cell culture

The human TSCC cell line Tca8113 was kindly provided by the Laboratory of Oral Tumor Biology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. Tca8113 cells were cultured in RPMI-1640 medium (GIBCO BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C, in a humidified 5% CO_2 atmosphere. Cells were used in logarithmic growth phase and when 95% were stain-resistant by 0.2% trypan blue staining.

Plasmids construction and transfection

pGL6 control vector containing Luc and Neo genes were purchased from Jiangsu Beyotime Biotechnology (China). According to the manufacture's instruction, pGL6 vector was amplified and purified, and purified again after Hind III digestion using CompactPrep Plasmid Maxi kit (Qiagen, Hilden, Germany). Tca8113 cells were transfected with pGL6 using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA).

Stable cell line creation and identification

Tca8113-luc cell colonies with stably expressed luciferase were established and cultured by G418 (400 μg/ml) resistance selection. Cell colonies were diluted using fold dilution method. Expression efficiency of luciferase gene in Tca8113-luc cells was subsequently evaluated by optical imaging system. The correlation between fluorescence intensity and cell numbers was analyzed and the growth curve of Tca8113-luc cells was plotted.

Human TSCC Tca8113-luc xenograft models establishment

Twenty-four BALB/c-nu (SPF) female mice (4-6 weeks old) weighing 15-18 grams were purchased from the Division of experimental animal science, Peking University Health Science Center (Beijing, China). All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Peking University Health Science Center (Beijing, China) and in conformity with the National Institutes of Health Guide for Care and Use of Laboratory Animals [6]. Nude mice were randomly and equally divided into Control, Scrambled-miR-21 and AS-miR-21 groups. A total of 200 μl of Tca8113-luc cell suspension (cell density at $1×10^7$/ml) in logarithmic growth phase were injected subcutaneously in the right flank of each nude mouse.

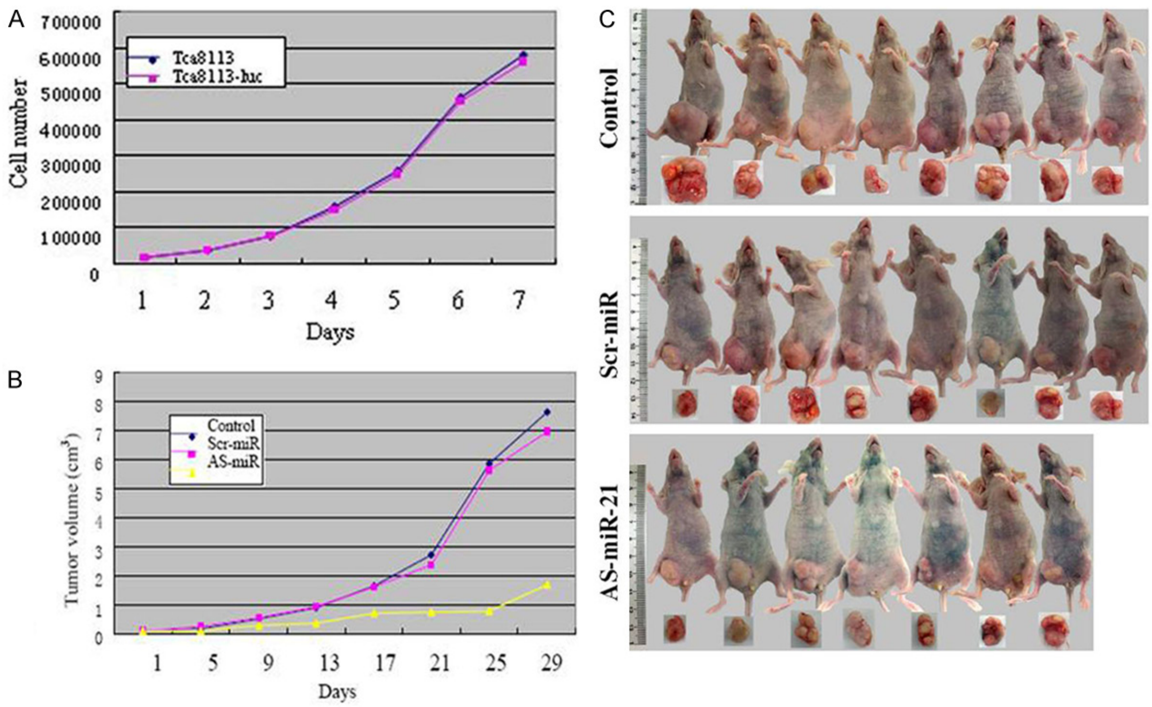


Figure 2. Growth curve of Tca8113-luc cells and Xenograft tumors. A. Growth curves of Tca8113 and Tca8113-luc cells; B. Tca8113-luc tumor growth according to treatment with AS-miR-21; C. Tca8113-luc xenograft tumors and nude mice in the three groups.

Tumorigenicity and therapeutic treatment in vivo

Fifteen days after cells grafting, 95.8% (23/24) of female nude mice had TSCC xenograft tumors. The mean volume of tumors was 0.109±0.076 cm^3. Therapeutic treatment was performed when tumors reached 0.2×0.2 cm. The sequence of human miR-21 was obtained from microRNA database (http://www.sanger.ac.uk). Sequences of AS-miR-21 and Srambled-miR-21 were subsequently identified and blasted. AS-miR-21 sequence was: 5'-GUCAACAUCAGUCUGAUAAGCUA-3'. Scrambled-miR-21 sequence was: 5'-AAGGCAAGCUGACCCUGAAGU-3'. Oligonucleotides with 2'O-Me modification were chemically synthesized by Shanghai Genepharma Company, Ltd. (Shanghai, China). Oligonucleotides (150 μl, 20 pmol/μl) were mixed with 60 μl of Oligofectamine™ Reagent (Invitrogen, Carlsbad, CA, USA) and 25 μl of Oligonucleotides and Oligofectamine™ Reagent mixtures were injected into each nude mouse at several intratumor sites. Treatment was delivered once every four days until observation phase (four weeks). Since the beginning of treatments, the length (a) and width (b) of tumor volume were measured before each injection. The volume was calculated with the formula $V=(ab^2)/2$ [7]. Xenograft tumors' boundary, invasion, necrosis, rupture, and infection were observed, as well as general body conditions.

In vivo imaging system

In vivo bioluminescence imaging system IVIS200 and airflow anesthesia system XGI-8 were purchased from Xenogen Corporation (Xenogen, Berkeley, CA, USA). Cells were observed using *in vivo* imaging system the day they were seeded and then once every week. Mouse was weighed using an electronic balance and injected with 150 mg/kg luciferase substrates (15 mg/ml) into abdominal cavity. After 10 min, the nude mouse was put into the camera and imaged. In addition, lymph node and distant metastases were also observed by blocking the tumor. Data were analyzed by Living Image® 2.50 software.

Real-time PCR analysis

After 28 days, all nude mice bearing tumors were sacrificed and dissected. RNA was extracted from xenograft tumors and miR-21 expression level was subsequently detected by real-time PCR.

Table 1. Tca8113-luc tumor growth according to treatment with AS-miR-21

Days	Tca8113-luc Tumor Volume (mean ± SD) cm³			F	P
	Control	Scrambled-miR-21	AS-miR-21		
1	0.103±0.092	0.129±0.093	0.096±0.044	0.359	0.703
5	0.201±0.155	0.249±0.162	0.128±0.072	1.44	0.26
9	0.512±0.319	0.552±0.347	0.306±0.252	1.314	0.291
13	0.89±0.606	0.929±0.499	0.382±0.34	2.715	0.091
17	1.658±1.344	1.632±1.159	0.705±0.587	1.543	0.239
21	2.741±1.732	2.381±1.016	0.748±0.501	4.763	0.023*
25	5.854±4.526	5.645±2.696	0.797±0.438	4.327	0.031*
29	7.628±4.347	6.971±2.829	1.71±0.57	4.436	0.032*

*A *P*-value <0.05 was considered as significantly significant.

Histopathological examination

Tumors and enlarged lymph nodes were routinely embedded in paraffin. Paraffin-embedded tissue samples were cut into 4 μm sections and each section was stained by H&E staining.

TUNEL apoptosis assay

The FITC-labeled TUNEL apoptosis assay (Keygene, China) was used to detect apoptosis in TSCC xenograft tumor tissue sections. Green fluorescence located in nucleus was indicative of apoptosis under fluorescence microscope at 495 nm excitation wavelength, while blue color was localized in all cellular nucleuses. Apoptotic cells were counted in eight random 400× fields (each field including more than 100 cells) and cellular apoptotic index was calculated in percentage.

Statistical analysis

All analyses were conducted using the SPSS software program (standard version 10.0; SPSS Inc., Chicago, IL, USA). Results of the real-time PCR analyses were calculated using one-way ANOVA. Multi-group continuous variables were compared using two-way ANOVA. Correlation between photon signals and cell numbers was analyzed using Pearson's bivariate correlation analysis. All tests were 2-sided, and *P*-values <0.05 were considered statistically significant.

Results

Detection of luciferase activity in Tca8113-luc cells

Stable expression of luciferase in Tca8113-luc cells was obtained by G418 selection. Cell colonies were diluted using fold dilution method as described in **Figure 1A**. Luciferase activity of 78 cells was identified as the lower detection limit. As shown in **Figure 1B**, correlation coefficient (R) between photon number and cell number was 1, revealing that Tca8113-luc cells with stable luciferase expression had strong luciferase activity.

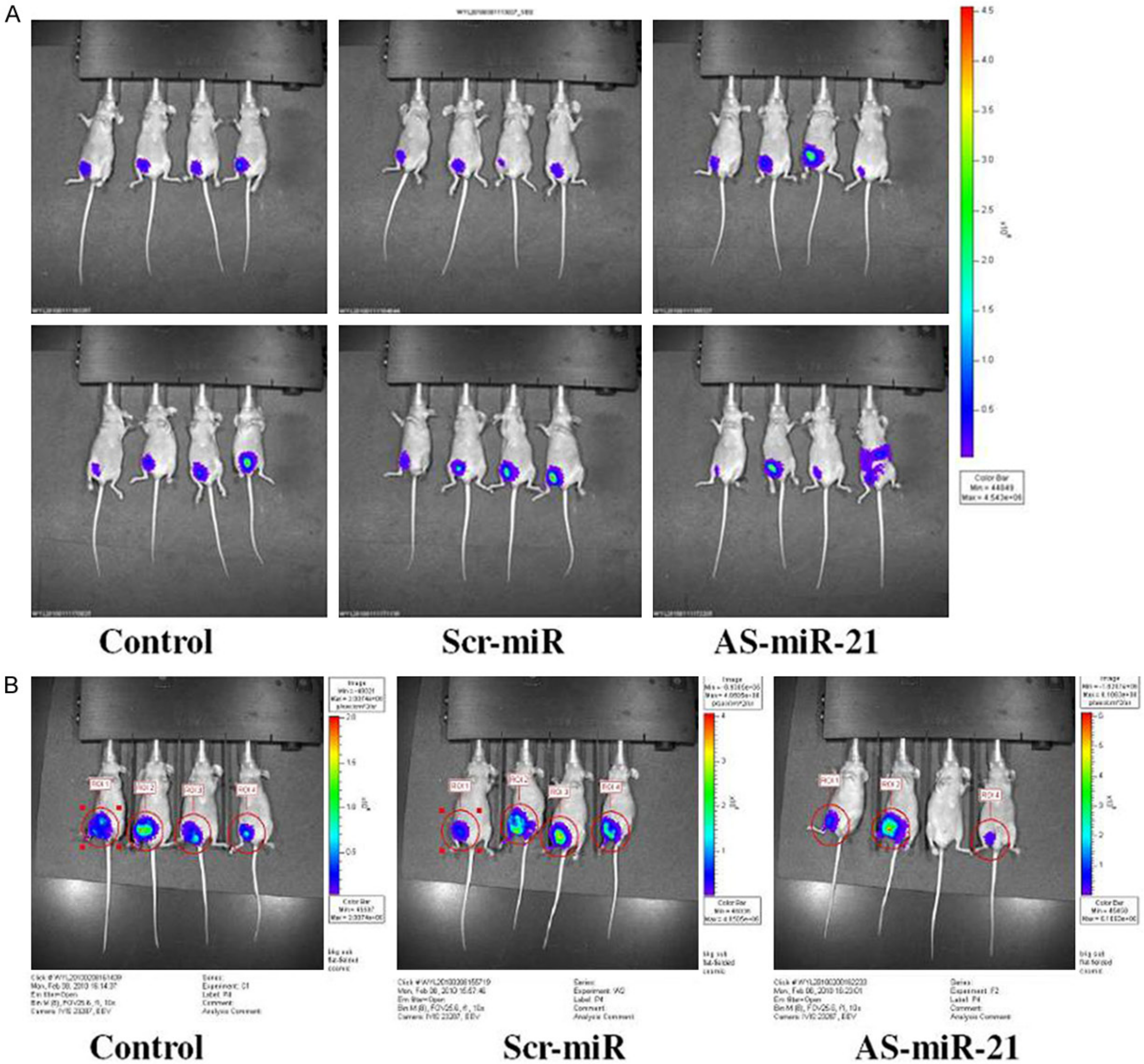
Growth curve of Tca8113-luc cells and xenograft tumors

Proliferation of Tca8113 and Tca8113-luc cells was observed by cell counting assay as shown in **Figure 2A**. No significant difference was observed in growth curves of both cell lines, suggesting that stable transfection of Tca8113 using pGL6-luc did not affect the proliferation potential of TSCC cells. At the treatment initiation point, tumors' boundary was clear and without invasion. Xenograft tumors in control and scrambled miR-21 groups grew rapidly, especially by the ninth day after seeding. There was no significant change in tumor volumes. The smallest tumors volume was observed in the AS-miR-21 group and the growth was slow. No significant difference was observed in the three groups before 21 days (*P*>0.05). From 21 days and later, there was a significant difference in tumors volume between the AS-miR-21 group and the other two groups (*P*<0.05) (**Table 1**; **Figure 2B** and **2C**).

No metastasis was observed. Xenograft tumors from control and scrambled miR-21 groups were hemorrhagic, deliquescent and necrotic. Mice from these two groups were bearing large, ulcerated tumors. They also showed poor quality of life and decreased weight. Xenograft tumors from the AS-miR-21 group were smaller, solid and without deliquescence or necrosis. Our data demonstrated that intratumor injection of Oligofectamine™ Reagent-mediated AS-miR-21 suppressed xenograft tumors growth.

Living imaging analysis was performed by optical imaging system IVIS200

Once Tca8113-luc cells were subcutaneously seeded into the right flanks of the 24 nude mice, *in vivo* living imaging assay was performed by optical imaging system.



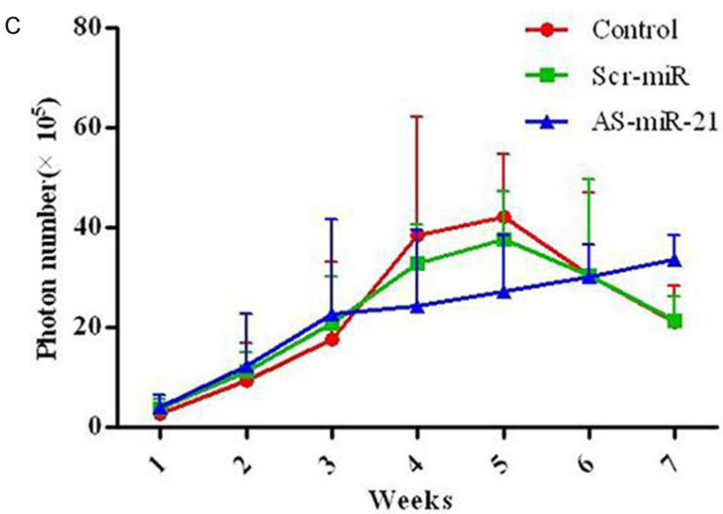


Figure 3. Living imaging analysis was performed by optical imaging system IVIS200. A. *In vivo* imaging of nude mice inoculated with Tca8113-luc cells; B. *In vivo* imaging 4 weeks after beginning treatment with AS-miR-21; C. *In vivo* imaging after beginning treatment with AS-miR-21.

As shown in **Figure 3A**, fluorescence imaging, as well as equal distribution of photon signals, was observed in each seeding site in nude mice, indicating that Tca8113-luc cells were capable of stable expression of luciferase activity.

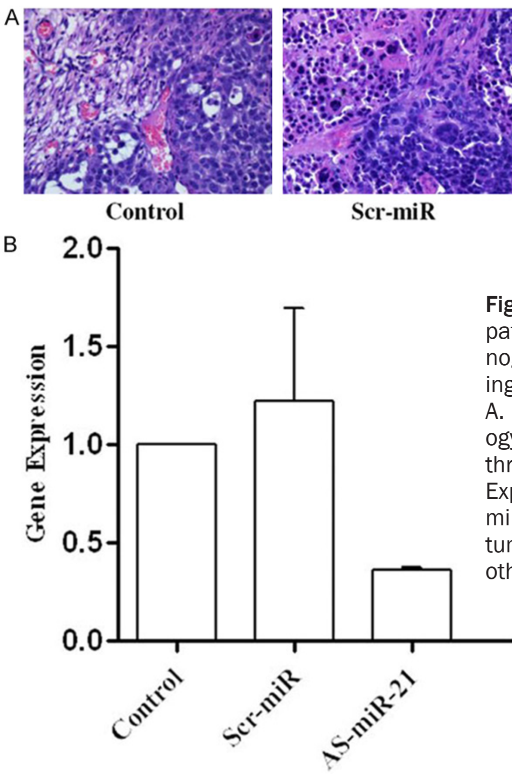


Figure 4. Detection of histopathological alterations in xenograft tumors by H&E staining and expression of miR-21. A. Histopathological morphology of xenograft tumors in the three groups (H&E 400×); B. Expression of miR-21 in AS-miR-21-treated Tca8113-luc tumors compared with the other two control groups.

As described in **Figure 3B** and **3C**, there was no difference in photon signals in the 23 xenograft tumors. Two weeks after beginning AS-miR-21 treatment, photon value was remarkably reduced in AS-miR-21 group compared with the other two groups, suggesting that AS-miR-21 decreased TSCC xenograft tumors growth. Three weeks after beginning AS-miR-21 treatment, photon values from the control and scrambled miR-21 groups were decreased and no significant difference was observed among the three groups. *In vivo* living imaging showed there was no distant or lymph node metastasis in all nude mice.

Detection of histopathological alterations in xenograft tumors by H&E staining and expression of miR-21

Xenograft tumors from the control and scrambled-miR-21 groups showed vigorous growth, irregular morphology, enlarged nucleuses, strongly stained nucleuses, lots of abnormal mitoses, obvious atypia, a large number of tumor giant cells, increased tumor angiogenesis and frequent necrosis foci, while tumors from the AS-miR-21 group revealed rare necrosis foci, small nucleuses, weakly stained nucleuses, reduced atypia and decreased tumor angiogenesis (**Figure 4A**). A small amount of inflammatory cells infiltration was observed in all xenograft tumors. The enlarged lymph nodes were diagnosed as normal lymph node tissues, without metastatic tumor.

Twenty-eight days after beginning of AS-miR-21 treatment, all nude mice were sacrificed and RNA was extracted from tumors. Expression of miR-21 assessed using real-time PCR in xenograft tumors from AS-miR-21 group was remarkably lowered compared to control and Scrambled-miR-21 groups (**Figure 4B**). Expression of miR-21 was reduced by 36% and 29.5% in AS-miR-21-treated group compared with control and scrambled-miR-21 groups, respectively. No difference was observed in miR-21 expression level between control and scrambled-miR-21 groups. Our results demonstrated that intratumor injection of Oligofectamine™-mediated AS-miR-21 significantly reduced miR-21 expression level in TSCC xenograft tumors.

Evaluation of apoptosis index by TUNEL apoptosis assay

FITC-labeled TUNEL apoptosis kit was used to investigate late apoptosis index of xenograft tumor sections under various treatments in nude mice (**Figure 5**). The apoptosis index was 37% in xenograft tumors from the AS-miR-21-treated group. There was a significant difference between the AS-miR-21 treated group and the other two control groups, indicating that intratumor injection of AS-miR-21 notably promoted the apoptosis potential of TSCC cells.

Discussion

It is well accepted that miRNAs are involved as regulatory elements in many cellular processes, including proliferation and differentiation. Targeting miRNAs as anti-tumor strategy will provide a novel insight in understanding the mechanisms of tumor initiation and progression. Emerging evidences suggest that down-

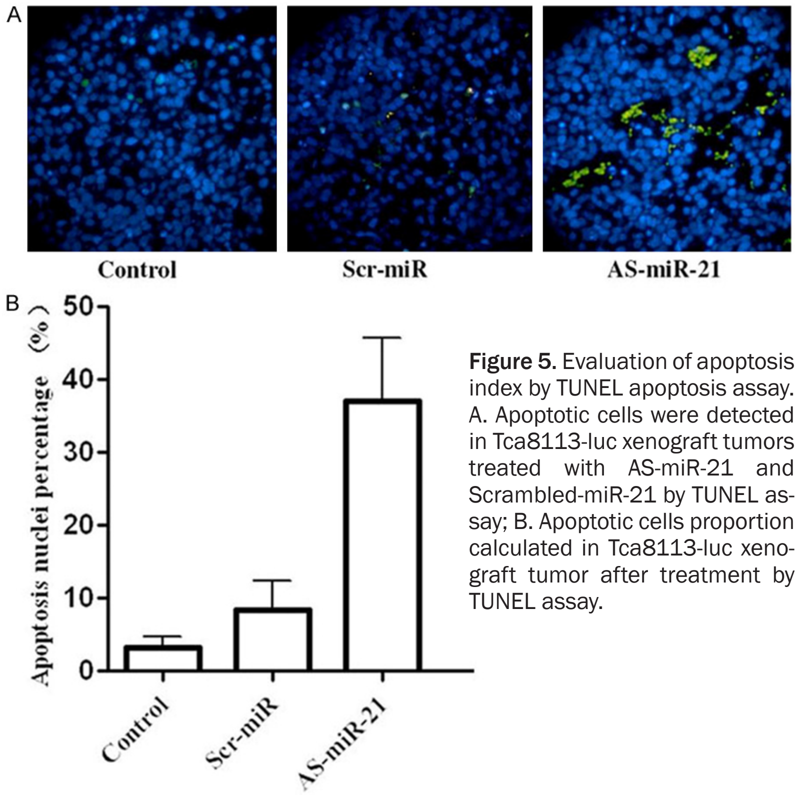


Figure 5. Evaluation of apoptosis index by TUNEL apoptosis assay. A. Apoptotic cells were detected in Tca8113-luc xenograft tumors treated with AS-miR-21 and Scrambled-miR-21 by TUNEL assay; B. Apoptotic cells proportion calculated in Tca8113-luc xenograft tumor after treatment by TUNEL assay.

regulation of some miRNAs expression acts as tumor suppressor. Also, some miRNAs can act as tumor suppressors and intratumor injection of viruses or liposomes-mediated miRNAs delivery mimics endogenous miRNAs overexpression in order to targetedly suppress growth of tumors, and induce apoptosis [8]. In the case of up-regulated miRNAs acting as oncogenes, miRNAs complementary antisense oligonucleotides were induced into cells to form hybridized double-strand inactive fragments, activating RNase H to degrade these fragments [9].

In one of our previous studies, we observed that miR-21 expression was aberrantly up-regulated in OSCC and that it possessed the ability to promote tumor initiation and progression by down-regulating tumor suppressors and/or by controlling cellular differentiation or apoptosis genes [10]. miR-21 was found to be involved in tumor cellular proliferation, apoptosis and invasion processes by virtue of directly regulating apoptotic and invasive-associated tumor suppressors. Targeting miR-21 with specific inhibitors might regulate a variety of cancer-dysregulated proteins to provide a novel therapeutic approach [11-14].

We established that the TSCC xenograft model by subcutaneous inoculation of Tca8113-luc cells stably transfected with pGL6-luc construct was a valid model of TSCC. Intratumor injection of AS-miR-21 to suppress growth of TSCC xenograft tumors was investigated. After 28 days of treatments, slower growing rate, smaller tumor sizes, rare necrosis foci, smaller nucleuses, weaker stained nucleuses, reduced atypia and decreased tumor angiogenesis were observed in the AS-miR-21 group. Our data also demonstrated that expression of miR-21 was markedly decreased in xenograft tumors in the AS-miR-21 group by real-time PCR analysis. TUNEL assays further showed that the apoptosis index was increased, suggesting that intratumor injection of Oligofectamine™-mediated AS-miR-21 significantly reduced miR-21 expression and inhibited growth of xenograft tumors by promoting apoptosis of TSCC cells. Our findings show the possibility of using AS-miR-21 as therapeutic agent in human OSCC.

In vivo imaging systems are widely used in life science, medical research and drug development [15, 16] In the current work, enzyme-digested pGL6 constructs were first transfected into Tca8113 cells by using liposomes, and Tca8113-luc cells with stable expression of luciferase activity were further screened using G418. TSCC xenograft models were created by subcutaneous inoculation of Tca8113-luc cells to perform *in vivo* imaging observation after injection with luciferase substrates. Only 100 of cells are sufficient to be monitored using the IVIS200 imaging system (Xenogen, Berkeley, CA, USA) [17-19]. Two weeks after Tca8113-luc cells seeding, luciferase was expressed in xenograft tumors, and no difference was observed in photon signals of xenograft tumors from the three groups. With the growth of xenograft tumors, photon signals were rapidly increased and reached saturation 14 days after treatment initiation. Twenty-one days after beginning treatment, necrosis was observed in the center of xenograft tumors and their fluorescence signal was then reduced. Compared with the two other groups, slower increasing of fluorescence values in the AS-miR-21 group was observed and there was no significant difference between AS-miR-21 groups and the other two groups 12 days after treatment beginning. Due to large sizes of some parts of tumors, liquefaction necrosis of xenograft tumors reduced fluorescence values, making the three groups no different at 21 days. Differences in tumor sizes were observed between the control groups and the AS-miR-21 group at the fourth week. There were contradictions between fluorescence signals and tumor sizes among the three groups in a later stage, probably due to a large amount of necrosis in all groups making the signals weaker. The repressive effect of AS-miR-21 on proliferation of tumors was not evaluated objectively and correctly in case we shorten the observation period. This problem remains to be further explored for solutions. In this study, we did not observe lymph node metastasis and distant metastasis using an *in vivo* imaging system in any group, probably because of tumor cells inoculation way. At present, subcutaneous injection into the right flanks of the nude mice instead of tail vein injection might reduce the probability of tumor metastasis. The effect of AS-miR-21 on OSCC metastasis warrants further investigation.

With the completion of the human genome project, the development of molecular biology and the improvement of other anti-tumor therapeutic approaches, gene therapy is to be expected to become a conventional treatment. Delivery of miR-21 inhibitors to tumor cells by cell-specific viruses or liposomes could be an effective therapeutic strategy. In conclusion, our findings demonstrated that intratumor injection of AS-miR-21 significantly inhibited the expression of miR-21, promoted tumor cell apoptosis and suppressed tumor growth.

Acknowledgements

This study was supported by National Natural Science Foundation of China (Grant No. 81172578).

Disclosure of conflict of interest

None.

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