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miR-221/222/PUMA Axis Promotes Oral Squamous Cell Carcinoma Apoptosis

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Abstract

Background: To investigate the therapeutic activity of the miR-221/222 inhibitor against OSCC *in vitro* and *in vivo*. **Materials and Methods:** HSC3 and HSC6 were treated with miR-221/222 inhibitor and the empty vector respectively. After the recombinants were transfected into HSC3 and HSC6 with Lipofectamine™ MAX, the expression of miR-221/222 and PUMA was analyzed by RT-PCR. The proliferation and migration of HSC3 and HSC6 were detected by CCK-8 assay and Wound-healing assay. Cell cycle and apoptosis were detected by flow cytometry. The effect of the miR-221/222 inhibitor was also assessed in OSCC xenografts in BALB/c-nu mice. **Results:** Transfection of the miR-221/222 inhibitor increased cell apoptosis and upregulated PUMA expression in OSCC cell lines HSC3 and HSC6 with the significantly reduced expression of miR-221 and miR-222. Furthermore, the miR-221/222 inhibitor suppressed cell growth and invasion and blocked the cell cycle at the G1 phase. Obvious anti-tumor activity was achieved in BALB/c-nu mice by treatment with the miR-221/222 inhibitor, together with the upregulation of PUMA protein in tumors retrieved from the mice. **Conclusions:** There was a significant inhibitory effect of the miR-221/222 inhibitor on the growth of OSCC both *in vitro* and *in vivo*, and there might be a regulatory loop between miR-221/222 and PUMA. These findings demonstrated that downregulation of miR-221/222 could induce cell apoptosis, and it might be considered as a candidate target for gene therapy of OSCC.

Keywords

miR-221/222, Apoptosis, Oral Squamous Cell Carcinoma, PUMA, Target

1. Introduction

As the most common cancer of the head and neck, oral squamous cell carcinoma

(OSCC) presents a poor prognosis, with a five-year survival rate. In recent years, gene therapy has begun to play an important role along with surgery, chemotherapy and radiotherapy [1] [2] [3]. In gene therapy, poor target activity is the pivotal reason restricting its clinical applications in treating cancers [4] [5]. Therefore, it is crucial to find sensitive gene targets and understand the molecular mechanisms involved in the aggressive growth characteristics of OSCC.

microRNAs (miRNAs), which could regulate protein expression by binding to partially complementary sequences in the 3'UTRs of target gene mRNAs, are small non-coding RNAs with a length of ~22-nt single-stranded RNA [6] [7]. In recent years, studies have demonstrated that miRNAs play an important role in the progression of cancers including OSCC [8] [9] [10]. miR-221 and miR-222 have the same seed sequence and are two highly homologous miRNAs encoded in tandem on the X chromosome. Overexpression of miR-221/222 has been observed in many advanced malignancies [11] [12]. Many studies have reported that miR-221/222 might be potential therapeutic targets in cancer development, either as oncogenes or as tumor suppressor genes, and several reports have suggested a key role of miR-221/222 in tumorigenesis [13] [14].

In fact, it has recently been shown that upregulation of miR-221/222 expression could confer resistance to TRAIL-induced cell death and enhance cell survival and proliferation of breast and lung cancer cells by targeting TIMP3 and PTEN [15] [16]. Moreover, it has been demonstrated that miR-221/222 could regulate prostate cancer cell proliferation, colony formation and invasion by directly targeting 3'UTR of the ARHI tumor suppressor gene [17]. In addition, one study has reported that miR-221/222 could also regulate radiosensitivity, cell growth and invasion of gastric cells by modulation of PTEN expression [18]. All these findings strongly support downregulation of miR-221/222 as a promising therapeutic option in many types of malignancies.

Our previous study has shown that PUMA (p53 upregulated modulator of apoptosis) was a direct target of miR-222, and that miR-222 could regulate the cell biological behavior of OSCC cell lines by targeting PUMA [19]. We have also found that the combination of miR-222 inhibitor transfection with the treatment of cisplatin (CDDP) could significantly inhibit OSCC cell growth by promoting apoptosis and reducing proliferation [20]. Since the therapeutic potential of the miR-221/222 inhibitor has been rarely investigated in OSCC, in the present study, we examined the biological effects and molecular mechanisms of miR-221/222 involved in the growth of OSCC *in vitro* and *in vivo*. Our results supported that the miR-221/222 inhibitor may be a novel agent for the treatment of OSCC.

2. Materials and Methods

2.1. Tissue Acquisition

OSCC tissues were obtained from 29 patients (including 14 males and 15 females) between October 2021 and March 2023 in Jining No. 1 People's Hospital (Shandong, China) with written informed consent as a prospective study. The inclusion criteria were as follows: 1) All tissues were confirmed by pathological ex-

amination, and radiotherapy or chemotherapy and other forms of antineoplastic therapy were not given before surgery. 2) Patients were between 40 to 65 years old. 3) According to the Union for International Cancer Control (UICC) standards in 2002, all the cases were classified as phases I to IV. Patients with an unclear diagnosis or who had radiotherapy or chemotherapy prior to surgery were excluded. In addition, 10 cases of normal oral tissue samples (aged 35 to 65, including 6 males and 4 females) were taken from patients with oral trauma, glossoplasty surgery and some non-neoplastic oral diseases were collected as control. The human material was performed in accordance with the Declaration of Helsinki and the experimental procedures were approved by the Research Ethics Committee of Jining No. 1 People's Hospital (2021-050).

2.2. Cell Culture and Transfection

Human oral squamous cell carcinoma cell lines HSC3 and HSC6 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and authenticated by short tandem repeat typing. DNA staining with Hoechst 33258 has been done to detect mycoplasma. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2 atmosphere. The miR-221/222 inhibitor and the empty vector were chemically synthesized by Gene Pharma Co., Ltd (Shanghai, China), and cell transfection was performed by the transfection system at 100 nm of miRNA inhibitor concentrations. Scramble sequences were used as negative control at the same concentration as miR-221/222 inhibitor. Cells were collected to be used for further analysis at different time points after transfection.

2.3. Real-Time qRT-PCR of miRNAs and mRNAs

Total miRNAs were extracted by means of the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), and mRNAs were extracted by means of the Total RNA Purification Kit (Qiagen GmbH) from HSC3 and HSC6 cells according to the manufacturer's instructions. Real-time qRT-PCR analysis was performed to validate miRNA and mRNA expression with the one-step RT-PCR kit. GAPDH (Invitrogen; Thermo Fisher Scientific, Inc.) and U6 were used as endogenous controls.

2.4. Western Blotting Analysis

After treatments for 48 h, HSC3 and HSC6 cells were washed three times with cold phosphate-buffered saline (PBS) and subjected to lysis in a buffer composed of RIPA (Beijing, China) and PMSF (Beijing, China) (100:1). Total proteins were heated at 95˚C for 10 min after the protein concentration was measured by means of the Enhanced BCA Protein Assay Kit (Shanghai, China). Equal amounts of heated proteins from each sample were separated by 12% SDS-PAGE gel (Bei-

jing, China) and transferred to polyvinylidene difluoride (PVDF) membranes (Beijing, China). The blots were blocked in TBST containing 5% non-fat milk for 1 h at room temperature and incubated with specific primary antibodies against PUMA, Bcl-2, Bax, Bak and GAPDH (Cell Signaling Technology, Inc.) overnight at 4˚C. Subsequently, the blots were rinsed three times with TBST and then incubated with the secondary antibody at room temperature for 1 h. The protein bands were imaged by the AlphaView SA western blot detection system (Carl Zeiss AG, Oberkochen, Germany) and quantified after normalization with the density of GAPDH (Cell Signaling Technology, Inc.) with Image J instrument software (National Institutes of Health, Bethesda, MD, USA). Each test was repeated in triplicate.

2.5. Immunofluorescence

After different treatments for 48 h, HSC3 and HSC6 cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100 and sequentially blocked in 1% BSA. Then the treated cells were incubated with primary antibodies (PUMA, Bcl-2, Bax and Bak) (Cell Signaling Technology, Inc.) and the corresponding secondary antibody according to the manufacturer's protocols. DAPI was used to stain nuclei. Images were captured by confocal laser scanning microscopy. Each test was repeated in triplicate.

2.6. Cell Proliferation Assay

Cell proliferation was measured by means of cell counting kit-8 (CCK-8) (Beijing, China) according to the manufacturer's instructions. After treatment with miR-221/222 inhibitor, HSC3 and HSC6 cells were seeded into 96-well plates at a density of 3000 cells/well and then allowed to attach overnight. After transfection, a 10-μl quantity of CCK-8 was added to each well at 0, 24, 48 and 72 h, and the cells were incubated for 1 h. Optical density (OD) was measured at a wavelength of 450 nm by spectrophotometric analysis. Each experiment was repeated in triplicate.

2.7. Wound-Healing Assay

The wound-healing assay was used to detect HSC3 and HSC6 cell migration. Twenty-four h after transfection, linear scratch wounds were created by means of a 200-μl pipette tip when cells grew to near-100% confluence in 6-well plates. Cells were maintained in serum-free medium to prevent cell proliferation. Images were taken at 0, 24, 48 and 72 h after scratching for visualization of wound healing and migrating cells. At least three areas were selected randomly from each well, and the data were derived from quintuplicate samples of three independent experiments.

2.8. Cell Migration Assay

After treatment, HSC3 and HSC6 cells were transferred to 8-μm-pore inserts and then placed in companion wells containing DMEM medium and 10% FBS. The

inserts were removed after a 12-hour incubation, and the non-migrating cells on the upper surface were harvested. HSC3 and HSC6 cells on the lower surface were fixed and stained and counted under a microscope. Each test was repeated in triplicate.

2.9. Cell Cycle Assay

HSC3 and HSC6 cells with different treatments were washed, harvested and stained with propidium iodide (PI, 20 μg/ml) (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Then the samples were analyzed by flow cytometry (Beckman Coulter GmbH, Krefeld, Germany), and the data were elaborated by Modfit software. Each test was repeated in triplicate.

2.10. Cell Apoptosis Assay

HSC3 and HSC6 cells were washed 3 times with PBS and resuspended in buffer at a concentration of 10^6/ml. Then the cells were mixed with 5 μl FITC-conjugated Annexin V reagent and 5 μl propidium iodide. After 15 min of incubation at room temperature in the dark, the samples were analyzed by flow cytometry (Beckman Coulter GmbH, Krefeld, Germany). Each test was repeated in triplicate.

2.11. Luciferase Assay

The 293T cells were co-transfected with 0.5 μg pMIR vectors containing PUMA 3'UTR (Shanghai, China) or mutPUMA 3'UTR (Shanghai, China) and 20 μM miR-221/222 mimics (Shanghai, China) or 20 μM miR-221/222 inhibitor (Shanghai, China) by Lipofectamine^{TM}RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.). Cells underwent lysis with Passive Lysis Buffer (Beijing, China) and were collected at 48 h post-transfection. The luciferase activity was detected with the dual luciferase reporter assay kit (Beijing, China) according to the manufacturer's protocol. Renilla luciferase activity was used for normalization.

2.12. Animals and *in Vivo* Models of Human OSCC

BALB/c-nu mice were selected for this study, and the experimental procedures were approved by the Research Ethics Committee of Jining No. 1 People's Hospital (2020-053). All BALB/c-nu mice used were maintained and handled under regulatory and institutional guidelines and surgical procedures. HSC3 and HSC6 cell suspensions (5×10^6) were injected subcutaneously into the interscapular area of the BALB/c-nu mice, and 30 mice in total were used in the study. The animal treatment was initiated after two weeks following HSC3 and HSC6 cell inoculation, when the xenograft tumors grew to approximately 40 mm^3. The treatments were performed by intra-tumor injection with 1 mg/kg per mouse of miR-221/222 inhibitor or inhibitor NC as control every two days for a total of seven injections. For nude mice that needed to be transfected, after the successful administration of anesthesia, the transplanted tumor sites of nude mice were disinfected with 75% alcohol, and needles were injected from 3 places, a 20-μl

quantity of transfection reagent was injected into the tumor and a 20-μl quantity of normal saline was injected into the control group. Tumor sizes were assessed before each treatment according to the formula: (maximum diameters × minimum diameters^2)/2. An inhibition ratio was calculated by the formula [1-(treated tumor average volume/untreated tumor average volume)] × 100%. After the experiment, the BALB/c-nu mice were euthanized by CO_2 euthanasia. The BALB/c-nu mice were placed in a euthanasia cage box, and CO_2 was filled at a balanced rate according to the CO_2 replacement rate of 30% - 70% container volume/min, so that the BALB/c-nu mice could quickly lose consciousness and minimize pain. Tumors were collected and placed for further analysis.

2.13. *In Situ* Hybridization (ISH)

OSCC tissues and xenograft tumors were fixed and embedded and then cut into 4-μm-thick sections. ISH analysis was performed on deparaffinized sections by means of the ISH tissue implementation kit (Beijing, China). OSCC tissues and xenograft tumor sections were incubated at 60˚C for 1 h and deparaffinized. Then the sections were rehydrated in decreasing concentrations of ethanol and washed in deionized water. The slides were incubated in 0.2 mol/l HCl at room temperature for 5 min and washed 3 times in PBS. Proteins were digested by the addition of pepsin solution at 37˚C for 15 min. Dehydration of sections was performed through 70%, 95% and 100% ethanol at each concentration for 1 min. The miR-221 and miR-222 probes labeled with digoxin were added to the hybrids. Sections were denatured at 90˚C for 4 min, followed by incubation in the ISH slide denaturation and hybridization system at 37˚C overnight. Finally, the slides were observed under an inverted microscope.

2.14. Immunohistochemical Staining

Immunohistochemistry was used to detect the expression of PUMA in OSCC tissues and PUMA, Bcl-2, Bax and Bak in xenograft tumors. The sections were deparaffinized with xylene and rehydrated by increased grades of ethanol. After antigen retrieval, primary rabbit polyclonal antibodies to PUMA, Bcl-2, Bax and Bak (Cell Signaling Technology, Inc.) at 1:200 dilution were added for treatment. Then immunohistochemical staining was performed by the recommended protocol.

2.15. Statistical Analysis

All the experiments were repeated at least 3 times, and the data analysis was performed with SPSS 19.0 software. The results were presented as means ± standard deviation (SD) with Student's t-test or one-way analysis of variance (ANOVA). The minimal level of significance was specified as $P < 0.05$.

3. Results

3.1. Expression of miR-221/222 and PUMA in OSCC Tissues and in HSC3 and HSC6 Cells

The expression of miR-221/222 and PUMA in OSCC tissues and normal tis-

sues was examined by ISH and immunohistochemical staining, respectively. As shown in Figure 1(A), miR-221/222 expression was higher in OSCC tissues than in normal tissues. By contrast, the expression of PUMA exhibited an opposite trend in OSCC tissues compared with the expression of miR-221/222. miR-221/222 and PUMA expression in HSC3 and HSC6 cells were analyzed by qRT-PCR. For knockdown of endogenous miR-221 and miR-222, miR-221 inhibitor and miR-222 inhibitor were synthesized and transfected into HSC3 and HSC6 cells. qRT-PCR analysis showed that miR-221/222 inhibitor transfection significantly reduced the miR-221 (Figure 1(B)) and miR-222 (Figure 1(C)) levels compared with control groups. In contrast, a notable upregulation of PUMA was observed in the miR-221/222 groups (Figure 1(D)). No significant differences between the empty vector transfection groups and the control groups were noted.

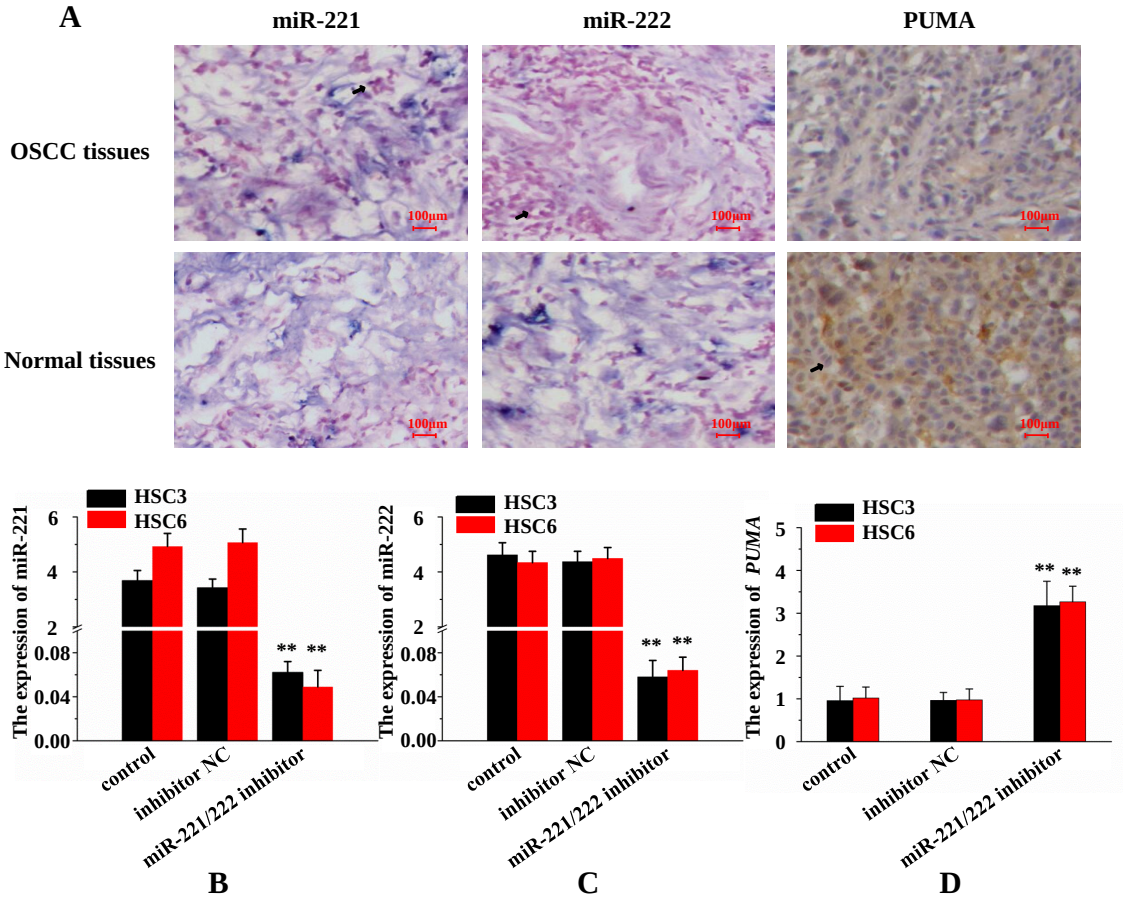

Figure 1. miR-221/222 and PUMA expression in OSCC tissues and in HSC3 and HSC6 cells. Expression of miR-221/222 and PUMA in OSCC tissues and in normal tissues (200×) (A). qRT-PCR analysis was used to detect the expression of miR-221/222 and PUMA in OSCC cell lines after treatment with the miR-221/222 inhibitor, the results showed significant downregulation of miR-221 (B) and miR-222 (C) in the miR-221/222 inhibitor groups compared with the control groups (**P < 0.01). The level of *PUMA* (D) expression following transfection with the miR-221/222 inhibitor was significantly increased compared with that of the control and inhibitor NC groups in HSC3 and HSC6 cells (**P < 0.01). Values were indicated as mean ± SD of three independent experiments. *Statistical significance (P < 0.05) and **obvious statistical significance (P < 0.01).

3.2. miR-221/222 Silence Altered the Expression of Apoptotic Proteins

miR-221/222 inhibitor transfection altered the expression of apoptotic proteins (PUMA, Bcl-2, Bax and Bak), and the apoptosis-related protein expression was measured by western blot and immunofluorescence. As shown in Figure 2, a significant increase of PUMA was observed in both HSC3 and HSC6 cells in miR-221/222 inhibitor groups. In contrast, Bcl-2 expression in miR-221/222 groups was downregulated relative to that in the control and inhibitor NC

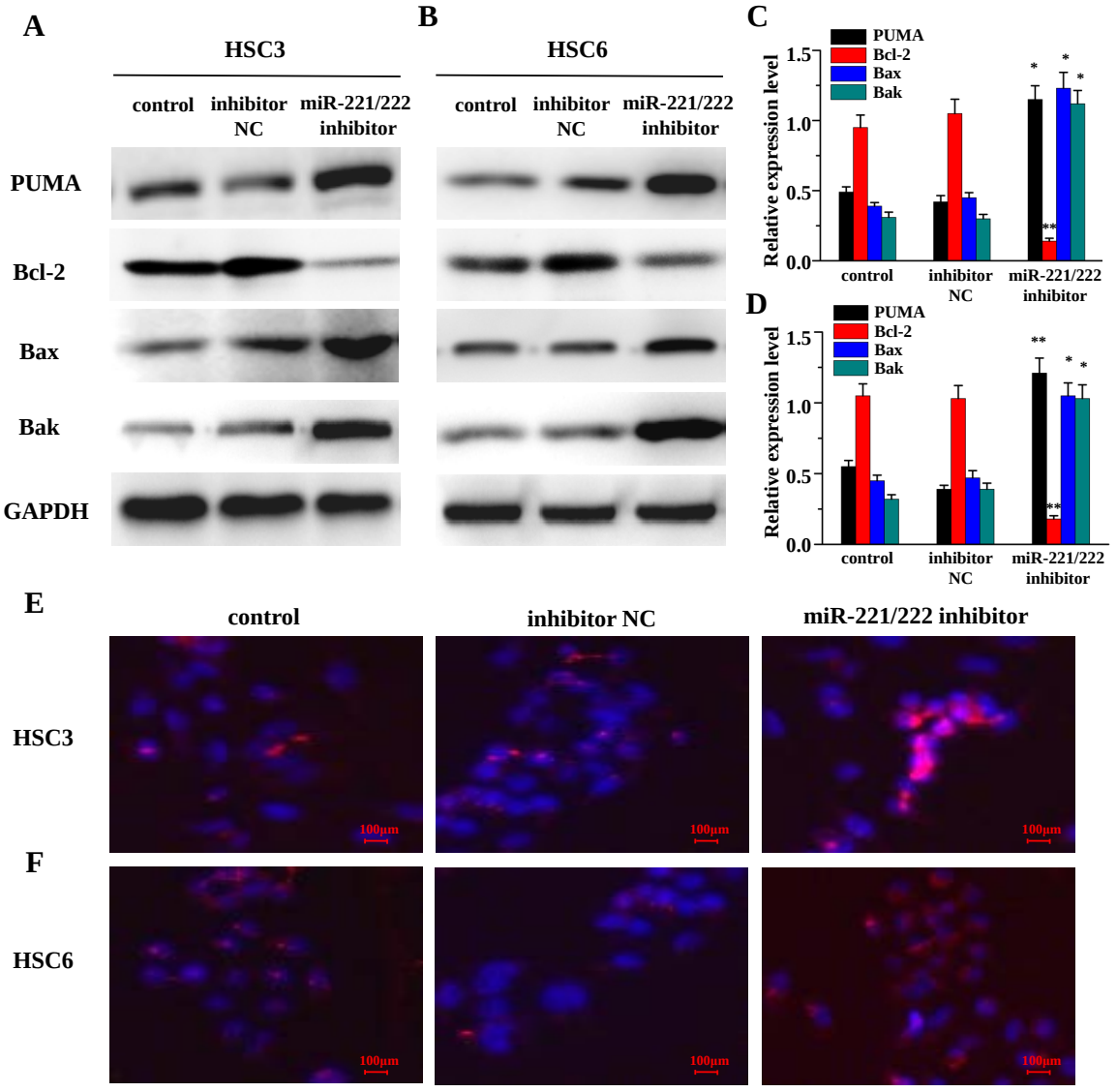

Figure 2. Expression of PUMA, Bcl-2, Bax and Bak in HSC3 and HSC6 cells with transfection of the miR-221/222 inhibitor. As determined by western blot, PUMA, Bax and Bak expression was observed to be upregulated in the miR-221/222 inhibitor groups compared with control and inhibitor NC groups ((A), (B)). In contrast, the expression of Bcl-2 exhibited an opposite trend. Relative expression levels of PUMA, Bcl-2, Bax and Bak were shown in HSC3 (C) and HSC6 (D) cells. Determination of PUMA expression was shown in HSC3 (E) and HSC6 (F) cells by immunofluorescence confocal microscopy (200×). Values were indicated as mean ± SD of three independent experiments. *Statistical significance ($P < 0.05$) and **obvious statistical significance ($P < 0.01$).

groups. The expression of Bax and Bak was increased as the result of Bcl-2 protein downregulation. Analysis of the data indicated that the miR-221/222 inhibitor could induce HSC3 and HSC6 cell apoptosis through altering the expression of PUMA, Bcl-2, Bax and Bak.

3.3. miR-221/222 Affected HSC3 and HSC6 Cell Proliferation and Invasion

The effect of miR-221/222 inhibitor on HSC3 and HSC6 cell proliferation and invasion was determined by CCK-8 assay, wound-healing assay and transwell assay. As shown in **Figure 3**, both the proliferation and the invasive abilities of HSC3 cells and HSC6 cells transfected with the miR-221/222 inhibitor were significantly reduced compared with those in the control groups ($P < 0.05$). There were no obvious differences between the control groups and inhibitor NC groups ($P > 0.05$).

3.4. miR-221/222 Inhibitor Induces HSC3 and HSC6 Cell Apoptosis

We assessed whether miR-221/222 inhibitor transfection modulated HSC3 and HSC6 cell apoptosis and cell cycle progress. The results showed that the apoptotic cells were significantly increased in cells treated with miR-221/222 inhibitor compared with control and inhibitor NC groups, suggesting that apoptosis was obviously induced in cells when treated with miR-221/222 inhibitor (**Figure 4(A)** and **Figure 4(B)**, $P < 0.01$). As for cell cycle progress, analysis of the data suggested that miR-221/222 silence induced a significant increase in the percentage of G1 phase cells in HSC3 and HSC6 cells (**Figure 4(C)** and **Figure 4(D)**).

3.5. Direct miR-221/222 Targeting of PUMA mRNA 3'UTR

PUMA 3'UTR and mutPUMA 3'UTR luciferase constructs were transfected into 293T cells with miRNA mimics NC, miRNAs inhibitor NC, miR-221/222 mimics or miR-221/222 inhibitor to validate whether PUMA was a direct target of miR-221/222. Luciferase activity was measured by means of a dual-luciferase reporter assay system. **Figure 5** showed that, compared with other groups, the luciferase activity of 293T cells transfected with PUMA 3'UTR and miR-221/222 mimics was reduced significantly ($P < 0.05$).

3.6. miR-221/222 Silence Inhibited the Growth of OSCC Cell Xenograft Tumors in BALB/c-nu Mice

In the previous experiment, we demonstrated the effect of miR-221/222 inhibitor on OSCC cells *in vitro*. Thus, we further evaluated the effect of miR-221/222 silence on the growth of OSCC cell xenograft tumors *in vivo*. As shown in **Figure 6**, the xenograft tumor volume of BALB/c-nu mice in the miR-221/222 inhibitor group was significantly decreased compared with that in the control group and the inhibitor NC group (**Figure 6(A)**, $P < 0.05$). The growth curve analysis

showed that the rate of xenograft tumor growth in the miR-221/222 inhibitor group was slower than that in the other two groups (Figure 6(B) and Figure 6(C)). The expression of miR-221/222 and apoptotic proteins was examined by ISH

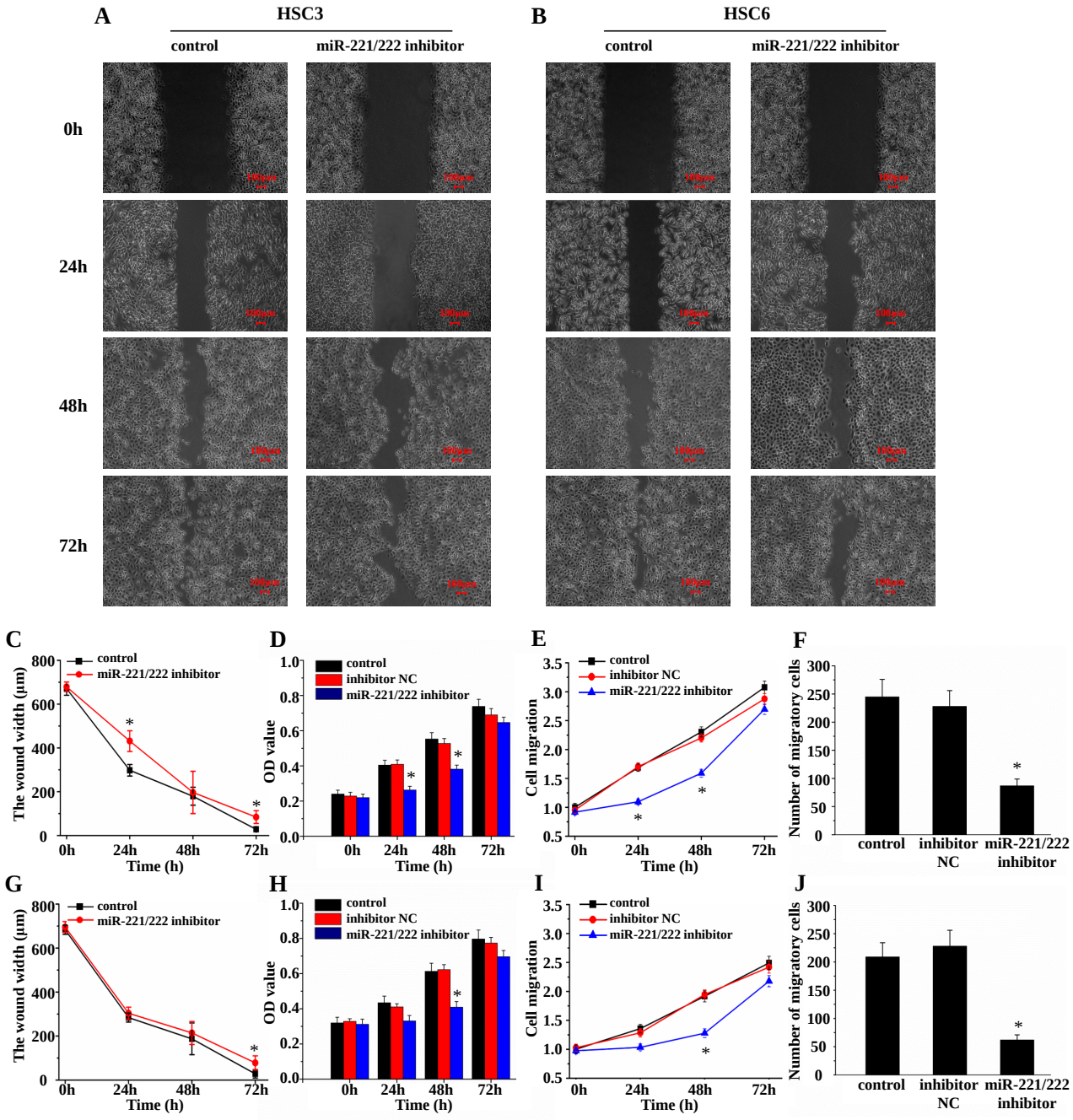

Figure 3. miR-221/222 influences HSC3 and HSC6 cell proliferation and invasion. Images and the wound width of HSC3 ((A) and (C)) and HSC6 ((B) and (G)) cells which were transfected with miR-221/222 inhibitor for the wound-healing assay. The CCK-8 assay and cell migration assay showed that HSC3 ((D) and €) and HSC6 ((H) and (I)) cells treated with miR-221/222 inhibitor proliferated at a significantly lower rate than in the control groups. The cell migration assay showed that HSC3 (F) and HSC6 (J) cells in the miR-221/222 inhibitor groups showed reduced migratory ability. No significant differences between the inhibitor NC groups and the control groups were noted. Values are indicated as mean ± SD of three independent experiments. *Statistical significance ($P < 0.05$) and **obvious statistical significance ($P < 0.01$).

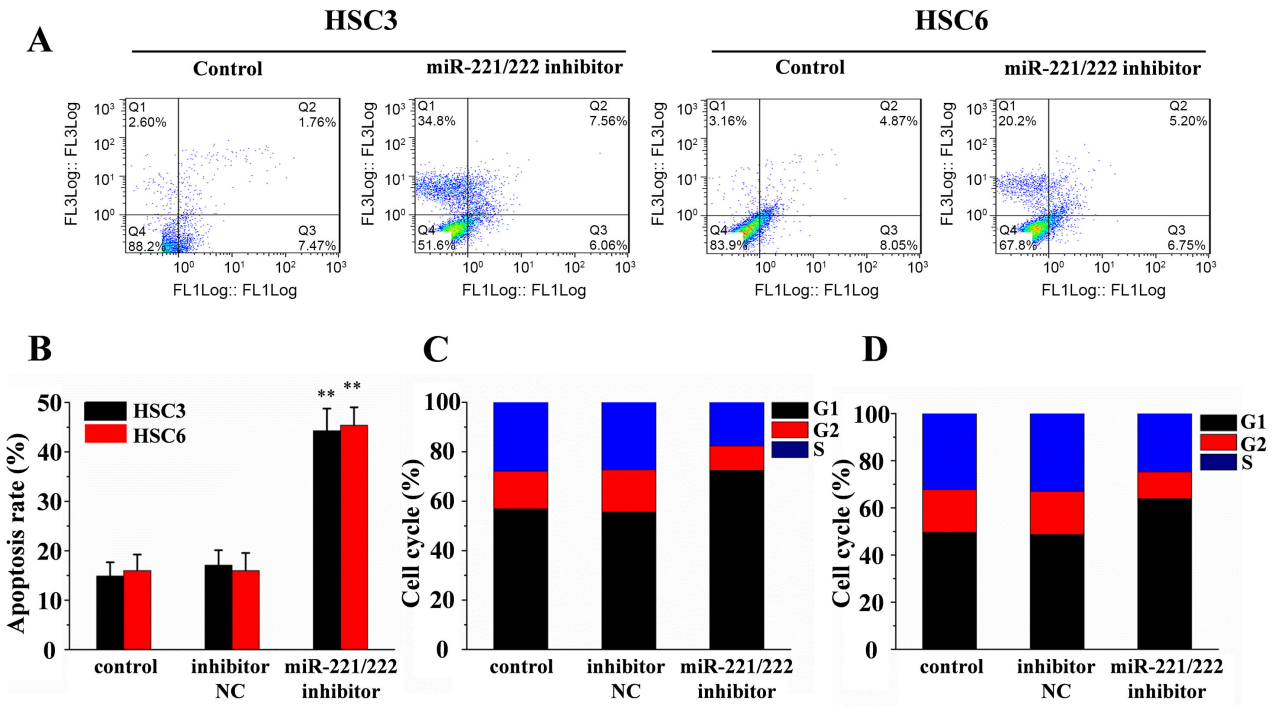

Figure 4. miR-221/222 silence induced HSC3 and HSC6 cell apoptosis. ((A), (B)) Percentages of apoptotic HSC3 and HSC6 cells in miR-221/222 inhibitor groups were significantly increased compared with control and inhibitor NC groups. Flow cytometry analyses were performed to detect (C) HSC3 and (D) HSC6 cell cycles in different groups. Values were indicated as mean ± SD of three independent experiments. *Statistical significance ($P < 0.05$) and **obvious statistical significance ($P < 0.01$).

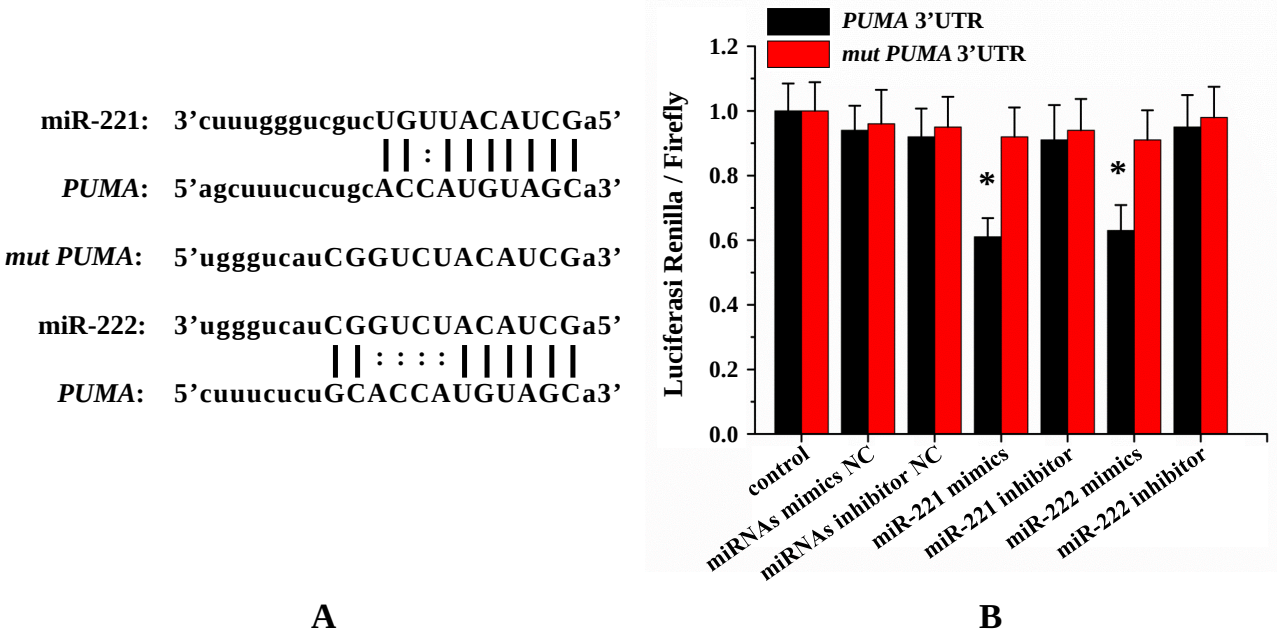

Figure 5. *PUMA* was confirmed to be a target gene of miR-221/222. (A) A panel that describes the binding sites between miR-221, miR-222 and PUMA 3′UTR and the sequence of *mutPUMA* 3′UTR. (B) Luciferase activity ratio of PUMA 3′UTR and mutPUMA 3′UTR luciferase constructs was determined after 293T cells were transfected with mimics NC, miR-221/222 mimics, inhibitor NC or miR-221/222 inhibitor. Values were indicated as mean ± SD of three independent experiments. * Denotes statistical significance ($P < 0.05$).

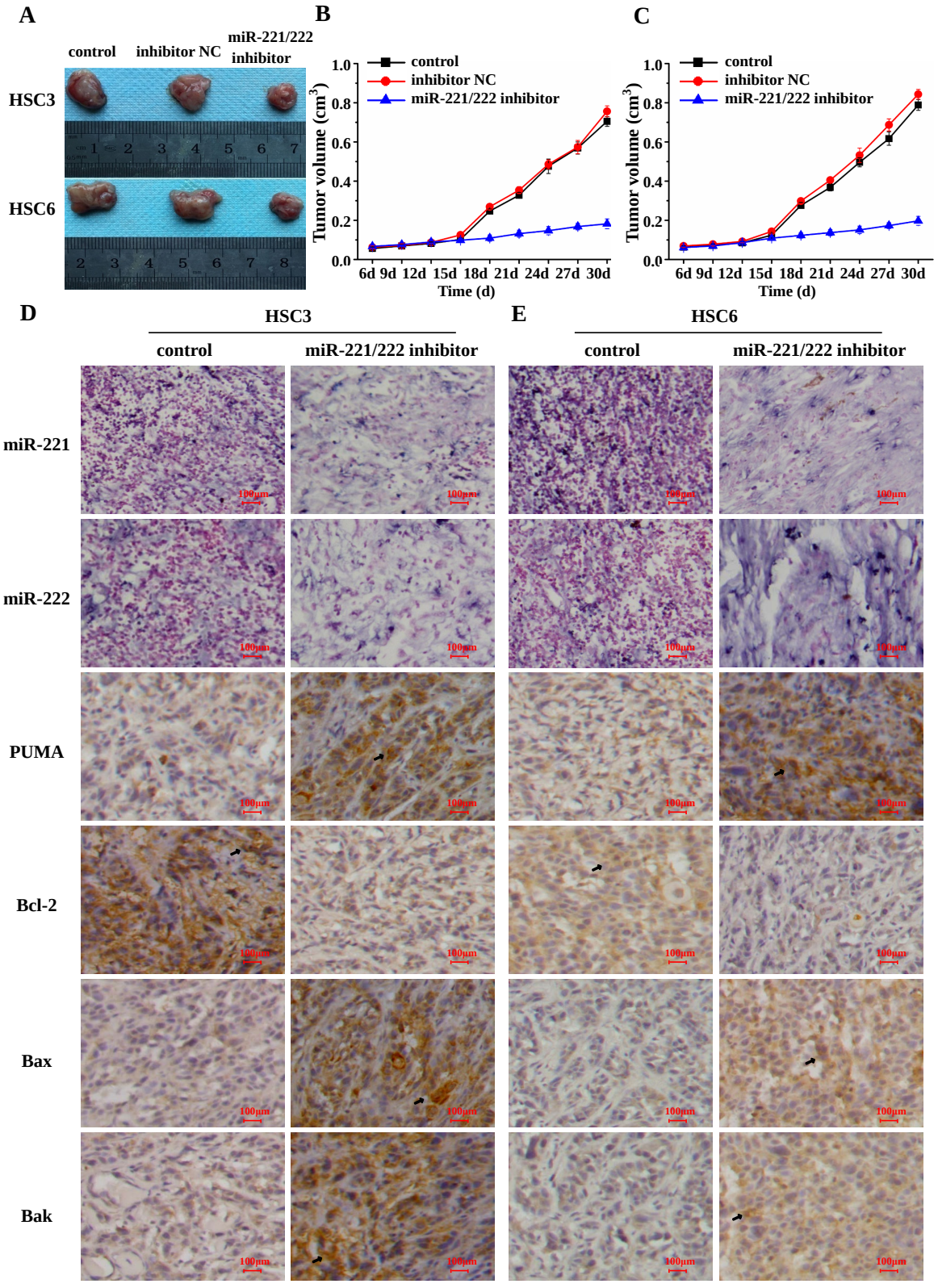

Figure 6. miR-221/222 silence suppressed the growth of HSC3 and HSC6 tumor xenografts. (A) The xenograft tumor volume of OSCC cell lines in the miR-221/222 inhibitor group and control group. ((B), (C)) The growth curve analysis showed the rate of xenograft tumor growth. ((D), (E)) The expression of miR-221/222 and apoptotic proteins was examined by ISH and immunohistochemical assay, respectively, in HSC3 and HSC6 tumor xenografts (200×).

and immunohistochemical assay, respectively, in both HSC3 and HSC6 tumor xenografts (**Figure 6(D)** and **Figure 6(E)**). The results suggested that miR-221/222 expression in the miR-221/222 inhibitor group was significantly lower than that of the control group. As for the apoptotic proteins, the expression of PUMA, Bax and Bak was increased, while Bcl-2 expression was decreased in the miR-221/222 inhibitor group.

4. Discussion

miRNAs, which are considered to be novel molecular targets in the diagnosis and treatment of human carcinomas, play important roles in the regulation of cell differentiation, proliferation, apoptosis, cell cycle and death [21]. miR-221/222, which act as oncomiRs, are known to promote the development and progression of various carcinomas. miR-221/222 overexpression could influence cell growth by targeting important tumor suppressor genes such as TIMP3, PTEN, p57, Bim and p27Kip1 [15] [18] [22] [23] [24]. Overexpression of miR-221/222 has been observed in many advanced malignancies, indicating that miR-221/222 could be potential therapeutic targets for epithelial cancer.

PUMA, which was newly discovered in 2001, is a key protein in apoptosis [25]. It belongs to the BH3-only subfamily of the Bcl-2 protein family and induces apoptosis through both p53-dependent and non-p53-dependent pathways. Although the specific mechanisms for inducing cell apoptosis need further investigation, PUMA has been confirmed to be a potential new target for gene therapy, since its pro-apoptotic function has achieved favorable results. Zhang *et al.* [26] reported that miR-221/222 could directly regulate apoptosis by targeting PUMA in glioblastoma; moreover, they found an inverse relationship between PUMA and miR-221/222 expression in glioma tissues *in vivo*. However, the influence of miR-221/222 and PUMA on the occurrence and development of OSCC needed to be further explored.

To detect the relationship between miR-221/222 and PUMA in OSCC, we transfected miR-221/222 inhibitor into HSC3 and HSC6 cells and real-time qRT-PCR results confirmed that the expression of miR-221/222 was significantly reduced in HSC3 and HSC6 cells transfected with miR-221/222 inhibitor compared with the control groups. In contrast, upregulation of PUMA gene expression was observed following treatment with the miR-221/222 inhibitor. As *in vivo*, the expression of miR-221/222 had a negative correlation with the expression of the PUMA protein. Analysis of the data suggested that miR-221/222 may negatively regulate the PUMA gene both *in vitro* and *in vivo* in OSCC. Our results were similar to Zhang's findings in glioblastoma [26].

Liu *et al.* [27] detected the clinical significance of miR-221/222 in retinoblastoma patients and explored its role in retinoblastoma cells. They found that overexpression of miR-221/222 was associated with tumor invasiveness in patients with retinoblastoma, and that targeting of miR-221/222 could induce apoptosis and inhibit Y79 cell proliferation, migration and invasion *in vitro*.

Wang *et al.* [28] investigated the role of miR-221/222 in cell proliferation and apoptosis in human prostate cancer cells. Their results showed that miR-221/222 could promote prostate cancer cell proliferation and repress apoptosis through suppressing caspase-10 and provided promising evidence for the miRNA-based therapeutic strategy of treating prostate cancers. In the present study, upregulation of the pro-apoptosis protein PUMA, Bax and Bak and downregulation of the apoptotic protein Bcl-2 were observed following treatment with the miR-221/222 inhibitor in OSCC cells and OSCC cell xenograft tumors. Furthermore, both bioinformatics and luciferase assays showed that miR-221/222 modulates PUMA expression by directly targeting the binding site within the 3'UTR. These findings suggest that PUMA is probably directly regulated by miR-221/222 in OSCC. In addition, our results showed that both the proliferation and the invasive abilities of HSC3 cells and HSC6 cells transfected with miR-221/222 inhibitor were significantly reduced compared with the control groups. However, cell apoptosis was obviously induced when treated with the miR-221/222 inhibitor. As for cell cycle progress, analysis of the data suggested that miR-221/222 silence induced a significant increase in the percentage of G1 phase cells in HSC3 and HSC6 cells. Based on the above experimental results, we knew that the miR-221/222 inhibitor might affect cell proliferation, migration and apoptosis by arresting the G1 phase of the cell cycle in OSCC.

As *in vivo*, the rate of OSCC xenograft tumor growth in the miR-221/222 inhibitor group was slower than that in the other two groups. Analysis of the western blot and immunohistochemical assay data suggested that cell proliferation, invasion and apoptosis might be associated with the expression of PUMA and the apoptotic proteins such as Bcl-2, Bax and Bak in OSCC cell lines and OSCC cell xenograft tumors. However, the specific mechanism still needs further confirmation.

5. Conclusion

The present study revealed that miR-221/222 inhibitor transfection could significantly inhibit the growth of HSC3 and HSC6 cells by reducing cell proliferation and promoting cell apoptosis both *in vitro* and *in vivo*. It was also demonstrated that PUMA was a direct target of miR-221/222 by the luciferase assays. Furthermore, the results indicated that the modulation of miR-221/222 activity could regulate the expression of PUMA and the apoptotic proteins. Overall, the present results indicated that the miR-221/222/PUMA axis might be a potential novel therapeutic target for OSCC.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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