Expression of Androgen Receptor Variant 7 (AR-V7) in Circulated Tumor Cells and Correlation with Drug Resistance of Prostate Cancer Cells

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Background: Prostate cancer is a common type of malignant tumor invading the male reproductive-urinary system, which has increasing incidence worldwide. Androgen receptor variant 7 (AR-V7) participates in regulating prostate cancer cell proliferation and gene expression. This study aimed to investigate the expression of AR-V7 in circulated tumor cells (CTCs) in patients with prostate cancer and to assess its correlation with drug sensitivity against enzalutamide or abiraterone.

Material/Methods: Blood samples of prostate cancer patients were collected for separating CTCs, in which mRNA expression level of full-length AR and AR-V7 was measured to analyze their correlation with enzalutamide or abiraterone resistance. Progression-free survival (PFS) of patients with different AR-V7 expression levels was compared. AR-V7 was overexpressed in transfected prostate cancer cells, and its effects on proliferation were analyzed by clonal formation assay.

Results: qRT-PCR showed AR-V7 overexpression in a total of 13 patients; 76.92% of these patients developed drug resistance, the distal metastasis of which was significantly higher than that in the group with AR-V7 downregulation, with lower PFS (p<0.01). In cultured prostate cancer cells, AR-V7 upregulation resulted in a significantly higher clonal formation rate than in the control group with enzalutamide-containing medium (p<0.05).

Conclusions: In prostate cancer cells, AR-V7 expression is correlated with drug resistance, as AR-V7 upregulation leads to enhanced proliferation potency of cancer cells, indicating unfavorable prognosis of patients.

MeSH Keywords: Drug Resistance • Prostate • Prostatic Diseases

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Background

Prostate cancer is a male-specific malignant tumor derived from prostate epithelial tissues. Prostate cancer has become the second most prevalent cancer in males [1], and has the highest incidence in American males. In China, prostate cancer incidence is still lower than in Western countries, but shows a rapidly increasing trend [2,3]. The pathogenesis of prostate cancer remains unclear, although it is commonly accepted that genetic factors, age, diet, and sexual activity are involved [4]. Currently, surgery is the favored treatment, as rapid shrinkage of tumors was found in about 70~80% of patients with prominent apoptosis. Further studies attributed those phenomena to the lower expression of androgen level and, consequently, hormone-dependent cancer cell apoptosis [5].

Castration surgery may be temporally effective, but only 10~20% of these surgical patients survived more than 5 years. The hormone-independent cancer cells lead to the development of castration-resistant prostate cancer [6]. Two novel drugs, enzalutamide or abiraterone, can suppress downstream signal pathway transduction targeting on androgen receptor (AR) via competitive binding [7,8] and enzalutamide can suppress translocation of AR into the nucleus [9]. However, there are still 20~40% of patients who are insensitive to drugs in clinical practice, developing drug resistance [10]. Some scholars proposed that the variable splicing isoform of AR, AR variant 7 (AR-V7), might have important roles in drug resistance to enzalutamide or abiraterone [11].

As an alternative splicing isoform of AR, AR-V7 lacks the C-terminal receptor-ligand binding structural domain of AR, but the N-terminal trans-activating domain remains intact and regulates nuclear gene expression [12]. A previous study showed that in patients with castration-resistant prostate cancer or those with resistance against enzalutamide or abiraterone, AR-V7 expression is higher in cancer cells than in non-drug-resistant patients [13], suggesting an essential role of AR-V7 in drug resistance. The present study thus investigated the correlation between AR-V7 expression in circulated tumor cells (CTCs) and drug resistance of prostate cancer.

Material and Methods

Recruitment of study objects

A total of 36 prostate cancer patients (average age=56.2±8.6 years) who were admitted to the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University from January 2013 to December 2015 with confirmed diagnosis were recruited in this study. None of the patients had received any related treatment before admission. Patients received bilateral

Table 1. PCR primer sequence.

Name	Sequence
AR-F	5'-CAGCCTATTGCGAGAGAGCTG-3'
AR-R	5'-GAAAGGATCTTGGGCACTTGC-3'
AR-V7-F	5'-CCATCTTGTCGTCTTCGGAAATGT-3'
AR-V7-R	5'-TGAATGAGGCAAGTCAGCCTTTCT-3'
Actin-F	5'-TGTCCCGATGGCGAGTGTTT-3'
Actin-R	5'-CCTGTTGGCCATAGTACTGC-3'

orchiectomy or tunica albuginea resection, and all transformed into castration-resistant prostate cancer. Among these patients, 20 received abiraterone (1000 mg oral daily, J&J, USA) and 16 received enzalutamide (160 mg oral daily, Astella, Japan). Cancer tissues were collected by biopsy surgery. Another matched cohort of 30 prostate hyperplasia patients was recruited as the control group (average age=52.3±9.5 years). Tissues collected from surgery were divided into 2 parts, of which one part was stored in liquid nitrogen for mRNA assay while the other part was embedded in paraffin sections for histopathology assay. This study was pre-approved and was supervised by the Ethics Committee of the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University. All participants signed informed consent.

Separation of CTCs

We collected 10-mL peripheral blood samples from all patients in the morning using EDTA-containing tubes (BD, USA), then mixed 7.5-mL blood samples with 6.5 mL of buffer and centrifuged the mixture at 8000 g for 10 min. The supernatant plasma was kept and mixed with buffer and immune-magnetic beads. After incubation at room temperature for 20 min, immune assay was performed. Excess reaction buffer and unresponsive magnetic beads were removed, and cells were washed from beads by elution buffer. Partial cell suspension liquids were mixed with APC-labelled CD45 antibody, PE-labelled CK8/18/19 antibody, and DAPI dye for staining. Excess dyes and antibody were removed. Stained cells were added into the MagNest sample chamber, which was incubated in the dark for 30 min. Within the MagNest sample chamber, cells dispersed into the analytical layer. CTCs were identified as CD45-CK+DAPI+. In morphology, CTCs showed clear cell contour, evenly distributed in plasma at more than 1 nucleus/cytoplasm ratio.

RT-PCR

qRT-PCR was used to quantify mRNA expression level of AR and AR-V7 in prostate cancer tissues. Firstly, qRT-PCR primers were designed based on sequences of AR and AR-V7 (GeneBank
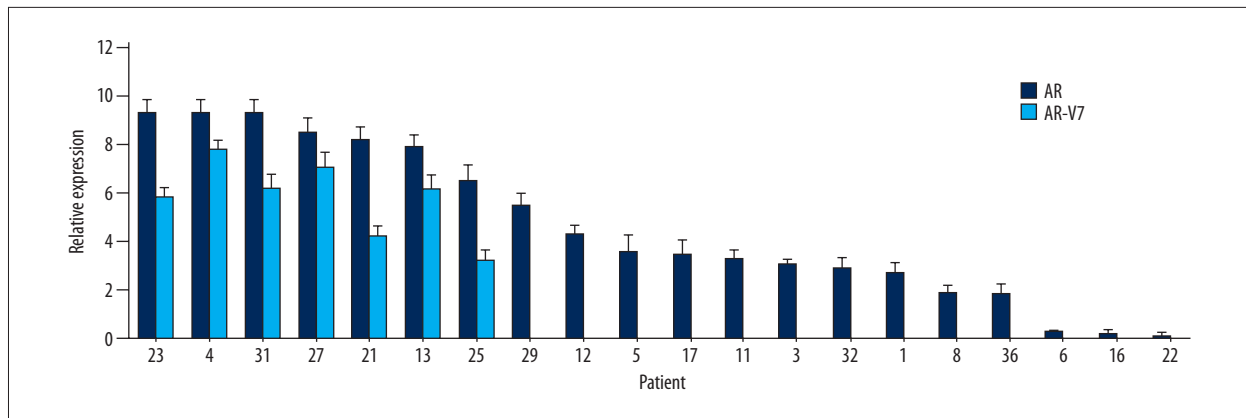
Figure 1. AR and AR-V7 expression levels in abiraterone treatment group.
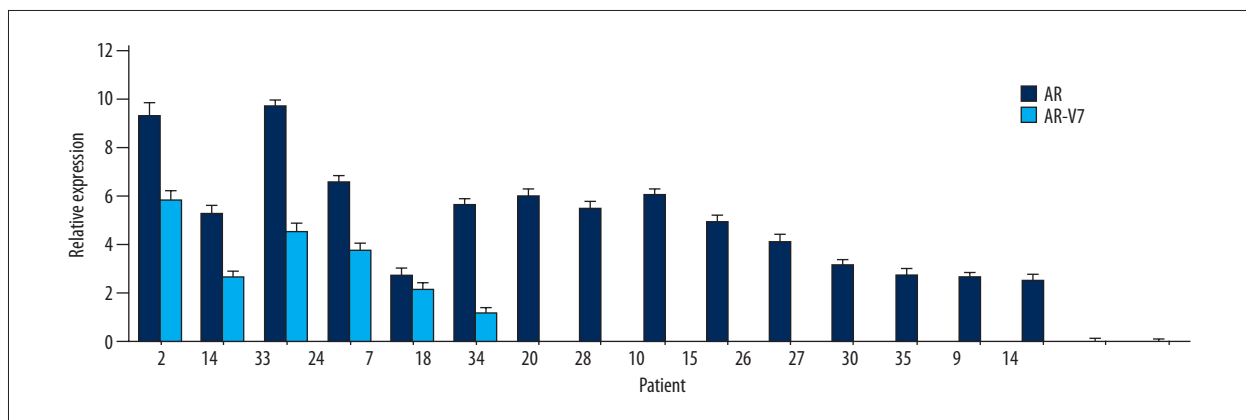

Figure 2. AR and AR-V7 expression levels in enzalutamide treatment group.

access No., AJ550426 and NR_30580, Table 1). Total RNA of CTCs was extracted using the RNAprep pure Tissue Kit (QIAGEN, USA). Total RNA extracted from breast gland hyperplasia tissues was set as the control, and qRT-PCR was performed using the Thermo OneStep RT-PCR Kit (Bioteke, USA) on a Real-Time PCR cycler (Bio-Rad). Reverse transcription was firstly performed at 42°C for 60 min, followed by PCR amplification under the following conditions: 95°C for 3 min, and 40 cycles each containing 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. Build-it software (V2.02) was used for data analysis by $2^{\Delta\Delta Ct}$ approach using β-actin as the internal control. Relative expression of those samples higher than 0.2-fold of AR-V7 was defined as AR-V7-positive and vice versa.

Cell culture and transfection

Human prostate cancer cell line LNCaP1 (CellBank, Shanghai Institute of Biological Sciences, Chinese Academy of Science) was incubated in RPMI 1640 medium containing 10% sterile fetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin, 50 U/mL streptomycin and 20 U/mL gentamycin, at 37°C with 5% CO_2.

Using pcDNA3.1 plasmid as the template, AR-V7 over-expression plasmid pcDNA3.1-AR-V7 was constructed based on the human AR-V7 mRNA sequence. LNCaP1 cells were transfected with over-expression plasmid by using the liposome transfection kit INTERFERin™ (Polyplus transfection, USA).

Drug resistance of cell line

LNCaP1 cells after transfection were cultured until log-growth phase and were inoculated into 100-mm cell culture dishes at the concentration of 10^4 cells per dish, using enzalutamide-containing medium. Cells were cultured until log-growth phase, digested by trypsin, and re-suspended into fresh culture medium. Cell suspensions were inoculated into cell culture dishes containing 10 mL fresh medium at the concentration of 100 cells per dish. The dish was gently swirled, and each group was performed in triplicate for 10 days of culturing. The supernatant was discarded and cells were rinsed in PBS, fixed in 2% paraformaldehyde for 15min, and stained in 5% crystal violet for 15 min. The staining buffer was discarded and the culture dish was inverted to cover a transparent grid. The number of clones was calculated for the clonal formation rate, which was equal to (clone number/100)×100%.

Table 2. Relationship between AR-V7 levels and pathological parameters.

	AR-V7 expression		P value
	High	Down	
Age (years)			
≥60	8	15	>0.05
<60	5	8	
Family history			
Yes	2	4	>0.05
No	11	19	
Drug resistance			
Yes	10	2	<0.01
No	3	21	
PSA level			
High	9	3	<0.01
Low	4	20	
Metastasis			
Yes	10	13	<0.05
No	3	10	

Statistical analysis

All results were processed by analysis of variance (ANOVA) and are presented as mean±standard deviation (SD). SPSS 20.0 was used for the *t* test to compare differences. Significant differences were defined as $p<0.05$ and $p<0.01$. All prognostic factors, including total survival span and progression-free survival (PFS), were analyzed by Kaplan-Meier approach.

Results

RT-PCR assay

qRT-PCR was used to detect expression of full-length AR and AR-V7 in CTCs from prostate cancer patients. AR-V7 up-regulation (35%) was observed in 7 (patients #4, #13, #21, #23, #25, #27, and #31) of the 20 castration-resistant prostate cancer patients receiving abiraterone treatment. Similarly, significantly higher expression level of full-length AR was found in those patients than in other patients. In patients receiving enzalutamide treatment, 37.5% (patients #2, #7, #14, #18, #24 and #33) had AR-V7 up-regulation. Among all patients, the overall up-regulation rate was 36.11% (Figures 1, 2).

Correlation between AR-V7 expression and clinical pathological parameters

Post-operative follow-up exams were performed on all prostate cancer patients (Table 2). During the treatment process of all 36 patients, 14 were found to have resistance to abiraterone or enzalutamide. In these 14 patients, AR-V7 up-regulation was detected among 10, but only 3 were non-resistant. Moreover, in patients with up-regulation of AR-V7, the PSA expression level was also significantly higher than those in patients with low levels of AR-V7 ($p<0.01$). We analyzed the distal metastasis of patients, finding that 10 out of 13 patients with high levels of AR-V7 had distal metastasis (76.92%), while this ratio was only 56.52% (13 out of 23) in patients with low expression of AR-V7.

Prognostic analysis

The Kaplan-Meier product limit method was used to predict PFS of patients with prostate cancer (Figure 3). Significantly shorter PFS was shown in patients with AR-V7 up-regulation,
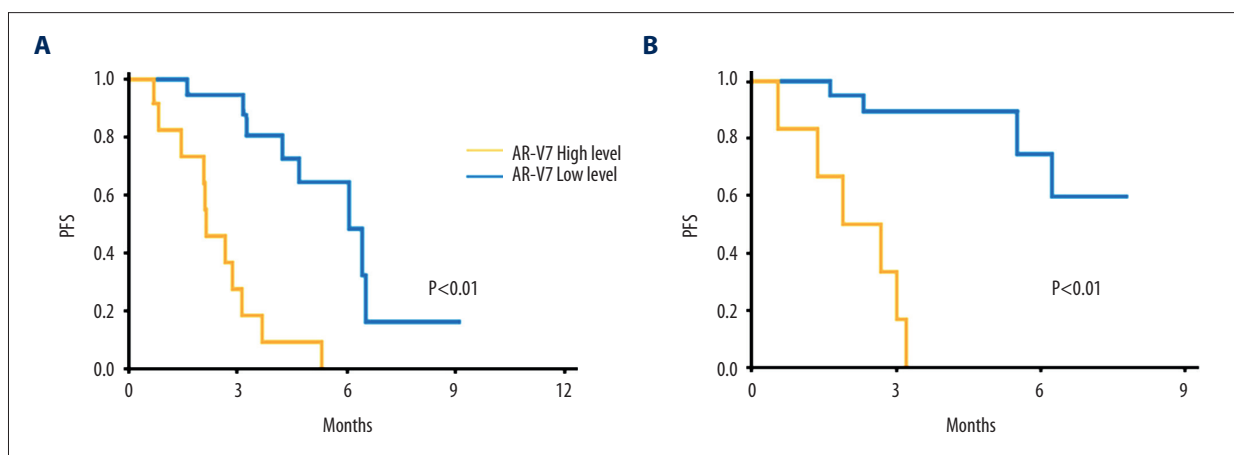
Figure 3. Effects of AR-V7 expression on prognosis of prostate cancer patients. (**A**) Survival rate of patients receiving abiraterone treatment; (**B**) Survival rate of patients receiving enzalutamide treatment.
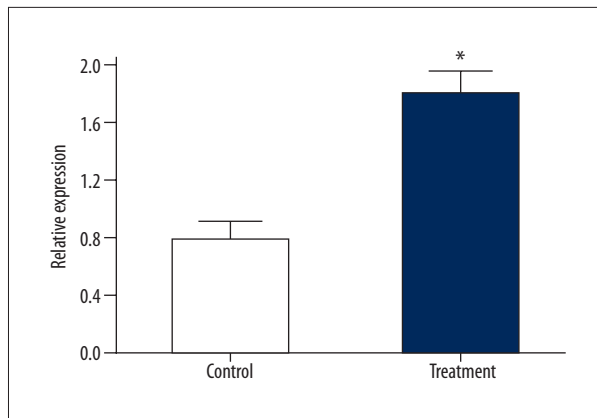
Figure 4. AR-V7 expression in LNVap cells after transfection. * p<0.05 compared to control group.

who either received abiraterone or enzalutamide treatment, compared to those with AR-V7 down-regulation, suggesting that the level of AR-V7 affected the PFS.

Over-expression of AR-V7 in prostate cancer cell line

QRT-PCR was used to test AR-V7 expression in LNCAP cells after transfection (Figure 4). Compared to that in untransfected cells, AR-V7 expression was significantly elevated in experimental cells (p<0.05), suggesting over-expression of AR-V7 *in vitro*.

Effects of AR-V7 expression on cell drug resistance

Cell clonal formation assay was used to assess abiraterone resistance of cells. As shown in Figure 5, over-expression of AR-V7 in the LNCAP cell line resulted in a significantly higher survival rate than that in the control group (p<0.05).

Discussion

Previous studies showed the correlation between treatment resistance of prostate cancer and AR expression [14,15]. The expression of androgen-independent constitutively and transcriptionally active androgen receptor splice variant-7 (AR-V7) remained high in the docetaxel-resistant castration-resistant prostate cancer (CRPC) cell subline Rv1-DR, and that it may be involved in acquired docetaxel-resistance of CRPC, suggesting it may be an important biomarker in the treatment of CRPC [14]. Androgen receptor variants (AR-V) expression in patient tissues or circulating tumor cells is associated with resistance to AR-targeting endocrine therapies and poor outcomes, which highlights the clinical value of inhibiting expression and chromatin binding of AR-Vs in prostate cancer [15]. The present study aimed to analyze AR-V7 expression in CTCs from different sub-groups of prostate cancer patients and to determine the correlation between AR-V7 expression and treatment resistance to abiraterone or enzalutamide in prostate cancer patients, based on pathological features of patients.

Abiraterone is an irreversible inhibitor for cytochrome CYP17, which exerts a blocking function on transition of cholesterol into dihydrotestosterone. A study has shown persistent activation of the AR signal pathway in patients resistant to abiraterone [16]. Cai et al. found that, although androgen level was decreased in patients after they received castration surgery, minor amounts of androgen can bind onto AR enhancer to potentiate its expression [17]. Moreover, transcriptional regulatory factors, including TIF-2, were found to be up-regulated in some prostate cancer patients. TIF-2 can activate AR expression in the absence of hormone stimulation to activate the AR signal pathway and potentiate proliferation potency of cancer cells, thus resulting in drug resistance of androgen inhibitor [18].
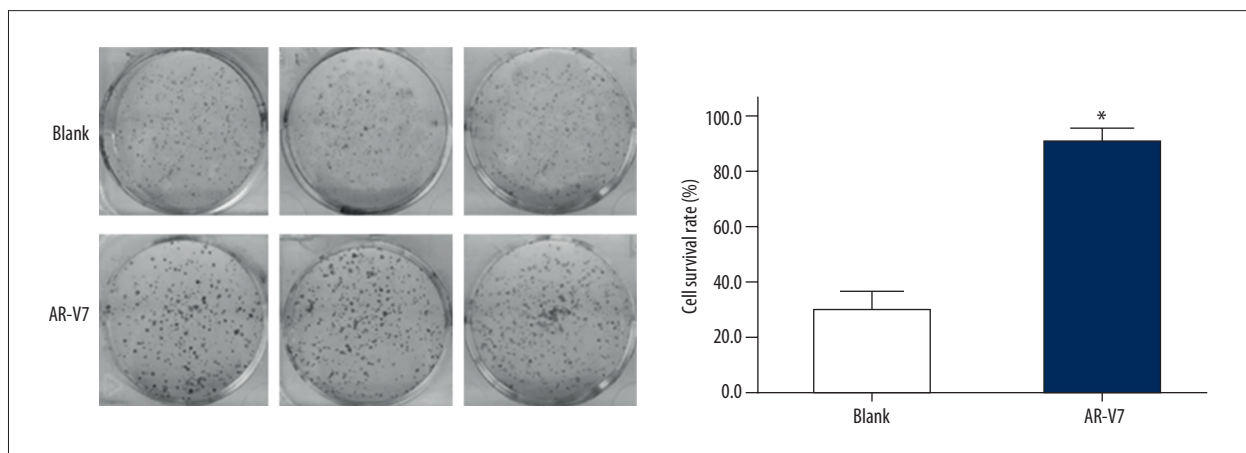
Figure 5. Cell survival rate after transfection. * p<0.05 compared to control group.

Enzalutamide serves as an AR-specific inhibitor by competitive binding, and can suppress AR nuclear translocation and interaction with DNA [19]. An *in vitro* study indicated that enzalutamide impeded prostate cancer cell proliferation, induced cell death, and decreased tumor volume in a mouse model of prostate xenograft [10]. By analyzing the interaction between enzalutamide and AR, we found that enzalutamide could bind onto the LBD structural domain of AR to disturb its binding onto androgen, thus inactivating the AR signal pathway [20]. In this study, AR-V7 up-regulation was found in 76.92% of patients resistant to enzalutamide, so we speculated there is a correlation between AR-V7 expression and occurrence of drug resistance. Compared to the full-length form of AR, AR-V7 lacks the C-terminal LBD structural domain but preserves the N-terminal domain with transcriptional activity intact. AR-V7 can be transported into the nucleus, where it exerts facilitating effects on cell proliferation and amplification [20]. Therefore, AR-V7 up-regulation may lead to the occurrence of drug resistance of patients. Our data showed that AR-V7 expression level was clearly increased in prostate cancer cells, as shown by *in vitro* transfection assay, and significantly higher proliferation potency of cells in medium containing enzalutamide was observed, consistent with previous reports [19,20].

Prostate cancer has a progressively increasing incidence, and severely affects men's health [1]. Abiraterone and enzalutamide

are common drugs used in treating prostate cancer resistant to castration, but the occurrence of drug resistance severely compromises treatment efficiency. This study found an elevated AR-V7 expression level in CTCs from drug-resistant prostate cancer patients. Cell transfection assay and drug-resistance assay confirmed the relationship between AR-V7 expression and drug resistance of prostate cancer, but assay of gene expression in CTCs can provide more evidence for predicting development of drug resistance in clinical practice [21], thus benefiting optimization of treatment and helping patients. A limitation of this study is its small sample size.

Conclusions

AR-V7 expression in cancer cells is correlated with drug resistance to abiraterone and enzalutamide, which provides insights into AR-V7 expression in CTCs as an indicator of patient drug resistance and prognosis. However, due to the limited number of patients enrolled in the present study, a large-cohort clinical study is required to confirm these findings.

Conflict of interest

None.

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