Androgen receptor-mediated upregulation of quaking affects androgen receptor-related prostate cancer development and anti-androgen receptor therapy

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Abstract. The androgen receptor (AR) has a crucial role in prostate cancer. RNA-binding protein-mediated post-transcriptional regulation is important in the initiation and development of cancer. The present study attempted to elucidate the mutual association of AR and RNA-binding protein quaking (QKI) in the development of prostate cancer. Dual-luciferase reporter demonstrated that AR can positively regulate the expression of QKI in prostate cancer cell lines due to its effective transcription regulating function. In addition, QKI may increase expression of AR by heat shock protein 90, which is a coactivator of AR, and silencing QKI can increase the sensitive of Casodex, which is an antagonist of AR in castration-resistant prostate cancer. This may be a new strategy for advanced prostate cancer.

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Abbreviations: AR, androgen receptor; QKI, quaking; PSA, prostate-specific antigen; CRPC, castration-resistant prostate cancer; PBS, phosphate buffered solution; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA; ADT, androgen deprivation therapy; DHT, dihydrotestosterone; HSP90, heat shock protein 90; CXD, Casodex

Key words: androgen receptor, prostate-specific antigen, quaking, prostate cancer, drug sensitive

Introduction

Prostate cancer is more prevalent in Western countries (1). However, in recent years increasing evidence suggests that it has started to become prevalent in the aging male Chinese population too (2). Androgen receptor (AR) is regarded as the main etiologic factor in prostate cancer initiation, and androgen deprivation therapy (ADT) serves as the most effective modality at the early stage of the disease. However, in time the majority of patients will eventually develop resistance, denoted as castration-resistant prostate cancer (CRPC), concurring with distant metastasis and lethal prognosis. Thus, understanding the in-depth mechanisms associated with AR signaling and its tumor promoting effects is useful for the control of this disease.

A recent study suggested that post-transcriptional regulations by various microRNA and mRNA-binding proteins appeared to be the important regulators in normal cell development and that their aberrant expression levels may participate in carcinogenesis in various ways, including affecting mRNA stability, alternative splicing of mRNA and translation efficiency (3).

Quaking (QKI) is a RNA-binding protein encoded by the quaking gene, which belongs to the signal transduction and activation of RNA family of proteins with important signal transduction and RNA activation functions (4). There are three isoforms, QKI-5, QKI-6 and QKI-7. The QKI-5 isoform possesses a noncanonical nuclear localization signal not present in the other QKI isoforms. This may assist in it being regulated by the AR gene, which is a nuclear receptor gene. Therefore, the QKI-5 isoform was the main subject of the present study. At the cellular level, it significantly modulates cell proliferation, apoptosis and differentiation. Notably, previous studies have identified its tumor suppressor roles (5) and the mechanical dysregulation of cell cycle with the involvement of apoptosis-associated signaling pathways (6,7). In colon epithelium, it positively promotes the expression of 26S proteasome non-ATPase regulatory subunit 9 (p27) by suppressing the transcription of β-catenin, which in turn suppresses cell proliferation and promotes

cell differentiation (8). During carcinogenesis in colon and gastric tissues, hypermethylation of QKI promoter regions are responsible for silenced QKI expression (8). In addition, aberrant alternative splicing, or even circular RNA changes, has been reported to be the unique mechanisms contributing to QKI-associated carcinogenesis (9,10).

In prostate cancer, our previous study demonstrated that QKI repression was also observed via hypermethylation in high staging prostate cancer samples and cell lines (11). There may be an association between QKI expression and positive androgen-associated signals in early-stage prostate cancer. In terms of AR-positive prostate cancer, the C4-2 cell line, denoted as CRPC cell line, developed an androgen agonist independent cell growth by having more constitutive active splicing isoforms of AR, including AR-V7 and ARv^{567es} (12). In addition, heat-shock protein (HSP)90 is known to be one of the most important HSPs responsible for various tumor invasion and metastasis via regulating cell growth and apoptosis processes (13). It is also a coactivator and molecular chaperone of AR, capable of stabilizing and enhancing AR functions (14). The combination of a heat shock protein (HSP)90 inhibitor with the anti-androgenic drug Casodex exhibits a synergistic anti-proliferative capacity on CRPC cells (15).

The present study explored the association between AR and QKI expression in prostate cancer, particularly the role of QKI in AR-induced carcinogenesis and anti-AR treatment effects.

Materials and methods

Tissue sample collection. The present study recruited 16 male unrelated individuals diagnosed with prostate cancer (age, 52-78 years; mean age, 68.25±5.39 years). Frozen tumor samples were collected from radical prostatectomy specimens from Xijing Hospital, the University of the Fourth Military Medical University (Xi'an, China) between May 2014 and December 2016. Tissue was frozen in liquid nitrogen within 30 min of resection. Non-neoplastic mucosa from colon was dissected free of muscle and histologically confirmed to be tumor-free by frozen sections. For cancer tissue, tumor purity of over 70% was confirmed by frozen sections for each case before submission for DNA and protein extraction. Information on patient age and sex in addition to Duke's tumor stage was collected. This study was approved by the Ethics Committee of the Fourth Military Medical University and the Institutional Review Board of Xijing Hospital. Informed written consent was obtained from patients prior to the study.

Cell lines and reagents. The LNCaP human prostate cancer cell line was sourced from the Chinese Academy of Sciences Shanghai Cell Repository (Shanghai, China), and the C4-2 human prostate cancer cell line was sourced from Dr Donghui Han (Department of Urologic Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an, China), who obtained from the China Center for Type Culture Collection (Wuhan, China). Fetal bovine serum (FBS) and charcoal stripped serum were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Gene-specific primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 2 mmol/l glutamine, 0.06 g/l penicillin, 0.1 g/l streptomycin and 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere of 5% CO_2.

Small interfering (si)RNA synthesis and transfection. All AR- and QKI-specific siRNA sequences were synthesized according to the literature by Shanghai GenePharma Co., Ltd. (Shanghai, China). AR siRNA interference sequences (16): AR RNAi-1 forward, 5'-AAGAAGGCCAGUUGUAUGGAC-3' and reverse, 5'-GUCCAUACAACUGGCCUUCUU-3'; AR RNAi-2 forward, 5'-AAGACGCUUCUACCAGCUCAC-3' and reverse, 5'-GUGAGCUGGUAGAAGCGUCUU-3'. QKI siRNA interference sequences (8): QKI RNAi-1 forward, 5'-GGCACCUAC AGAGAUGCCAACAUUA-3' and reverse, 5'-UAAUGUUGG CAUCUCUGUAGGUGCC-3'; QKI RNAi-2 forward, 5'-CCU UGAGUAUCCUAUUGAACCUAGU-3' and reverse, 5'-ACU AGGUUCAAUAGGAUACUCAAGG-3'.

The quality was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), as detailed in a subsequent section. The transfection procedure was carried out in accordance with the protocol of the Liposome 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for adherent cells. At 4-6 h post-transfection, the cells were resuspended in fresh medium containing 10% FBS. For the Casodex sensitivity test, $5x10^3$ cells were seeded per well into a 96-well plate (3599; Corning Incorporated, Corning, NY, USA), in 100 μl fresh medium containing 10% FBS per well, and after 24 h of culture, a final concentration of 10^{-5} mol/l Casodex (98%; Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) was added to the appropriate treatment groups as a 10^{-4} mol/l stock solution in phosphate buffered solution (PBS). After 48 h, a final concentration of 10% Cell Counting kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc., Shanghai, China) was added to determine proliferative capacity.

Androgen deprivation treatment. RPMI-1640 medium with the addition of 20% charcoal stripped serum was used to cultivate the LNCaP and C4-2 cells. The cells were first cultured for 24 h, following which the medium was removed to prevent any remaining androgens from influencing the experiment. Subsequently, the cells were transferred into 6-well plates (3596; Corning Incorporated) with 2 ml medium per well, and assigned to experimental and control groups. After 36 h, protein and RNA samples were obtained, and the remaining cells were transferred to fresh 1640 medium with 20% charcoal stripped serum and a physiological concentration of 10^{-9} mol/l dihydrotestosterone (DHT) (98%; Sigma-Aldrich; Merck KGaA) for another 36 h of continued cultivation, following which the final protein and RNA samples were measured.

Western blotting and determination of protein contents. Cells were lysed on ice for 30 min using NP-40 cell lysis solution (Abiocenter, Shanghai, China) and the resultant lysate harvested by centrifugation at 13,400 x g and 4˚C for 5 min. The Bicinchoninic Acid assay (Thermo Fisher Scientific, Inc.) was used to determine protein concentrations with 10% bovine serum albumin (CWBio, Beijing, China) as a standard, following which the measured protein concentrations

were adjusted to 2.5 μg/μl with SDS (Guidechem, Shanghai, China) loading buffer. Aliquots comprising 50 μg total protein were separated using 12% SDS-PAGE, the proteins were then transferred onto a nitrocellulose membrane (0.2 μm; Invitrogen; Thermo Fisher Scientific, Inc.), which was blocked with 5% skim milk powder in tris-buffered saline (TBS) at room temperature for 1 h. Detection was conducted using rabbit primary antibodies directed against AR (cat. no. A9853; 1:500), prostate-specific antigen (PSA) (cat. no. SAB4501531, 1:1,000), QKI (cat. no. HPA019123; 1:300) purchased from Sigma-Aldrich (Merck), p27 (cat. no. D121177; 1:500), p21 (cat. no. D153391, 1:250) purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and mouse primary antibodies directed against caspase-12 (cat. no. ab10455; 1:500) purchased from Abcam (Shanghai, China). All membranes were incubated with the primary antibodies overnight at 4˚C. Following washing with TBST (0.1% Tween-20), the membranes were incubated with an IgG-IRDye® 800CW fluorescent secondary antibody solution (cat. no. ab216773; 1:15,000 in TBS; Abcam) for 1 h at 37˚C. The protein bands were visualized using an Odyssey Infrared Imaging Laser scanning imaging system (LI-COR Biosciences, Lincoln, NE, USA). Monoclonal mouse anti-β-actin antibodies (cat. no. D190606; 1:500; Sangon Biotech Co., Ltd.) were incubated at 4˚C overnight and used as an internal reference.

RT-qPCR determination of mRNA levels. A total of $1x10^6$ cells were used for RT-qPCR analysis. Total RNA was isolated using the TRIzol kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cDNA for AR, QKI and PSA was synthesized using SYBR PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The cDNA for all other mRNA were synthesized using GoScript™ Reverse Transcriptase system (Promega Corporation, Madison, WI, USA). The RT reaction was as follows: 37˚C for 15 min and 85˚C for 5 sec. qPCR analyses were performed using the SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.); the thermocycler used for AR, QKI and PSA mRNA analysis was the LightCycler 480 system (Roche, Basel, Switzerland) and the thermocycler used for the analysis of all other mRNAs was the Applied Biosystems® 7500 Fast Real-time PCR system (Thermo Fisher Scientific, Inc.). The thermal cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec, 60˚C for 35 sec and 72˚C for 45 sec, followed by a final elongation step at 72˚C for 10 min. The PCR primers were as follows: Human AR gene forward, 5'-GACTTCACCGCA CCTGATGT-3' and reverse, 5'-GCAGTCTCCAAACGCAT GTC-3'; human PSA gene forward, 5'-ATCTGTGGAGCT GGATTCTGG-3' and reverse, 5'-AAGACCCAGTGTGCC CTAAG-3'; human QKI gene (8) forward, 5'-GGGGAAATG GAAACGAAGG-3' and reverse, 5'-TTGAGCCTTTGCCTC GGAC-3'; human β-actin gene (8) forward, 5'-AGCGGGAAA TCGTGCGTGAC-3' and reverse, 5'-TGGAAGGTGGACAGC GAGGC-3'; human ARv567es splicing variant (17) forward, 5'-CCTTGCTCTCTAGCCTCAATGAA-3' and reverse, 5'-CTTGATTAGCAGGTCAAAAGTGAACT-3'; human AR-V7 splicing variant forward, 5'-CCATCTTGTCGTCTT CGGAAATGTTATGAAGC-3' and reverse, 5'-TTTGAATGA GGCAAGTCAGCCTTTCT-3'; human UBE2C gene forward, 5'-TGGTCTGCCCTGTATGATGT-3' and reverse, 5'-AAA AGCTGTGGGGTTTTTCC-3'; human HSP90 gene forward, 5'-GGGGGATCCCCAGCTATGAACTCCTTCTCC-3' and reverse, 5'-GGGGTCGACCTACATTTGCCGAAGAGCC CT-3'. Each sample was replicated in three wells of a 96-well plate (3599; Corning Incorporated). Relative mRNA expression was calculated using $2^{-\Delta\Delta Cq}$ as previously described (18).

Flow cytometry for the determination of cell cycle status and apoptosis. LNCaP and C4-2 cells ($1x10^6$) were trypsinized, washed twice with PBS and fixed in 70% ice-cold ethanol (Sangon Biotech Co., Shanghai, China) for 1 h at 4˚C. The samples were centrifuged at 300 x g for 5 min at 4˚C, the ethanol was removed and they were incubated with 100 mg/ml RNaseA (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. For cell-cycle distribution analysis, 400 ul PI solution (BestBio Co., Ltd., Shanghai, China) was added to the cells. The cells were vortexed and incubated in the dark for 30-60 min at 2-8˚C. For cell apoptosis analysis, cellular DNA was stained with the Annexin V-FITC/PI Apoptosis Detection kit (cat. no. BB-4101-2; BestBio Co., Ltd., Shanghai, China). Briefly, the cells were centrifuged at 300 x g for 5 min at room temperature and resuspended in Annexin V Binding Buffer. A total of 5 μl FITC Annexin V was added to the suspension, they were then gently vortexed and incubated for 15 min at 2-8˚C in the dark. The cells were then incubated with 10 μl of PI solution, gently vortexed and incubated for 5 min at 2-8˚C in the dark. Cell-cycle distributions and cell apoptosis were determined by flow cytometry using a BD FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA) and data was analyzed using the ModFit software version 4.1 (Verity Software House, Inc., Topsham, ME, USA).

MTT cell proliferation assay. Following siRNA transfection, cells were transferred into 96-well plates (3599; Corning Incorporated) in triplicate wells. After 48 h, 10 μl CCK-8 was added to each well, and the cells incubated at 37˚C in an atmosphere comprising 5% CO_2 for 1-4 h, following which the reaction product, which accumulated in proportion to cell viability, was dissolved in RPMI-1640 medium and measured using a 96-well plates reader (Sunrise; Tecan Group Ltd., Männedorf, Switzerland) at 450 nm.

Luciferase reporter assay. To detect the interaction between AR 3'UTR (forward, 5'-CAGTACAACTTGGC-3' and reverse, 5'-GCCAAGTTGTACTG-3'; Shanghai GenePharma Co., Ltd.) and QKI, LNCaP cells were seeded in 24-well plates and transfected with 400 ng pGL3-AR in combination with 400 ng pcDNA3.1-QKI and the internal control vector 40 ng pRL-TK, respectively, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. siRNAs targeting AR were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and were dissolved in diethypyrocarbonate-treated H_2O at a concentration of 20 μmol/l as a stock. Cells were co-transfected with 50 ng pBIND (Promega Corporation, Madison, WI, USA), a plasmid constitutively expressing *Renilla* luciferase, to normalize for the transfection efficiency. After 48 h transfection, cells were lysed using passive lysis buffer and analyzed for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reagent

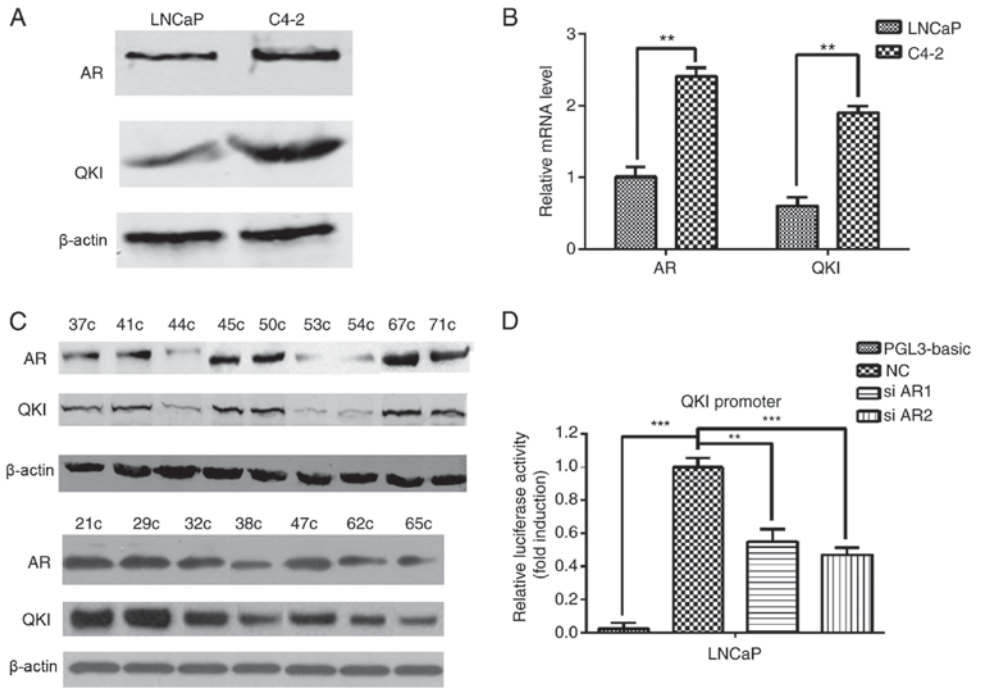

Figure 1. Expression of AR and QKI in prostate cancer cell lines, clinical samples and AR binding elements in QKI promoter region. (A) Protein levels of QKI in the two prostate cancer cell lines LNCaP and C4-2 were detected by western blotting. β-actin served as an internal control to ensure equal loading. (B) mRNA expression levels of QKI in the above cell lines were quantified by reverse transcription-quantitative polymerase chain reaction, and β-actin served as an internal control to ensure equal loading. (C) The protein expression of AR and QKI in prostate cancer samples. (D) AR binding elements in the QKI promoter region were detected by a dual-luciferase reporter system. Data are expressed as the mean ± standard deviation (n=3). **P<0.01 and ***P<0.001. AR, androgen receptor; QKI, quaking; si, small interfering.

Assay kit (Promega Corporation) according to the manufacturer's protocols.

Statistical analysis. Data are expressed as the mean ± standard deviation. All experiments were performed at least in triplicate. SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA) was used to perform a direct-probability t-test, one-way analysis of variance with a Dunnett post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Associations of QKI expression with AR level in prostate cancer clinical samples and different prostate cancer cell lines. The present study used 16 clinical prostate cancer samples and 2 prostate cancer cell lines; one an androgen-dependent prostate cancer cell line (LNCaP) and the other a CRPC cell line (C4-2). In order to determine the expression levels of AR and QKI in the clinical samples and cell lines, protein and mRNA expression levels were detected by using western blot analysis and RT-qPCR assays, respectively. C4-2 cells had increased AR and QKI protein (Fig. 1A) and mRNA (Fig. 1B) expression levels compared with LNCaP cells, indicating their possible association in prostate cancer development. Meanwhile, in clinical samples, positive AR expression often co-existed with higher QKI expression levels too (Fig. 1C). Dual-luciferase reporter demonstrated that there are AR binding elements in the QKI promoter region, and AR can regulate the expression of QKI positively (Fig. 1D). These data suggested that there are positive associations between AR and QKI expression levels in prostate cancer development.

Dynamic changes of QKI with ADT. As ADT is an optional treatment for prostate cancer, following androgen deprivation and re-addition, positive altered expression levels of QKI and PSA were observed in parallel with androgen levels, since PSA is the known positive target gene of AR (Fig. 2A-C). These phenomena suggested that QKI may function as novel target gene in AR signaling pathways. Notably, even castration-resistant C4-2 cells still retained the partial sensitivity to exogenous AR alterations, although to a lesser extent, compared with LNCaP cells. In addition, the aberrant splice variants AR, including AR-V7 and ARv^{567es} mutations, contribute to the progression of prostate cancer ending with the castration-resistant state (19). Therefore the RNA levels of ARv^{567es} and AR-V7 were determined, in addition to the target gene UBE2c; the results demonstrated that RNA levels of all three genes were significantly increased following androgen removal, in contrast with reductions following androgen replenishment (Fig. 2D). These results demonstrated there are negative associations between androgen levels and the aberrant AR splicing forms in C4-2 cells.

Influential effects of AR silencing on the expression of QKI and prostate cancer proliferation and apoptosis. The above data demonstrated the positive associations between AR and QKI. In order to validate this, an siRNA sequence was used to specifically silence AR expression and then observe its regulatory role on QKI (16). As a control, the expression of the AR known target gene, PSA, was inhibited following AR gene silencing. The expression levels of QKI were demonstrated to be markedly inhibited in both cell lines (Fig. 3). On the whole, the positive association observed between QKI and AR

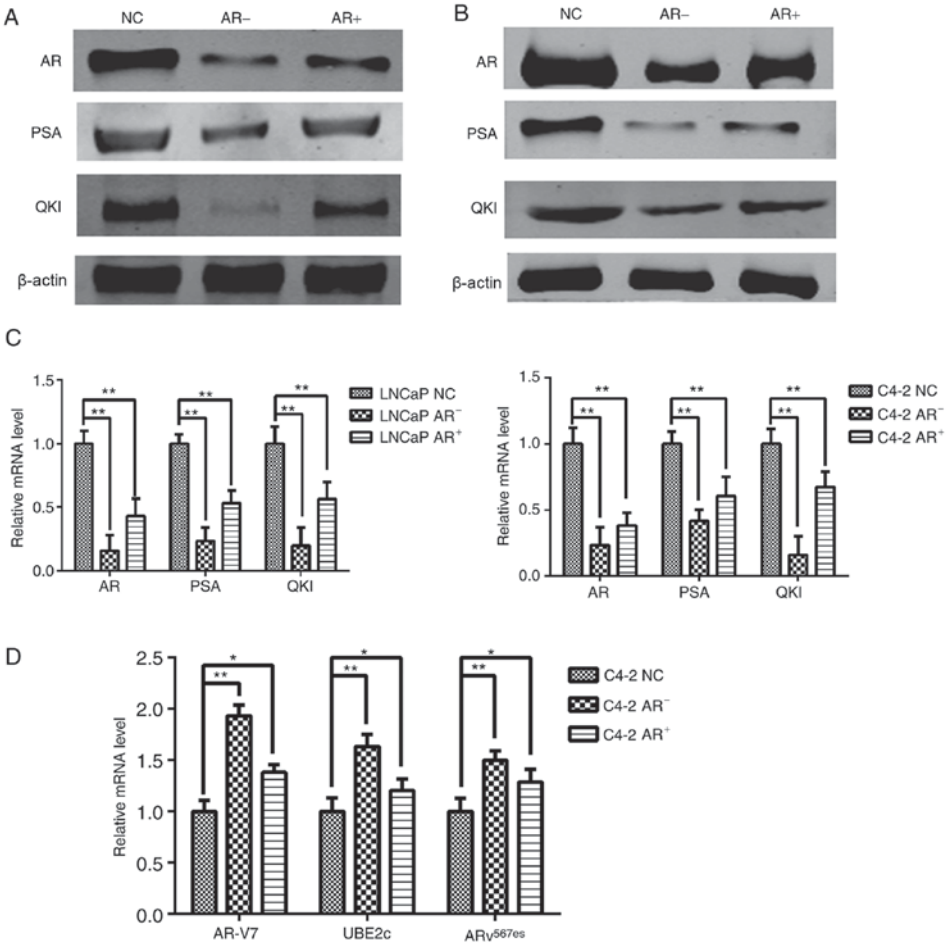

Figure 2. Dynamic changes of QKI with androgen deprivation therapy. Representative western blot images of protein expression levels of AR, PSA and QKI in (A) LNCaP and (B) C4-2 cells. (C) The mRNA expression levels of AR, PSA and QKI were determined using RT-qPCR in LNCaP and C4-2 cells. (D) The mRNA expression levels of ARv567es, AR-V7 and their target gene UBE2c in C4-2 cells upon androgen deprivation treatment determined using RT-qPCR in C4-2 cells. Data are expressed as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01. AR^{-}, cells cultured with charcoal stripped serum; AR^{+}, cells cultured with charcoal stripped serum and dihydrotestosterone; QKI, quaking; AR, androgen receptor; PSA, prostate-specific antigen; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

expression level, combined with the concomitant changes of QKI in androgen deprivation and AR silencing conditions, suggesting that androgen and the relevant AR signals aid the enhancement of QKI expression at mRNA and protein levels.

Prior to considering the role of QKI in AR related tumorigenesis, the present study first investigated AR effects in prostate cancer cells. Flow cytometry demonstrated that in LNCaP cells, AR silencing arrested cell cycle at G1/S phase, while in C4-2 cells, knocking down AR effectively retained the cell cycle at G2/M phase. The cell-cycle G2/M phase gene UBE2C is over-expressed in various solid tumors including CRPC, whereas UBE2C is a target gene of AR spliced variants, which is a significant difference between LNCaP and C4-2. In addition, the two cell lines demonstrated increased rates of apoptosis (Fig. 4). These data further supported the anti-apoptosis and proliferation-promoting effects of AR.

Effects of QKI gene silencing on prostate cancer cells and AR antagonist drug Casodex. In agreement with the above data, QKI was confirmed as a novel target gene of AR, although its functions in AR-positive prostate cancer remain to be elucidated. In the present study, two differing sets of RNA interference sequences against QKI were employed, effectively knocking down the QKI gene (8), and expression levels of PSA and AR were mildly reduced (Fig. 5). In addition, identical cell cycle patterns were detected in LNCaP and C4-2 cells following QKI reductions (Fig. 6), as was the case in AR silencing. In particular, LNCaP cells mainly exhibited G1/S phase arrest, while C4-2 cells exhibited a G2/M retention. Corresponding cell-cycle regulators, p27 and p21 demonstrated upregulations additionally (Fig. 7A and B). Furthermore, knock down QKI enhanced the susceptibility of prostate cancer cells to death in fluorescence-activated cell sorting assays, accompanied with higher level of caspase-12, an apoptosis indicator (Fig. 7A and B). In conclusion, these data indicated that QKI exerted a tumor promoting effect in AR-associated prostate cancers.

Finally, a Casodex-sensitivity assay was conducted; prostate cancer cells were treated with QKI siRNA 24 h in advance, and the AR antagonist drug Casodex was added and cultivated for another 48 h. MTT results demonstrated that C4-2 cells were more resistant to Casodex treatment compared with LNCaP cells, whereas QKI reduction synergistically sensitized androgen resistant cells C4-2 to Casodex treatment (Fig. 7C). This may be associated with HSP90 repression; QKI silencing dramatically reduced HSP90 expression.

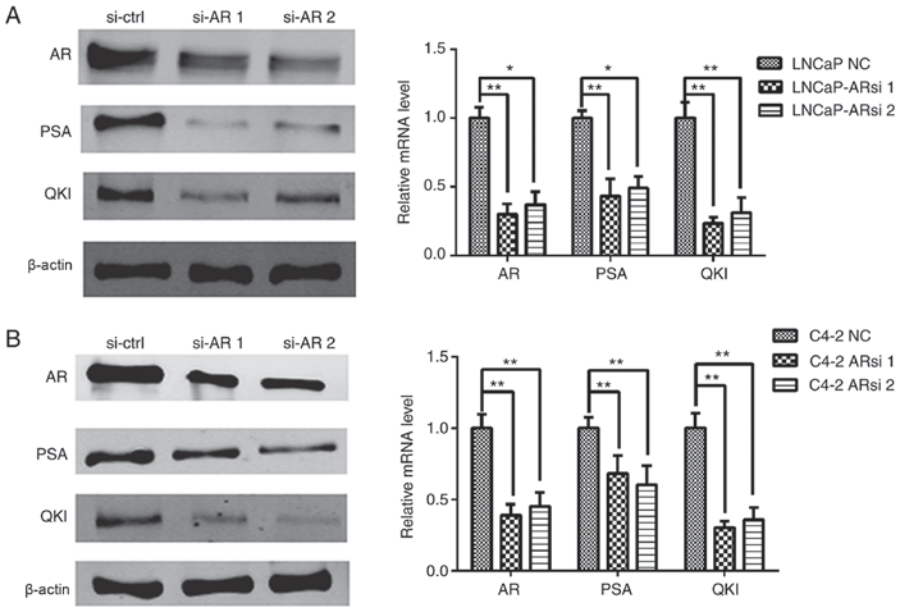

Figure 3. Influential effects of AR silencing on the expression of QKI. The protein and mRNA expression levels of AR, PSA and QKI were detected by western blotting and reverse transcription-quantitative polymerase chain reaction, respectively, in (A) LNCaP and (B) C4-2 cells. β-actin served as an internal control to ensure equal loading. The data are expressed as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01. AR, androgen receptor; QKI, quaking; PSA, prostate-specific antigen; si, small interfering.

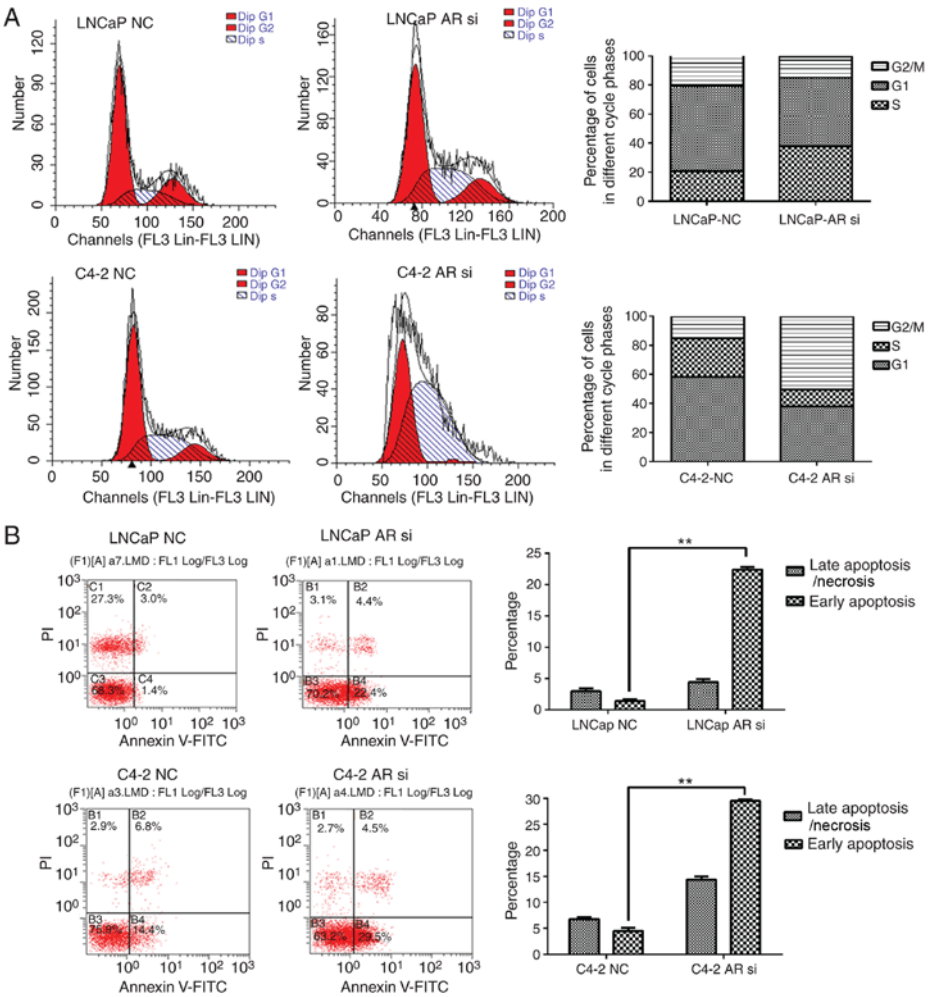

Figure 4. Influential effects of AR silencing on the function of prostate cancer cell proliferation and apoptosis. (A) The effects of AR silencing on the cell cycle. FCM demonstrated that the LNCaP cells were predominantly arrested in the G1/S phase while the C4-2 cells predominantly arrested in the G2/M phase. (B) The effect of AR silencing on apoptosis. FCM demonstrated that apoptosis increased upon AR silencing. The data are expressed as the mean ± standard deviation (n=3). **P<0.01. AR, androgen receptor; si, small interfering; FCM, flow cytometry; NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate.

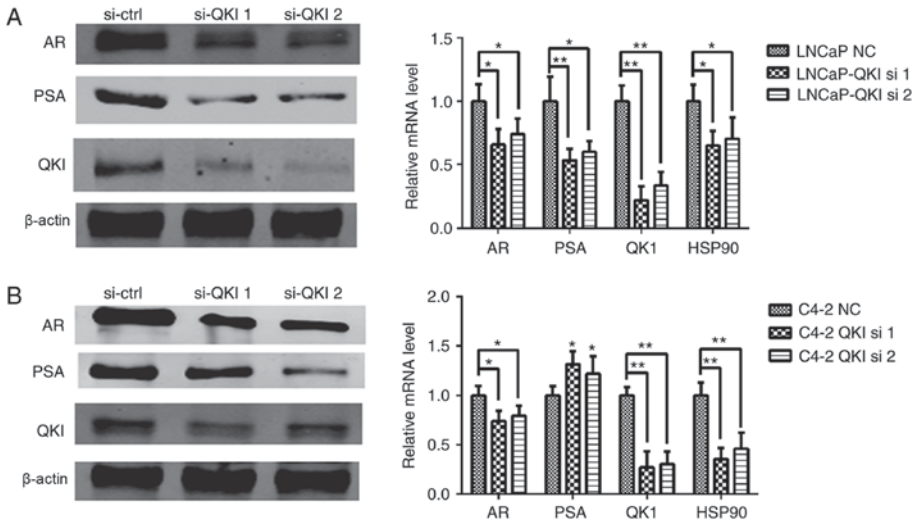

Figure 5. Influential effects of QKI silencing on the expression of AR in prostate cancer cells. The effect of QKI silencing in (A) LNCaP and (B) C4-2 cells, as detected by western blotting and reverse transcription-quantitative polymerase chain reaction. The data are expressed as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01. QKI, quaking; AR, androgen receptor; PSA, prostate-specific antigen; HSP90, heat shock protein 90; si, small interfering; NC, negative control; ctrl, control.

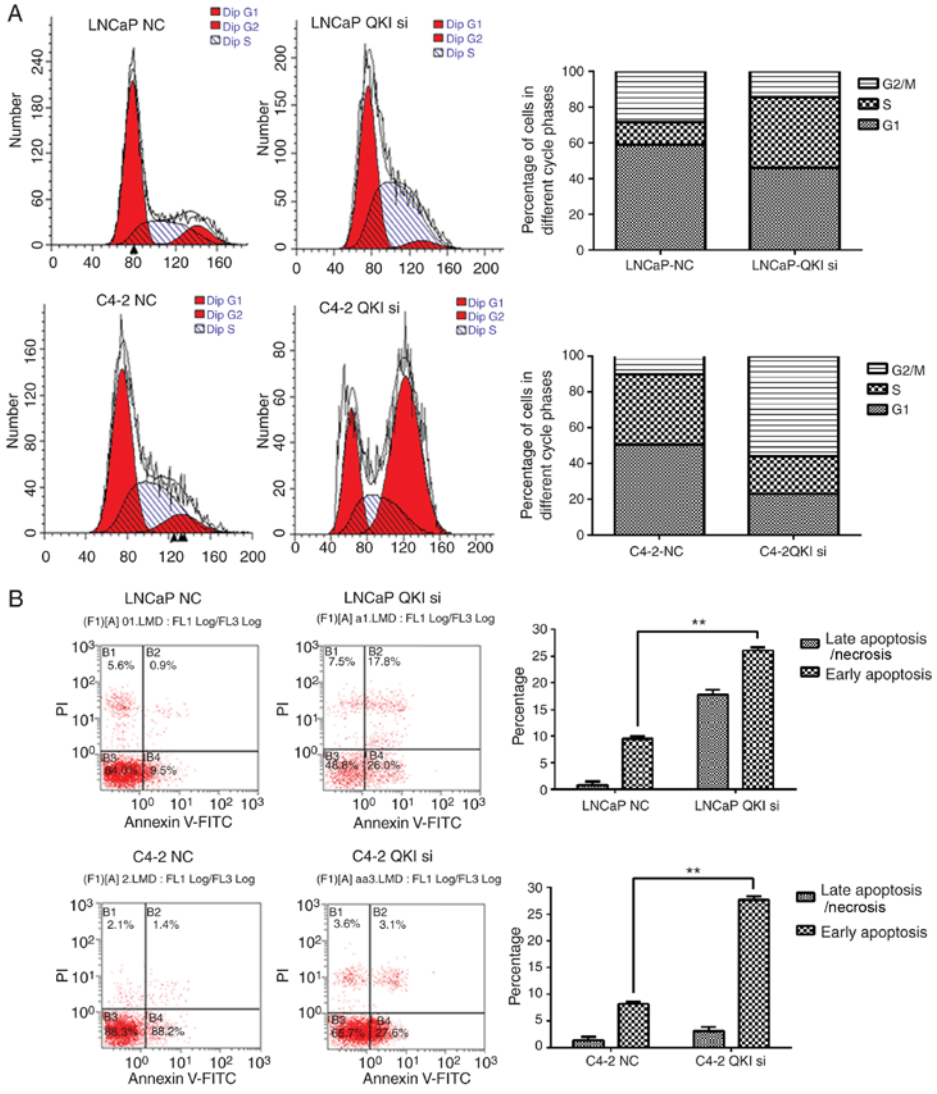

Figure 6. Influential effects of QKI silencing on the function of prostate cancer cell proliferation and apoptosis. (A) The effect of QKI silencing on the cell cycle. FCM demonstrated that the LNCaP cells were predominantly arrested in the G1/S phase while the C4-2 cells arrested predominantly in the G2/M phase. (B) The effects of QKI silencing on apoptosis. FCM analysis demonstrated that apoptosis was increased when QKI was silenced. The data are expressed as the mean ± standard deviation (n=3). **P<0.01. QKI, quaking; si, small interfering; NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate.

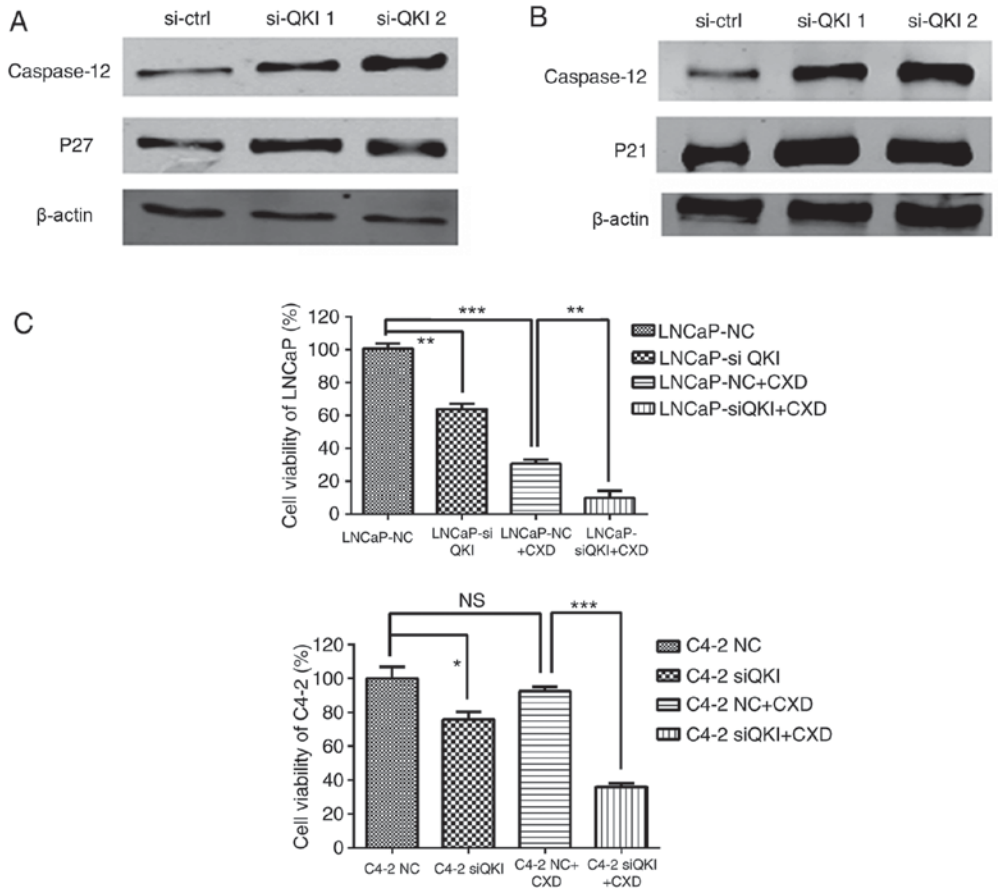

Figure 7. Effects of QKI gene on cell cycle- and apoptosis-associated proteins and the AR antagonist drug Casodex in prostate cancer cells. The effects of QKI silencing on the cell cycle- and apoptosis-associated proteins in (A) LNCaP and (B) C4-2 cells. (C) The effects of QKI silencing on bicalutamide sensitivity. The data are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001. QKI, quaking; si, small interfering; ctrl, control; CXD, Casodex.

Discussion

The incidence of prostate cancer is increasing among Chinese males, and studies on its underlying mechanism are becoming more important. QKI is an RNA-binding protein closely associated with cell cycle and differentiation regulation (20). Aberrant hypermethylation at promoter GC island regions of QKI is responsible for the reduced expression in the higher staging prostate cancers, particularly in those AR negative cell lines, such as PC-3 and DU-145. Previous functional characterizations in prostate cancer have suggested tumor suppressing effects for QKI.

The present study demonstrated a positive association between QKI and AR in AR positive cell lines and clinical samples. Either altered AR or androgen levels were able to affect QKI expression levels positively in androgen sensitive LNCaP and resistant C4-2 cells. Dual-luciferase reporter gene results demonstrated that QKI promoter activity could be regulated by AR, and future studies may elucidate this interaction further. This is the first study, to the best of our knowledge, to report that QKI is a positive target gene associated with AR signals in prostate cancer.

The data from the current study suggested that QKI mediated tumor-promoting effects in AR positive prostate cancer deriving from the cell cycle and cell apoptosis assays and relevant molecular markers. The present study indicated that QKI has a tumor suppressor role in inhibiting cell proliferation and enhancing apoptosis (8,20). The level of QKI expression has been demonstrated to be high in ER-β positive breast cancer cells (21), increasing with the degree of malignancy. However, QKI may not serve a tumor suppressor function in all cancer cells. The results of the present study demonstrated that QKI may facilitate ADPC to CRPC in prostate cancer progress. The reason for this phenomenon remains to be elucidated. More importantly, HSP90, known to be responsible for AR relevant signals and a number of cell cycle regulations, was significantly diminished following QKI silencing. AR protein stability is known to be also affected by the presence of HSP90. Therefore, it is possible that QKI exerted tumor promoting effects by improving the anti-stress efficiency in response to AR signals. Further experiments are required to clarify this.

Casodex resistance is a severe problem in prostate cancer treatment (22), and the findings of the present study provided a novel option for overcoming that resistance. QKI reduction markedly enhanced the efficiency of Casodex in the resistant C4-2 cell line, as did a combination of Casodex and HSP90 inhibitors (23); therefore, HSP90 repression following QKI silencing through a mechanism yet to be elucidated, may contribute to the increased sensitivity of the C4-2 cells to Casodex.

The present study reported that QKI, defined as a novel target gene downstream of AR signaling, functions as a positive regulator in AR signaling to induce cell growth and anti-apoptosis effects in the early stage of prostate cancer. Knocking down QKI may enhance the anti-AR efficiency.

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