Increased efficiency of testicular tumor chemotherapy by ultrasound microbubble-mediated targeted transfection of siMDR1

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Received March 23, 2015; Accepted April 21, 2015

DOI: 10.3892/or.2015.4262

Abstract. The MDR1 gene encoding P-glycoprotein (P-gp) is an ATP-dependent drug efflux transporter and is related to drug resistance of yolk sac tumors. Drug resistence may be an important factor for the low efficiency of chemotherapy in the treatment of testicular tumors. P-gp, encoded by the MDR1 gene, is an ATP-binding cassette transporter. P-gp exhibits high expression in capillary endothelial cells of the testis and prevents the intracellular accumulation of chemotherapy agents in testicular tumor cells, resulting in drug resistance. In the present study, we aimed to use specific siRNA to silence the expression of the MDR1 gene and P-gp, leading to the reversal of multidrug resistance of testicular tumors and contributing a suitable condition for chemotherapy. Ultrasound microbubble-mediated delivery is a safe and effective tool for gene delivery. In the present study, we demonstrated that ultrasound microbubble-mediated delivery effectively improved the siMDR1 gene transfection in interstitial capillary endothelial cells of the testis, inhibited the expression of P-gp and increased daunorubicin accumulation. The testis tumor model was successfully constructed by injecting $1x10^7$ yolk sac tumor cells in 3-week-old Sprague-Dawley rats. Ultrasound microbubble-mediated siMDR1 gene therapy improved the effect of chemotherapy on the testicular tumors. The testicular volume was reduced, the number of tumor cells within the testicular tissues decreased, and pathological changes were mostly recovered. Therefore, the present study indicated that ultrasound microbubble-mediated siMDR1 gene therapy *in vivo* reversed drug resistance by regulating P-gp expression, providing a promising method for the treatment of testicular tumors.

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Key words: yolk sac tumor, ultrasound microbubble-mediated delivery, RNA interference, multiple drug resistance gene

Introduction

Testicular tumors are common malignant solid tumors of the urinary and reproductive system. Chemotherapy is the most common method to treat testicular cancer (1,2). However, low therapeutic efficiency is a crucial factor affecting the therapeutic results (3). It has been found that P-glycoprotein (P-gp), as a multidrug resistance-associated protein, is highly expressed in capillary endothelial cells of the testis to form a biological barrier, blocking access of chemotherapy agents to tumor sites, playing an important role in the drug resistance of testicular tumors (4,5). P-gp, encoded by the MDR1 gene, is a membrane protein that functions as an ATP-dependent efflux pump, pumping out water insoluble toxic substances from cells, and impeding the delivery of chemotherapeutic drugs to the testis (6,7). Therefore, breaking through the blood-testis barrier and the reversal of drug resistance are key goals for the treatment of testicular tumors (8).

Thus, we aimed to use RNA interference technology to silence the expression of the MDR1 gene and inhibit expression of P-gp, to improve the responsiveness to chemotherapeutic drugs of testicular tumors *in vitro* (9,10).

The first and most important problem of gene therapy is development of a safe and effective gene therapy carrier, as well as an effective way to delivery genes into target cells. However, commonly used traditional transfection methods are not satisfactorily translatable to *in vivo* conditions (11). Recent studies have shown that microbubble contrast agents can carry and release genes under ultrasonic action, promoting the *in vitro* and *in vivo* transfection of target genes. Ultrasound microbubble-mediated gene therapy is expected to be a safe, efficient and non-invasive gene therapy (12,13).

In a previous study, we revealed that ultrasound microbubble-mediated gene delivery effectively assisted the entry of siMDR1 into L2RYC cells, inhibited the expression of the MDR1 gene and P-protein, as well as the efflux pump function of P-protein, increasing the aggregation of chemotherapy drugs in cells, thus resulting in a more effective antitumor effect (9). In the present study, we demonstrated that the siMDR gene-carrying polymer coated microbubble contrast agent reached the testis through intravenous injection, and

destruction of microbubbles under the ultrasonic action successfully transfected the siMDR1 gene into rat testicular capillary endothelial cells. Silencing of MDR1 inhibited the expression and function of P-gp, so that the anticancer drug daunorubicin entered more easily into testicular tumors. Ultrasound microbubble-mediated gene therapy combined with RNAi, has a good application prospect in the treatment of testicular tumors.

Materials and methods

Cell culture and chemicals. The L2RYC cell line was obtained from ATCC (Manassas, VA, USA), and maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Ultrasound microbubble-mediated gene transfection. Sprague-Dawley (SD) male rats, 4-weeks of age (n=25), were randomly divided into five groups: group 1, plasmid only; group 2, ultrasound + plasmid; group 3, microbubble + plasmid; group 4, ultrasound + microbubbles + plasmid; and group 5, untreated control. The siMDR1-loaded lipid microbubble was prepared as described (ref?). Plasmid (50 μg) in 100 μl phosphate-buffered saline (PBS) or lipid microtubule was injected into the tail vein of the experimental rats, and the right testis was exposed to 300 kHz-ultrasound irradiation at the acoustic intensity of 2 W/cm^2 for 10 min. After 2 weeks, frozen sections of the right testis were observed. Green fluorescent protein (GFP) expression was considered as an indicator of efficiency of gene delivery.

Real-time PCR analysis. As previously described (14), freshly prepared testis tissues were minced and homogenized in TRIzol reagent and total RNA was extracted by TRIzol method. cDNA templates were generated with 10 μg of total RNA and hexamer using SuperScript II reverse transcriptase (Invitrogen). The PCR primers specific for detecting MDR1 (sense, 5'-GAGAACATCGCCTACGG-3' and antisense, 5'-GCTTCCTGGACGACCTT-3'); and GAPDH (sense, 5'-TGGATGGTCCCTCTGGAA-3' and antisense, 5'-GTGAG CTTCCCGTTCAGC-3') were designed using the Primer 3.0 program. Real-time PCR reactions were carried out using a Bio-Rad protocol as follows: 94˚C for 20 sec, 55˚C for 20 sec, 70˚C for 20 sec for 40 cycles, reading plates after each cycle. Data are reported as the fold-change with endogenous GAPDH normalization.

Western blot analysis. Western blotting was performed as previously described (14,15). Freshly prepared testis tissues were homogenized in liquid nitrogen and lysed in RIPA buffer with phenylmethanesulfonyl fluoride (PMSF). Approximately 20 μg of total proteins/lane were subjected to 10% SDS-PAGE gel and then electrically transferred to Immobilon-P membranes (Millipore). The membranes were blocked with 5% fat-free skimmed milk in Tris-buffered saline and Tween-20 (TBST) buffer at room temperature for 1 h, followed by incubation with anti-MDR1 or anti-β-actin at 4˚C overnight. After washing with TBST, the membranes were probed with the appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, USA) at room temperature for 1 h. The expression of the proteins was visualized using enhanced chemiluminescent substrate (Kaiji Biotechnology, China) and exposed under the Syngene G:BOX Imaging System.

Daunorubicin accumulation. Daunorubicin accumulation was detected at 2 days after the SD rat treatment. Daunorubicin (2 ml) at a concentration of 0.1 mg/ml was administered via tail vein injection. Testis tissues were collected after 2 h and prepared into frozen sections. Red fluorescence which was emitted by daunorubicin indicated the accumulation/concentration of daunorubicin and was detected under a fluorescence microscope.

Establishment of the testicular tumor model. Sixty SD male rats 3-weeks and 3-months of age were collected; 10 rats in each group. After being anesthetized by chloral hydrate, the right testis of the rats was exposed by opening the skin, meat membrane and tunica vaginalis. A cell (10 μl) suspension containing 1x10^6, 1x10^7 or 1x10^8 cells was injected into the testis via the connection part of the ductuli efferentes testis (16). Then the testis was put back into the scrotum, the spermatic cord and meat membrane were fixed to prevent retraction, and finally the scrotal incision was sutured. The survival and general situation of the rats were dynamically monitored. The testis volume was detected every 7 days to draw a growth curve. Testis was scanned with Color Doppler Ultrasound Diagnostic instrument to observe the change in testicular size, blood supply and sonographic characteristics. Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed to evaluate tumor progression.

Ultrasound microbubble-mediated siMDR1 transfection combined with chemotherapy in the treatment of testis tumors. Forty SD male rats 3-weeks of age were collected to set up the testicular tumor model as described above. After 3 weeks, successful constructed testicular tumor-bearing rats were randomly divided into 4 groups: group 1, chemotherapy only; group 2, blank microbubbles + ultrasound + chemotherapy; group 3, siMDR1 plasmid-loaded microbubbles + ultrasound + chemotherapy; and group 4, saline control. Microbubbles or siMDR1 plasmid-loaded microbubbles were constantly injected into the tail vein and the right testis was exposed to an ultrasound wave field at 300 kHz, 2 W/cm^2, irradiation for 10 min. At 1 day after microbubble transfection, the rat models were treated with vincristine chemotherapy by intravenous injection 1 time/week, for 2 weeks. Surface area was calculated on the basis of the Meeh-Rubner formula: A (m^3) = 9.1 x W (g)$^{2/3}$/10,000; rat and human dose conversion formula: trial dose in rats (mg/kg) = dose in human (mg/kg) x 36/6 (human dose conversion factor/rat dose conversion factor). The survival and general situation of rats was dynamically monitored. The volume of the testis was measured every week. Models were sacrificed at 1 week after chemotherapy for H&E staining, immunohistochemistry and Fas/p53 gene detection.

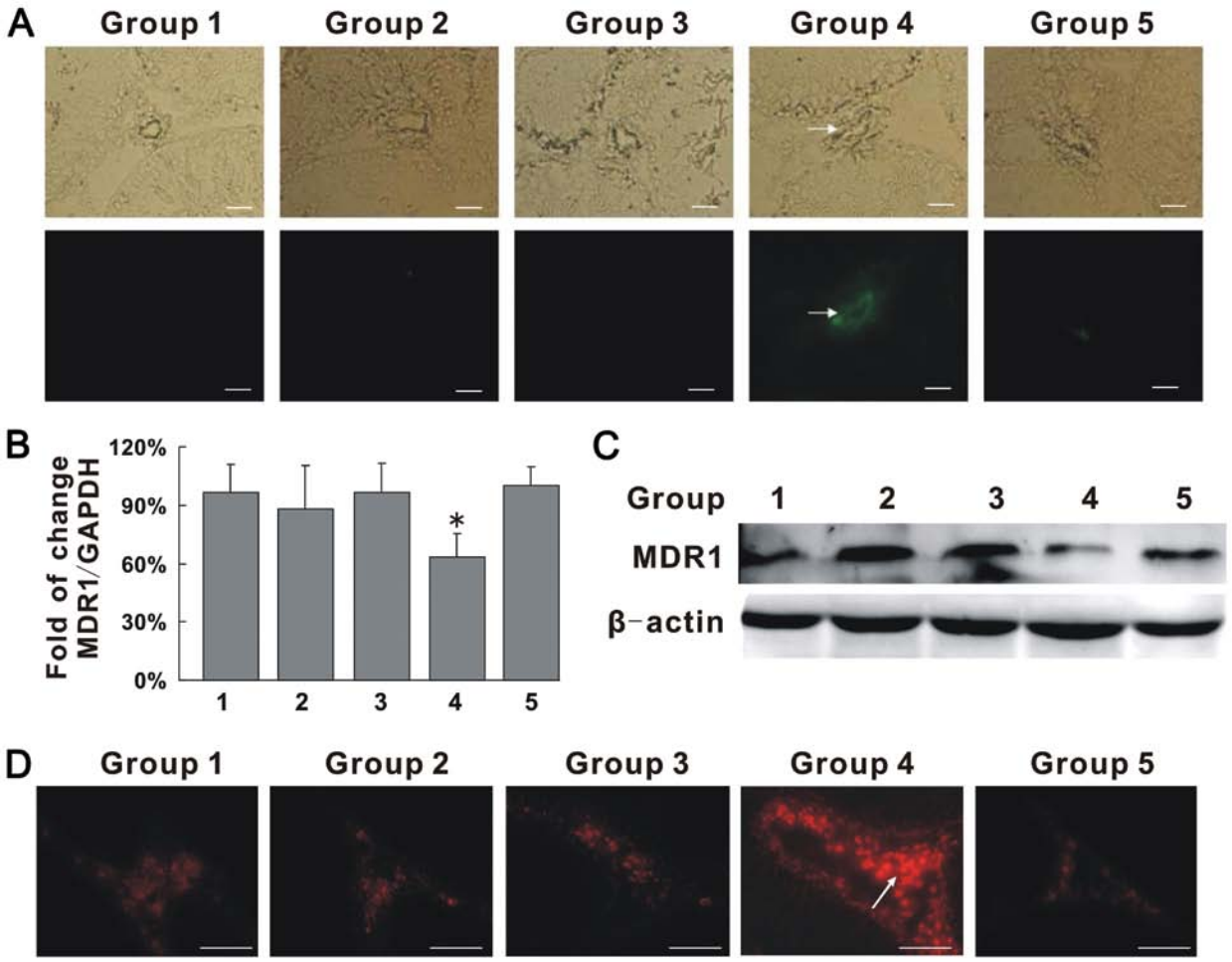

Figure 1. Ultrasound microbubble-mediated gene delivery promotes siMDR1 gene transfection *in vivo*. Twenty-five Sprague-Dawley male rats 4-weeks of age were randomly divided into five groups: group 1, plasmid only; group 2, ultrasound + plasmid; group 3, microbubble + plasmid; group 4, ultrasound + microbubbles + plasmid; group 5, untreated control. The pSEB-siMDR plasmid, microbubbles or siMDR1-loaded lipid microbubbles were injected into the tail vein of the experimental rats, and the right testis was exposed to ultrasound irradiation. After 2 weeks of treatment, the tissues were harvested for detection. (A) Ultrasound microbubble-mediated gene delivery efficiently promoted the transfection of pSEB-siMDR1 into testicular vascular endothelial cells. GFP expression indicates the efficiency of gene delivery (scale bar, 200 μm). (B) The mRNA expression of the MDR1 gene in testis tissues was detected by real-time PCR. All samples were normalized to GAPDH in triplicate, and the PCR results were confirmed in at least 3 sets of independent experiments ($^{*}P<0.05$ compared with group 1). (C) The protein expression of MDR1 in testis tissues was detected by western blotting using the anti-MDR1 or β-actin antibody. Proteins were collected and lysed at 2 weeks after treatment and subjected to SDS-PAGE and western blotting using the anti-MDR1 antibody. Equal loading of the samples was normalized by β-actin detection. (D) Daunorubicin accumulation was increased in the tissues treated with siMDR1-loaded lipid microbubble transfection. Red fluorescent cells were observed under a microscope. Cells in group 4 exhibited more red granular fluorescence in the cytoplasm (indicated by a white arrow; scale bar, 200 μm). GFP, green fluorescent protein.

H&E staining and immunohistochemistry. The harvested tumor tissues were fixed with 10% formalin, embedded in paraffin, and serially cut into 5-μm sections. Sections of each specimen were stained with H&E. For immunohistochemistry, the sections were dewaxed in xylene, rehydrated in graded ethanol solutions, and denatured in water at 60˚C for 1 h, followed by incubation with α-1-antitrypsin (ATT) antibody at 4˚C overnight. After washing in PBS 3 times, the sections were incubated with anti-goat IgG HRP-conjugated secondary antibody at 37˚C for 1 h, colored with DAB and counterstained with hematoxylin.

Statistical analysis. Quantitative data are presented as the mean ± SD. Statistical analysis was performed using the two-tailed Student's t-test or analysis of variance. The probability value of $P<0.05$ was considered to indicate a statistically significant result.

Results

Ultrasound-targeted microbubble destruction promotes siMDR1 gene delivery in vivo. The MDR1 gene which is highly expressed in the testicular capillary wall, hinders chemotherapy drugs into the testis (4,17). Herein, we aimed to use ultrasound-targeted microbubble destruction to deliver the siMDR1 gene into *in vivo* target testicular capillaries *in vivo*, to explore whether the expression and function of the MDR1 gene and P-gp were effectively suppressed.

Since the pSEB-siMDR1 plasmid contains the GFP gene sequence, GFP is an indicator to measure the gene transfection efficiency. As shown in Fig. 1A, GFP expression in testicular interstitial capillary endothelial cells, was only observed in the microbubbles + ultrasound group (group 4), suggesting that the pSEB-siMDR1 plasmid was successfully transfected. There was no GFP expression in the other 4 groups. We

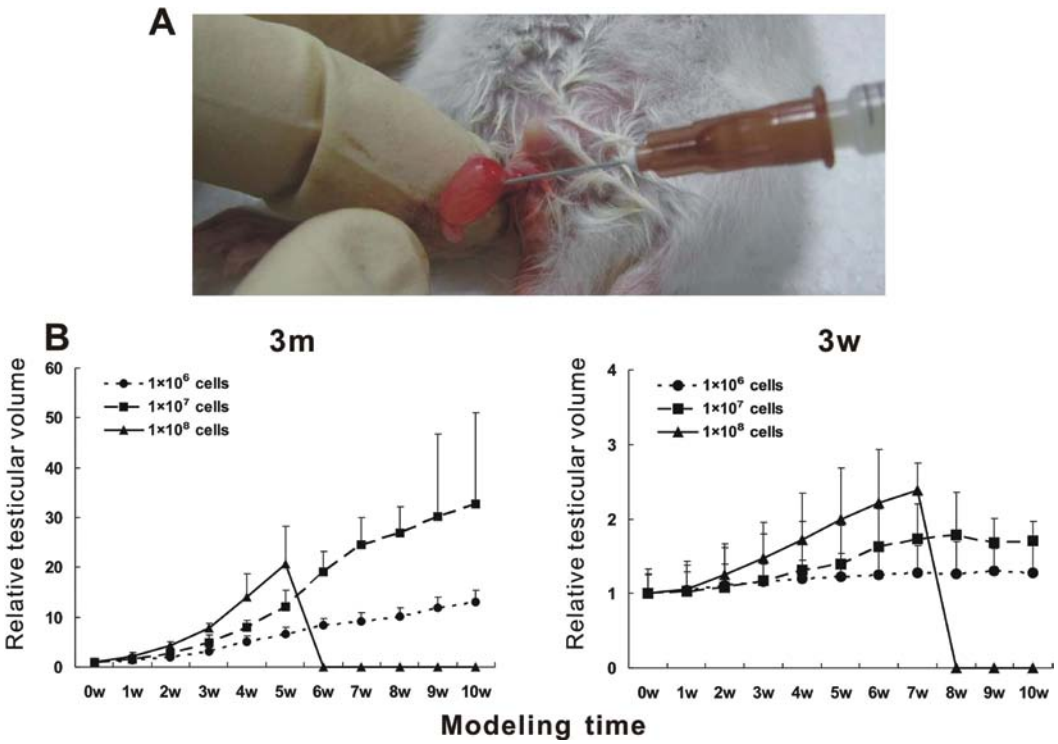

Figure 2. Rats (3-week-old) are suitable for the establishment of the testicular tumor model. (A) Schematic diagram of the operation of the injection of L2RYC cells into the testis tissues. (B) Relative testicular volume of testis tissues injected with different numbers of L2RYC cells. Cells ($1x10^6$, $1x10^7$ or $1x10^8$) in 10 μl of cell suspension were injected into the testis via the connecting region of the ductuli efferentes testis. Testicular volume was detected every 7 days. 3m, 3-month-old rats; 3w, 3-week-old rats.

Table I. Testicular tumor formation rate and the survival time of the tumor bearing rats.

	Injected cell no.	No. of samples	Tumor formation/no. of surviving rats after 1-week injection	Tumor formation/no. of surviving rats after 3-week injection	Tumor formation/no. of surviving rats after 6-week injection	Tumor formation/no. of surviving rats after 10-week injection
3-Week-old SD rats	$1x10^6$	10	0/10	0/10	0/9	0/9
	$1x10^7$	10	6/9	9/9	7/7	2/2
	$1x10^8$	10	8/8	4/4	0/0	0/0
3-Month-old SD rats	$1x10^6$	10	0/9	0/9	0/9	0/9
	$1x10^7$	10	1/8	1/8	1/8	0/7
	$1x10^8$	10	9/9	7/7	2/2	0/0

SD, Sprague-Dawley rats.

further demonstrated that the mRNA expression of the MDR1 gene (Fig. 1B) and protein expression (Fig. 1C) of P-gp were reduced in the microbubble + ultrasound group only. No difference was found between the other 3 intervention and control groups. Daunorubicin as a P-gp substrate, spontaneously emits red fluorescence. At 1 h after daunorubicin injection via tail vein, red fluorescence indicating increased daunorubicin accumulation was observed in the frozen sections of testis in the microbubble + ultrasound group (Fig. 1D), suggesting that the function of P-gp was inhibited and the drug accessed the testis easier. The above results indicated that the combination of ultrasound and microbubbles is an effective method to mediate gene transfection *in vivo*.

These data suggest that ultrasound microbubble-mediated gene delivery effectively promoted the plasmid DNA transfection *in vivo*.

Establishment of the testicular tumor model. Rat yolk sac tumor L2 cells were injected into testicular tissue of the SD rats system to establish the testicular tumor model (Fig. 2A), and then we evaluated the model feasibility and application value by observing the biological characteristics of the tumors, growth rate and morphology. In rats of different ages, stages and different planting cell concentrations, the testicular tumor formation rate and the survival time of the tumor-bearing rats were different (Table I). In the group with implanted cells at

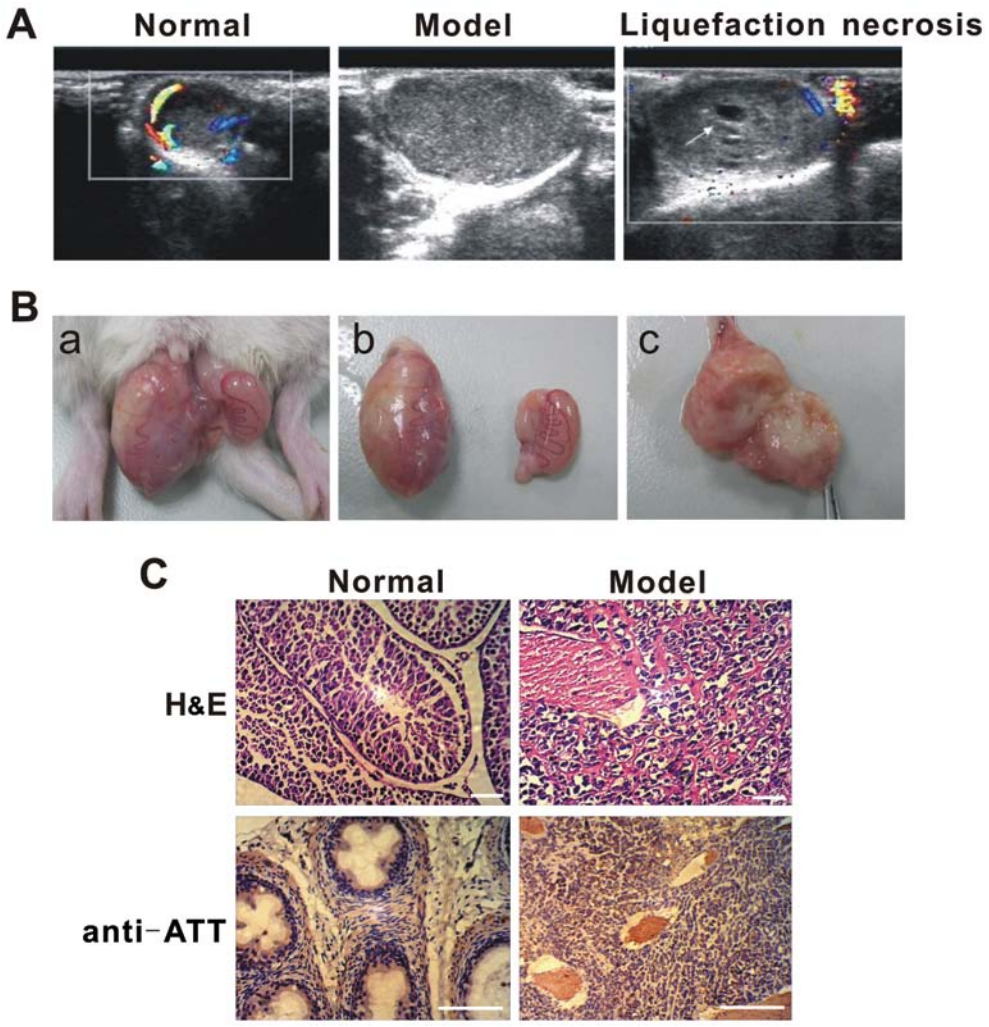

Figure 3. Testicular tumor model was successfully established. L2RYC tumor cells ($1x10^7$) were implanted into testis tissues of 3-week-old SD rats and tumors were detected after 10 weeks. (A) Image of testis tissues using the Color Doppler Ultrasound Diagnostic system. (B) Image of testis tumor tissue. (a) General image of the testis; (b) the volume of testis tumors was much larger than that of the normal testis tissue; (c) cross section of testis tumor tissue. (C) H&E staining and ATT immunohistochemistry of the normal and tumor testis tissues (scale bar, 200 μm). SD, Sprague-Dawley rats; H&E, hematoxylin and eosin; ATT, α-1-antitrypsin.

the concentration of $1x10^6$/ml, no tumors were present at the end of the experiment. At 3 weeks after implantion of cells at the concentration of $1x10^7$, the tumor formation rate of the 3-week-old rats was 100%, and increased gradually with time after planting, and reached a peak at the 6th week with regions of ulceration and erosion; some rats began to die. The tumor formation rate of the 3-month-old rats was only 12.5%, and tumors increased more slowly. When plantation of the cells was at the concentration of $1x10^8$, the tumor formation rate of both 3-week- and 3-month-old rats were 100%, yet the tumors grew very quickly with a high mortality rate.

The growth curve of testicular tumors (Fig. 2B) showed that the testicular volume of the rats injected with $1x10^6$ cells was similar to that of the control group. With injection of $1x10^7$ tumor cells, the relative volume of the testis was significantly larger than that of the control group, particularly the 3-week-old rats. With injection of $1x10^8$ tumor cells, the testis volume increased rapidly; all rats died after 5 weeks in the 3-week-old rats and 7 weeks in the 3-month-old SD rats, respectively. Thus, we chose to establish the testicular tumor model with the 3-week-old rats and implanted cells at the concentration of $1x10^7$. Ultrasound imaging showed that compared with the control group, the testicular volume of the model side increased significantly; the internal echo was medium and uniform, with small punctuate and cord-like high echo. Part of the testicular tissue exhibited liquefied necrosis with no echo area (Fig. 3A). The affected testis was much larger than the normal side; the testicular mass was suborbicular, cystic and enveloped. Transverse section of the tumor was pale yellow, soft fleshy with partial necrosis (Fig. 3B). Pathological sections under the microscope exhibited loose reticulate structure, regions of adenoid structure, eosinophilic granular and Schiler-Duval bodies by H&E staining. Immunohistochemistry showed that AAT was positively stained (Fig. 3C). Therefore, the testis tumor model was successfully constructed.

Ultrasound microbubble-mediated siMDR1 gene therapy improves the effect of chemotherapy on the testicular tumors. This experiment aimed to investigate the treatment efficiency of chemotherapy drugs on testicular tumors following the inhibition of P-gp. Following treatment with chemotherapy drugs, tumor growth was inhibited. The survival rate was

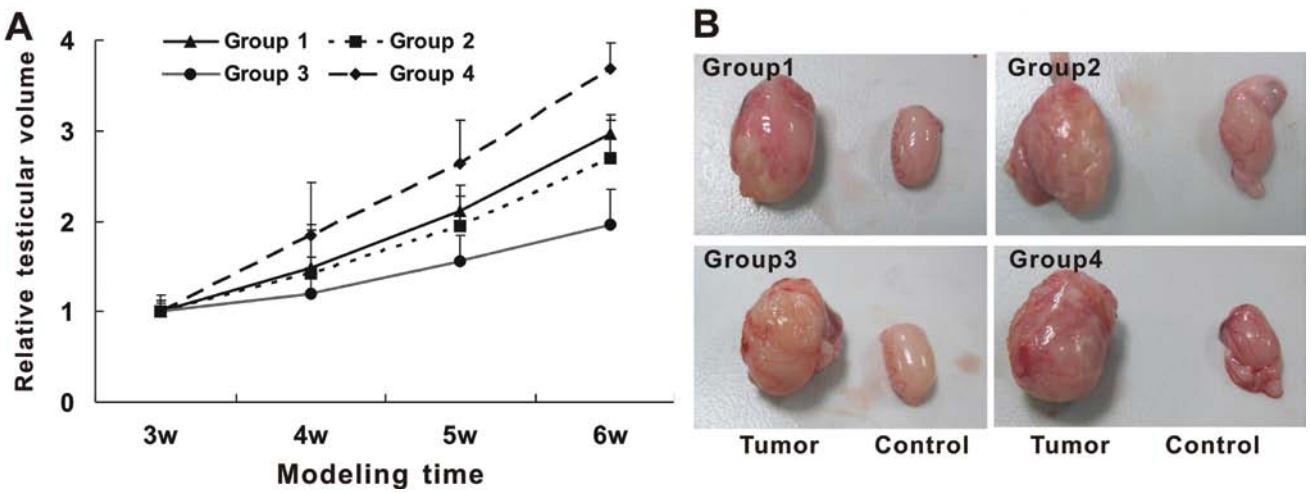

Figure 4. Ultrasound microbubble-mediated siMDR1 gene therapy effectively promotes the effect of chemotherapy on testicular tumors. Testicular tumor model rats were treated with group 1, chemotherapy only; group 2, blank microbubbles + ultrasound + chemotherapy; group 3, siMDR1 plasmid loaded microbubbles + ultrasound + chemotherapy; group 4, saline control. (A) Relative testicular volume of the different treatment groups. (B) Testicular volume of the tumors compared with the controls at 6 weeks.

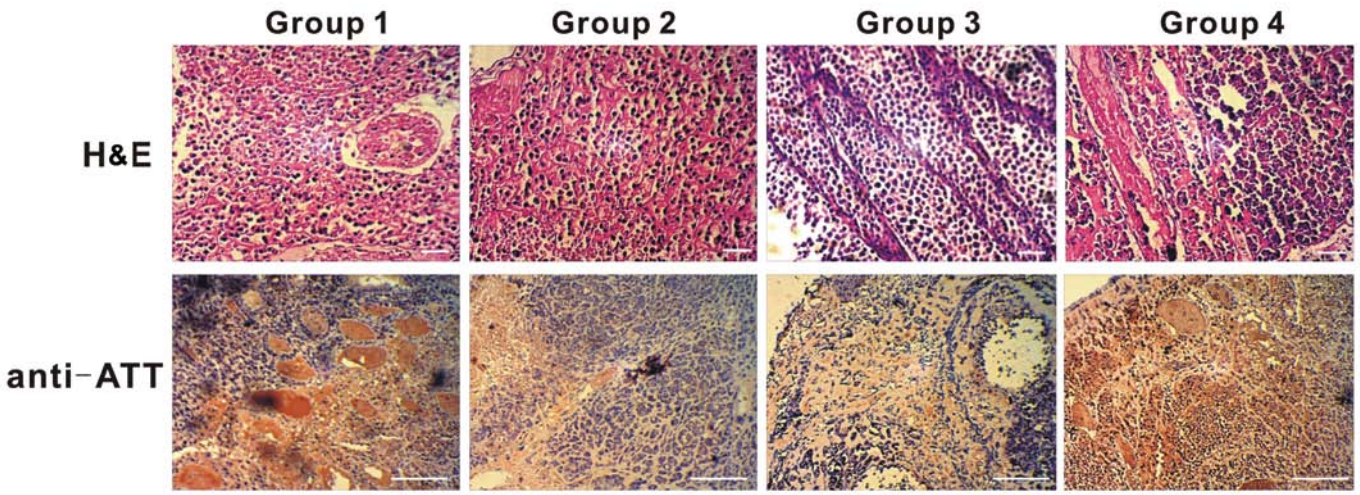

Figure 5. Ultrasound microbubble-mediated siMDR1 gene therapy effectively improves the pathological changes in the testicular tumor tissues. Testicular tumor model rats were treated as described in Fig. 4. H&E staining and ATT immunohistochemistry of the different treated testis tumor tissues were carried out at 6 weeks of the modeling treatment process (scale bar, 200 μm). H&E, hematoxylin and eosin; ATT, α-1-antitrypsin.

improved obviously when compared with the control group at the same time-point. At the end-point, the survival rate of group 3 was increased to 60%, which was significantly different with the other groups ($P<0.05$). Chemotherapy was reported to inhibit the growth of tumors (18). Relative testis volume was significantly smaller than that of the control group. In our groups, the testicular volume of the ultrasound microbubble-mediated siMDR1 therapy after chemotherapy was the smallest (Fig. 4A and B), indicating the best efficiency of tumor suppression.

H&E staining showed that the numbers of tumor cells within the testicular tissues of group 1-3 were significantly decreased compared with that of group 4, without tumor-specific adenoid structure, and no eosinophilic bodies and Schiler-Duval bodies. Particularly group 3 had visible lumen-like structure in testis tissues. Immunohistochemistry results showed that positive AAT expression of testicular tissues in group 1-3 was lower than that in group 4 (Fig. 5). Therefore, we further confirmed that ultrasound combined with microbubble transfection of siMDR1 in the testicular capillary wall provided easier access of vincristine to the testis tissues, and thus improved the effect of chemotherapy on testicular tumors.

Discussion

Testicular cancer is one of the most common types of cancer of the urinary and reproductive system. The effect of chemotherapy still requires improvement (1,2,19,20). The blood-testis barrier hinders chemotherapy drugs into the testis tissues, which is an important factor that affects treatment outcome (21,22). A recent study has found that an ATP-dependent drug efflux pump protein is expressed in the capillary endothelium of the testis and is related to the formation of the blood-testis barrier (23). Previous studies have demonstrated that this protein associated with tumor multidrug-resistance is P-glycoprotein (P-gp), which is strongly expressed in testicular interstitial capillary endothelial cells (24). P-gp is a glycoprotein encoded by

the MDR1 gene, is located on the cell membrane and is an ATP-dependent efflux pump, which pumps the insoluble toxic substances out of the cell, so that chemotherapy drugs cannot easily enter into the testis (4,5,25). In an MDR1-knockout rat model, the concentration of P-gp substrates in the testis was significantly higher than that in the normal rat (26). The structure of the blood-brain barrier is similar to the blood-testis barrier. The rate of positive P-gp expression was found to be 65.8% in the brain tissues of children with intracranial tumors, while the positive rate of P-gp in brain tissues of children with non-intracranial tumors was only 10% (27). Hendrikse *et al* (28) used isotype-labeled P-gp reversal agents to block the P-gp function in the blood-brain barrier, and found that the concentration of intracranial drug increased by 13-fold, indicating that P-gp plays an important role in the blood-brain barrier, and possible also in the blood-testis barrier. P-gp-mediated reverse efflux is not only a part of the biological function of the blood-testis barrier, yet also may be associated with the application of chemotherapy drugs in testicular tumors. Therefore, breaking through the barrier has a great effect on the treatment of testicular cancer (5,8,29,30).

In the present study, we aimed to use RNA interference technology to silence the mRNA expression of the endogenous MDR1 gene, resulting in inhibition of P-gp expression and resistance reversal in testicular tumors, providing a suitable condition for chemotherapy drug treatment. The main concern of *in vivo* gene therapy is how to successfully transfect and express the objective gene in target cells (31). In recent studies, it has been demonstrated that ultrasound-mediated gene transfer is a safe, efficient and non-invasive gene therapy as a possible alternative to viral gene transfer. It plays a significant role in gene therapy-based approaches to the treatment of diseases (32-34). We previously demonstrated that ultrasound microbubble-mediated destruction could effectively delivery siRNA specific for the MDR1 gene into L2-RYC cells using ultrasound microbubble-mediated destruction. We successfully inhibited MDR1 expression and function of P-gp. L2-RYC cells with MDR1 silencing became more sensitive to anticancer drugs, vincristine and dactinomycin (9). In the present study, we further confirmed that ultrasound microbubble-mediated destruction led to transfection of pSEB-siMDR1 into rat testis capillary endothelial cells. The endogenous expression of the MDR1 gene and P-protein decreased. P-gp function was also suppressed, altering the high-resistance state of testicular tumors in response to chemotherapy drugs.

Since the origin and classification of testicular tumors are markedly complex, the animal models are not, strictly speaking, valid for the study of testicular tumors. Usually the transplanted tumor model is established in nude rats; however, nude rats have characteristics of immunodeficiency, therefore the biological characteristics of testicular tumors are different compared with normal immune rats (35). In this study, we implanted a rat yolk sac tumor cell line.

L2RYC cells were injected into the testis tissues of normal immune system SD rats to establish the animal model. Graft rejective reaction is the most important problem of a cell implanted animal model and the use of immunosuppressants is needed. However, in tumor therapy, immunosuppressants are also used as antitumor medicines. The efficiency of chemotherapeutics on tumors may be affected using immunosuppressants (36-38). Thus, in the present experiment, we used 3-week- and 3-month-old SD rats to set-up the animal model. The establishment of a cell transplantation tumor model prior to development of an adaptive immune system may be feasible.

The number of inoculated cells is another important factor affecting the establishment of a transplanted tumor model (39). Usually in the nude rat model, $1x10^6$ inoculated cells form a tumor (40). However, no tumor formation was found in the 3-week- or 3-month-old SD rats following $1x10^6$ L2RYC cell transplantation. When $1x10^7$ cells were implanted into the testis, tumors were formed in the 3-week-old rats but not in the 3-month-old rats. This result may be associated with host immune graft rejection suggesting that more cells are required to establish the xenografts in normal animals compared to the nude rats. If the number of cells was excessive ($1x10^7$), tumor cells were strongly invasive, leading to the quick death of the host rat. The pathological manifestations mainly exhibited testicular necrosis, which was different from the clinical course and performance, and not suitable for tumor study. At 4 weeks after $1x10^7$ L2RYC cells were inoculated in the 3-week-old SD rats, ultrasound imaging and histopathological characteristics were similar to clinical testicular yolk sac tumors, indicating that this method can successfully establish a feasible model of testicular tumors.

Next, we assessed the feasibility of ultrasound microbubble-mediated destruction in delivering chemotherapy drugs in the tumor model. By intravenous injection of microbubbles, the permeability of the cell membrane was enhanced under local ultrasonic action, thus promoting the transfection of target genes or drugs (41). Through the observation of tumor growth, the survival rate of tumor-bearing rats, and detection of pathological changes, we found that ultrasound microbubble-mediated destruction followed by chemotherapy treatment was most effective for the treatment of testis tumors. Compared with the chemotherapy alone group, ultrasound microbubble-mediated destruction combined with chemotherapy had a better effect, probably since the permeability of testicular capillary transiently increased under ultrasound microbubble-mediated destruction. The drug concentration in the testis was slightly higher than that in the chemotherapy alone group. Expression of an exogenous gene mediated by transfection sustains for 2-3 weeks (42). MDR1 expression was suppressed during this time window. The permeability of the testicular vascular was selectively increased following suppression of the P-gp substrate, and then drugs easily entered into the testis tissues, thereby enhancing the effect of chemotherapy.

In summary, the present study mainly focused on the issue that chemotherapy drugs cannot easily enter testicular tumors. We used ultrasound microbubble-mediated destruction method to increase the permeability of the capillary endothelial cell membrane, and achieved efficient *in vivo* transfection of the siMDR1 gene and inhibited P-gp production. The reversal of chemotherapy drug resistance in testicular tumors improves the treatment effect, which is expected to provide an effective method for the treatment of testicular tumors.

Acknowledgements

The present study was supported in part by a research grant from the National Natural Science Foundation of China (nos.

81001030 to Y.H. and 81301300 to X.-J.J.). This study was also supported in part by the Special Fund of Chongqing Key Laboratory (CSTC) (to G.-H.W.), Program for Innovation Team Building at Higher Education Institutions in Chongqing, China (to G.-H.W.) and National Clinical Key Subject Construction Project (AXA medical office letter [2013]544).

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