Regular Article

Artesunate Suppresses the Growth of Prostatic Cancer Cells through Inhibiting Androgen Receptor

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Received November 23, 2016; accepted January 24, 2017

Prostatic cancer (PCa) is a leading cause of cancer related death in males and is often regarded as a kind of androgen-sensitive cancer. *Artesunate* (ART), a semi-synthetic derivative of the Chinese herb *Artemisia annua*, is such an anti-cancer agent. However, the effects and mechanism of ART on PCa cells remains unclear. The study aims to elaborate the mechanism of the involvement of androgen receptor (AR) in anti-prostatic cancer (PCa) of *artesunate* (ART). PCa cells 22rvl were used *in vivo* and *in vitro*, and the viability and apoptosis were conducted using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, respectively. Ectopic expressions of AR and DNA methyltransferase (DNMT) were detected in cells in overexpression or interference of AR or DNMT3b. ART dose-dependently suppressed tumor growth, inhibited cell viability, enhanced apoptosis, decreased AR expression, and increased the expression and the catalytic activity of DNMT3b in 22rv1 cells either in transplanted mice or *in vitro*. Furthermore, AR downregulated DNMT3b expression, and overexpression of AR or interference of DNMT3b could reverse ART-induced cytotoxicity and apoptosis in 22rvl cells, whereas overexpression of DNMT3b could not change the effect profiles of ART on the cells. The results indicated that ART suppressed tumor growth of prostatic cancer cells through AR-DNMT3b pathway, underlying ART will allow for the utilization of this Chinese therapeutic agent for the potential treatment of prostate cancer.

Key words *artesunate*; prostatic cancer; androgen receptor; DNA methyltransferase; apoptosis

Prostatic cancer (PCa) is the most common cause of cancer-related death in males. Morbidity and mortality of PCa underscore the need for novel treatment options. Although improved therapies have been recently developed to treat and prevent PCa, prostate cancer still remains refractory disease. Lately, herbs and phytochemicals[1] have been attractive as therapeutic agents for tumorigenesis suppression. Understanding how the production of these Chinese herbs is regulated during cancer progression would help develop new cancer therapeutic strategy.

Artesunate (ART) is a semi-synthetic derivative of artemisinin, the active principle of the Chinese herb *Artemisia annua*. ART has been revealed remarkable activity against otherwise multidrug-resistant *Plasmodium falciparum* and *P. vivax* malaria. With the recognition of ART, it has now been analyzed for its anti-cancer activity in various tissues-specificity. For example, Tran *et al.* found that enhancement of ART activity could effectively induce the apoptosis of breast cancer cells.[2] Moreover, ART was proved to be as a novel therapeutic agent for metastatic renal cell carcinoma due to its attenuation action on tumor growth, metastasis.[3] Recently, Michaelsen *et al.* clinically treated PCa patients with long-term *Artemisia annua* capsules *via* oral administration combined with short-term ART injection and concluded that this treatment was a considerable strategy for advanced metastasized prostate carcinoma.[4] However, the potential mechanism by which ART suppresses PCa remains to be elaborate.

Androgen receptor (AR) is a steroid receptor transcription factor that governs the expression of genes required for development and physiologic function of the prostate.[5] Due to its critical role in prostatic carcinogenesis, it has been regarded as a potential target for PCa. AR antagonists aroused *in vivo* and clinical therapeutic approach.[6] Although the emergence of hormone-refractory prostate cancer obstacle to the use of androgen deprivation therapy, AR and it associated signals remained the critical pathway for PCa.[7] AR controlled signalling pathway can be triggered in castration-resistant prostate cancer (CRPC).[8] Therefore, therapeutic strategy focusing on inhibiting AR signalling pathway continues being a trend of research and clinical treatment.

Gene methylation modification that mediated by DNA methyltransferase (DNMT) is a ubiquitous event in the pathological process of cancers.[9] DNMTs can catalyze Pca-related gene nuclear factor-E2-related factor 2 (Nrf2)[10] and RASSF1A[11] methylation that promotes tumor progression. Gravina *et al.* analyzed the correlation between ectopic expression of DNMT1, DNMT3a and DNMT3b and tumorigenic capacity of prostate cancer cells and concluded that the increased methylation in more aggressive tumors supporting the use of DNMTs in advanced prostate cancer.[12] Interestingly, DNMTs activity can be negatively regulated by AR in human prostate cancer cells.[13] Therefore, the present study used the prostatic cancer cells 22rv1 and investigated the effects of ART on the growth in mice and cell viability and apoptosis

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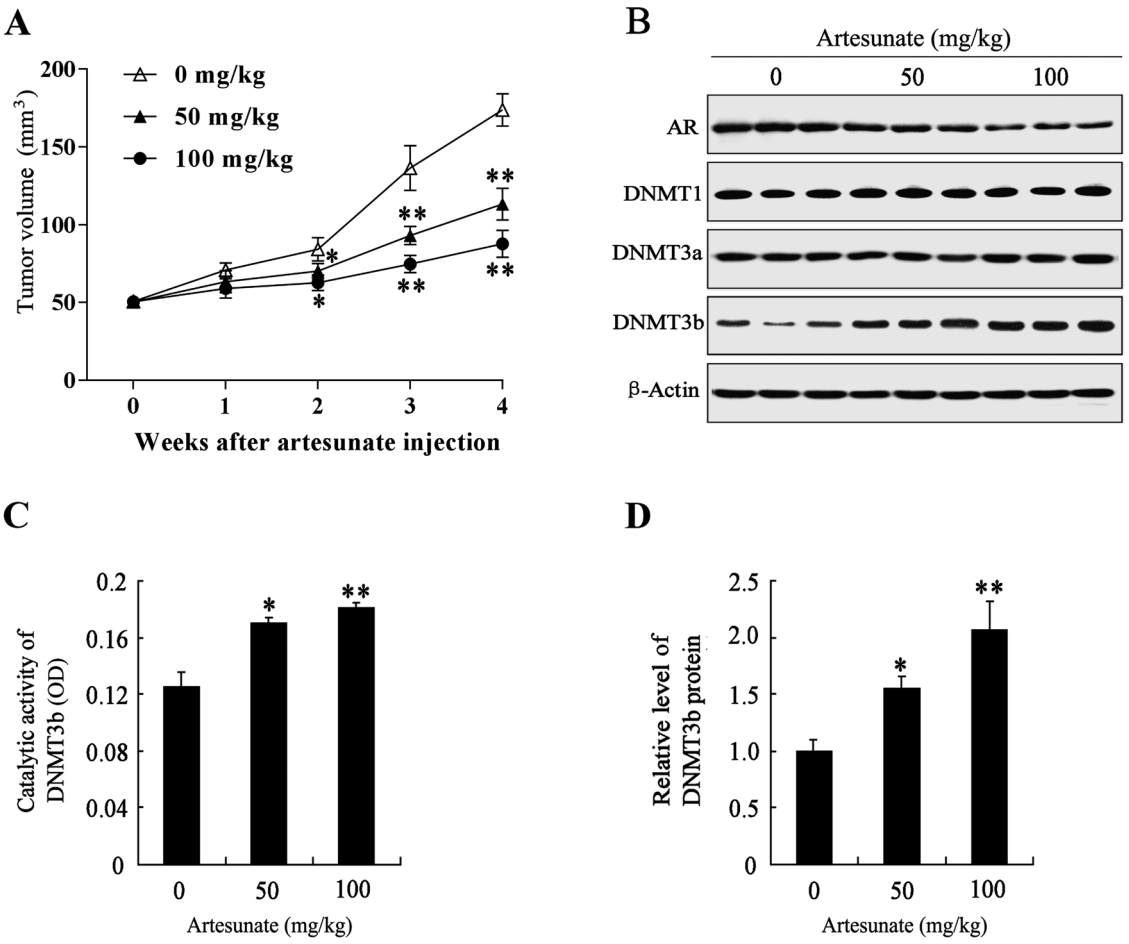

Fig. 1. Effects of ART on Tumor Growth and the Expression of AR and DNMTs in 22rvl Cells Transplanted Mice

(A) Tumor growth. (B) Expression of androgen receptor (AR), DNMT1, DNMT3a and DNMT3b proteins using Western blotting. (C) Catalytic activity of DNMT3b. (D) Relative level of DNMT3b protein by the densitometric analysis. Data are presented as the mean±S.D. of six mice in each group. *$p<0.05$, **$p<0.01$ compared with the corresponding 0 mg/kg ART treatment group of each week.

in vitro, and the expressions of AR and DNMTs as well, aiming to demonstrate the role of AR and it regulated DNMTs in anti-PCa action of ART.

MATERIALS AND METHODS

Mice and Tumor Transplantation Male BALB/c nude mice aged 7 weeks were obtained from Chinese Academy of Sciences, Shanghai Laboratory Animal Center. For preparing mice bearing tumor experiments, freshly-prepared admixtures of prostatic cancer cells 22rv1 were suspended at 1×10^6 cells/mL in 50% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50% Matrigel. Total 100 μL of admixtures was then injected subcutaneously in the right flank of mice. Tumor volume was monitored per week by measuring with calipers and calculating tumor volume using the formula length (mm)×width (mm)×width (mm)×0.5. After tumors reached 100 mm^3, mice were received subcutaneous injection with 50 or 100 mg/kg *artesunate* (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 5% sodium bicarbonate in dorsal lateral tumor surrounding area, and equal volume of sodium bicarbonate was used as control. After 4 weeks, mice were euthanized and tumors were removed for the investigation of tumor growth and related protein expressions and acitivity. All protocols for mouse experiments were subject to be approved by the Ethical and Research Committee of Nanjing Medical University.

Cell Culture Prostatic cancer cells 22rv1 were obtained from American Type Culture Collection (ATCC) and cultured in DMEM containing 10% FBS supplemented with glutamine and antibiotics and maintained in 5% CO_2 incubator under 37°C. To evaluate the role of ART on cells viability and apoptosis, cells were incubated with 50 or 200 μmol/L ART for 48 h for the further analysis.

Plasmid Construction and Transfection Sequences for AR and DNMT3b were amplified with PCR and reconstituted into pcDNA plasmid (pcDNA3.1, Invitrogen, Shanghai, China) to generate pcDNA-AR and pcDNA-DNMT3b. To overexpress AR and/or DNMT3b in 22rvl cells, cells were transfected pcDNA-AR and/or pcDNA-DNMT3b recombinant plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions by using empty pcDNA transfection as control.

Small Interfering RNA (siRNA) Transfection Prostatic cancer cells 22rvl were plated in 24-well plates (4×10^5 cells/well) and grown in phenol red-free DMEM containing 10% charcoal-stripped serum for 2 d. Then, 22rv1 clones were transfected by AR-1 siRNA or AR-2 siRNA (Dharmmacon, Lafayette, U.S.A.) or DNMT3b-1 siRNA or DNMT3b-2 siRNA (Santa Cruz Biotechnology, Inc., TX, U.S.A.) accord-

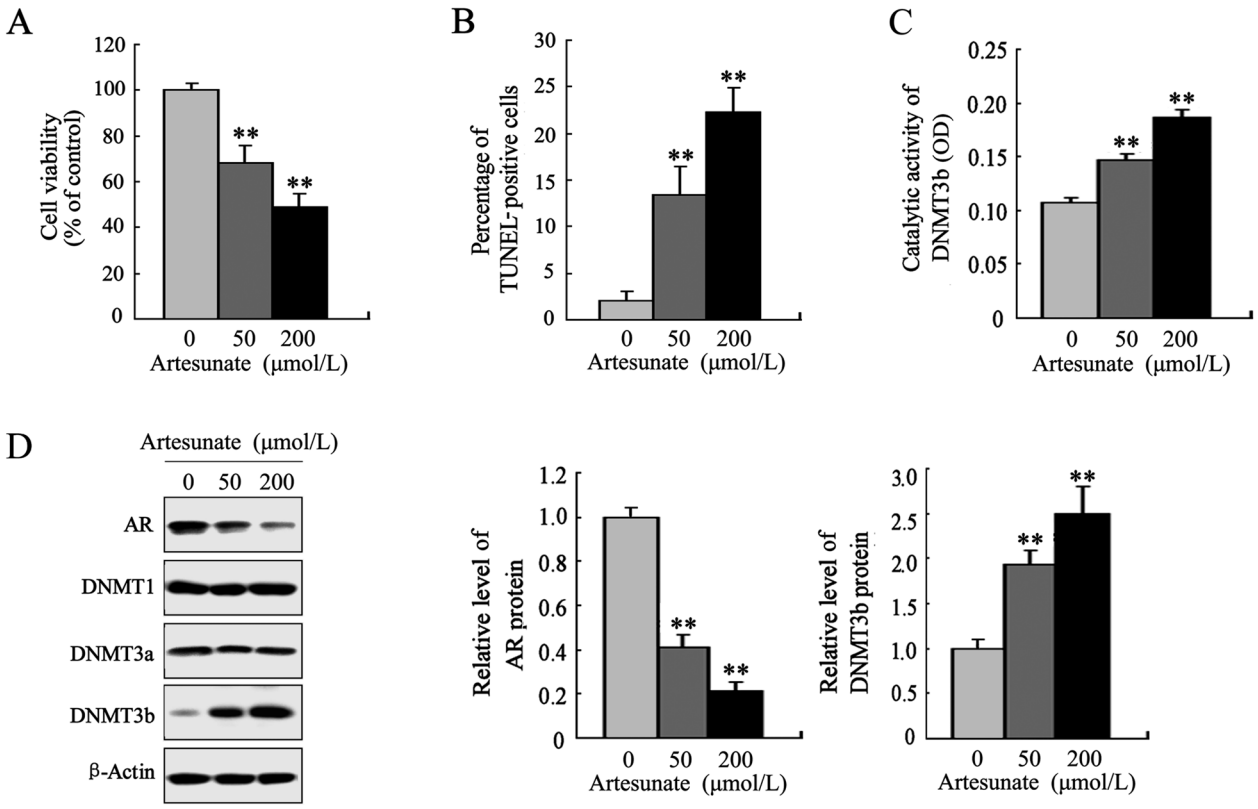

Fig. 2. Effects of ART on Cell Viability, and the Expression of AR and DNMTs in 22rvl Cells

(A) Cell viability by MTT assay. (B) Cell apoptosis by TUNEL assay. (C) Catalytic activity of DNMT3b. (D) Expression of AR, DNMT1, DNMT3a and DNMT3b by Western blotting and densitometric analysis. Data are presented as the mean±S.D. of three independent experiments with triplicate samples. $^{**}p<0.01$ compared with 0 mg/kg ART treatment group.

ing to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen). The sequences of siRNA were as follows: si-DNMT3b-1: 5′-CCU CAA GAC AAA UUG CUA U-3′; si-DNMT3b-2: 5′-GCU ACA CAC AGG ACU UGA C-3′; si-AR-1: 5′-GUA GUU GUA AGU AUC AUG A-3′; si-AR-2: 5′-GCU ACU CUU CAG CAU UAU U-3′. The scrambled siRNA control (si-control) for AR or DNMT3b was used as control.

Cell Viability Analysis by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium Bromide (MTT) Assay MTT assay was used to evaluate cell viability of 22rv1 cells. Cells were pretreated with ART and/or pcDNA-AR/pcDNA-DNMT3b or si-DNMT3b, and the seeding density of 22rvl cells was 5×10^3 cells/well. After 48 h incubation, the cell viability was detected using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, U.S.A.) following the manufacturer's instruction. Absorption intensity was measured using enzyme-linked immunosorbent assay reader at 540 nm.

Apoptotic Analysis by Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay Prostatic cancer cells 22rvl were plated at a density of 3×10^4 cells per well in 48-well tissue culture plates. About 24 h culture, the cells were treated with 50 or 200 μmol/L of ART, companying by transfection of the siRNA(s) or recombinant plasmids for 48 h. Cells were harvested and washed three times with phosphate-buffered saline. For TUNEL analysis, cells were subjected to the fixture with 4% formaldehyde and TUNEL assay with the *in situ* Apoptosis Detection (Thermo Fisher Scientific). The fixed cells were subsequently stained with TUNEL reaction buffer. Cell apoptosis was observed by fluorescent microscopy. The percentage of TUNEL-positive cells was counted.

DNMT Activity Assay After treatment of ART, the 22rvl cells or the tumor tissues in 22rv1 cells transplanted mice were harvested, and the nuclear extracts were prepared using EpiQuik nuclear extraction kit (Epigentek, U.S.A.) following the manufacturer's protocol. DNMT activity was assayed using the EpiQuik DNMT activity assay kit (Epigentek, U.S.A.) following the protocol instructions. Briefly, after incubation, capturing, and developing enzyme activity for samples and controls, absorbance (optical density (O.D.) value) was measured on a microplate reader (Infinite M200, Switzerland) at 450 nm.

Western Blotting Proteins were extracted, and assessed using the BCA kit method, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane by electroblotting. Immunoblottings were performed with the following antibodies: anti-DNMT1 (17), anti-DNMT3a (D15), anti-DNMT3b (4H84), anti-AR (N-20) and (C-19), and anti-β-actin (all from Santa Cruz Biotechnology, Inc.). Peroxidase conjugate anti-mouse or anti-rabbit immunoglobulin G (IgG) was used for enhanced chemiluminescence's detection in SmartGel (Beijing Sage Creation, Beijing, China). The densitometric analysis was conducted for the protein bands using ImageMaster™2D Platinum (Version 5.0, Amersham Biosciences, Piscataway, NJ, U.S.A.).

Statistical Analysis Continuous variables were sum-

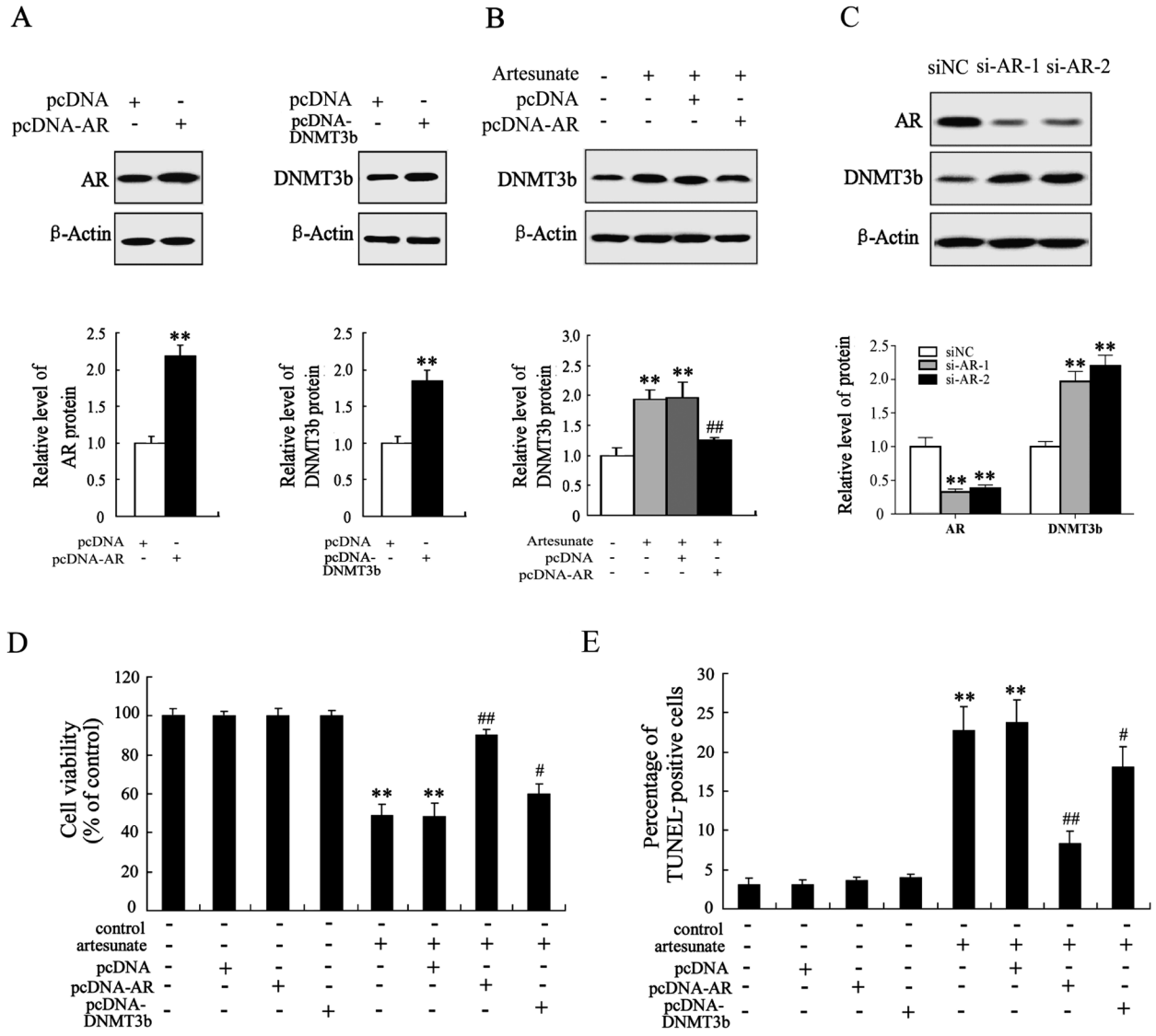

Fig. 3. Effects of AR on DNMT3b Expression and Cell Viability and Apoptosis in 22rvl Cells Treated with ART

(A) Expression of AR or DNMT3b protein in 22rv1 cells transfected with pcDNA-AR or pcDNA-DNMT3b. (B) Expression of DNMT3b in 22rv1 cells transfected with pcDNA-AR. (C) Expression of DNMT3b and AR in 22rvl cells transfected with si-AR. (D) Effects of ART on cell viability of 22rvl cells transfected with pcDNA-AR or pcDNA-DNMT3b. (E) Effects of ART on apoptosis of 22rvl cells transfected with pcDNA-AR or pcDNA-DNMT3b. 22rvl cells were transfected with pcDNA-AR, pcDNA-DNMT3b or pcDNA empty plasmid for 24 h, and then treated with 200 μmol/L ART for 48 h. Data are presented as the mean±S.D. of three independent experiments with triplicate samples. $^{**}p<0.01$ compared with control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with the cells transfected with pcDNA.

marized as the mean±standard deviation (S.D.). Significant differences were analyzed with one-way analysis of variance (ANOVA) and the Student–Newman–Keuls multiple comparison test using SPSS software (version 16.0, Chicago, IL, U.S.A.). p values of less than 0.05 were considered statistically significant.

RESULTS

Effects of ART on Tumor Growth and the Expressions of AR and DNMTs in 22rv1 Cells Transplanted Mice To evaluate the effect of ART on prostatic tumor growth, ART was injected in mice with prostatic cancer cell line 22rv1 xenograft and examined tumor volume once a week. The result showed that the growth of tumor volume was significantly suppressed by 50 and 100 mg/kg ART in a dose-dependent manner since two weeks after ART injection ($p<0.05$ or $p<0.01$) (Fig. 1A). Additionally, ART dose-dependently inhibited the expression of AR whereas enhanced DNMT3b expression (Figs. 1B, D), and increased the catalytic activity of DNMT3b (Fig. 1C). However, there was no apparently changed in DNMT1 and DNMT3a expression compared with the control (untreatment of ART) (Fig. 1B).

Effects of ART on Cell Viability and Apoptosis and the Expressions of AR and DNMTs in 22rvl Cells To further study the mechanism by which ART mediated tumor growth suppression of prostatic cancer, we performed cell viability and ectopic protein expression of 22rv1 cells in response to 50 and 200 μmol/L of ART. After 48 h ART incubation, cell viability was dose-dependently inhibited (Fig. 2A), accordingly, the percentage of apoptosis cells increased (Fig. 2B). Similar to the results in 22rvl cell transplanted mice, ART dose-dependently inhibited the expression of AR, and enhanced DNMT3b expression as well as the catalytic activity of DNMT3b, whereas no change was observed in DNMT1 and DNMT3a expression (Figs. 2C, D), suggesting that AR

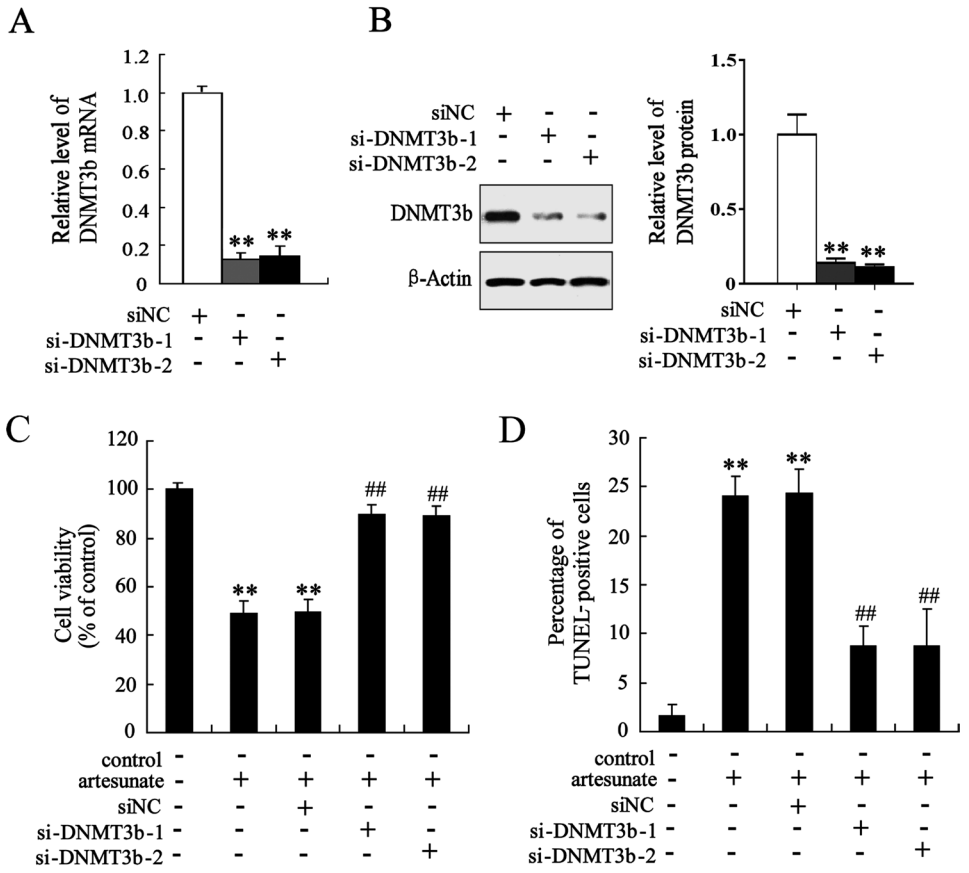

Fig. 4. Effects of Interference of DNMT3b on ART-Induced Cell Viability and Apoptosis in 22rvl Cells

(A) Expression of DNMT3b mRNA. (B) Expression of DNMT3b protein. (C) Cell viability. (D) Cell apoptosis. 22rvl cells were transfected with si-DNMT3b for 24 h, and then treated with 200 μmol/L ART for 48 h. Data are presented as the mean±S.D. of three independent experiments with triplicate samples. $^{**}p<0.01$ compared with control, $^{\#\#}p<0.01$ compared with the cells transfected with si-NC (negative control).

mediated ART-induced the cytotoxicity of 22rvl cells through DNMT3b rather than DNMT1 or DNMT3a.

AR-DNMT3b Mediated the Effects of ART on Cell Viability and Apoptosis in 22rvl Cells To confirm the regulation of AR in DNMT3b expression, AR was overexpressed or interfered in 22rv1 cells with ART treatment. The results showed that overexpression of AR (pcDNA-AR) could decrease the ART-induced the expression of DNMT3b (Figs. 3A, B), while interference of AR (siRNA-AR) increased the expression of DNMT3b in 22rvl cells (Fig. 3C), indicating that DNMT3b may act as the downstream signals of AR in ART treatment in prostatic cancer cells.

The effects of expressions of AR and DNMT3b on ART-induced cell viability and apoptosis in 22rvl cells were further investigated. As shown in Figs. 3D and E, overexpression of AR almost rescued ART-induced the decrease in cell viability and the increase in apoptosis in 22rvl cells, whereas very slight effects were observed in overexpression of DNMT3b in the presence of ART. Moreover, interference of DNMT3b (si-DNMT3b) could reverse ART-induced the decrease in cell viability and the increase in apoptosis in 22rvl cells (Fig. 4).

DISCUSSION

The anticancer activity of ART is an issue of ongoing discussion. Recent clinical trials indicated that the tumor suppression action of ART on advanced breast cancer,[14] colorectal cancer[15] and pancreatic cancer cells.[16] ART increasingly attracts the attention of cancer researchers due to a broad range of anti-cancer activity, such as pro-apoptosis attenuation of tumor growth, metastasis, and angiogenesis.[2,3,17] The present study further supported that ART could dose-dependently suppress tumor growth of prostatic tumor-bearing mice.

The present study sought to elucidate the potential involvement of signals by which ART inhibited tumor growth. By tumor tissues' analysis, we confirmed that in the process of inhibiting tumor growth, ART attenuated the expression of androgen receptor (AR). AR had been reported to play important roles in prostatic carcinogenesis. For example, Stelloo *et al.* identified AR and chromatin interaction as potential prognostic markers for prostate cancer outcome.[18] During prostate development, stromal AR induced and promoted epithelial cell growth, as observed from mouse knockout studies. During prostate carcinogenesis and progression, the stromal cells begin to lose AR expression as early as at the stage of high-grade prostatic intraepithelial neoplasia.[19] Increasing evidences pointed that AR was a critical mediator for anti-prostate cancer.[20–22] In view of this, researchers tended to explore agents to antagonize AR as improved therapeutic methods for prostatic cancer. A natural prenylflavonoid, also known as icaritin (ICT), inhibited AR-regulated genes in AR-positive prostate cancer cells *via* AR-depended pathways.[23] All these studies suggested the role of AR in metastatic prostate cancer. Thus, we manipulated cellular AR level and examined wheth-

er AR overexpression abrogates anti-PCa activity of ART. Expectedly, the reduction of cell viability and the enhancement of cell apoptosis in 22rvl cells transfected with pcDNA-AR were found in the presence of ART treatment.

Cellular responses to AR activation by androgen represent change of downstream signals that involved in metabolism, cell proliferation and prostate differentiation. Chu *et al*. found that there existed the negative correlation between AR and total DNMT activity, indicating that DNMTs are such responder for this process and can be negatively controlled by AR.[13] In addition, ART could induce the marked sustained increase of $[Ca^{2+}]_i$, and subsequently triggered the apoptosis of prostatic cancer cells.[24] While DNMT3b resulted in the increase in apoptosis of prostatic cancer cells, DNMT3b should be positively related to ART, which was approved by our analysis of DNMTs expression of PCa tumor and PCa cells that both of the expression and the catalytic activity of DNMT3b were upregulated when AR was restrained in the presence of ART. DNA methylation might be the earliest somatic genome changes in prostate cancer, it also plays an important role in the process of tumor invasion, growth and metastasis. The observation of abnormal DNMT3b responding to ART or ART plus pcDNA-AR suggested that AR-DNMT3b signals constitute a potential pathway for anti-PCa of ART. To address the involvement of DNMT3b, we examined cell viability and cell apoptosis in ART and si-DNMT3b co-transfected cells. DNMT3b silencing restored cell viability and substantially suppressed cell apoptosis in presence of ART. Furthermore, DNMT3b overexpression partly abrogated the pcDNA-AR induced reduction in cell apoptosis in the presence of ART, suggesting cross-talk between AR and DNMT3b in anti-PCa activity of ART.

In summary, the present study demonstrated that *artesunate* suppressed the growth of prostatic cancer cells through inhibiting androgen receptor and subsequent regulation of DNMT3b expression. The study not only illustrated the important role of AR-DNMT3b in ART-suppressed the growth of prostatic tumor, but also provided a clue that ART could be as a potential drug in the therapy of prostatic tumor.

Acknowledgments This work was supported by the National Natural Science Foundation of China (81573194, 81372956), Natural Science Foundation of Jiangsu province (BK20151555), Natural Science Foundation of Jiangsu Universities (14KJA330002), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), China.

Conflict of Interest The authors declare no conflict of interest.

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