

Original Article

Targeting silencing androgen receptor gene by shRNA with low-intensity focused ultrasonic irradiation inhibits growth of prostate cancer xenografts in nude mice

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Abstract: The androgen receptor (AR) plays a pivotal role in prostate cancer, making it a potential therapeutic target. Short-hairpin RNA (shRNA) inhibits gene expression and offers a novel strategy to eradicate disease. Ultrasound-mediated gene transfection is a promising gene delivery method. This study sought to determine whether targeting silencing androgen receptor gene by shRNA with low-intensity focused ultrasonic irradiation could be used as effective therapy for prostate cancers in vivo. A plasmid-based short-hairpin RNA combined with low-intensity focused ultrasonic irradiation approach was used to specifically knock down the expression of AR in prostate cancer 22RV1 cells in vivo. The growth of 22RV1 tumors that had been subcutaneously xenografted was evaluated and expression level of AR was determined by immunohistochemical staining. The proliferative index (PI) and the apoptotic index (AI) were respectively derived from the percentage of positive cells by Ki-67 immunohistochemical staining and TUNEL assay. The plasmid-based AR shRNA administrated intravenously significantly inhibited the tumor growth and AR expression. These inhibitory effects of AR shRNA were augmented when the region of tumor received low-intensity focused ultrasound irradiation. Immunohistochemical staining and TUNEL assay confirmed AR shRNA with low-intensity focused ultrasonic irradiation exhibited growth-inhibitory, antiproliferative, and apoptotic effects on prostate cancer xenografts. The authors showed for the first time that the knockdown of AR expression by plasmid-based AR shRNA with low-intensity focused ultrasonic irradiation significantly suppressed the tumor growth of prostate cancer in vivo.

Keywords: Prostate cancer, androgen receptor, gene silencing, focused ultrasound

Introduction

Prostate cancer is the second most frequently diagnosed cancer in the male population worldwide [1]. In developed countries, the incidence rate of prostate cancer appears much higher. In the United States, prostate cancer has overtaken lung cancer as the most commonly diagnosed malignancy and the second leading cause of cancer death in males for decades [2]. Meanwhile, the morbidity and mortality are rising in many developing countries such as China due to population aging and lifestyle changes [3]. Androgen receptor (AR) is expressed not simply in androgen-dependent prostate cancer (ADPC), but also in most castration-resistant prostate cancer (CRPC) where the AR remains active and is necessary to prostate cancer cells [4]. Therefore, AR is a potential target for treatment of prostate cancers both at the androgen-dependent and the castration-resistant stages.

Through the RNA interference (RNAi) mechanism, small interfering RNAs (siRNAs) or the precursors to siRNA known as short-hairpin RNAs (shRNAs) can target complementary mRNA strands for degradation, thus specifically down-regulating gene expression [5]. The ability of siRNA/shRNA to inhibit gene expression offers a novel strategy to eradicate disease.

Ultrasound could facilitate cellular uptake of macromolecules owing to its sonoporation and cavitation effect [6, 7]. It has been proven by numerous groups, that the cellular uptake of drugs and genes is sharply increased, when the region of interest is under sonication. Hence the ultrasound could be exploited to transfer gene into targeted tumor tissue efficiently [8, 9].

In the present study, we investigated whether targeting silencing androgen receptor gene by

shRNA with low-intensity focused ultrasonic irradiation could be used as effective therapy to treat prostate cancers *in vivo*. To test this, a plasmid-based short-hairpin RNA combined with low-intensity focused ultrasonic irradiation approach was used to selectively knock down the expression of AR in prostate cancer 22RV1 cells *in vivo*. Our results showed that AR shRNA combined with low-intensity focused ultrasonic irradiation could down-regulate the expression of AR, suppress proliferation, and induce apoptosis of human prostate cancer cells, and thereby inhibit the growth of prostate cancer xenografts in nude mice.

Materials and methods

Cell line and culture conditions

The human prostate cancer cell line 22RV1 was received from Institute of Biophysics, Chinese Academy of Science and was cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO). The cells were maintained in 75 cm² tissue culture flasks (Corning) under sterile conditions at 37°C in water-saturated air containing 5% carbon dioxide.

Experimental animals

Male athymic nude mice ages 5-6 weeks were purchased from the Vital River Laboratories (VRL, China). These animals were provided with sterilized food and water *ad libitum*, housed in negative-pressure isolators (Laboratory Animal Science Center of Peking university People's Hospital, Beijing, China), and subjected to 12-hour light and dark cycles. Mice were acclimated to our vivarium for 1 week prior to their use in study protocols. All animal procedures were performed under sterile conditions. All animal studies were approved by the Peking University People's Hospital Committee on Use and Care of Animals and conducted in accordance with local humane animal care standards.

Construction of an AR shRNA and preparation of recombinant plasmids

First, a 54-nucleotide (nt) DNA sequence targeting AR was designed and constructed. The sense oligonucleotide sequence was 5'-GATCCTATCCCAGTCCCAGTTCGATCGAGCAAGTGGGA-

CT GGGATAGGGCTTTTT-3', and the antisense oligonucleotide sequence was 5'-AGCTAAAAA GCCCTATCCCAGTCCCAGTTCGATCGATCAAGTGGGACTGGGATAG-3'. Synthetic sense and antisense oligonucleotides were used to create the template for generating RNA composed of 2 identical sequence motifs in an inverted orientation separated by a loop spacer to form a double-stranded short-hairpin RNA. Next, 50 pmol of each oligonucleotide was annealed for 3 minutes at 95°C and then for 1 hour at 37°C, and 2 µg of pRNAT-U6.1/Neo plasmids (Invitrogen) which contain the ampicillin resistance gene and the U6 promoter were linearized with 1 µL of BamHI (MBI), 1 µL of HindIII (MBI) and 2 µL of 10× tango buffer (MBI) for 3 hours at 37°C. After digestion, 30 µL of the linearized plasmids were recovered by column DNAback (Tiandz, Inc) following the manufacturer's instructions. Then, the ligation was performed between the recovered linearized plasmids which carried sticky ends left by BamHI and HindIII and the annealed double-stranded oligonucleotides which had un-base paired single stranded regions capable of recognizing the sticky ends of linearized plasmids. 5 µL of digested plasmids were mixed with 1 µL of T4 DNA ligase (MBI), 1 µL of 10× T4 DNA ligase buffer, and 1 µL of annealed double-stranded oligonucleotides, then incubated for 12 hours at 16°C. Finally, these recombinant plasmids were cloned in chemically competent *Escherichia coli* cells (Invitrogen) according to the manufacturer's instructions. Several colonies were picked up, and the proper insert was confirmed by automated sequencing. After identifying clones with the correct shRNA sequence, the colony was cultivated in 5 mL Luria broth (LB) medium overnight at 37°C in an orbital shaker. The recombinant plasmids were massively extracted with the plasmid maxi kit (QIAGEN) following the manufacturer's instructions and gave us 1 mg of plasmids.

Tumor implantation and growth

Human 22RV1 prostate cancer cells (5×10⁶ cells) in 100 µl PBS were inoculated subcutaneously into the right flank of athymic nude mice. Tumor growth was monitored every other day, using vernier calipers to measure the length (l) and width (w) of each lesion. Tumor volume was calculated using the following formula: volume = $\pi/6(l \times w)^{3/2}$ [10]. For experiments, mice were randomly segregated into 4 groups, each

of which consisted of 5 animals. The four groups were respectively called control group, ultrasound treated group, shRNA treated group, and combined treatment group according to the treatments the nude mice would receive.

Tumor treatments

After 4 weeks, when the tumors had reached an average volume of approximately 300 mm³, recombinant plasmids in 200 µL of phosphate-buffered saline (PBS) were injected into the tail vein of the tumor-bearing nude mice in the combined treatment group at a dose of 2 milligrams per gram as soon as the region of tumors received low-intensity focused ultrasound irradiation. The ultrasonic parameters included acoustic power (20 W), pulse duration (100 ms), duty ratio (50%), total acoustic exposure time (40 s). At the same time, mice in the shRNA treated group were simply treated with the same dose of recombinant plasmids intravenously as the combined treatment group and mice in the ultrasound treated group only received low-intensity focused ultrasound irradiation to the tumor sites with the same parameters as the combined treatment group. Mice in the control group were injected with 200 µL of PBS intravenously as control. The length (l) and width (w) of tumors were measured every other day with vernier calipers and the tumor volumes in cubic millimeter were calculated with the formula: volume = $\pi/6(l \times w)^{3/2}$ [10]. Fourteen days after treatment, mice were sacrificed, and tumors were harvested, weighed, fixed in formalin, and embedded in paraffin for the analysis of AR, Ki-67 levels and apoptotic activity. All animal experiments in the present study complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Immunohistochemical staining of AR

Four-micron sections were obtained by microtome and transferred onto glass slides. They were deparaffinized in xylene and rehydrated sequentially in a graded series of ethanol (100%, 90%, and 70%). Antigen retrieval was performed using heat treatment under pressure in Tris EDTA buffer (pH=8) (Beijing XiYa JinQiao Biological Technology co., Ltd) for 3 minutes and subsequently nonspecific labeling

was blocked with goat serum blocking solution (ZSGB-BIO) for 30 minutes. Tissue sections were then incubated with mouse monoclonal antibody against AR antigen (1:400 dilution) (Santa Cruz Biotechnology) at 4°C overnight in a humidified chamber, followed by washing with PBS three times and incubation with polymer horseradish peroxidase (HRP) antimouse IgG (ready to use) (Beijing Xiya Jinqiao Biological Technology co., Ltd) for 30 minutes at room temperature. As a negative control, primary antibody was omitted and replaced with PBS. Immunoreactivity was observed with the DAB kit (ZSGB-BIO) following the manufacturer's instructions and the nuclei were counterstained with haematoxylin.

Proliferative cell labeling with a Ki-67 antibody

As described above, slide mounted, paraffin-embedded tumor tissue sections were deparaffinized in xylene, rehydrated sequentially in a graded series of ethanol (100%, 90%, and 70%). Antigen retrieval was performed by heating to 96°C for 20 minutes in citrate-based antigen retrieval solution (pH=6) (ZSGB-BIO) and subsequently blocked with goat serum (ZSGB-BIO) for 30 minutes. Tissue sections were then incubated with mouse monoclonal antibody against Ki-67 antigen (1:200 dilution) (ZSGB-BIO) at 4°C overnight in a humidified chamber, followed by washing with PBS three times and incubation with polymer horseradish peroxidase (HRP) antimouse IgG (ready to use) (Beijing Xiya Jinqiao Biological Technology co., Ltd) for 30 minutes at room temperature. As a negative control, primary antibody was omitted and replaced with PBS. Immunoreactivity was observed with the DAB kit (ZSGB-BIO) following the manufacturer's instructions and the nuclei were counterstained with haematoxylin.

Apoptotic cell labeling by TUNEL assay

The TUNEL assay was performed with the In Situ Cell Death Detection Kit, POD (Roche Applied Science) according to manufacturer's instructions. Briefly, as described above, slide mounted, paraffin-embedded tumor tissue sections were deparaffinized in xylene, rehydrated sequentially in a graded series of ethanol (95%, 90%, 80% and 70%). Permeabilization was performed with proteinase K working solution (15 µg/ml in 10 mM Tris/HCl, pH 7.5) for 15 min at 25°C. Tissue sections were then incubated

with TUNEL reaction mixture which was prepared by adding 50 µl of enzyme solution (vial 1) to 450 µl of label solution (vial 2) for 60 min at 37°C in a humidified chamber, followed by washing with PBS three times and incubation with Converter-POD for 30 minutes at 37°C in a humidified chamber. As a negative control, TUNEL reaction mixture replaced with label solution (without terminal transferase). As a positive control, permeabilized tissue sections were incubated with DNase I recombinant for 10 min at 20°C to induce DNA strand breaks, prior to adding the TUNEL reaction mixture. Staining was observed with the DAB kit (ZSGB-BIO) following manufacturer's instructions and the nuclei were counterstained with haematoxylin.

Quantification of AR, proliferative cells and apoptotic cells

Initially, all sections were scored blindly by 2 independent observers without any prior knowledge of the experimental mice data or treatment-related outcomes. The scores were then compared to confirm consistency between observers, and a final score was agreed after simultaneous viewing of slides by both observers. The mean of the two observers' scores were used for statistical analysis.

AR expression was evaluated in a semiquantitative manner known as the HSCORE system [11] whereby the levels of expression are represented as the percentage of positive cells and the intensity of the staining. HSCORE were calculated using the following equation: HSCORE = (0 × % cells staining negative) + (1 × % cells staining weakly positive) + (2 × % cell staining moderately positive) + (3 × % cells staining strongly positive) with a score between 0 and 300.

As a specific biomarker for cell proliferation, Ki67 was evaluated for tumors proliferative index (PI) [12, 13]. Cells were counted for the determination of PI (Ki67-LI) in 5 randomly selected high power fields (×400 magnification) each slide. Any nuclear staining, regardless of intensity, was considered positive for Ki67. The PI (Ki67-LI) was expressed as a percentage of immunoreactive tumor cells to the total counted tumor cells.

TUNEL is a common method to detect apoptosis and the staining level of apoptotic cells was

expressed as apoptotic index (AI) [13]. Likewise, cells were counted for the determination of AI in 5 randomly selected high power field (×400 magnification) each slide. Any nuclear staining, regardless of intensity, was considered positive for apoptosis. Like PI, the AI was expressed as a percentage of staining tumor cells to the total counted tumor cells.

Statistical analysis

The results of the study were statistically analyzed using SPSS 18.0 for Windows. Numerical data are presented as means ± SD. For numerical data that obeyed the normal distribution, the ANOVA was used for the comparison of variables in four separate groups, The LSD test was used for the comparisons of two independent groups. For numerical data that did not have the normal distribution, Kruskal-Wallis test was used for the comparison of variables in four separate groups. Mann-Whitney U test was used for the comparisons of two independent groups. Statistical significance was set at P<0.05.

Results

AR shRNA inhibits human prostate tumor growth in nude mice

At the time of treatment, the tumor volumes in mice in the four groups were similar, which were not significantly different (P=0.978). The effect of treatment on prostate cancer xenograft in nude mice was continuously monitored for 14 days. The average tumor volume over the total 14 days of study and tumor wet weight measured at 14 days among these four groups is shown in **Figure 1**. In **Figure 1**, it is fairly easy to see that tumor volumes (P=0.685) and tumor weights (P=0.572) were not significantly different between mice in control group and in ultrasound treated group. As the graph of tumor volume revealed in **Figure 1A**, mice intravenously administered AR shRNA with or without low-intensity focused ultrasound had sustained a significant tumor growth arrest compared with mice in control group (P<0.001). According to the significant tumor volume reduction in mice with combined treatment when compared with AR shRNA simply treated mice (P<0.001), low-intensity focused ultrasonic irradiation to the region of tumor significantly enhanced the inhibitory effect of AR shRNA. Consistent with

shRNA and ultrasound inhibit prostate cancer

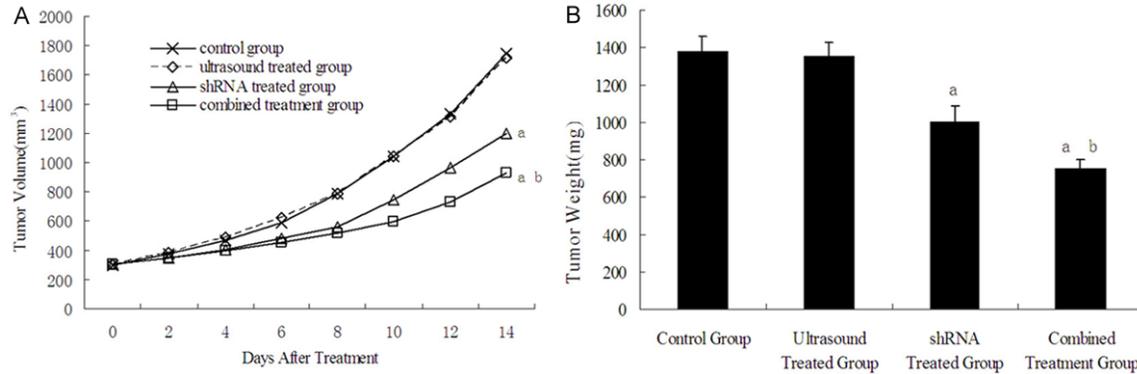


Figure 1. Effects of treatment on the tumor growth in each group. A. A graph shows tumor volume as calculated on the indicated days. The curve representing the tumor volume in the ultrasound treated group almost coincided with that in the control group ($P=0.685$). However, significant reduction in tumor volume was observed in mice intravenously administered AR shRNA with or without ultrasound compared with controls ($P<0.001$). In addition, the tumor volume significantly decreased in the mice in the combined treatment group compared with mice only treated with AR shRNA ($P<0.001$). B. A representative histogram of tumor weight revealed the difference between mice in control group and ultrasound treated group was not significant ($P=0.572$). As is consistent with tumor volume data, tumor weight was significantly reduced in mice intravenously administered AR shRNA with or without ultrasound compared with controls ($P<0.001$), as well as significantly decreased in the mice in combined treatment group compared with mice in shRNA treated group ($P<0.001$). a $P<0.001$, compared with control group; b $P<0.001$, compared with shRNA treated group.

tumor volume data, the average wet weight of the tumor was significantly reduced ($P<0.001$) in mice treated with AR shRNA combined with or without ultrasound, compared with mice in the control group (**Figure 1B**). The tumor wet weight significantly decreased in the mice in combined treatment group compared with mice in shRNA treated group ($P<0.001$), as is similarly shown in **Figure 1B**. No gross adverse effects such as loss of body weight, infection and so on were observed and no mice died during the experimental period.

AR shRNA suppresses the expression of AR in human prostate cancer xenografts

To investigate whether the inhibition of tumor growth is associated with the down-regulation of AR expression, the xenograft tumors were excised and processed for immunohistochemical staining of AR protein expression. The immunohistochemical staining showed the positive staining for AR was observed in the nuclear region. AR protein was highly expressed in human prostate cancer tissues in control group and ultrasound treated group (**Figure 2A, 2B**). The AR HSCORE values between these two groups were not significantly different ($P=0.173$), as presented in **Table 1**. AR protein was markedly down-regulated in AR shRNA

administered xenografts (**Figure 2C**), compared with pretty high AR expression in the control group (**Figure 2A**), which coincided with AR HSCORE values in **Table 1** between these two groups ($P=0.009$). Tumor tissues in mice with combined treatment showed no or extremely weak AR protein expression (**Figure 2D**). The AR HSCORE value of combined treatment group significantly declined compared with shRNA treated group ($P=0.009$) and control group ($P=0.008$) (**Table 1**).

AR shRNA reduces cell proliferation in human prostate cancer xenografts

To test whether AR shRNA-mediated inhibition of human prostate cancer growth in vivo was associated with reduced cell proliferation, tumor tissues were processed and probed for Ki-67 expression. These results were consistent with the results of the AR expression in human prostate cancer xenografts. The immunohistochemical staining showed positive staining for Ki-67 was observed in the nuclear region. Ki-67 protein expressions were very high in tumor tissues both in control group and ultrasound treated group (**Figure 3A, 3B**). The proliferative index (PI) as determined by Ki67 staining between these two groups were not significantly different ($P=0.462$), as presented

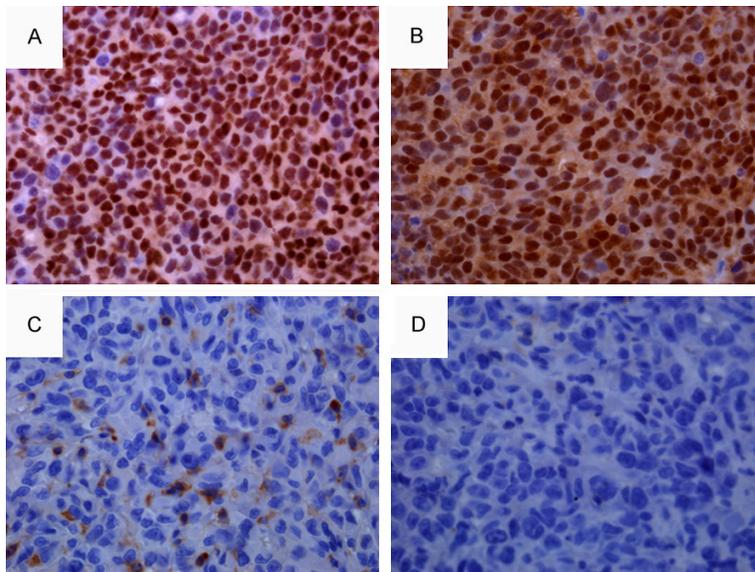


Figure 2. Expression of AR Protein in Each Group of Xenografts. (A, B) Tumor tissues of the mice in both control group and ultrasound treated group demonstrated that AR protein was expressed at a markedly high level. In contrast, (C) tumor tissues from shRNA-treated mice showed significantly decreased AR protein expression and (D) tumor tissues of mice with combined treatment showed no or extremely weak AR protein expression. Original magnification, $\times 400$; objective, $\times 40$.

Table 1. AR HSCORE value, proliferative index, and apoptotic index in each group

	AR HSCORE value	Proliferative index (%)	Apoptotic index (%)
Control group	268.8 \pm 18.74	87.6 \pm 7.92	27.2 \pm 3.96
Ultrasound treated group	259.4 \pm 16.56	84.0 \pm 8.88	29.6 \pm 2.61
shRNA treated group	25.8 \pm 6.68 ^a	26.0 \pm 3.07 ^a	55.6 \pm 7.86 ^a
Combined treatment group	2.0 \pm 1.87 ^{a,b}	7.8 \pm 2.38 ^{a,b}	83.6 \pm 8.29 ^{a,b}

^aP<0.01, compared with control group; ^bP<0.01, compared with shRNA treated group.

in **Table 1**. A significant reduction of Ki-67 expression was observed in tumors from AR shRNA-treated mice (**Figure 3C**) compared with controls (**Figure 3A**), which was in keeping with PI in **Table 1** between these two groups (P=0.009). There was markedly decreased cell proliferation observed in **Figure 3D** in the combined treatment group whose PI significantly reduced compared with shRNA treated group (P=0.009) and control group (P=0.008) (**Table 1**).

AR shRNA induces apoptosis in human prostate cancer xenografts

To determine whether AR shRNA-mediated inhibition of human prostate cancer growth in vivo

was associated with induced apoptosis, tumor tissues were processed for TUNEL assay. The TUNEL assay showed the positive staining for apoptosis was observed in the nuclear region too. As revealed in **Figure 4A, 4B**, no significant differences were observed in the degree of apoptosis in human prostate cancer xenografts treated with ultrasound when compared with that in xenografts untreated (control group). As presented in **Table 1**, the apoptotic index (AI) as determined with TUNEL method between these two groups were not significantly different (P=0.169). In **Figure 4C, 4D**, the apoptosis was considerably high both in shRNA treated group and combined treatment group. As shown in **Table 1**, AIs between the shRNA treated group and the control group were significantly different (P=0.009) as well as that between the combined treatment group and the control group (P=0.008). AR shRNA treatment alone yields an intermediate level of increased apoptosis when compared with the combined treatment group (P=0.009). The difference was statistically significant.

Discussion

Prostate cancer is androgen dependent, and endocrine therapy is one of the main treatment modalities in the clinical management of prostate cancer patients. Since Huggins and Hodges [14] demonstrated the responsiveness of prostate cancer to androgen deprivation therapy (ADT), androgen-suppressing strategies has been the preferred treatment for advanced prostate cancer for nearly 70 years. Prostate cancer is initially dependent upon androgens and androgen withdrawal has been considered a significant therapeutic approach at this stage. Androgen ablation therapy generally induces a remission in 80-90% of patients with advanced prostate cancer, and results in a

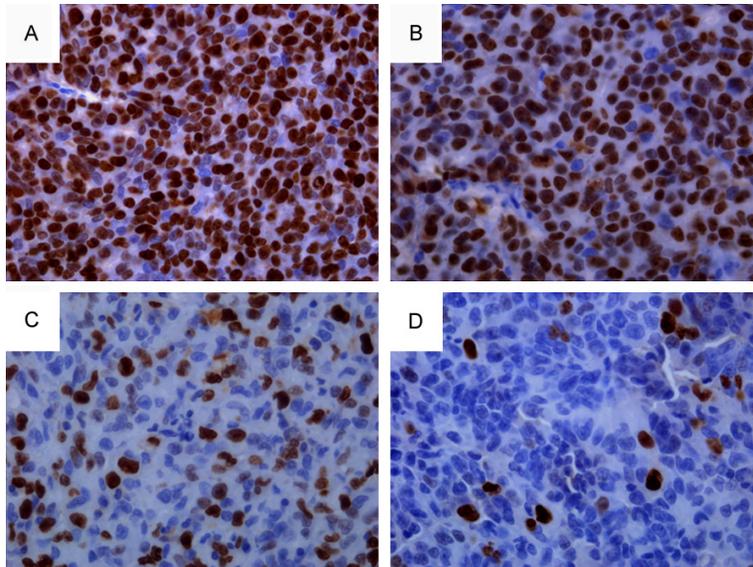


Figure 3. Expression of Ki-67 Protein in Each Group of Xenografts. (A, B) Tumor tissues from the mice in both control and ultrasound treated group showed significantly higher Ki-67 protein expression. Whereas, (C) tumor tissues in shRNA-administered mice revealed reduced Ki-67 protein expression and (D) tumor tissues in mice with combined treatment demonstrated very weak Ki-67 protein expression. Original magnification, $\times 400$; objective, $\times 40$.

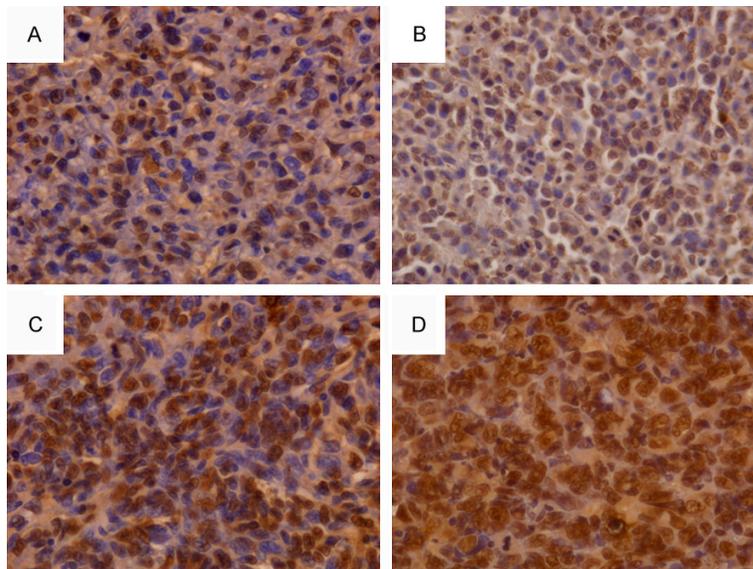


Figure 4. Results of TUNEL Assay in Each Group of Xenografts. (A, B) Specimens from the control and ultrasound treated tumor showed weak apoptosis. In contrast, (C) tumor tissues of shRNA-treated mice showed moderate apoptosis and (D) numerous apoptotic cells appeared in tumor tissues of the mice in combined treatment group. Original magnification, $\times 400$; objective, $\times 40$.

median progression-free survival of 12-33 months [15]. Unfortunately, most patients ultimately develop progressive castration-resis-

tant prostate cancer (CRPC), which is characterized by continued tumor proliferation in the absence of testicular androgen production. The best treatment for CRPC remains elusive and the patients die of metastasis or other complications within a few years [16]. Therefore, novel therapeutic strategies prostate cancers are required.

Androgen receptor (AR) is a member of nuclear hormone receptor superfamily which is activated in a ligand-inducible manner. The activated AR could interact with other transcription factors and regulates target gene expression [17]. AR plays a pivotal role both in ADPC and CRPC [17-19]. In ADPC, AR functioning through the binding of androgen promotes prostate cancer cell growth and proliferation [18, 20]. In contrast, in CRPC, AR doesn't regulate cells growth in the form of receptor-ligand binding, but probably in the form of being constitutively activated. Even so, there was strong evidence that most if not all CRPC cells still express AR which was thought to turn more active and be indispensable to tumor survival through a variety of potential mechanisms including AR amplification, AR mutation, increased androgen sensitivity, changed expression of AR regulatory factor [21-27]. Furthermore, it was proven that AR signaling is integral to the growth of CRPC and decreasing levels of AR protein expression reduces both ADPC and CRPC growth in model system [19, 22]. AR, as a critical transcription factor, appears to be an

effective target for the containment of tumor growth both in the pathogenesis of early ADPC and late-stage CRPC [28, 29]. Moreover, a dra-

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matic paradigm shift has taken place recently in the treatment of patients with advanced CRPC where the androgen receptor becomes a central therapeutic target [30, 31]. Therefore, it is a potential approach to treat human prostate cancer by silencing androgen receptor gene [32, 33].

Small interfering RNAs (siRNAs) or the precursors to siRNA known as short-hairpin RNAs (shRNAs) can specifically inhibiting gene expression, through the RNA interference (RNAi) mechanism which is an endogenous gene-silencing mechanism that involves double-stranded RNA-mediated sequence-specific mRNA degradation. The ability of siRNA/shRNA to inhibit gene expression offers a promising strategy for therapeutic product development. Their wider application with high efficiency and specificity [34] could have high impact and clinical relevance to any disease by manipulating gene expression. There is an intense research effort aimed at developing siRNAs/shRNAs as therapeutics against various diseases such as viral infections, gene disorders, and cancers [35]. To date, many clinical trials have been conducted to test the siRNAs/shRNAs for the treatment of diseases [36, 37]. In this study, a shRNA against human AR successfully inhibited the expression of human AR by human prostate cancer 22RV1 cells, leading to significant suppression of tumor growth in the xenograft model. Our findings suggest that the shRNA-directed transcriptional silencing is a promising tool for suppression of AR gene function and illustrate the potential of its application in prostate cancer treatment.

There were several reports that show the *in vitro* efficacy of siRNAs/shRNAs in targeting prostate cancer cells [38, 39]. Nevertheless, most of these siRNAs/shRNAs have not made it past cell culture studies because of several reasons, such as the unclear systemic toxicity associated with these siRNAs/shRNAs or their vectors, and the most importantly, nonbioavailability of siRNAs/shRNAs when tested under *in vivo* conditions. Snoek and colleagues [40] utilized lentiviral vector carrying AR-targeted shRNAs to transduce prostate cancer C4-2 cells expressing tetracycline repressor which were generated previously and inoculated C4-2 cells containing inducible shRNAs into mice to establish a xenograft model. Through successful treating the tumor with doxycycline orally, they proved that knocking down AR *in vivo* is an effective therapy to treat prostate cancers that

have progressed to the hormone refractory state for the first time.

Ultrasound (US)-mediated gene transfection is a recently developed and promising non-viral gene delivery method. Fechheimer et al [41] transfected mammalian cells with plasmid DNA by sonication loading for the first time in 1987. As a promising strategy for gene delivery, ultrasound has attracted increasing attention of the scientific community from then on. Ultrasound could facilitate cellular uptake of macromolecules [42] owing to its sonoporation and cavitation effect which lead to increased permeability of cell membrane and capillary. It has been proven by numerous groups, that the cellular uptake of drugs and genes is sharply increased, when the region of interest is under sonication. Accordingly, the ultrasound could be exploited for clinical applications to transfer gene into targeted tumor tissue safely and efficiently [43, 44]. The key feature of ultrasound-enhanced gene delivery is that DNA delivery can be targeted.

Our xenograft tumor studies showed that AR shRNA injected intravenously is not only safe but also effective in knocking down AR and preventing growth of 22RV1 tumors. Upon immunohistochemical examination of AR and Ki67 and TUNEL assay, it showed that inhibiting the expression of human AR suppressed proliferation and induced apoptosis of human prostate cancer cells, and thereby inhibited the growth of the prostate cancer *in vivo*. Furthermore, we used ultrasound as an effective delivery auxiliary for shRNAs as described above. Our findings showed that shRNA, when injected intravenously into a tumor which was under sonication suppress AR protein more efficiently and perform anti-tumor effort more effectively than when injected intravenously without ultrasonic irradiation.

In conclusion, our results further strengthened the significant effect of AR suppression on human prostate cancer growth and suggested that AR was a molecular target for cancer treatments. Silencing AR gene could reduce proliferation, induce apoptosis and eventually inhibit the growth of prostate cancer. The application of low-intensity focused ultrasound could achieve the desired targeted gene delivery *in vivo* and assist AR shRNA in treating prostate cancer more effectively. To our knowledge, it is the first time that the targeting silencing androgen receptor gene by shRNA combined with

shRNA and ultrasound inhibit prostate cancer

low-intensity focused ultrasonic irradiation significantly suppressed the tumor growth of prostate cancer in vivo.

Conclusion

In sum, the AR shRNA could be injected intravenously to suppress the expression of AR and hence inhibit prostate cancer growth. The low-intensity focused ultrasound could achieve the desired targeted gene delivery in vivo, having a promising future in its application. Targeting silencing androgen receptor gene by shRNA with low-intensity focused ultrasonic irradiation represents a viable therapeutic strategy for the treatment of prostate cancer.

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Disclosure of conflict of interest

None.

Abbreviations

AR, Androgen receptor; ADPC, androgen-dependent prostate cancer; CRPC, castration-resistant prostate cancer; siRNA, small interfering RNA; shRNA, short-hairpin RNA; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PI, proliferative index; AI, apoptotic index; ADT, androgen deprivation therapy.

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