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ORIGINAL RESEARCH

Codelivery of doxorubicin and MDR1-siRNA by mesoporous silica nanoparticles-polymerpolyethylenimine to improve oral squamous carcinoma treatment

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Abstract: Oral cancer is a type of head and neck cancer that is the seventh most frequent cancer and the ninth most frequent cause of death globally. About 90% of oral cancer is of squamous cell carcinoma type. Surgery and radiation with and without chemotherapy are the major treatments for oral cancer. Better advanced treatment is still needed. Multidrug resistance plays an important role in failure of oral cancer chemotherapy. In this study, we tried to fabricate a novel nanoparticle that could carry both MDR1-siRNA to block MDR1 expression and doxorubicin (DOX), a chemotherapy drug, into cancer cells in order to directly kill the cells with little or no effect of multidrug resistance. Results showed that mesoporous silica nanoparticles (MSNP) can be modified by cationic polymerpolyethylenimine (PEI) to obtain positive charges on the surface, which could enable the MSNP to carry MDR1-siRNA and DOX. The transfection efficiency assays demonstrated that the MSNP-PEI-DOX/MDR1-siRNA was efficiently transfected into KBV cells in vitro. KBV cells transfected with MSNP-PEI-DOX/MDR1-siRNA could effectively decrease gene expression of *MDR1* (~70% increase after 72 hours posttreatment) and induce the apoptosis of KBV cells (24.27% after 48 hours posttreatment) in vitro. Importantly, MSNP-PEI-DOX/MDR1-siRNA dramatically reduced the tumor size (81.64% decrease after 28 days posttreatment) and slowed down tumor growth rate compared to the control group in vivo ($P<0.05$). In the aggregate, newly synthesized MSNP-PEI-DOX/MDR1-siRNA improves cancer chemotherapy effect in terms of treating multidrug-resistant cancer compared to DOX only, clearly demonstrating that MSNP-PEI-DOX/MDR1-siRNA has potential therapeutic application for multidrug-resistant cancer in the future.

Keywords: oral squamous carcinoma, doxorubicin, MDR1, mesoporous silica nanoparticles, polymerpolyethylenimine

Introduction

Oral squamous cell carcinoma is the most common maxillofacial malignant neoplasm. Chemotherapy is an important treatment to cure or prevent tumors from relapsing and metastasizing. One of the major problems, however, is the development of multidrug resistance (MDR) in squamous cancer cells, resulting in the failure of cancer treatment. Multiple drug resistance protein 1 (MDR1) is also known as P-glycoprotein 1, ATP-binding cassette-sub-family B member 1, or cluster of differentiation.[1] MDR1 is a cell membrane protein that plays an important role in pumping foreign substances out of cells. MDR1 is constitutively expressed in normal cells, such as intestinal

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epithelium, liver cells, or kidney capillary endothelial cells to pump unused substances out of cells and body.[2–6] Unfortunately, some tumors also express higher levels of MDR1, resulting in drug resistance because the higher level of MDR1 in cancer cells prevents intracellular buildup of chemotherapeutic agents.[7–9] Multidrug resistance has always been a difficult problem in cancer chemotherapy.[10] According to the American Cancer Society, 61% of multidrug reistances are related to primary drug resistance, and 33% are related to acquired drug resistance among patients who die from cancer every year. Hence, it is of great significance to reverse or inhibit multidrug resistance effect during cancer treatment.

siRNA production is a biological process in cells where 20–25 base pairs of RNA molecules are involved in regulating specific gene expression by posttranscriptional gene silencing. Recently, siRNA technology has been widely used in basic research to understand the function of a specific gene and has also been used as a clinical therapeutic approach to cure a specific disease.[11–14]

Doxorubicin (DOX) is a common chemotherapy medication used to treat many kinds of cancers, such as breast cancer, bladder cancer, and lymphoma.[15–19] DOX interacts with DNA leading to a break in the DNA strand, which blocks DNA transcription and replication, resulting in apoptosis of cancer cells.[20]

Silica nanoparticles are one of the most promising inorganic nano-biomaterials and are approved by the US FDA for cancer therapy clinical trials.[21] Mesoporous silica nanoparticles (MSNP) contain a phosphonate surface, and can be coated with cationic polyethylenimine (PEI) polymers to enable them to carry DNA or siRNA.[22] MSNP-PEI polymer attachment leaves the porous interior free for antitumor drug binding and delivery, such as DOX.[23] Also, MSNP possess many unique advantages, such as extensive and uniform mesoporosity, tunable particle size and wide ranges of pore diameter, flexible morphology, high surface area and large pore volume, facile surface functionalization, excellent biocompatibility, easy modification, and biodegradation.[24–28] Therefore, these characteristics allow MSNP to have a distinctively high payload of anticancer drugs and controllable release feature.

To overcome or prevent the difficulty of multidrug resistance during squamous cancer treatment, herein we investigate if MSNP could be modified, and then efficiently carry/deliver MDR1 siRNA to block gene expression of MDR1 in cancer cells, and also efficiently carry/deliver DOX into cancer cells leading to killing of the cancer cells at the same time in order to improve and achieve optimum cancer treatment effect.

Materials and methods

Particle synthesis, PEI coating, and characteristics of MSNP

MSNP were synthesized according to sol-gel procedure.[22,29] Briefly, 100 mg of cetyltrimethyl ammonium bromide (Sigma-Aldrich Co., St Louis, MO, USA) was dissolved in 48 mL of ddH_2O plus 0.35 mL of sodium hydroxide (2 M) and heated on a block heater at 80°C. Next, 0.5 mL of tetraethyl orthosilicate (Beijing Chemical Reagents Company, Beijing, People's Republic of China) was added into the solution, and then 15 minutes later 150 µL of 3-trihydroxysilylpropyl methylphosphonate was added into the solution to perform phosphonate modification. The solution was continuously stirred on the block heater for 2 hours. MSNP were collected by centrifugation at 18,000 rpm for 20 minutes. The synthesized MSNP were dispersed alternately with 20 mL of anhydrous ethanol or deionized water by centrifugation (at 18,000 rpm for 15 minutes) three times. Finally, the MSNP were dispersed in 20 mL of anhydrous ethanol plus 1 mL of 37% of HCl, and heated on the block heater at 70°C for 24 hours to remove cetyltrimethyl ammonium bromide surfactants from the pores.

To coat MSNP with PEI, 5 mg of phosphonate-modified MSNP were dispersed in 1 mL of anhydrous ethanol containing 2.5 mg of PEI (25 kD; Sigma-Aldrich Co.), sonicated and stirred for 30 minutes, and dispersed alternately with 20 mL of anhydrous ethanol or deionized water by centrifugation (at 18,000 rpm for 15 minutes) two times. After these procedures, ~10% of the surface of MSNP were coated by PEI polymer.

Morphology of the final modified MSNP was examined with a transmission electron microscope (H-800; Hitachi Ltd., Tokyo, Japan). The average of the hydrodynamic diameter and the zeta potential of the nanoparticles were obtained using dynamic light scattering at 25°C.

Cell culture

In this study, human oral squamous carcinoma DOX-resistant cell line (KBV) was used, which was obtained from Nanjing Keygen Biotech Company (Nanjing, People's Republic of China). KBV cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (ThermoFisher Scientific, Waltham, MA, USA), together with 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in 5% CO_2, in a 95% humidified incubator.

Cytotoxicity assays in vitro

KBV cells were plated/cultured at 3×10^3 cells/well in a 96-well plate with the MSNP-PEI at 0, 5, 10, 20, 40, and 50 µg/mL for 24 hours. Then, the cells were incubated with 20 µL of MTT

solution (5 mg/mL; AMRESCO, Solon, OH, USA) for 4 hours at 37°C. After incubation, medium was removed and replaced with 150 μL dimethyl sulfoxide and mixed thoroughly. The plate was read at 490 nm wavelength with a microplate reader (RT-6000; Lei Du Life Science and Technology Co, Shenzhen, People's Republic of China). Percentage of cell viability was calculated compared to no MSNP-PEI control wells.

Drug loading rate and release assays

Five milligrams of DOX (Sigma-Aldrich Co.) was dissolved in 5 mL phosphate-buffered saline (PBS, pH 7.4), then 10 mg of freeze-dried MSNP-PEI was added, stirred at room temperature for 24 hours, spun to collect supernatant, washed with PBS two times, and all supernatants collected. The OD of supernatants were measured at 480 nm, and unloaded DOX was calculated according to standard curve. One milligram of MSNP-PEI-DOX was suspended in 1 mL PBS (pH 7.4) and oscillated constantly at 37°C. The OD of supernatant was measured after 1, 2, 4, 8, 10, 12, 24, 48, 72, 96, and 120 hours. Accumulated DOX release was calculated at each time point.

Agarose gel retardation assay

To determine the optimal ratio of MSNP-PEI to siRNA, plasmid pACCMV-EGFP was used to mimic siRNA. Ratios of MSNP-PEI to pACCMV-EGFP (w/w, μg) from 1:0 to 25:1 were tested. Each ratio was 10 μL with 2 μL of loading buffer, and then 12 μL of sample was loaded into 1% of agarose gel for electrophoresis.

Transfection assays

Transfection assays were set up for six groups, PBS (control), MSNP-PEI/scrambled siRNA (negative control [NC]), MSNP-PEI/MDR1-siRNA (MSNP-PEI/siRNA), MSNP-PEI-DOX, MSNP-PEI-DOX/siRNA (MSNP-PEI-DOX/siRNA), and DOX only (DOX) groups. MDR1-siRNA and scrambled siRNA were obtained from Shanghai GenePharma Co., Ltd (Shanghai, People's Republic of China), and DOX was obtained from Sigma-Aldrich Co. Forty-five micrograms of MSNP-PEI was mixed with 75 pmol of scrambled siRNA at 37°C for 30 minutes to form NC. Forty-five micrograms of MSNP-PEI was mixed with 75 pmol of MDR1-siRNA at 37°C for 30 minutes to form MSNP-PEI/MDR1-siRNA. We used 51.6 μg of MSNP-PEI-DOX mixed with 75 pmol of MDR1-siRNA at 37°C for 30 minutes to form MSNP-PEI-DX/MDR1-siRNA. The MDR1-siRNA was labeled with carboxyfluorescein (FAM) and then mixed with MSNP-PEI to be used to evaluate transfection efficiency. Forty-five micrograms of MSNP-PEI was mixed with 75 pmol of MDR1-siRNA^{FAM} at room temperature for 30 minutes to form MSNP-PEI/MDR1-siRNA^{FAM} complex.

The KBV cells were seeded/cultured in six-well plates at a density of 3×10^5 cells/well for 24 hours prior to transfection, and then the medium was replaced with 1 mL of serum-free medium. Subsequently, 100 μL of PBS, 45 μg of NC, 45 μg of MSNP-PEI/MDR1-siRNA, 51.6 μg of MSNP-PEI-DOX, 51.6 μg of MSNP-PEI-DOX/MDR1-siRNA, or 3.6 μg of DOX was added into the corresponding well. Forty-five micrograms of MSNP-PEI/MDR1-siRNA^{FAM} was also added into the corresponding well. After 48 and 72 hours of transfection, the cells were collected for further gene expression assays and apoptosis assay. After 48 hours of transfection, the cells treated with MSNP-PEI/MDR1-siRNA^{FAM} were used to assess transfection efficiency by flow cytometry (BD Biosciences, San Jose, CA, USA).

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assay

To evaluate effects of MDR1-siRNA on MDR1 gene expression, RT-qPCR assays were used. KBV cells from different transfection groups were used to extract total RNA using an RNeasy mini purification kit (Qiagen, Valencia, CA, USA), and cDNAs were synthesized using iScript™ cDNA synthesis kit (Takara Bio, Tokyo, Japan). Primers (MDR1 F: 5′-TGACTCAGGAGCAGAAGTTTGAACA-3′ and MDR1 R: 5′-AAATACATCATTGCCTGGGTGAAG-3′) were used to check MDR1 expression. Primers (β-actin F: 5′-TGGCACCCAGCACAATGAA-3′ and β-actin R: 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′) were used as internal control. The qPCR assays were performed using SYBR-Green Premix Ex Taq (Takara Bio) and MxPro M ×3005P real-time PCR detection system (Agilent Technologies, Santa Clara, CA, USA). All experiments were repeated three times.

Apoptosis analysis using flow cytometry

The above transfected KBV cells from different groups were used to perform apoptosis analysis using fluorescein isothiocyanate (FITC)-labeled Annexin V (Annexin-V-FITC) and 7-amino-actinomycin D (7-AAD) double staining (Nanjing Keygen Biotech Company). Briefly, after 48 hours of transfection, cells from each group were collected, washed with cold PBS and Annexin-binding buffer, and 5 μL Annexin-V-FITC and 5 μL 7-AAD working solution were added to each cell suspension. The mixture was incubated at room temperature for 15 minutes, then 400 μL of binding buffer was added, and these stained cells were analyzed with flow cytometry at 530 nm and at 575 nm using 488 nm excitation.

Animal experiments

To further evaluate therapeutic effects of MSNP-PEI-DOX/MDR1-siRNA in vivo, 20 male Balb/c-nude mice (4–6 weeks old) were used to create subcutaneous tumors by injecting KBV cells (1.5×10^6 cells per mouse) into the dorsal flank. After tumor diameters reached about 5 mm, tumor-bearing mice were randomly divided into four groups (n=5): PBS, MSNP-PEI/MDR1-siRNA (150 μg MSNP-PEI plus 16.5 μg MDR1-siRNA), MSNP-PEI-DOX (150 μg MSNP-PEI-DOX), and MSNP-PEI-DOX/MDR1-siRNA (150 μg MSNP-PEI-DOX plus 16.5 μg MDR1-siRNA). Components from the MSNP-PEI/MDR1-siRNA and MSNP-PEI-DOX/MDR1-siRNA groups were separately dissolved in 50 μL of PBS and from the MSNP-PEI-DOX group in 100 μL of PBS and administered into the tumor by intratumoral injection with an insulin syringe once a day for 5 days. The size of the tumor was measured every 3 days until 28 days posttreatment. Tumor volume was calculated with the formula $a \times b^2 \times 0.5$, (where a is the largest diameter and b is the smallest diameter. After 28 days, tumor xenograft, heart, liver, and lung were collected, fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). For immunohistochemical detection, the anti-MDR1 monoclonal antibody (Proteintech Group, Inc, Wuhan, People's Republic of China) was used at a dilution of 1:500. The anti-Ki67 antibody (Proteintech Group, Inc) was used at a dilution of 1:500. The anti-Bcl-2 antibody (Proteintech Group, Inc) was used at a dilution of 1:200. All immunohistochemistry experiments were carried out using UltraSensitive TM^S-P kit (Maixin Biotech Co., Ltd., Fu-zhou, People's Republic of China) according to the manufacturer's instructions. Animal use in this study was approved by the Institutional Animal Care and Use Committee of Jilin University, Changchun, People's Republic of China. The animal experiments were performed in accordance with the requirements of the Experimental Animal Ethics and Welfare guidelines (Permit Number: 20160205).

Statistical analysis

All in vitro experiments were repeated three times. Results are expressed as mean ± standard deviation. One-way analysis of variance followed by Tukey post hoc analysis was used to test for statistical significance. *P*-value <0.05 was considered to be significantly different.

Results

Characteristics of MSNP and MSNP-PEI nanoparticles

Dynamic light scattering results show that sizes (hydrodynamic diameter) of MSNP and MSNP-PEI nanoparticles were 150.4±6.9 nm and 170.5±3.8 nm, respectively (Table 1). Zeta potential of MSNP was −24.3±2.7 mV, while zeta potential of MSNP-PEI was +34.7±0.9 mV (Table 1). The transmission electron microscopy images showed that both MSNP and MSNP-PEI nanoparticles were spherical (Figure 1A and B). The surface mesoporous diameter of both MSNP and MSNP-PEI nanoparticles was 3–5 nm (Figure 1A–D).

Table 1 Particle size and zeta potential

Samples	Average particle size (nm), mean ± SD	Zeta potential (mV), mean ± SD
MSNP	150.4±6.9	−24.3±2.7
PEI	12.9±0.7	+41.1±1.3
MSNP-PEI	170.5±3.8	+34.7±0.9

Abbreviations: MSNP, mesoporous silica nanoparticles; PEI, polymerpolyethylenimine.

The surface of mesoporous silica nanoparticles had a phosphonate group which allowed DOX to bind to the porous interior by electrostatic binding and also allowed exterior coating with the cationic polymer, PEI, resulting in MSNP-PEI with the ability to contemporaneously bind and deliver MDR1-siRNA.

Cytotoxicity assays

Cytotoxicity was assessed on KBV cells via MTT assays. Results show that both MSNP and MSNP-PEI had similar mild effects on the KBV cells (Figure 1E). These weak effects were only seen after the dose was increased to over 40 μg/mL (Figure 1E), and the effects at 50 μg/mL were significantly different compared with the no-treatment control group (Figure 1E) ($P<0.05$).

DOX loading rate and release rate

By calculating the percentage of drug encapsulated in MSNP-PEI nanoparticles from the total amount of nanoparticles, the DOX loading rate of MSNP-PEI was found to be 7.15%±1.43% (w/w), while the DOX loading rate of MSNP was 6.6%±0.81% (w/w) (Figure 1F). Data of in vitro cumulative release of DOX show that DOX was released from the MSNP-PEI nanoparticles in two phases, one was an exponential release phase within the first 12 hours, and the second phase was a slow plateau phase between 12 and 120 hours (Figure 1G). The DOX cumulative release rate from MSNP-PEI was slow, ~30% at 12 hours, ~50% at 48 hours, and ~73% at 120 hours (Figure 1G).

Determination of optimal carry amount of MDR1-siRNA

To find out the optimal carry amount of siRNA by MSNP-PEI, different ratios of MSNP-PEI to plasmid pACCMV-EGFP

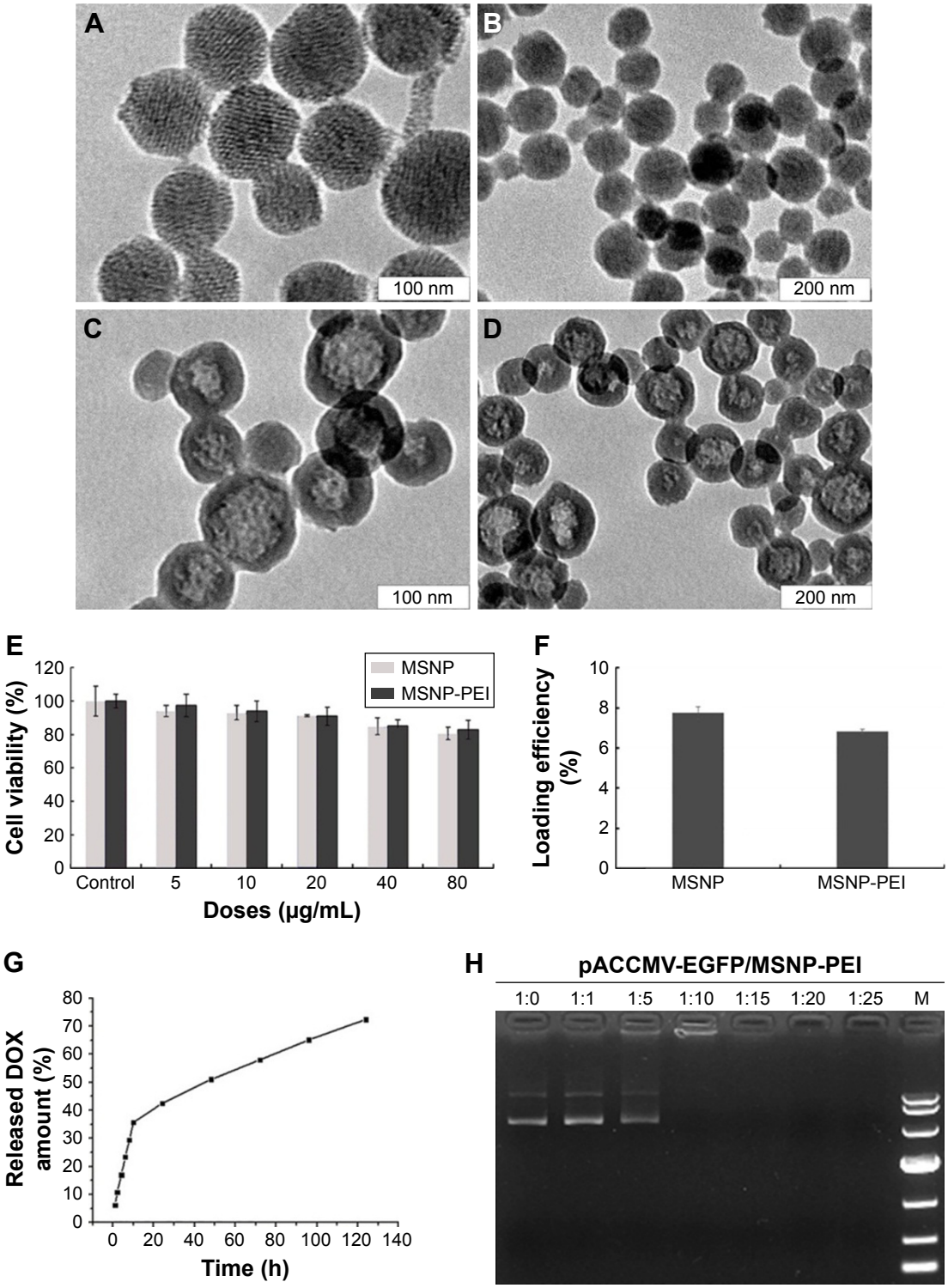

Figure 1 Characteristics of the MSNP and MSNP-PEI nanoparticles.
Notes: (**A** and **B**) TEM images of MSNP. (**C** and **D**) TEM images of MSNP-PEI. (**E**) Cell viability assays. (**F**) Loading ability of MSNP and MSNP-PEI for DOX. (**G**) Release profile of DOX from MSNP-PEI. (**H**) Agarose gel electrophoresis retardation assay for MSNP-PEI/pACCMV-EGFP.
Abbreviations: DOX, doxorubicin; MSNP, mesoporous silica nanoparticles; PEI, polymerpolyethylenimine; TEM, transmission electron microscopy.

(from 1:0 to 25:1) were used to perform agarose gel electrophoresis. Figure 1H clearly shows that the migration of pACCMV-EGFP was completely retarded at the ratio of 10:1. This suggests that the complex of MSNP-PEI/pACCMV-EGFP nanoparticles becomes neutral when the w/w ratio of MSNP-PEI and pACCMV-EGFP reached 10:1, a ratio which can allow PEI on the MSNP to carry the maximum amount of plasmid or MDR1-siRNA.

Transfection efficiency of MSNP-PEI-DOX/MDR1-siRNA

To evaluate transfection efficiency of MSNP-PEI-DOX/MDR1-siRNA, KBV cells were transfected with MSNP-PEI-DOX/MDR1-siRNA^{FAM} at the ratio of 15:1, and observed under inverted fluorescence microscope. The transfected KBV cells were indicated by blue fluorescence (DAPI-labeled nucleus), green fluorescence (FAM-labeled siRNA), and red fluorescence (DOX) (Figure 2A–D). The results show that MSNP-PEI can effectively carry DOX and siRNA into KBV cells, and the transfection efficiency of MSNP-PEI-DOX/MDR1-siRNA^{FAM} was 32.44%±2.54% as assessed by flow cytometry 48 hours posttransfection.

Assessment of MSNP-PEI-DOX/MDR1-siRNA biological effects in vitro

The assays of transfection efficiency showed that MSNP-PEI-DOX/siRNA can efficiently transfect KBV cells. Next, we evaluated the biological effects of MDR1-siRNA and DOX from MSNP-PEI-DOX/MDR1-siRNA by gene expression and apoptosis assays. The qPCR results showed that the MDR1-siRNA in the MSNP-PEI/MDR1-siRNA and MSNP-PEI-DOX/MDR1-siRNA effectively blocked gene expression of MDR1 at 48 and 72 hours posttransfection compared to the NC, MSNP-PEI-DOX, and DOX groups (Figure 2E and F) ($P<0.05$); especially, MDR1-siRNA in MSNP-PEI-DOX/MDR1-siRNA could dramatically decrease the gene expression of MDR1 ~50% at 48 hours and ~70% at 72 hours compared to the control group (Figure 2E and F) ($P<0.01$).

Thereafter, we further evaluated the apoptotic effect of DOX in MSNP-PEI-DOX/MDR1-siRNA using an Annexin-V-FITC assay. The data from flow cytometry demonstrated that the apoptosis rates of KBV cells were 6.75%±1.57% for the control group, 8.01%±0.87% for the NC group, 9.27%±1.17% for the MSNP-PEI/MDR1-siRNA group, 9.97%±2.4% for the DOX group, and 11.31%±1.98% for the MSNP-PEI-DOX groups 48 hours posttransfection (Figure 2G). The DOX from MSNP-PEI-DOX only slightly increased the rate of apoptosis compared to DOX (~1.34%) (Figure 2G). Interestingly, the DOX in MSNP-PEI-DOX/MDR1-siRNA significantly elevated the rate of apoptosis to 24.27%±3.05% at 48 hours posttransfection (Figure 2G) ($P<0.05$). These results indicate that MSNP-PEI nanopaticles can efficiently deliver DOX and MDR1-siRNA into KBV cells in vitro, thus resulting in inhibition of gene expression of MDR1 to enhance the killing effect of DOX.

Assessment of therapeutic effects of MSNP-PEI-DOX/MDR1-siRNA in vivo

In this study, we tried to create a nanoparticle to carry both DOX and MDR1-siRNA in order to effectively block tumor growth. Therefore, in vivo therapeutic assay was a major experiment to prove our hypothesis. The in vivo experiment included four groups: control, MSNP-PEI/MDR1-siRNA, MSNP-PEI-DOX, and MSNP-PEI-DOX/MDR1-siRNA. Tumor growth rates and sizes were not significantly different between control and MSNP-PEI/siRNA groups 28 days post-treatment (Figure 3) ($P>0.05$). Tumor growth rates and sizes from MSNP-PEI-DOX and MSNP-PEI-DOX/MDR1-siRNA groups were, however, significantly different from control and MSNP-PEI/MDR1-siRNA groups 28 days posttreatment (Figure 3) ($P<0.05$). Tumor size decreased to 58.67%±2.37% in the MSNP-PEI-DOX group and 81.64%±3.17% in the MSNP-PEI-DOX/MDR1-siRNA group compared to the control group (Figure 3). Tumor sizes were significantly different between MSNP-PEI-DOX and MSNP-PEI-DOX/MDR1-siRNA groups on day 28 posttreatment (Figure 3) ($P<0.05$). These data further suggest that MSNP-PEI-DOX/MDR1-siRNA can enhance the killing effect of DOX through the inhibition of MDR1 gene expression.

H&E staining showed that tumor cells actively grew in the control and MSNP-PEI/MDR1-siRNA groups, and their nuclei were stained dark blue with more mitotic cells and less apoptotic cells (Figure 4). The tumor growth in the MSNP-PEI-DOX and MSNP-PEI-DOX/MDR1-siRNA groups, however, was not active, with many dead cells which had undergone karyorrhexis (fragmentation) and karyolysis (dissolution) resulting in the formation of a uniform pink area (Figure 4). These results indicate that MSNP-PEI-DOX and MSNP-PEI-DOX/MDR1-siRNA have potential therapeutic effects on tumor therapy, especially MSNP-PEI-DOX/MDR1-siRNA, which has a stronger killing effect. H&E staining of heart, liver, spleen, lung, and kidney did not show a difference between groups, suggesting that MSNP-PEI nanoparticles have no side effects on other tissues (Figure 5).

Immunohistochemical staining showed that the gene expression of MDR1 in the MSNP-PEI/MDR1-siRNA and MSNP-PEI-DOX/MDR1-siRNA groups reduced significantly compared to the control and MSNP-PEI-DOX groups. The results indicate that the MDR1-siRNA can effectively block gene expression of MDR1 (Figure 6). We also checked the expression of the cancer biomarker Ki67 by immunohistochemical staining. There was no difference before and after treatment with DOX and MDR1-siRNA (Figure 6).

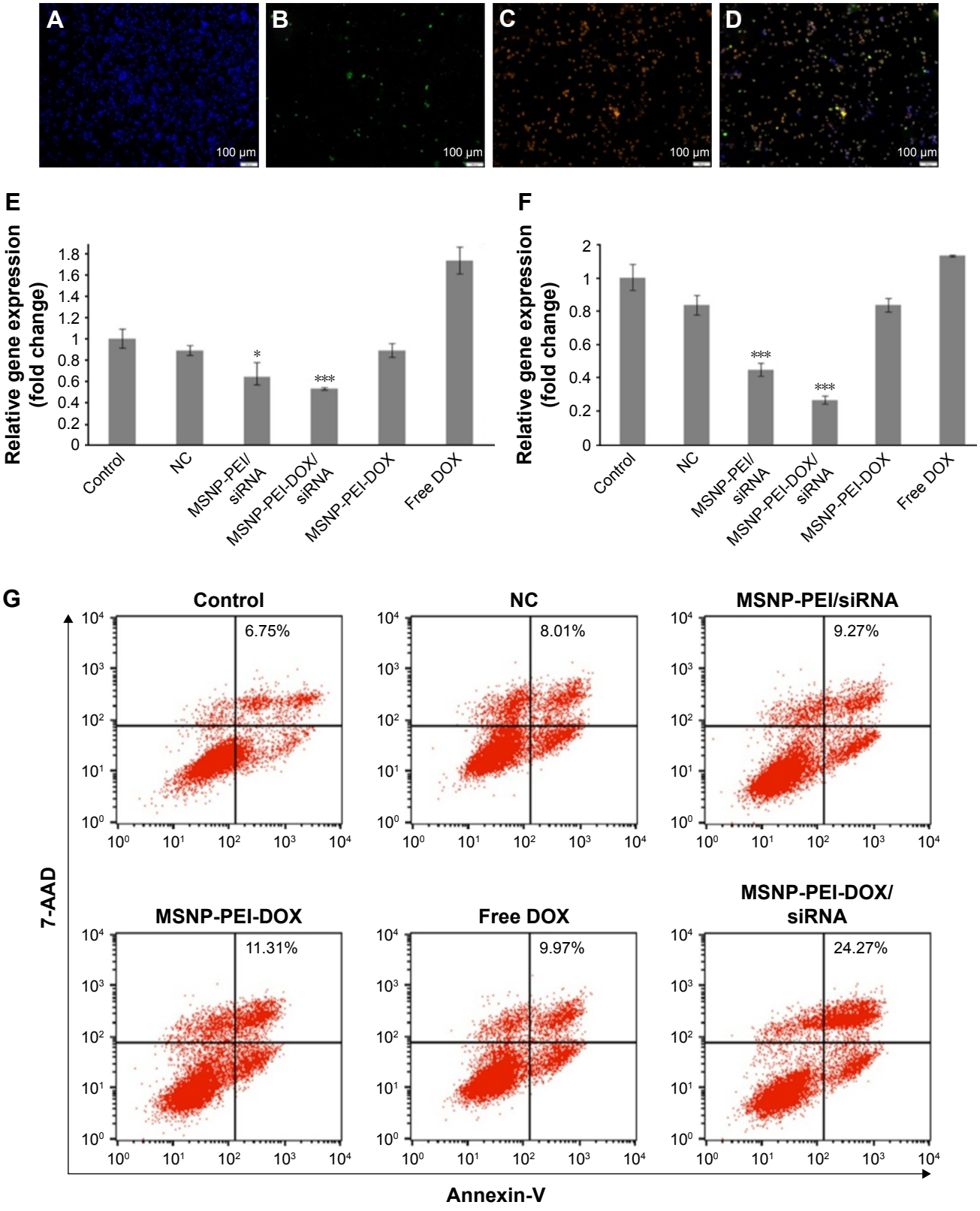

Figure 2 Transfection efficiency and biological effects of MSNP-PEI-DOX/MDR-siRNA.

Notes: (**A**–**D**) Transfection efficiency assay of MSNP-PEI-DOX/MDR1-siRNA^{FAM} 48 hours posttransfection. (**E**) Gene expression of MDR1 48 hours posttransfection. (**F**) Gene expression of MDR1 72 hours posttransfection. *P<0.05, ***P<0.001. (**G**) KBV cell apoptosis assays 48 hours posttransfection. All results shown are mean value ± SEM (n=3/group).

Abbreviations: DOX, doxorubicin; MDR, multidrug resistance; MSNP, mesoporous silica nanoparticles; NC, negative control; PEI, polymerpolyethylenimine; SEM, standard error of the mean; siRNA, small interfering RNA.

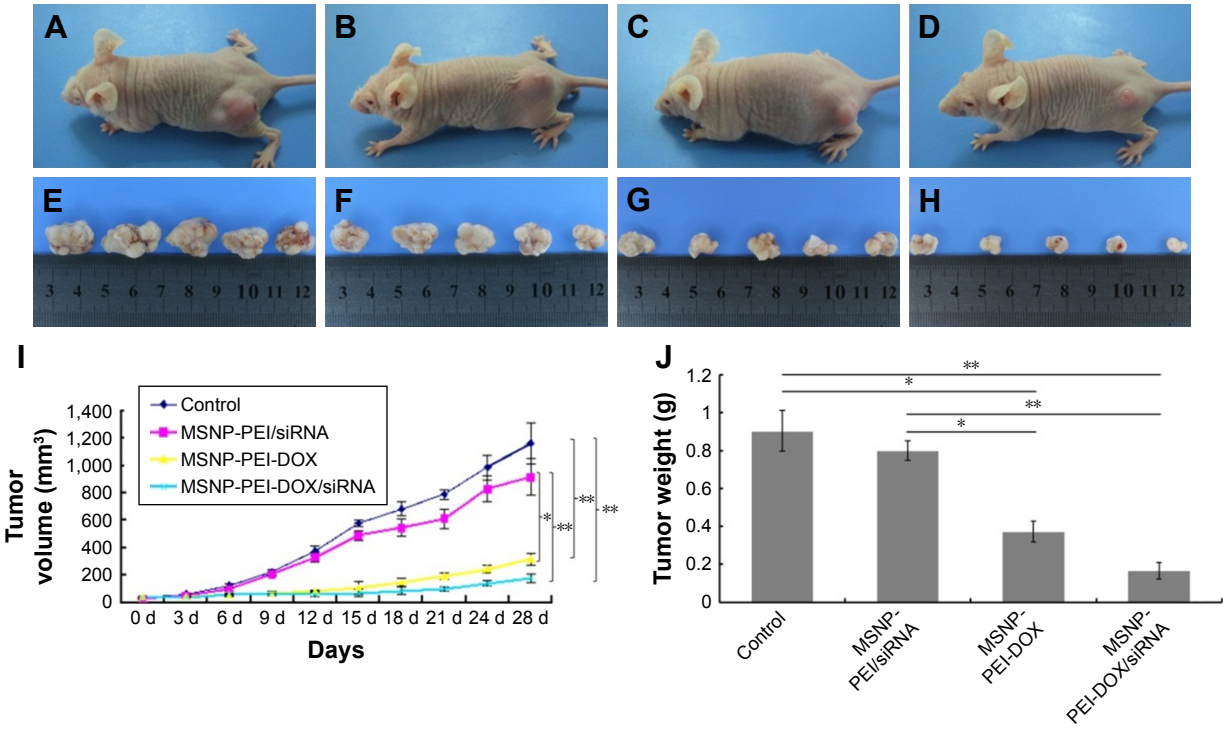

Figure 3 Tumor size measurements: photographs of typical mouse bearing tumor and removed KBV tumor xenografts.
Notes: (**A** and **E**) Control group. (**B** and **F**) MSNP-PEI/siRNA group. (**C** and **G**) MSNP-PEI-DOX group. (**D** and **H**) MSNP-PEI-DOX/siRNA group. (**I**) Time course of tumor size measurements. (**J**) Tumor weights of KBV xenografts at 28 days posttreatment. *$P<0.05$, **$P<0.01$. All results shown are mean value ± SEM (n=5/group).
Abbreviations: DOX, doxorubicin; MSNP, mesoporous silica nanoparticles; PEI, polymerpolyethylenimine; SEM, standard error of the mean.

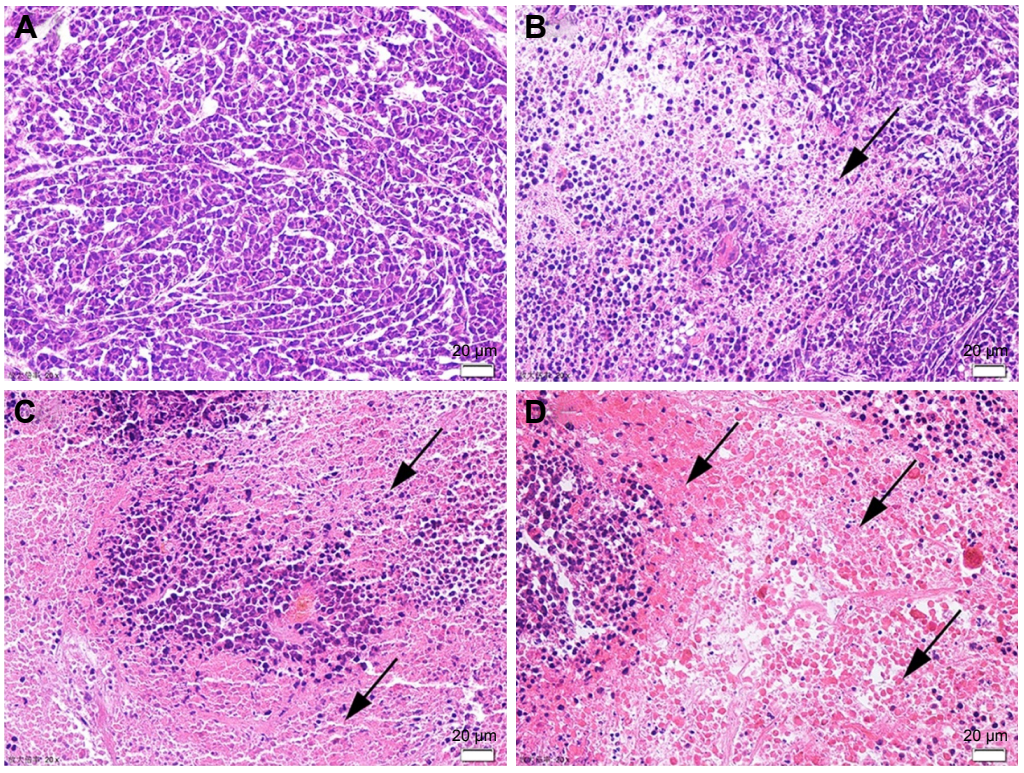

Figure 4 Morphologies of KBV tumor by H&E staining.
Notes: (**A**) Control; ×200. (**B**) MSNP-PEI/MDR1-siRNA; ×200. (**C**) MSNP-PEI-DOX; ×200. (**D**) MSNP-PEI-DOX/MDR1-siRNA; ×200. The black arrows indicate dead cells resulting in the formation of a uniform pink area.
Abbreviations: DOX, doxorubicin; H&E, hematoxylin and eosin; MDR, multidrug resistance; MSNP, mesoporous silica nanoparticles; PEI, polymerpolyethylenimine.

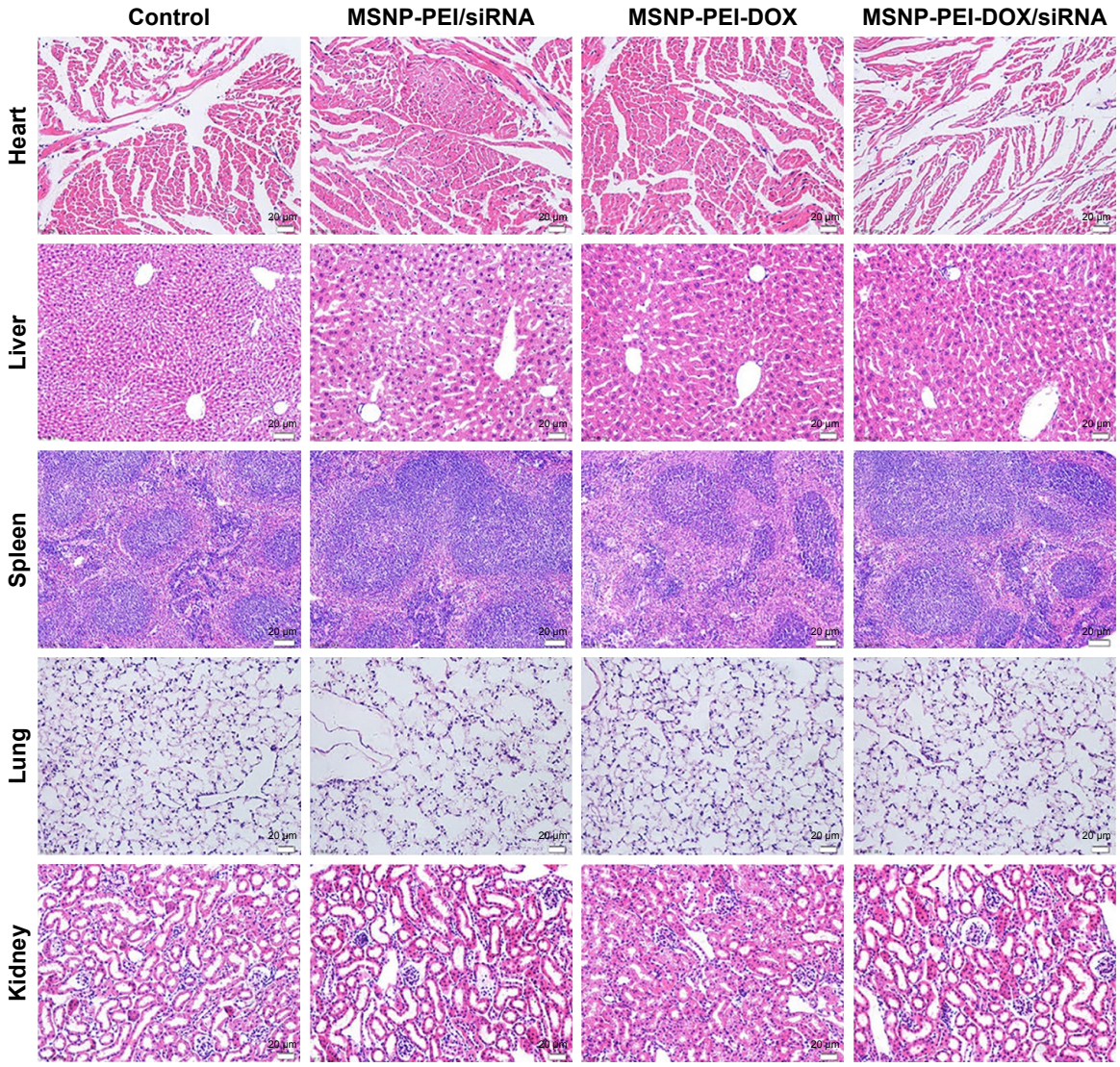

Figure 5 H&E staining of heart, liver, spleen, lung, and kidney; ×200.
Abbreviations: DOX, doxorubicin; H&E, hematoxylin and eosin; MDR, multidrug resistance; MSNP, mesoporous silica nanoparticles; PEI, polymerpolyethylenimine.

At the same time, we detected the expression of the apoptosis-related gene Bcl-2. The result showed that the gene expression of Bcl-2 increased significantly after treatment of DOX and MDR1-siRNA (Figure 6). These results indicate that MSNP-PEI-DOX/MDR1-siRNA can reverse multidrug resistance significantly and kill tumor cells.

Discussion

Oral squamous cell carcinoma is the highest incidence of oral maxillofacial malignant tumor.[30,31] Traditional treatment is mainly surgical resection, which often leads to patients experiencing postoperative maxillofacial tissue or organ defects, thus seriously affecting the patients' life quality.[32,33] Despite the improvement in oral squamous carcinoma treatment, the 5-year survival rate has reached ~64%; for terminal-stage cancer patients, the 5-year survival rate is still low, only about 30%.[34] Therefore, comprehensive treatment, namely surgery combined with radiotherapy, chemotherapy, or biological treatment, becomes a principle approach for oral maxillofacial squamous cell carcinoma treatment, especially in the middle and late stages.

Chemotherapy is an important treatment for oral squamous carcinoma. Multidrug resistance, however, can be a major problem for chemotherapy. The MDR1 gene plays key roles in multidrug resistance phenomenon. Overexpression of MDR1 gene increases MDR1 protein, a plasma membrane glycoprotein, which can pump out chemotherapy drugs from the intracellular to extracellular milieu in order to eliminate the effects of the chemotherapy drugs in the cancer cells.[35,36] A previous study demonstrated that adenoviral vector-carried

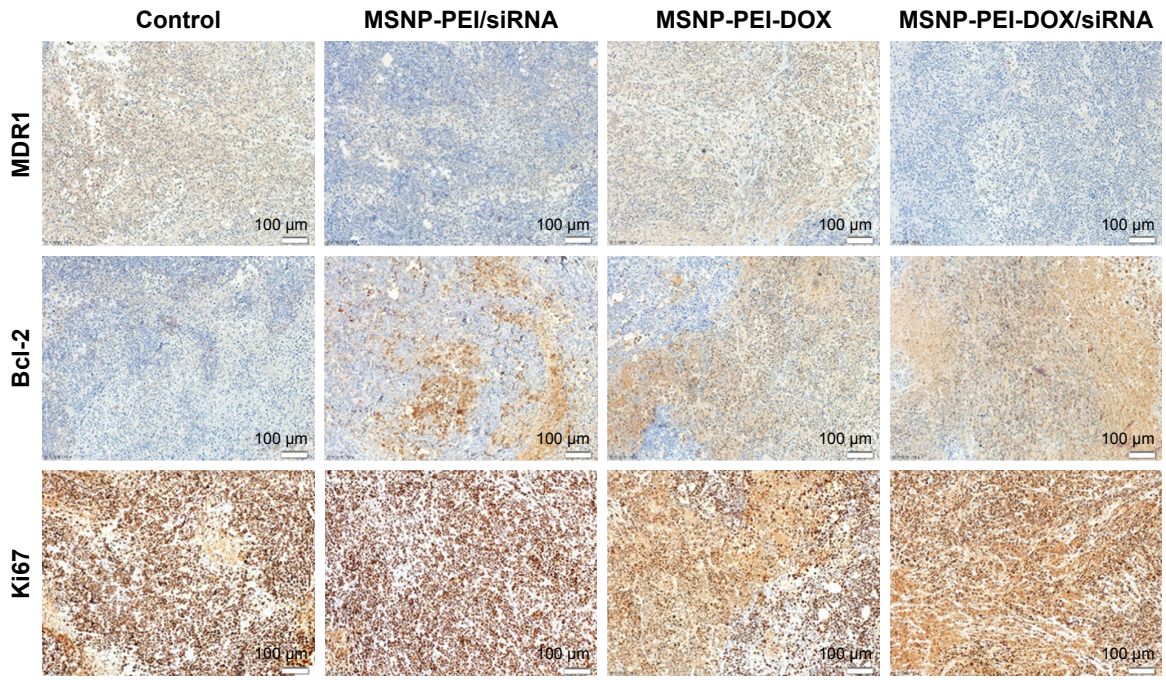

Figure 6 Immunohistochemical staining of MDR1, Bcl-2, and Ki67; ×200.
Abbreviations: DOX, doxorubicin; MDR, multidrug resistance; MSNP, mesoporous silica nanoparticles; PEI, polymerpolyethylenimine.

MDR1-siRNA can effectively inhibit expression of MDR1 gene and reduce MDR1 P-glycoprotein in human breast cancer cells and oral cancer cells.[29] In our current study, we tried to create a carrier or vector which was able to carry both MDR1-siRNA, to block gene expression of MDR1, and the chemotherapy drug DOX, to induce target cancer cell death (apoptosis), without or with less effects from MDR1.

Recently, the nanoparticle drug delivery system has become a useful means to deliver therapeutic drug to target cells.[37,38] Herein, we used MSNP to absorb PEI on its phosphonate surface and hoped these new nanoparticles could carry DOX via the phosphonate surface and bind MDR1-siRNA through cationic PEI. Our data demonstrated that the zeta potential of MSNP-PEI was +34.7 mV, with slight increase in size and very mild cytotoxicity when it is over 40 μg/mL, while the zeta potential of MSNP was −24.3± 2.7 mV (Table 1 and Figure 1). MSNP can effectively carry DOX at 18.5%±0.5% (w/w) and release DOX over a period of 120 hours (Figure 1). The MSNP-PEI-DOX/MDR1-siRNA can efficiently transfect KBV cells (32.44%±2.54%), decrease ~50% or 70% of gene expression of MDR1 48 or 72 hours posttransfection which reduces DOX efflux from cytoplasm, and enhance DOX killing effect leading to dramatically increased apoptosis of KBV cells (24.27%±3.05%) in vitro (Figure 2). These findings suggest that the MSNP-PEI-DOX/MDR1-siRNA created herein can efficiently transfect KBV cells, effectively inhibit MDR1 gene expression to block DOX efflux from the intracellular to extracellular milieu, and release DOX from the lysosome leading to effective induction of apoptosis of KBV cancer cells. MSNP-PEI-DOX/MDR1-siRNA have been shown to have expected characteristics, as well as the dual function of blocking multidrug resistance effects as well as enhancing the cancer killing effect.

Importantly, our data from the tumor model in vivo demonstrated that MSNP-PEI-DOX/MDR1-siRNA dramatically decreased tumor size (81.64%±3.17% reduction) and tumor growth rate compared to control group, all without causing cytotoxicity in heart, liver, spleen, lung, and kidney (Figures 3 and 4). H&E staining of tumor sections revealed that the MSNP-PEI-DOX/MDR1-siRNA induced more cell death in tumor tissue (Figure 4). These in vivo assays further strongly support the fact that MSNP-PEI-DOX/MDR1-siRNA can play dual roles in cancer therapy, by blocking MDR1 function and enhancing killing effect on the tumor cell not only in vitro but also in the in vivo model. These results indicate that MSNP-PEI-DOX/MDR1-siRNA has potential clinical application.

Conclusion

Our results clearly demonstrate that the newly created MSNP-PEI-DOX/MDR1-siRNA succeed in carrying DOX and MDR1-siRNA with high efficiency, and could transfect human oral squamous carcinoma DOX-resistant KBV cells,

and effectively play dual functions of multidrug resistance blocking and cell death promotion in vitro and in vivo. The MSNP-PEI-DOX/MDR1-siRNA provide a new dual delivery system for chemotherapy of multidrug-resistant cancers in future clinical cancer treatments.

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Disclosure

The authors report no conflicts of interest in this work.

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