Inhibition of miR-21 promotes cell apoptosis in oral squamous cell carcinoma by upregulating PTEN

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Abstract. MicroRNA-21 (miR-21) has been identified as an oncogene and confirmed to serve an important role in carcinogenesis in various types of cancer. However, the effect and mechanism of miR-21 in oral squamous cell carcinoma (OSCC) has not been fully elucidated. In the present study, miR-21 inhibitor and empty vector were transfected into OSCC cells, and non-transfected cells were used as a blank control. The results indicated that when compared with the control and scramble groups, miR-21 inhibitor suppressed the expression of miR-21. Conversely, phosphatase and tensin homolog deletion on chromosome 10 (*PTEN*) was markedly upregulated, and a dual luciferase reporter assay revealed *PTEN* to be a target gene of miR-21. Furthermore, miR-21 inhibitor decreased the proliferation and invasion and enhanced the apoptosis of OSCC cells. There was no significant difference in cell proliferation, invasion and apoptosis between the control and scramble groups. The present data suggested that there may be a regulatory loop between miR-21 and PTEN, and that miR-21 inhibition affected the proliferative, invasive and apoptotic abilities of OSCC cells. These findings indicate that miR-21 may be a possible novel target in the treatment of OSCC.

Introduction

In recent years, increasing data have indicated the importance of microRNAs (miRNAs) in the progression of cancers including oral squamous cell carcinoma (OSCC) (1-4). miRNAs are small non-coding RNAs of ~22 nucleotides in length, existing as single stranded RNAs that act by binding to partially complementary sequences in the 3'untranslated regions (UTRs) of target gene mRNAs to regulate protein expression. miR-21, which is considered to be a proto-oncogene, is frequently reported as overexpressed in various cancer types and has been implicated in tumorigenesis (5,6). In addition, miR-21 has been reported to serve an important role in several signaling pathways, including Wnt/β-catenin and phosphatase and tensin homolog deletion on chromosome 10 (PTEN)/phosphatidylinositol 3-kinase (PI3K)/Akt (7,8). Furthermore, miR-21 may negatively regulate multiple target genes, including programmed cell death protein 4 (PDCD-4), tissue inhibitor of metalloproteinases-*3*, integrin subunit β 4 and PTEN (9-12).

PTEN is a tumor suppressor gene that has frequently been reported to be involved in the regulation of cell proliferation, invasion, migration and apoptosis in many types of cancer, and the expression of PTEN is downregulated in a wide range of malignancies, including breast cancer, glioblastoma, colorectal carcinoma, pancreatic cancer and OSCC (13-17). Previous studies have demonstrated that the PI3K/Akt signaling pathway is involved in multiple biological processes and that PTEN functions as a tumor suppressor by negatively regulating the PI3K/Akt signaling pathway, which reduces cell growth and increases cell apoptosis (18,19). miRNAs, including miR-136, miR-181a and miR-221/222, have been reported to regulate the expression of PTEN (20-22). However, the role of miR-21 with regard to the expression of PTEN in OSCC is not well established.

Since the therapeutic potential of miR-21 inhibitor, to the best of our knowledge, has not been investigated in OSCC, in the present study the biological effects and molecular mechanism of miR-21 involved in the apoptosis of OSCC cells were evaluated. The results obtained support that miR-21 inhibitor, via its influence on PTEN expression, may be a novel agent for the treatment of OSCC.

Materials and methods

Tissue acquisition. Surgically resected OSCC specimens were obtained from 35 patients (aged 45 to 70, including 19 males and 16 females) with OSCC between June 2015 and June 2017 at Jining No. 1 People's Hospital (Jining, China) with written informed consent. According to the Union for International Cancer Control (UICC) standards in 2002, all the cases were classified as phases I-IV. In addition, individual oral tissue samples from 10 normal subjects (aged 35-65, including 6 males and 4 females) were collected as controls

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with written informed consent. The experimental procedures were approved by the Research Ethics Committee of Jining No. 1 People's Hospital.

Hematoxylin and eosin (H&E) staining. H&E staining is a popular staining method in histology and it is one of the most widely used staining in medical diagnosis. The prepared 4-μm paraffin sections were incubated for 2 h in a 60˚C incubator. The sections were deparaffinized by turpentine oil (TO) and sequentially soaked in 100, 95, 85 and 70% alcohol solutions for 2 min, and then hydrated using distilled water for 5 min. The sections were stained with hematoxylin for 5-15 min. Then, the excess dyeing solution on the slide was washed with water. Next, the sections were stained with eosin for 1-5 min. Subsequently, the sections were dehydrated using 70, 85, 95 and 100% alcohol, respectively, and were transparent through TO. Finally, the excess TO transparent agent around the section was wiped off, and a suitable amount of neutral resin was added, and the slide was covered.

Immunohistochemical staining. Immunohistochemistry was used to detect PTEN expression in the OSCC specimens. The samples were fixed, embedded and cut into 4-μm thick sections. The sections were deparaffinized using xylene and rehydrated by increased grades of ethanol. A total of 0.5 μg primary rabbit polyclonal antibodies against PTEN (1:200; cat. no. 9188S; Cell Signaling Technology, Inc., Danvers, MA, USA) were added following antigen retrieval and incubated at 4˚C overnight. Subsequently, immunohistochemical staining was performed using VECTASTAIN® ABC immunohistochemistry kit (Cowin Biosciences, Co., Ltd., Beijing, China) according to the manufacturer's instructions.

In situ hybridization (ISH). ISH analysis was performed on deparaffinized 4-μm OSCC tissue sections using an ISH tissue implementation kit. OSCC sections were incubated at 60˚C for 1 h and deparaffinized. Subsequently, the sections were rehydrated in decreasing concentrations of ethanol and washed in deionized water. The slides were incubated in 0.2 mol/l HCl for 5 min at room temperature and washed 3 times in phosphate-buffered saline (PBS) for 5 min. Proteins were digested for 15 min at 37˚C following the addition of pepsin solution. Dehydration of sections was performed through incubation with 70, 95 and 100% ethanol for 1 min at each concentration. miR-21 probes labeled with digoxin were added to the hybrids. Sections were denatured at 90˚C for 4 min, followed by incubation in ISH slide denaturation and hybridization solution at 37˚C overnight. Post-incubation, coverslips and glue were removed and the slides were washed. Finally, the slides were observed under an inverted microscope.

Cell culture and transfection. The OSCC cell lines SCC15 and SCC25 were obtained from the American Type Culture Collection (ATTC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2 environment. miR-21 inhibitor and empty vector were chemically synthesized by Suzhou GenePharma Co., Ltd. (Suzhou, China) and transfected into SCC15 and SCC25 cells using Lipofectamine™ RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the recommended protocol. Cells in which Lipofectamine™ RNAiMAX was only added were used as a control group. Both SCC15 and SCC25 Cells in each of the 3 groups (control, scramble and miR-21 inhibitor) were collected to be used for subsequent analysis at different time-points following transfection.

Dual luciferase reporter assay. 293T cells were obtained from ATTC and cultured in 24-well plates and co-transfected with 0.5 μg pMIR vectors containing PTEN 3'UTR or mutant (mut) PTEN 3'UTR and 20 μM miR-21 mimics or 20 μM miR-21 inhibitor using Lipofectamine® RNAiMAX. Cells were lysed with Passive Lysis Buffer and collected at 48 h post-transfection. Luciferase activity was detected with a dual luciferase reporter assay kit according to the manufacturer's protocol. *Renilla* luciferase activity was used for normalization.

Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of miRNAs and mRNAs. Total miRNAs and mRNAs were extracted from SCC15 and SCC25 cells using an miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and Total RNA Purification kit (Qiagen GmbH) according to the manufacturer's instructions. Real-time RT-qPCR analysis was performed to validate miRNA and mRNA expression using a One-Step RT-PCR Kit. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Invitrogen; Thermo Fisher Scientific, Inc.) were used as endogenous controls. Each test was repeated in triplicate.

Western blot analysis. After transfection for 48 h, SCC15 and SCC25 cells were washed 3 times with cold PBS and lysed in a buffer composed of radio-immunoprecipitation assay buffer (Cowin Biosciences Co., Ltd.) and phenylmethanesulfonyl fluoride (Cowin Biosciences Co., Ltd.) (100:1). Total proteins were heated at 95˚C for 10 min following measurement of protein concentration with an Enhanced BCA Protein Assay kit (Cowin Biosciences Co., Ltd.). Equal quantities (25 μg) of heated proteins from each sample were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were blocked in Tris-buffered saline with Tween-20 (TBST) containing 5% non-fat milk at room temperature for 1 h, then incubated with specific primary antibodies against against PTEN (1:1,000; cat. no. 9188S), phosphorylated (p)Akt (1:2,000; cat. no. 4060T), Akt (1:1,000; cat. no. 4691T) and GAPDH (1:1,000; cat. no. 5174S) (Cell Signaling Technology, Inc.) overnight at 4˚C. Subsequently, the blots were rinsed 3 times with TBST and incubated with the secondary antibody for 1 h at room temperature. Protein bands were imaged using the AlphaView SA Western blot detection system (Carl Zeiss AG, Oberkochen, Germany) and quantified following normalization to the density of GAPDH using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Each test was repeated in triplicate.

Cell proliferation assay. Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8; Cowin Biosciences Co., Ltd.) according to the manufacturer's instructions. Following treatment with miR-21 inhibitor, SCC15 and SCC25 cells were

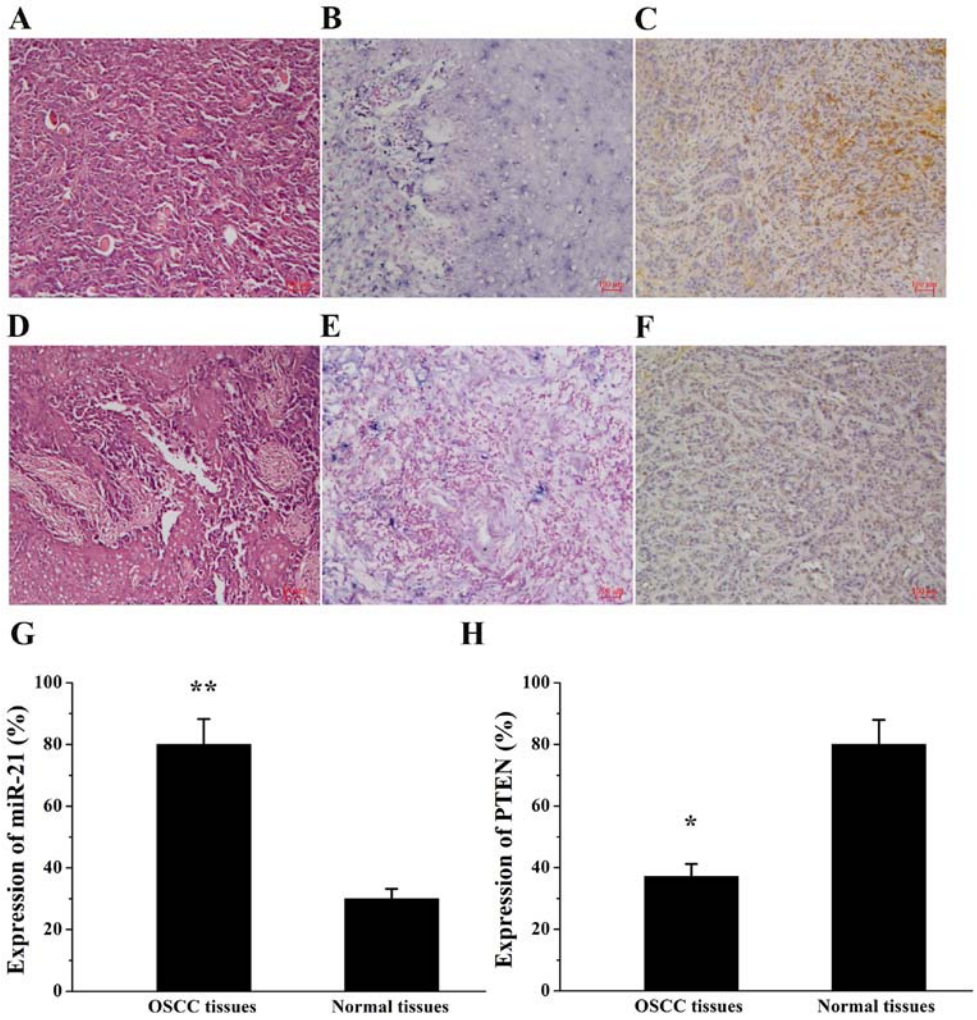

Figure 1. Expression of miR-21 and PTEN in OSCC samples. Hematoxylin and eosin staining results in (A) normal tissues and (D) OSCC tissues. miR-21 expression in (B) normal tissues and (E) OSCC tissues detected by *in situ* hybridization. Immunohistochemical staining of the expression of PTEN in (C) normal tissues and (F) OSCC tissues. (G and H) miR-21 and PTEN expression in OSCC and normal tissues. *P<0.05 and **P<0.01. miR-21, microRNA-21; PTEN, phosphatase and tensin homolog deletion on chromosome 10; OSCC, oral squamous cell carcinoma.

seeded into 96-well plates at a density of 4,000 cells/well and subsequently allowed to attach overnight. A 10 μl quantity of CCK-8 was added to each well at 0, 24, 48 and 72 h after transfection, and the cells were incubated for 1 h. Optical density (OD) was determined by spectrophotometric analysis at a wavelength of 450 nm. Each experiment was repeated in triplicate.

Transwell cell migration assay. After transfection, SCC15 and SCC25 cells were transferred to 8-μm pore inserts and placed in companion wells containing DMEM and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The inserts were removed after a 12-h incubation, and the non-migrated cells on the upper surface were harvested. Cells on the lower surface were fixed with 5% glutaraldehyde (Beyotime Institute of Biotechnology, Shanghai, China) and stained with Giemsa (Beyotime Institute of Biotechnology) and counted under a fluorescent microscope. Each test was repeated in triplicate.

Cell apoptosis assay. SCC15 and SCC25 cells were washed with PBS and resuspended in buffer at a concentration of 10^6 cells/ml. Cells were mixed with 5 μl fluorescein isothiocyanate-conjugated Annexin V reagent and 5 μl propidium iodide (PI) (Invitrogen; Thermo Fisher Scientific, Inc.). At 15 min after incubation in the dark at room temperature, the samples were analyzed by flow cytometry (Beckman Coulter GmbH, Krefeld, Germany). Each test was repeated in triplicate.

Statistical analysis. All experiments were repeated at least 3 times and the data were presented as the mean ± standard deviation (SD). The results were analyzed by Student's t-test for the comparison of two and ANOVA (followed by Tukey's post hoc test) for the comparison of multiple samples using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant result.

Results

Expression of miR-21 and PTEN in OSCC tissues. The expression of miR-21 and PTEN was examined by ISH and immunohistochemical staining, respectively, in human OSCC tissues and normal tissues (Fig. 1). As depicted in Table I, miR-21 expression was observed in 80.0% (28/35) of OSCC

Table I. miR-21 and PTEN expression in OSCC and normal tissues.

		miR-21 expression			PTEN expression		
Group	No. of tumor specimens, n	n	χ^2	P-value	n	χ^2	P-value
OSCC tissues	35	28	9.073	0.003	13	5.740	0.017
Normal tissues	10	3			8		

miR-21, microRNA-21; PTEN, phosphatase and tensin homolog deletion on chromosome 10; OSCC, in oral squamous cell carcinoma.

Table II. Associations of miR-21 and PTEN expression with clinicopathological profiles.

		miR-21 expression			PTEN expression		
Clinicopathological profile	No. of tumor specimens, n	n	χ^2	P-value	n	χ^2	P-value
Sex							
Male	19	16	0.461	0.497	8	0.438	0.508
Female	16	12			5		
Age, years							
>60	20	17	0.729	0.393	7	0.092	0.762
≤60	15	11			6		
Stage							
I+II	17	11	4.833	0.028	10	6.655	0.010
III+IV	18	17			3		
Differentiation							
Well	9	7	0.043	0.979	3	0.092	0.955
Moderate	16	13			6		
Poor	10	8			4		
Lymph node metastasis							
Negative	23	18	0.127	0.722	9	0.114	0.736
Positive	12	10			4		
Total	35	28			13		

miR-21, microRNA-21; PTEN, phosphatase and tensin homolog deletion on chromosome 10.

tissues and in 30.0% (3/10) of normal tissues (P=0.003). By contrast, PTEN expression exhibited an opposite trend in OSCC tissues (37.1%, 13/35) and normal tissues (80.0%, 8/10; P=0.017) compared with the expression of miR-21. Furthermore, the association between miR-21 and PTEN expression and the clinicopathological profiles of patients was evaluated (Table II). The data indicated that the expression of miR-21 and PTEN was associated with tumor stage. miR-21 expression was lower in early stages (I+II) than in advanced stages (III+IV; P=0.028) and the expression of PTEN was significantly higher in early stages (I+II) than in advanced stages (III+IV; P=0.010). miR-21 and PTEN expression levels were not significantly associated with patient sex or age, tumor differentiation or lymph node metastases (P>0.05).

miR-21 and PTEN expression in SCC15 and SCC25 cells. To knockdown endogenous miR-21, miR-21 inhibitor was synthesized and transfected into SCC15 and SCC25 cells. The expression of miR-21 and PTEN was then analyzed by RT-qPCR. The data indicated that miR-21 inhibitor efficiently silenced the expression of miR-21 compared with that in the control groups (Fig. 2A; P<0.01). By contrast, the gene expression of PTEN was notably upregulated in the miR-21 inhibitor group (Fig. 2B; P<0.01). No significant differences between the scramble and control groups were noted (P>0.05). The results indicated that miR-21 may be negatively associated with PTEN expression in SCC15 and SCC25 cells.

miR-21 inhibitor transfection altered the expression of PTEN, Akt and pAkt. To investigate the molecular mechanism of miR-21 in the apoptosis of SCC15 and SCC25 cells, western blot analysis was performed following miR-21 silencing to assess the expression of PTEN, Akt and pAkt. The expression of PTEN was significantly increased (P<0.01) and the

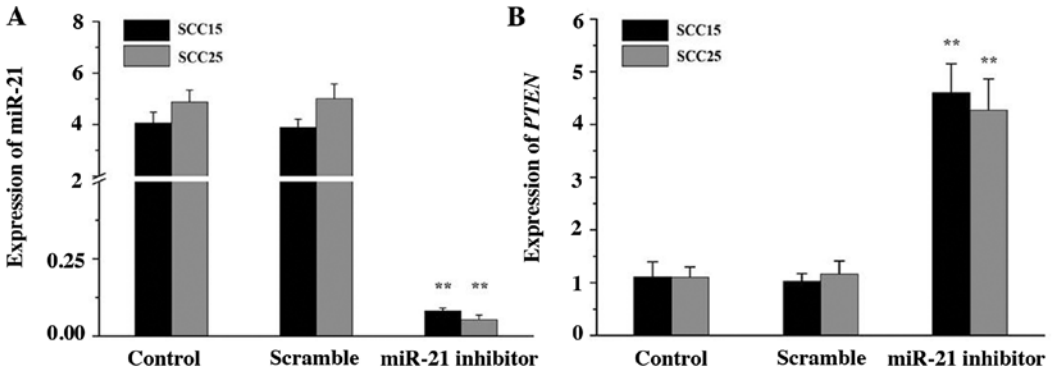

Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis of the expression of (A) miR-21 and (B) PTEN in SCC15 and SCC25 cells transfected with miR-21 inhibitor. The results demonstrated significant downregulation of miR-21 and marked upregulation of PTEN in the miR-21 inhibitor groups compared with those in the control groups in both SCC15 and SCC25 cells. **P<0.01. miR-21, microRNA-21; PTEN, phosphatase and tensin homolog deletion on chromosome 10.

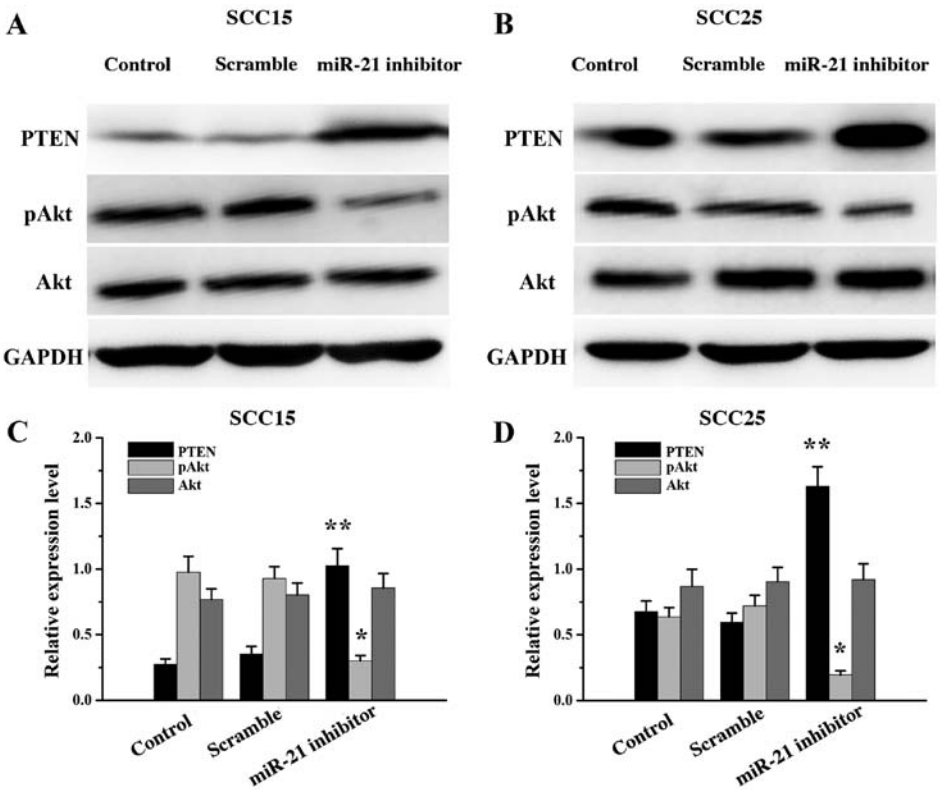

Figure 3. (A-D) Influence of miR-21 silencing on PTEN, pAkt and Akt signaling. Expression of PTEN, pAkt and Akt in (A and C) SCC15 and (B and D) SCC25 cells determined by western blot analysis following treatment with miR-21 inhibitor or empty vector. PTEN was observed to be overexpressed in the miR-21-inhibitor groups compared with that in the control groups in both SCC15 and SCC25 cells. By contrast, when compared with the expression of PTEN, pAkt expression exhibited an opposite trend. *P<0.05 and **P<0.01. miR-21, microRNA-21; PTEN, phosphatase and tensin homolog deletion on chromosome 10; p, phosphorylated.

expression of pAkt decreased (P<0.05) in the miR-21 inhibitor groups compared with that in the control groups. There were no significant differences in Akt expression between the miR-21 inhibitor and control groups (P>0.05; Fig. 3). The results indicated that the level of PTEN expression in SCC15 and SCC25 cells was negatively regulated by miR-21.

miR-21 silencing inhibits SCC15 and SCC25 cell proliferation and invasion. The effect of miR-21 inhibitor on the proliferation and invasion of SCC15 and SCC25 cells was determined by CCK-8 and Transwell assays, respectively. As depicted in Fig. 4, both the proliferative and invasive abilities of SCC15 and SCC25 cells in the miR-21 inhibitor groups were significantly suppressed compared with those in the control groups (P<0.05). There were no significant differences between the control and scramble groups (P>0.05).

miR-21 inhibitor induces SCC15 and SCC25 cell apoptosis. To detect the effect of miR-21 inhibitor on SCC15 and SCC25 cell apoptosis, Annexin V/PI analysis was performed. The data indicated that the percentage of apoptotic cells was markedly induced in the miR-21 inhibitor groups compared with that

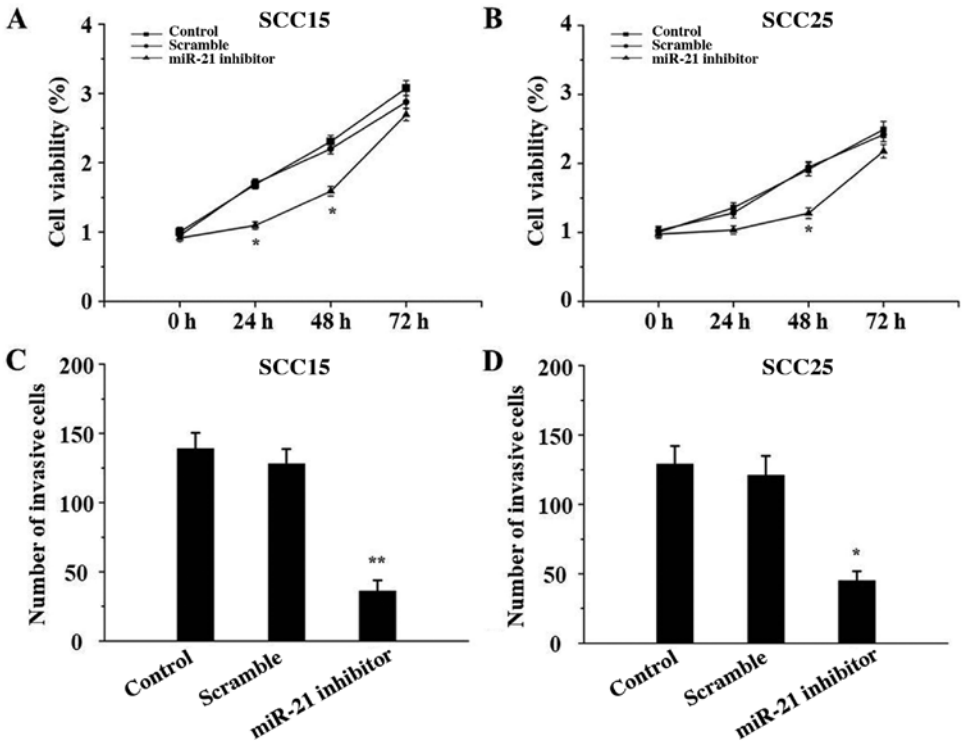

Figure 4. (A-D) miR-21 influences SCC15 and SCC25 cell proliferation and invasion. Cell Counting Kit-8 and Transwell assays indicated that in (A and C) SCC15 and (B and D) SCC25 cells, miR-21 silencing significantly inhibited cell proliferation and invasion compared with those in the control groups. *P<0.05 and **P<0.01. miR-21, microRNA-21.

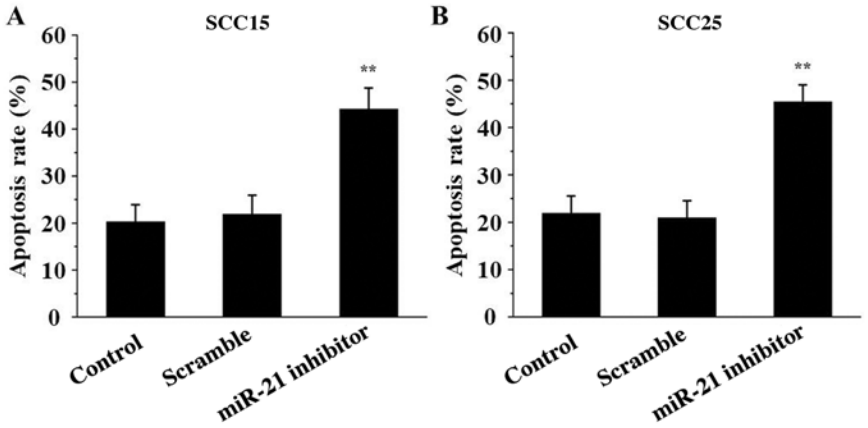

Figure 5. Percentages of apoptotic SCC15 and SCC25 cells following treatment with miR-21 inhibitor. The apoptotic rates of (A) SCC15 and (B) SCC25 cells were analyzed by flow cytometry at 48 h post-treatment with miR-21 inhibitor or empty vector. The data indicated that the percentage of apoptotic cells was notably increased in the miR-21-inhibitor groups compared with that in the control and scramble groups. **P<0.01. miR-21, microRNA-21.

in the control groups (P<0.01; Fig. 5), indicating that miR-21 inhibitor transfection induced cell apoptosis.

miR-21 acts directly on PTEN mRNA 3'UTR. To validate whether PTEN is a direct target of miR-21, *PTEN* 3'UTR and mut*PTEN* 3'UTR luciferase constructs were transfected into 293T cells with negative control (NC) mimics, miR-21 mimics, inhibitor NC or miR-21 inhibitor. Luciferase activity was assessed using a dual-luciferase reporter assay system. As illustrated in Fig. 6, compared with the cells in the other groups, the luciferase activity of 293T cells transfected with miR-21 mimics and *PTEN* 3'UTR was significantly reduced (P<0.05).

Discussion

OSCC is the most common cancer of the head and neck and presents a poor prognosis, with a 5-year survival rate of <60% (23,24). As one of the current treatment strategies for OSCC, gene therapy serves an important role alongside other treatment modalities including surgery, chemotherapy and radiotherapy. However, poor target activity is a pivotal reason that restricts the clinical applications of gene therapy in treating cancers. Therefore, there is a critical need to identify sensitive gene targets and to understand the molecular mechanism involved in the aggressive growth characteristics of OSCC.

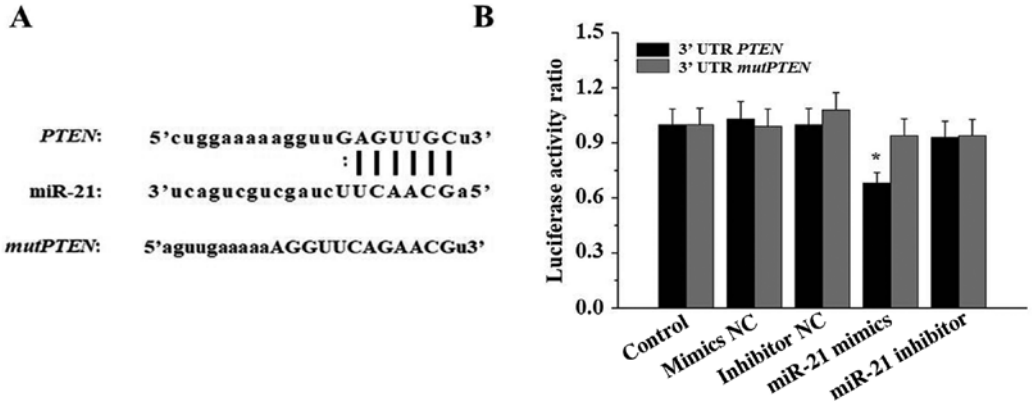

Figure 6. PTEN was identified as a target gene of miR-21. (A) Sequences of miR-21, PTEN 3'UTR and mutPTEN 3'UTR, including the sites involved in the binding between PTEN 3'UTR and miR-21. (B) Luciferase activity ratio was detected 48 h after 293T cells were transfected with mimics NC, inhibitor NC, miR-21 mimics or miR-21 inhibitor. *P<0.05. miR-21, microRNA-21; PTEN, phosphatase and tensin homolog deletion on chromosome 10; UTR, untranslated region; mut, mutant; NC, negative control.

miRNAs are considered to be important regulators of cell proliferation, differentiation, the cell cycle and cell death. They are also considered to be novel molecular targets in the diagnosis and treatment of human carcinomas. As a member of the oncomiRs, miR-21 has been confirmed to be overexpressed in various types of tumors and to be capable of negatively regulating multiple target genes. Li *et al* (25) demonstrated that miR-21 was overexpressed in tongue SCC (TSCC) tissues compared with that in the adjacent normal tissues, and that it may regulate TSCC development by inhibiting TSCC cell apoptosis in part via tropomyosin α-1 chain silencing. Koenig *et al* (26) compared the expression of miR-21 targets in 377 patients with liver cancer and revealed that the levels of 402 miR-21 targets were altered in hepatocellular carcinoma. Their analysis identified novel miR-21 targets (CAMSAP1, DDX1, MARCKSL1 and RMND5A) that appeared likely to serve a causal role in hepatocarcinogenesis. Yan *et al* (27) reported that miR-21 may promote salivary adenoid cystic carcinoma (SACC) progression through PDCD-4, PTEN and B-cell lymphoma 2, and suggested that miR-21 may be a novel target for SACC therapy.

The present study on OSCC determined that the expression of miR-21 had a negative association with the expression of PTEN protein. In OSCC cells, real-time RT-qPCR results revealed that the expression of miR-21 was significantly reduced in SCC15 and SCC25 cells transfected with miR-21 inhibitor compared with that in the control groups. By contrast, upregulation of PTEN gene expression was observed following the treatment with miR-21 inhibitor. The data indicated that miR-21 may negatively regulate PTEN gene expression in both OSCC tissues and cells. Furthermore, bioinformatics and luciferase assays indicated that miR-21 modulates PTEN expression by directly targeting a binding site within the mRNA 3'UTR. Collectively these findings revealed that PTEN is directly regulated by miR-21.

As the first tumor suppressor gene identified with phosphatase activity, PTEN has been confirmed as a target gene in colorectal, gastric, cervical and non-small cell lung cancer. Wu *et al* (28) reported that miR-21 could modulate malignant phenotypes including proliferation, invasion, cell cycle progression and anti-apoptosis in colorectal cancer cells by downregulating PTEN protein expression. A previous study indicated that the PI3K/Akt signaling pathway was activated in multiple types of cancers, and notably that the mechanisms activating PI3K/Akt signaling included loss of function of PTEN (29). Wang *et al* (30) observed that miR-155 suppressed PTEN expression, enhanced PI3K/Akt/mTOR signaling and inhibit human osteosarcoma MG-63 cell apoptosis and autophagy was induced by adrenomedullin. In the present study, upregulation of PTEN and downregulation of pAkt proteins was observed following treatment with miR-21 inhibitor. Additionally, the results revealed that when the expression of miR-21 was suppressed, the proliferative and invasive abilities of SCC15 and SCC25 cells were inhibited, while cell apoptosis was promoted. These data indicated that cell proliferation, invasion and apoptosis may be associated with the expression of PTEN and the PI3K/Akt signaling pathway in OSCC cell lines.

In conclusion, the present study revealed that miR-21 inhibitor transfection significantly inhibited the growth of SCC15 and SCC25 cells by reducing cell proliferation and promoting cell apoptosis. It was also revealed by luciferase assay that PTEN was a direct target of miR-21. Furthermore, the results indicated that the modulation of miR-21 activity may regulate the expression of PTEN, and that the PI3K/Akt signaling pathway may be involved through this targeting of PTEN. Overall, these present results indicate that the miR-21/PTEN axis may be a potential novel therapeutic target in OSCC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ, JX and HM conceived and designed the study. JX, FJ, YL and GC performed the experiments. YL, GC and HM analyzed the data. YZ and FJ wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The experimental procedures were approved by the Research Ethics Committee of Jining No. 1 People's Hospital and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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