

RESEARCH ARTICLE

Ultrasound Targeted Microbubble Destruction for Novel Dual Targeting of HSP72 and HSC70 in Prostate Cancer

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Abstract

The aim was to determine whether ultrasound targeted microbubble destruction (UTMD) promotes dual targeting of HSP72 and HSC70 for therapy of castration-resistant prostate cancer (CRPC), to improve the specific and efficient delivery of siRNA, to induce tumor cell specific apoptosis, and to find new therapeutic targets specific of CRPC. VCaP cells were transfected with siRNA oligonucleotides. HSP70, HSP90 and cleaved caspase-3 expression were determined by real-time quantitative polymerase chain reaction and Western blotting. Apoptosis and transfection efficiency were assessed by flow cytometry. Cell viability assays were used to evaluate safety. We found HSP72, HSC70 and HSP90 expression to be absent or weak in normal prostate epithelial cells (RWPE-1), but uniformly strong in prostate cancerous cells (VCaP). UTMD combined with dual targeting of HSP72 and HSC70 siRNA improve the efficiency of transfection, cell uptake of siRNA, downregulation of HSP70 and HSP90 expression in VCaP cells at the mRNA and protein level, and induction of extensive tumor-specific apoptosis. Cell counting kit-8 assays showed decreased cellular viability in the HSP72/HSC70-siRNA silenced group. These results suggest that the combination of UTMD with dual targeting HSP70 therapy for PCa may be most efficacious, providing a novel, reliable, non-invasive, safe targeted approach to improve the specific and efficient delivery of siRNA, and achieve maximal effects.

Keywords: Ultrasound targeted microbubble destruction - siRNA - hormone refractory prostate cancer - targeted therapy

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Introduction

Prostate cancer is the most frequent malignancy and a leading cause of cancer-related deaths in American men, accounting for an estimated 238, 590 new cases and 29,720 deaths in 2013 (Siegel et al., 2013; DeSantis et al., 2013). It also is the second leading cause of cancer deaths among men, with an estimated 903, 000 newly diagnosed cases and 258,000 deaths per year worldwide. Recently, the incidence of PCa increases steadily in China (Jemal et al., 2011). Androgen deprivation therapy (ADT) provides an effective therapy for patients with advanced PCa. Although most patients with advanced PCa show an initial response to ADT, a significant percentage of patients invariably progress to hormone refractory prostate cancer (HRPC) and castration-resistant prostate cancer (CRPC) (Bitting et al., 2013; Garcia et al., 2012). Responses to systemic chemotherapy are frequently poor (Di Lorenzo et al., 2007).

Therefore, additional therapies are needed. Another challenge is that PCa are also associated with poor prognosis and androgen independence. Effective targeting

treatment for advanced CRPC and HRPC remains a critical clinical challenge. Thus, there is a clear need for novel targeted therapeutic strategies for the treatment of advanced PCa. siRNA have provided us with dawn to tackle this issue. The properties of siRNA that are attractive for therapeutics include (i) stringent target-gene specificity, (ii) relatively low immunogenicity of siRNA and (iii) simplicity of design and testing of siRNA (McNamara JO 2nd et al., 2006).

The HSP70 family includes at least eight members with diverse biochemical functions, including nascent protein folding, preventing denatured protein aggregation, and modulating assembly/disassembly of protein complexes (Daugaard et al., 2007; Zuiderweg et al., 2013). The exact role of the HSP70 family in cancer remains to be elucidated. Firstly, The two major cytoplasmic isoforms are HSC70 and HSP72. Generally, HSC70 is abundantly and ubiquitously expressed in nontumor tissues, whereas HSP72 is present at relatively low levels in the absence of stress. Secondly, HSC70 and HSP72 expression may reduce sensitivity to HSP90 inhibitors. Combinatorial modulation of these two HSP70 isoforms could therefore

be doubly advantageous (Davenport et al., 2010; Powers et al., 2010; Meng et al., 2011; Stangl et al., 2011; Evans et al., 2010; Balaburski et al., 2013; Rérole et al., 2011).

However, the specific and efficient delivery of siRNA into cancer cells *in vivo* remains a major obstacle (Yao et al., 2012). Therefore, development of a useful multi-modal approach or multiple pathways in targeted delivery will further improve siRNA efficacy.

UTMD has been recognized as an efficient modality for drug and gene delivery *in vivo* and *in vitro*. Microbubble destruction by ultrasound exposure generates microstreams or microjets that create shear stress on cells and open transient pores in cell membranes (Li et al., 2009; Tachibana et al., 1999; Xie et al 2010; Suzuki et al., 2011; Zheng et al., 2012).

In the present study, we tested whether UTMD combined with dual targeting of HSC70 and HSP72 could promote the specific and efficient delivery of siRNA, and to explore its molecular mechanism.

Materials and Methods

Cell and Cell culture

The human prostate carcinoma cell line VCaP and prostate epithelial cell RWPE-1 cell line were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured accordingly. VCaP cell lines were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen) and maintained in a humidified atmosphere of 5% CO₂, 95% air at 37 °C. RWPE-1 cells were grown in Keratinocyte serum-free medium (K-SFM) medium (Invitrogen) supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract under standard cell culture conditions.

Experimental groups

VCaP cells were divided into the following 7 groups for gene transfection experiment. The control group (NC); HSP72 siRNA, HSC70-siRNA, HSP72/HSC70-siRNA; UTMD+HSP72-siRNA, UTMD+HSC70-siRNA, UTMD+HSP72/HSC70-siRNA.

UTMD exposure protocols

A therapeutic ultrasound machine (PHYSIOSON-Basic, PHYSIOSON Elektro-medizin, Germany) was used to emit ultrasound at the frequency of 1 MHz. The area of the ultrasound probe was 2.5 cm². The ultrasound transducer was placed at the bottom of plates or dishes with coupling medium on the surface of the transducer. The adjustable sonication parameters included ultrasound intensity, exposure time, pulse frequency, and duty cycle. Microbubbles (SonoVue, Bracco, Milan, Italy) were lipid-shelled ultrasound contrast agents containing sulfur hexafluoride gas (diameter 2.5 - 6.0 μm) and used at a concentration of ~2×10⁸ bubbles/mL. The volumetric ratio of microbubbles to medium dictated the choice of contrast agent dose.

After protocol optimization with several various settings, the following UTMD parameters were used:

ultrasound intensity, 1 W/cm²; exposure time, 60 s; pulse frequency, 100 Hz; duty cycle, 20%; volumetric ratio of microbubbles: medium, 1:5.

siRNA-mediated gene knockdown

siRNA (Shanghai GenePharma Co., Ltd) were designed against the open reading frame of HSP72 (HSPA1A; accession number NM_005345) or HSC70 (HSPA8; accession number NM_006597). Two active sequences were used for studies against HSP72 or HSC70 (designated HSP72 or HSC70). Active sequences for HSP72 and HSC70 are as follows: HSP72 (sense:GGACGAGUUUGAGCACAAGTT, antisense: CUUGUGCUCAAACUCGUCCTT), HSC70 (sense:CCAAGCAGACGCAGAUCUUTT, antisense: AAGAUCUGCGUCUGCUUGGTT), Negative control (sense:UUCUCCGAACGUGUCACGUTT, antisense: ACGUGACACGUUCGGAGAATT). For all experiments cells were transfected with siRNA (200 nM for single transfections or 100 nM for combinatorial transfections) in Opti-MEM® medium without serum according to the Lipofectamine® 2000 protocol (Invitrogen). An equal amount of scrambled Stealth siRNA was used as a negative control. Six hours after the cells were transfected, the medium was replaced with fresh culture medium. All experiments were performed 48 h after transfection and repeated three times.

Gene transfection efficiency

Following gene transfection, the efficiency of transfection was evaluated by fluorescence microscopy and flow cytometry. Green fluorescence was detected using inverted fluorescence microscopy (Zeiss Axiovert S100; Carl Zeiss, Jena, Germany). The percentage of FAM expression of the transduced VCaP cells was quantitatively measured by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA)

Cell viability assays

A cell counting kit-8 assay (CCK-8, Dojindo Laboratories) was used to evaluate relative cell viability. At 48 hours after siRNA transfections, RWPE-1 and VCaP cells were plated in 96-well microtiter plates at a density of 5×10³ cells per well, then the live cell count was assayed using CCK-8 according to the manufacturer's instructions. Briefly, 10 μL of CCK8 solution was added to each well, and the absorbance at 450 nm was measured using a Thermomax microplate reader (Molecular Devices, Hercules, CA) after 1-4 hour incubation. Relative cell viability was calculated as a percentage of untreated control cells.

Flow Cytometry

Cells were trypsinized and resuspended in Opti-MEM medium and plated on 6-well plates at 1,000 cells/well after transfection with siRNA for 48 h. For apoptosis detection, cells were stained with Annexin V and propidium iodide using the Annexin V-FITC Apoptosis Detection kit (Invitrogen), and the percentage of apoptotic cells was determined by flow cytometry (Beckman Coulter).

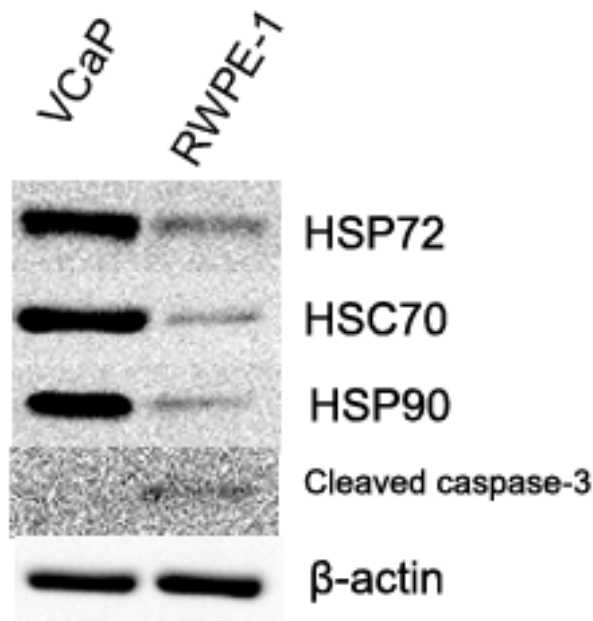


Figure 1. HSP72, HSC70, and HSP90 is Overexpressed in VCaP Compared with RWPE-1. Representative images showing expression of HSP72, HSC70, and HSP90 protein in VCaP and RWPE-1 cell as analyzed by Western blotting ($P < 0.05$). All experiments were repeated three times with similar results

Quantitative Real-Time PCR analysis

Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen) and reverse transcription was performed by PrimeScript® RT Master Mix Perfect Real Time (Takara) according to the manufacturer's instructions. RNA integrity was examined by agarose gel electrophoresis. Quantitative RT-PCR was performed in triplicate using the SYBR-Green (Takara) method on an Applied Biosystems Fast Sequence Detector System. Conditions for each target were validated by standard and melting curve analyses. We used β -actin expression as an internal control. Expression was normalized to β -actin and determined using the Applied Biosystems 7500. All primers were designed using Primer 5 and synthesized by Shanghai Sunny Biotechnology Co., Ltd. The following primers were used: HSP72 (forward, 5'-TGGAGTCCTACGCCTTCAAC-3', reverse, 5'-AGC CACGAGATGACCTCTTG-3'), HSC70 (forward, 5'-CG CAAGCATAAGAAGGACATCA-3', reverse, 5'-GAAC AGGTCAGCATTGAGTTCT-3'), β -actin (forward, 5'-GGAGATTACTGCCCTGGCTCCTA-3', reverse, 5'-GACTCATCGTACTCCTGCTTGCTG-3'). Caspase-3 (forward, 5'-GAACTGGAC TGTGGCATTGAGA-3' reverse, 5'-ATGGCACAAAGCGACTGGATGA-3'). HSP90 (forward, 5'-TCTTGGCACCACTACTCTTGT -3', reverse, 5'-CATCACCGATCAACCGTTCAGT-3').

Western blot analysis

Prostate cells transfected with siRNA were washed twice in ice-cold phosphate buffer solution (PBS), harvested and then lysed using RIPA buffer (Sigma). Protein concentrations were quantified using the BCA Protein assay kit (Thermo, IL, USA). Equal amounts of

protein and PageRuler™ Prestained molecular weight markers (Thermo Scientific) were separated by 8% or 10% SDS-PAGE and transferred to 0.45 μ m pore size PVDF membrane (Millipore). Membranes were blocked in TBS with Tween-20 containing 5% skim milk (Bright Dairy) and incubated with the primary antibody at room temperature for 1 h in blocking buffer. Antibodies and dilutions were 1: 5,000 for anti-HSC70, (#1776-1, Epitomics); 1: 2,000 for anti-HSP72 (C92F3A-5, Stressgen Biotech Corp) 1: 2,000 for anti-HSP90 (#2877-1, Epitomics), 1: 2,000 for anti-cleaved caspase3 and anti- β -actin (#9664, #4970, Cell Signaling Technology) antibodies. Washing steps after hybridization were three times at room temperature for 10 min for all other antibodies. Membranes were incubated with secondary HRP-conjugated antibodies diluted 1: 2,000 in blocking buffer and at room temperature for 1 h. After three washing steps of 10 min at room temperature, detection of the signals was performed using the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Statistical analysis

Statistical analysis was carried out with SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA). All data were represented as mean \pm SD and analyzed by One-way ANOVA. Values were considered statistically significant at $P < 0.05$. Results were representative of more than three individual experiments.

Results

HSP72, HSC70, and HSP90 is overexpressed in human prostate cancer cells compared with human prostate epithelial cells

We compared gene expression profiles of VCaP and RWPE-1 *in vitro*, and found that HSP72, HSC70, and HSP90 was overexpressed in VCaP cells. Western blot analysis revealed that VCaP cells expressed higher levels of HSP72, HSC70, and HSP90 protein *in vitro* compared with RWPE-1, which expressed undetectable levels. VCaP and RWPE-1 cells expressed very low levels of cleaved caspase-3 (Figure 1).

Effects of HSP70 expression after transfection at different time points

To confirm the optimal time points in HSP70 expression *in vitro* after transfection, we selected 24 h, 48 h, 72 h, 96 h to quantify the expression of HSP70 protein in VCaP cells. As shown in Figure 2, optimal time points of efficacy of silencing HSP70 gene by siRNA is 48 h. Therefore, we set up 48 h to further investigate after transfection *in vitro*.

Gene transfection efficiency and apoptosis

Figure 3 showed the UTMD+HSC70/HSP72-siRNA group had maximal transfection percentage (73.15 \pm 0.53) and it was significantly higher than HSC70/HSP72-siRNA group (40.37 \pm 0.65), UTMD+HSC70-siRNA group (28.31 \pm 0.60) and UTMD+ HSP72-siRNA group (24.25 \pm 0.53). In accordance with the results of transfection analysis, the apoptosis percentage of the UTMD+HSC70/

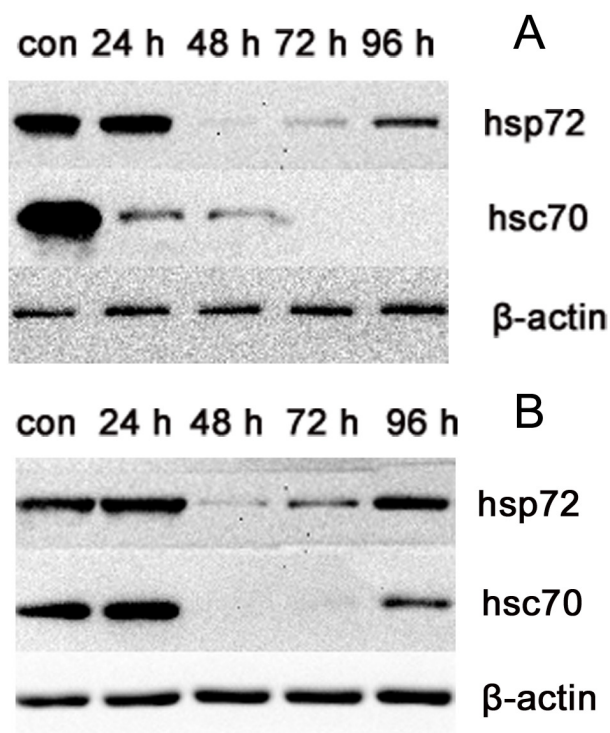


Figure 2. Optimal Time Points of Efficacy of Silencing HSP70 Gene by siRNA-transfected. (A) HSP72 siRNA transfection; (B) HSC70 siRNA transfection. The best time points of efficacy of silencing HSP70 gene was at 48 h ($P<0.05$)

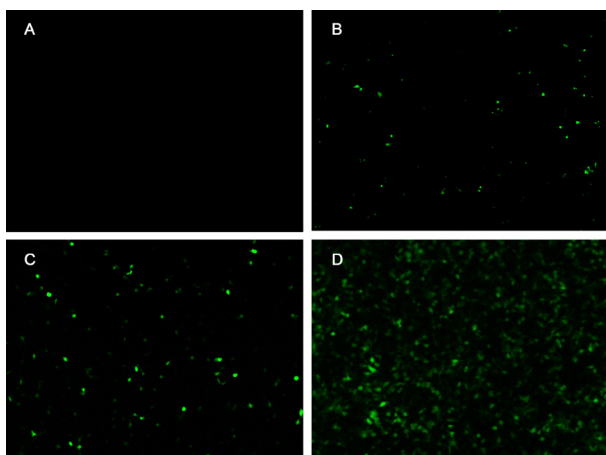


Figure 3. The Transfection Percentage of UTMD+HSC70/HSP72-siRNA Group had been Improved ($P<0.05$). (A) control; (B) HSC70-siRNA; (C) HSP72/HSC70 siRNA; (D) HSP72/HSC70 siRNA+UTMD

HSP72-siRNA group was the highest (47.16%) and the other 6 groups had significant difference between each other ($P<0.05$). It seems that the UTMD+HSC70/HSP72-siRNA could serve as a gene delivery system and silencing HSP70 and HSP90 expression induced extensive cell apoptosis (Figure 4A).

UTMD is a safety and noninvasive approach

In order to investigate the safety of UTMD, cellular viability was monitored using the cell counting kit-8 assay for 48 hours. The cellular viability of VCaP cells transfected with HSP72 and HSC70 siRNA was delayed compared with that of the control groups after UTMD. No

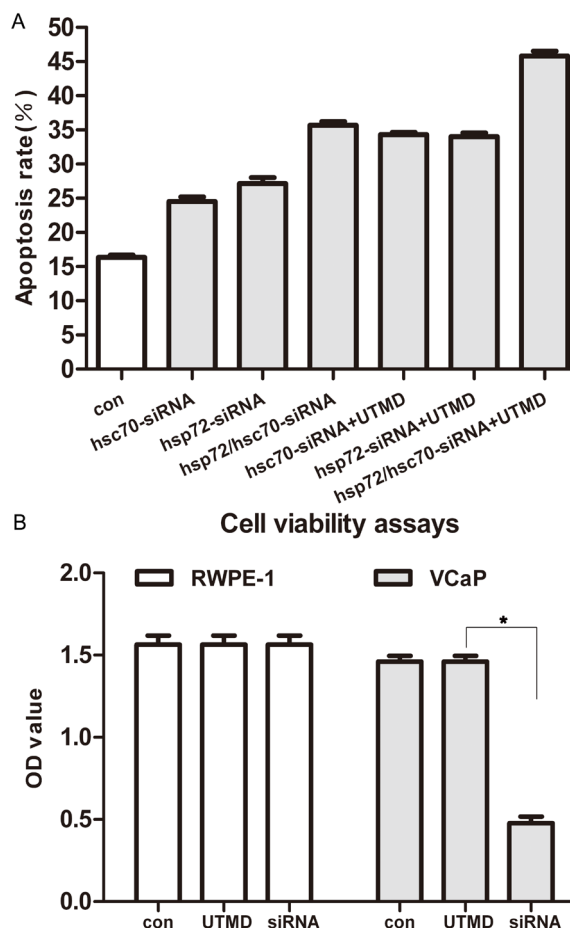


Figure 4. Apoptosis and Viability Assay of RWPE-1 and VCaP Cells in Presence of UTMD and siRNA. (A) Apoptosis rates of 3 groups shown. Compared with siRNA group and UTMD plus siRNA group ($P<0.01$). (B) The cellular viability of VCaP cells transfected with HSP72 and HSC70 siRNA was delayed compared with that of the control groups after UTMD ($P<0.01$). UTMD is a safety and noninvasive approach for RWPE-1

significant difference was found in the cellular viability between the RWPE-1 and VCaP of the control group and the two group treated with UTMD (Figure 4B).

To investigate the molecular mechanisms by which the combination of UTMD and HSP70-siRNA restrains PCa in the context of HRPC, we process a set of relevant experiment *in vitro* system. RT-qPCR revealed 3.1-fold higher levels of HSP72, HSC70, and HSP90 mRNA in NC control compared with HSP72/HSC70-siRNA treatment (Figure 5). The group of UTMD combined HSP72/HSC70-siRNA expressed undetectable levels of HSP70 and HSP90 mRNA. This is consistent with the results of Western Blot in VCaP cells (Figure 6). To better understand the biological processes that underlie what appears to be HSP70, HSP90, and cleaved caspase-3 in VCaP cells, We next sought to define the mechanism by which HSP70 regulates cleaved caspase-3 expression. we used flow cytometry and found that the cleaved caspase-3 gene that was most significantly enriched in the group of UTMD combined HSP72/HSC70-siRNA (Figure 4). Therefore, we tested the ability of the combination of UTMD with HSP72/HSC70-siRNA to induce tumor

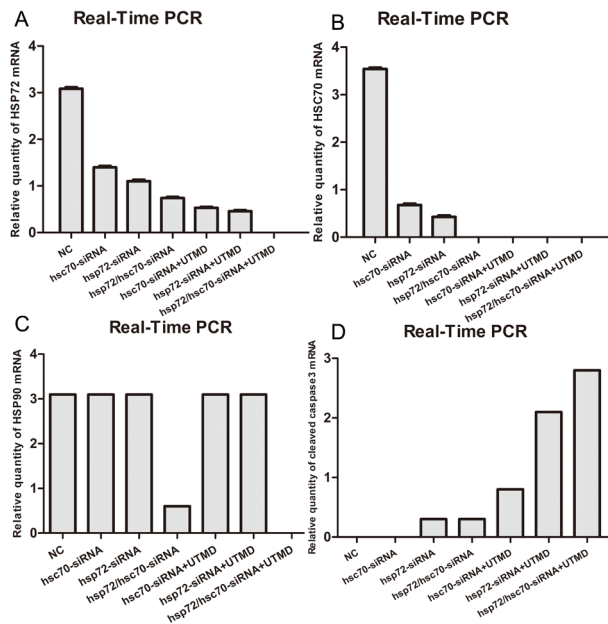


Figure 5. Real-time qPCR Analysis of mRNA HSP72, HSC70, HSP90, and Cleaved Caspase-3 Expression in VCaP Cells. (A) HSP72 mRNA expression; (B) HSC70 mRNA expression. (C) HSP90 mRNA expression; (D) cleaved caspase-3 mRNA expression. UTMD combination with dual targeting HSP72 and HSC70 siRNA had better gene silencing efficiency than other group ($P < 0.01$)

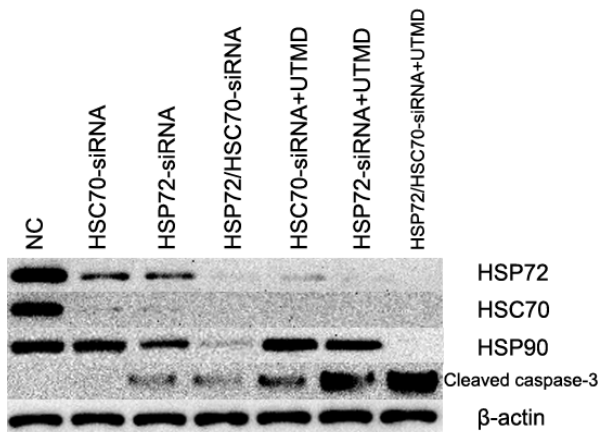


Figure 6. Western Blot Analysis of Expression of HSP72, HSC70, HSP90, and Cleaved Caspase-3 Protein in VCaP Cells at 48 Hours after Transfection. One of three independent experiments is shown

specific apoptosis.

Discussion

Silencing of gene expression by siRNA is rapidly becoming a powerful tool against cancer, efficient delivery of siRNA into tumor cells remains a key obstacle (Pai et al., 2006). HSP90 is an exciting therapeutic target in cancer because inhibition of this single protein causes the simultaneous degradation of multiple oncoproteins and combinatorial blockade of numerous oncogenic pathways. HSP70 molecular chaperones are of interest when considering modulation of HSP90 (Whitesell et al., 2005). HSP70 is the major therapeutic target in advanced PCa (Alaiya et al., 2001; Garrido et al., 2003; Lebret et

al., 2003; McConnell et al., 2013; Matthias et al., 2013). However, targeting HSP70 alone can result in off-target effects, drug resistance and disease recurrence. Therefore, simultaneous targeting of a multi-modal approach or multiple pathways in targeted delivery could in principle be an effective approach to treating PCa. With respect to the functional role of HSP70 isoforms in the HSP90 chaperone, The data we presented here demonstrate that silencing HSC70 or HSP72 individually has no effect on HSP90 protein level of VCaP prostate cancer cells, while dual targeting of HSC70 and HSP72 inhibit the cellular chaperone activity of HSP90. Additionally, UTMD combined with simultaneous targeting of HSC70 and HSP72 may further enhance silencing efficacy of the expression of HSP70, HSP90 protein and mRNA. In agreement with our findings, Powers et al. (2008) also found that dual targeting of HSC70 and HSP72 silence HSP90 protein in human HCT116 colon and A2780 ovarian cells.

In the present study, we have shown that silencing HSC70 or HSP72 individually has no effect on apoptosis by flow cytometry. Using combinatorial siRNA approach, we revealed that dual silencing of HSC70 and HSP72 considerably increased the apoptotic efficacy (Figure 4A, 6), UTMD may further induce extensive tumor-specific apoptosis. our apoptosis results were in accord with the study of Rérole et al. (2011). The data of apoptosis is nearly consistent with the cleaved caspase-3 of quantitative Real-Time PCR and western blot. Importantly, the safety and noninvasive of the combinational targeting strengthen our confidence further. And these data indicate that this is a promising strategy for delivering siRNA as cancer therapeutics.

The most important attribute of UTMD combination with siRNA is their specific, efficient delivery, which leads to concentration in the target tumor and avoidance of distribution to irrelevant normal tissues. Specific delivery into the intended target cell will likely reduce the dose required for antitumor activity as well as limit toxicity.

An exciting implication of our research is the attractiveness of combinatorial targeting of HSC70 and HSP72 as an alternative means to achieve HSP90 inhibition, with the added advantage of avoiding the antiapoptotic effects of HSP70 isoform induction that limit the use of current pharmacologic inhibitors. Interestingly, UTMD may promote the effect of silencing HSP90. The approach of UTMD combined siRNA was used here as a tool to silence the expression of HSC70, HSP72, and HSP90. It is possible that UTMD combined with RNA interference may be developed as a potential therapeutic approach.

Our findings also reveals further details about the relationship between UTMD and siRNA, especially in the context of HRPC. The reason of UTMD combined with dual targeting of HSC70 and HSP72 improve the efficacy of silence are as follows: Firstly, UTMD promote the efficient delivery of siRNA. Secondly, UTMD also may activate the active region of HSP70, HSP90 or increase crosstalk between distinct signaling pathways. Further work is required to identify the molecular and cellular mechanisms whereby UTMD interact with siRNA *in vitro*

and in vivo from the results presented here.

In summary, this study provide experimental support for the efficacy and therapeutic potential of combination UTMD with dual targeting HSP70-siRNA for the treatment of HRPC *in vitro*. Because of the specificity and efficacy of delivery, we were able to target a gene accurately, without any obvious toxicity. Our work supports the potential role for HSP70 as biomarker targeting in PCa (Powers et al., 2008; Goloudina et al., 2012). The multi-modal delivery strategy is versatile because the same delivery agent may be better aimed to target cells, an ideal, promising approach for delivering siRNA as cancer targeted therapeutics.

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