A NOVEL CAPILLARY NANO-IMMUNOASSAY FOR ASSESSING ANDROGEN RECEPTOR SPLICE VARIANT 7 IN PLASMA. CORRELATION WITH CD133 ANTIGEN EXPRESSION IN CIRCULATING TUMOUR CELLS. A PILOT STUDY IN PROSTATE CANCER PATIENTS.

Authors: Juan Luis García^{a*}, Rebeca Lozano Mejorada^b, Irena Misiewicz-Krzeminska^a, Patryk Krzeminski^a, Sara Alfonso Hernández^b, Rosa Ana Marcos^c, Rocío García Domínguez^b, Francisco Gómez-Veiga^d, Álvaro Virseda Rodríguez^d, Manuel Herrero Polo^d, David Olmos^{e,f}, Juan Jesús Cruz-Hernández^{b*}

Affiliations:

a Institute of Biomedical Research of Salamanca (IBSAL) - Centro de Investigación del Cáncer-IBMCC (USAL-CSIC), Paseo de San Vicente, 58-182, 37007 Salamanca, Spain.

b. Medical Oncology Service, Hospital Universitario de Salamanca Institute of Biomedical Research of Salamanca (IBSAL), Paseo de San Vicente, 58-182, 37007 Salamanca, Spain.

c Dpto. de Oncología Médica, Complejo Asistencial Nuestra Señora de Sonsoles. Av. Juan Carlos I, s/n, 05071 Ávila, Spain

d Urology Department, Hospital Universitario de Salamanca Grupo de Investigación Traslacional de Urología (GITUR), Institute of Biomedical Research of Salamanca (IBSAL), Paseo de San Vicente, 58-182, 37007 Salamanca, Spain.

e Prostate Cancer Clinical Research Unit, Spanish National Cancer Research Centre (CNIO), C/ Melchor Fernández Almagro, 3, 28029 Madrid, Spain.

f CNIO-IBIMA Genitourinary Cancer Clinical Research Unit, Medical Oncology Department, Hospital Universitario Virgen de la Victoria y Regional de Málaga, Campus de Teatinos S/N, 29010 Málaga, Spain.

*Corresponding authors:

Juan Jesús Cruz-Hernández

Medical Oncology Service, University Hospital of Salamanca-IBSAL, Paseo de San

Vicente 5-182, Salamanca, 37007, Spain, Tel: +34-923291100 Ext: 55749

E-mail address: jjcruz@usal.es

Juan Luis García

Institute of Biomedical Research of Salamanca (IBSAL) - Centro de Investigación del Cáncer-

IBMCC (USAL-CSIC), Paseo de San Vicente, 58-182, 37007 Salamanca,

Spain. Tel: +34-923294812

E-mail address: jlgarcia@usal.es

Short title: Novel capillary nano-immunoassay for assessing AR V-7

ABSTRACT

Purpose

Androgen receptor (AR) splice variant 7 (AR-V7) has been related with both a higher risk of prostate cancer (PC) progression and differential responsiveness to hormonal agents versus chemotherapy. The objective of this study was to investigate the feasibility of a novel capillary nano-immunoassay in assessing AR-V7 in plasma from PC patients.

Methods

Patients with either localized or advanced PC were included in the study. Assessment of AR-V7 in plasma was performed through a capillary nano-immunoassay platform. Correlation with clinical data, stem cell biomarkers (such as CD133+), AR amplification and PTEN status was identified.

Results

The study included 72 PC patients. AR-V7 signal was detected in 21 (29%) patients: 17 (81%) had a Gleason score ≥ 7 , 15 (71%) castration-resistant prostate cancer (CRPC) and 18 (86%) metastatic disease. CD133 was expressed in 69 (96%) patients. The median CD133+ expression in circulating tumour cells CTCs was higher among the 21 AR-V7 positive cases versus AR-V7-negative (7 vs. 3). Androgen Receptor and PTEN fluorescence in situ hybridization (FISH) on CD133+ captured cells were performed: 37 cases showed $\geq$ four CD133+ CTCs, of which 81% showed an increased AR copy number. This percentage was similar in both AR-V7 positive and AR-V7 negative patients. A total of 68% of the cases showed deletion of PTEN: 70% were ARV-7 positive vs. 67%, which were AR-V7negative.

Conclusions

Assessing the presence of AR-V7 in plasma from PC patients is feasible by a novel capillary nano-immunoassay. AR-V7 was observed in 29% of the tumours and is more frequent in aggressive tumours.

Key words:

Androgen receptor; AR-V7; circulating tumour cells; capillary nano-immunoassay; CD133; PTEN

INTRODUCTION

Prostate cancer is an androgen-dependent malignant tumour. However, after an initial response to androgen deprivation therapy (ADT), patients with advanced disease will develop castration-resistant prostate cancer (CRPC), a late-stage of the disease, which is associated with worse prognosis. Multiple adaptation processes such as androgen receptor (AR) gene amplification, AR gene mutation, alternative splice variants or alternative AR activation through bypass pathways [1] allow persistent activation of androgen receptor (AR) signalling pathways in CRPC. The past decade has seen the rapid development of several agents for CRPC, which has resulted in improved overall survival. These newer therapeutic agents have not been compared with each other. Therefore, the best sequence remains unclear and new biomarkers are urgently needed to guide treatment decisions. Because mutations in the AR gene are an escape mechanism that can lead to CRPC, these mutations have been examined in circulating tumour cells (CTCs). Indeed, patients whose CTCs tested positive for AR splice variant 7 (AR-V7), a truncated form of the AR, were found to be resistant to enzalutamide and abiraterone. This finding could make AR-V7 a marker for treatment selection [2]. On the other hand, CD133 -a glycoprotein associated with stem cell and migratory properties and vasculogenic mimicry circulating tumour cells-has been used as a stem cell biomarker identifying infrequent prostate epithelial progenitor cells that play a role in development and tumour biology. Recent studies have demonstrated that CD133+ CTCs are present in patients with advanced prostate cancer, thus providing greater proliferative potential compared to CD133 negative cells [3,4]. Taking serial biopsies to capture changes in tumour biology may be troublesome for patients. Furthermore, a single biopsy may not represent the heterogeneity of the disease. Taking a liquid biopsy at different time-points may be an opportunity to perform this serial assessment without patient discomfort. CTCs can be obtained from a non-invasive routine blood test.

Indeed, the CTC count number has emerged as an important prognostic tool in CRPC [5]. Even more promising than enumeration of CTCs is the ability to molecularly characterise these CTCs [6]. Validation of potential biomarkers in blood is difficult because blood contains proteins from many tissue sources in small concentrations and these proteins are under numerous mechanisms for blood clearance. For this reason, novel technology based on automated capillary nano-immunoassay (CNIA) has been developed [7]. The WES™ system (ProteinSimple) enables a rapid and quantitative analysis of specific proteins from small quantities of sample (dependent on cell size and percentage of protein). Uniquely, the system can examine numerous proteins from a small cell sample as it requires very small amounts (as low as 80 ng) of protein per capillary. The aim of this study was to evaluate and validate the presence of AR-V7 in plasma from patients with different stages of prostate cancer using the WES™ platform. Firstly, we used the WES™ platform on prostate cancer cell lines to standardise the assays for determining AR-V7. The association between AR-V7 with stem cell biomarkers (such as CD133+) and genomic changes in these CTCs (AR amplification and PTEN status) were also investigated.

MATERIAL AND METHODS

Design and variables

This was an exploratory study to evaluate and validate the presence of AR-V7 in the plasma of prostate cancer patients. The design of this study comprised evaluation of the presence of AR-V7 assessed by TM WESTM in different groups of samples (prostate cancer cell lines, plasma of healthy donors and plasma from patients with prostate cancer). Then, the correlation of the results with clinical characteristics of the patients, expression of CD133 in CTCs, AR amplification and PTEN status was analyzed. Patient clinical characteristics, such as age, serum PSA level, Gleason score and metastatic sites were collected.

Patients

Patients with either localized or advanced prostate cancer were eligible for the study. The samples of patients with localized prostate cancer were extracted before the radical treatment (prostatectomy or radiotherapy). Men with metastatic prostate cancer could have received standard-of-care therapy with ADT (LHRH agonists +/- peripheral antiandrogen therapy), abiraterone, enzalutamide, chemotherapy (docetaxel, cabazitaxel) or corticosteroids. Both castration-sensitive and castration-resistant patients were eligible. In those patients with advanced disease, samples were collected before or during the course of therapy. The study was carried out in accordance with the ethical standards of the Declaration of Helsinki and was approved by the Institutional Review Board of the University Hospital of Salamanca (Spain). Written informed consent was obtained from each patient before any study-specific procedure was conducted.

Samples

Whole blood (10 ml) was collected and processed in EDTA tubes. Within 1 hour of collection, plasma was removed after centrifugation at 1,600 rpm at 4°C (2×10 minutes) and stored in 1 ml aliquots at -80°C. The peripheral blood mononuclear cells (PBMC) were used for fluorescence-activated cell sorting (FACS).

Western Blot

The human prostate cell lines PC3 and VCaP (ATCC, Manassas, VA, USA) were used to standardize the assays for AR-V7 through the WES™ platform. Cell lines were grown in recommended media and maintained in a humidified 5% CO₂ incubator at 37°C. For Western Blot analysis the cells were lysed in RIPA buffer (Santa Cruz Biotech. USA) supplemented with protease and phosphates inhibitors (Roche). Then, 8% SDS-PAGE was performed followed by protein transfer on nitrocellulose membrane. Next, the membranes were blocked in 5% non fat milk in PBS with 1% TWEEN 20 and incubated over night at 4°C with appropriate antibodies (AR-V7 1:1000 Abcam USA; AR 1:1000

Santa Cruz Biotech. USA; GAPDH 1:50000, Novusbio USA). Next day, the membranes were incubated with appropriate secondary antibody conjugated with Alexa-780, washed 3 times in PBS with 1% TWEEN 20 and visualized in LI-COR Odyssey scanner. Then, blood samples from eleven healthy donors were analyzed.

Capillary Electrophoresis Immunoassay

Capillary Electrophoresis Immunoassay or SimpleWestern analyses were performed using the WES™ machine (ProteinSimple Santa Clara, CA) according to the manufacturer's protocol. With this technique, proteins are separated by size, immobilised and probed with specific antibodies. Signal intensity is detected by a horseradish peroxidase-conjugated chemiluminescence system and the data is shown as an electropherogram. In brief, 4.8 ul of 1:100 pre-diluted plasma samples in 0.1x Sample Buffer (ProteinSimple) were mixed with a master mix (Protein Simple) to a final concentration of 1x sample buffer, 1x fluorescent molecular weight markers and 40 mM dithiothreitol (DTT) and then heated at 95°C for 5 min. The samples, blocking reagent, wash buffer, primary antibody, secondary antibody and chemiluminescent substrate were dispensed into designated wells in the manufacturer-provided microplate. After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated. Simple Western analysis was carried out at room temperature. The primary antibody used was AR-V7 (Abcam ab198394). The data were analyzed by Compass Software. Quantitative analysis for AR-V7 protein peak was done automatically as area under the peak.

Fluorescence-activated cell sorting (Flow Cytometry)

For FACS (FACS Aria, BD Biosciences, San Jose, CA, USA), the PBMC were subjected to erythrocyte lysis using NH₄CL 0.38% ammonium chloride for 30 minutes on RT (dilution 2 ml: 20 ml). Then, the suspension was washed in Ca⁺⁺/Mg⁺⁺-free phosphate-buffered saline (PBS) for 5 minutes at 2000 rpm. FACS was used to isolate and evaluate the expression of CD133 CTCs using the following murine-anti-human antibodies: CD45-FITC (BD Pharmingen, San Diego, USA), CD34-PerCP (BD Pharmingen, San Diego, USA), CD16 PECy7 (BD Pharmingen, San Diego, USA) and

CD133 APC (AC133.Miltenyi Biotech). Cells suspended in Ca⁺⁺/Mg⁺⁺-free phosphate buffered saline (PBS) supplemented with 1% FBS were labelled with the antibody (approximately 1 µg/10⁶ cells) for 30 minutes on ice. Cell fluorescence was sorted and analyzed with the FACS Aria sorter (Becton Dickinson, New Jersey, USA) and FACSDiVa software (Becton Dickinson Biosciences (BDB)). The acquisition was performed in two consecutive steps. In the first step, 50,000 cells corresponding to the total cellularity were acquired; and in the second step, only the information on CD133 + cells was stored in order to increase assay sensitivity. Then, CD34-, CD133+ and CD45- events were selected and separated into an Eppendorf tube containing methanol: acetic (3/1) for further studies

In situ Hybridization:

In the CD133+ enriched fraction of the FACS sorted samples, FISH analysis was performed for AR-LSI (Androgen Receptor Gene (Xq12)) and PTEN (PTEN/CEP10) (Vysis ProVysion, Abbott Molecular Inc.). The cell suspensions were dropped on cold and wet slides and left to dry at room temperature overnight. FISH was carried out according to the manufacturer's protocol. Probes were placed on slides, sealed with rubber cement and incubated at 37°C in a moisture chamber overnight. After washing, the slides were mounted in Vectashield antifade solution

(Vector Laboratories, Burlingame, CA, USA). The slides were coded and stored at 4°C in the dark until analysis. The automated scanning system Metafer 4, Version 3.2.1, from MetaSystems (Attusheim, Germany) with a motorized Axio Imager Z1 microscope (Carl Zeiss, Germany) was used for scanning, classifying and detecting cells under fluorescent illumination. A minimum of FISH results on four CTCs was required to ensure that the result was technically reproducible.

RESULTS:

Assay setup

In order to show the antibody specificity, we used cell lines whose expression of the AR-V7 isoform was previously known: VCaP [8] as positive control and PC3 as negative control [9]. The specificity of AR-V7 antibody was tested by standard Western Blot. As shown in Figure 1 only one band corresponding to AR-V7 was detected in positive control. Subsequently, capillary Electrophoresis Immunoassay detected no AR-V7 signal for PC3 cells even using high antibody concentration, as shown in Figure 2 A. On the other side, a strong, specific peak was observed for VCaP cell lines. Using this cell line, we were able to titrate the antibody for saturation and determined the antibody dilution to be used at 1:50. The peak detected corresponded to the MW about 97kDa in size-CNIA (Figure 2B). We then performed the titration using serial dilution of two samples and the average peak area was quantified for each dilution. Linear regression analysis confirmed that the assay is linear from 250,000 to 2,000 AU of peak area of chemiluminescence (Figure 2C).

Serum samples analysis

Since human serum contains a high concentration of proteins, we pre-diluted the samples in 0.1x Sample Buffer provided in the kit. All analyses were done automatically in the WES™ system, detecting previously established peaks of AR-V7 protein in serum samples at migration

in capillaries that corresponded to a size around 97kDa. To allow the standardization between each capillary and between distinct runs, the system control (SC) antibody was used as well. The peak from the SC was used to automatically normalize the AR-V7 signal in each capillary. As previously established, the limit of detection was around 2000 AU, thus any signal below this value was considered as negative.

Blood samples from eleven healthy donors were analyzed to establish the presence or absence of this protein in blood. No cases of previously established peaks of AR-V7 protein that correspond to a size around 97kDa were detected.

From January 2014 to November 2015, seventy-two patients with localized or advanced prostate cancer who were treated at the University Hospital of Salamanca were enrolled in the study. AR-V7 signal was detected in 21 cases (29%). Fifty-one samples (71%) were considered as negative. The clinical characteristics of these patients, shown by AR-V7 expression, are described in Table 1. Of the 21 patients with expression positive for AR-V7, a Gleason score ≥ 7 was observed in 17 patients (81%), 15 had CRPC (71%) and 18 (86%) had metastatic disease.

AR-V7 and CD133

We found CD133 to be expressed in CTCs from 69 of 72 patients (96%). The median of the CD133+ cell number was 4 (range 1-1922) in a median of 20×10^6 sorting cells. In half of patients the number of CD133+ CTCs was higher than the median (37/72 [51%] ≥ 4), the number of CD133+ CTCs was ≥ 4 . Three cases did not show CD133+ expression in their CTCs (two cases of metastatic CRPC and one case of castration-sensitive prostate cancer). The median CD133+ CTCs of the 21 AR-V7+ cases was 7 (range 1-81) whereas in AR-V7-negative cases (51 cases), the median CD133+ was 3 (range 0-1922). The number of cases with more than 4 CD133+ CTCs was higher in the AR-V7+ tumours compared with AR-V7-negative tumours (66% versus 47%).

In situ Hybridization

In the FISH analysis, the number of signals observed on the total of the separate cells (hybridized to AR and PTEN) was analyzed. FISH for AR was successfully performed on samples from 60 out of 72 patients (83%). Increased AR copy number was detected in 40 out of 60 patients (67%). We have taken into account only cases presenting more than the median of CD133+ in the entire group (≥ 4 CD133+ cells). In the group of 37 cases with more than four CD133+ CTCs, 30 (81 %) showed gains for AR whereas the remaining seven had no abnormality. FISH analysis for PTEN status assessment was performed on samples from 59 patients, 19 of whom showed ≤ 3 CD133+ CTCs. PTEN gene status was available in 40 cases with more than four CD133+CTCs, 27 of whom (68%) showed loss of PTEN, 3 (8%) were polyploid for CEP10/PTEN and 10 (25%) showed no changes.

We performed FISH analysis on the basis of the patterns of the signals for CEPX/AR and CEP10/PTEN (Table 2). The patterns of distribution of CD133+ CTCs concerning AR relative gains, which are well known in prostate cancer, showed that among the 1,846 CTCs analyzed for AR copy number (Table 3), the majority (1562; 85%) showed normal patterns (1R1G) and 209 (11%) showed AR gains (2R2G). CTCs with trisomy or polyploidy patterns (3R3G or 4R4G) were observed in 33 CTCs (1.7%) and 39 (2.1%) showed a $2G \geq 4R$ pattern. CEP10/PTEN patterns of distribution were analyzed using FISH in all cases with CD133+ CTCs. Of the 1,620 CTCs from 62 patients, 1,134 (70%) were diploid for CEP10/PTEN, 372 (23%) showed PTEN loss and 114 (7%) showed polyploidy. Therefore, no abnormalities by CEPX/AR and CEP10/PTEN were shown in the majority of the CD133+ CTCs. Regarding AR-V7 expression, Table 2 shows the distribution

of cells in cases of AR-V7+ versus AR-V7-negative. There are no differences in terms of CEPX/AR between the groups (16% gains in AR-V7negative cells versus 13% gains in AR-V7+ cells). For PTEN/CEP10 results, a higher number of cell deletion was observed in AR-V7-negative cells (24% vs 18%), whereas among those that were AR-V7+, the number of cells with polyploidy patterns for PTEN was higher (10% vs 6%).

DISCUSSION

This study describes, for the first time, the feasibility of detecting AR-V7 expression in plasma (liquid biopsies) from patients with prostate cancer at different stages by using an automated capillary nano-immunoassay “WESTM ”(ProteinSimple). Previous studies have shown that detection of AR-V7 in CTCs from men with CRPC is associated with resistance to abiraterone and enzalutamide [2], but not to taxane chemotherapy [2,10]. These studies used a CTC-enriched platform followed by multiplexed reverse transcription PCR to detect full-length androgen receptor and AR-V7 transcripts. However, this technique is difficult to implement in daily practice, and it is only able to detect ARV7 positive patients if they have CTCs, so a high burden disease. The present study describes the possibility to detect ARV7 expression in different stages of prostate cancer from plasma, with a simpler technique. We observed AR-V7 expression in 29% of patients with prostate cancer. Of note, there was a high percentage of patients with CRPC, with metastatic disease and with a high Gleason score in the group of cases with positive expression of AR-V7. The poor prognosis of patients with AR-V7 expression (frequently associated with the acquisition of the CRPC-phenotype) is probably related to constitutive activity of ARV7 [11–13]. However, previous experimental evidence showed that AR-V7 is expressed in both prostate cancer cells and normal prostate epithelial cells [14] and AR-V7 is more frequently expressed in CRPC compared with hormone sensitive prostate cancer [13], as we have shown in our preliminary study. The transcriptional activity of AR-V7 is not regulated by androgens or antiandrogens. Moreover, AR-V7 is significantly upregulated during progression of prostate cancer [15] and AR-V7 expression level is correlated with the risk of

tumour recurrence after radical prostatectomy [16]. We have only 27 patients castration-naïve, and our results show that 29% of these patients showed expression of AR-V7 in plasma, before they became castration-resistant. Therefore, AR-V7 may be a novel mechanism underlying ablation-independence during progression of prostate cancer, which supports the suggestion that AR-V7 may serve as a prognostic and predictive biomarker in response to hormonal therapy [16–18]. What is more, detection of AR-V7 on CTCs has been shown to be associated with superior survival in patients with CRPC who received taxane-based treatment over patients that received hormonal therapy [19].

Previous studies have documented the presence of CD133+ CTCs in samples from patients with advanced CRPC as well as from patients with localised prostate cancer after local therapy (4,20). Putative prostate cancer stem cells and other cancer stem cell populations express the stem cell-associated antigen CD133. We observed an association between CD133 expression, genomic changes for AR and PTEN with the presence of AR-V7 in the plasma of these patients. Indeed, 96% of our patients had detectable populations of CD133+ CTCs, which is in line with results of previous studies [3]. The number of cells detected depended on the stage and the number of previous treatments (Table 3) . Regarding the expression of AR-V7, AR-V7+ patients with tumours had a higher number of CD133+ CTCs than AR-V7-negative patients.

Furthermore, in those AR-V7+ cases, the median CD133+ CTC count was higher in patients with CRPC as compared with patients with castration-sensitive tumours. The expression of the stemness antigen CD133 suggests that these cells may have acquired properties of stemness during their migration into the bloodstream. Indeed, evidence suggests that AR variants could contribute to progression of prostate cancer through induction of an epithelial-to-mesenchymal transition phenotype and acquisition of stem cell characteristics [21]. The genetic

characterization of the CD133+ population allows us to suggest that the acquisition or progression to a more aggressive phenotype was associated with the presence of genomic alterations for AR and PTEN gene in the ARV7+ cases. All but one of the AR-V7+ cases (CRPC or castration-sensitive) showed abnormalities in these genes.

CONCLUSIONS

Our results demonstrate the feasibility of assessing the presence of AR-V7 and the genomic characterization in a single-tube assay of CTCs from patients with prostate cancer. These findings lay the foundations for liquid biopsy as a means of obtaining biological data in a more convenient way for the patient. These data are needed for personalized treatment decisions, which can prevent patients from receiving inefficient systemic therapy.

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Conflict to interest

We declare that we have no conflict of interest

REFERENCES

1. Nadiminty N, Gao AC. Mechanisms of persistent activation of the androgen receptor in CRPC: recent advances and future perspectives. *World J Urol.* 2012;30(3):287–95.
2. Antonarakis ES, Lu C, Wang H, Lubber B, Nakazawa M, Roeser JC, Chen Y, Mohammad TA, Chen Y, Fedor HL, Lotan TL, Zheng Q, De Marzo AM, Isaacs JT, Isaacs WB, Nadal R, Paller CJ, Denmeade SR, Carducci MA, Eisenberger MA, Luo J. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *N Engl J Med.* 2014;371(11):1028–38.
3. Reyes EE, Gillard M, Duggan R, Wroblewski K, Kregel S, Isikbay M, Kach J, Brechka H, Weele DJV, Szmulewitz RZ, Griend DJV. Molecular analysis of CD133-positive circulating tumor cells from patients with metastatic castration-resistant prostate cancer. *J Transl Sci.* 2015;1(1).
4. Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bittling RL, Turnbull JD, Herold CI, Marcom PK, George DJ, Garcia-Blanco MA. Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. *Mol Cancer Res MCR.* 2011;9(8):997–1007.
5. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LWWM, Pienta KJ, Raghavan D. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2008;14(19):6302–9.
6. Punnoose EA, Ferraldeschi R, Szafer-Glusman E, Tucker EK, Mohan S, Flohr P, Riisnaes R, Miranda S, Figueiredo I, Rodrigues DN, Omlin A, Pezaro C, Zhu J, Amler L, Patel P, Yan Y, Bales N, Werner SL, Louw J, Pandita A, Marrinucci D, Attard G, de Bono J. PTEN loss in circulating tumour

cells correlates with PTEN loss in fresh tumour tissue from castration-resistant prostate cancer patients. *Br J Cancer*. 2015;113(8):1225–33.

7. Chen J-Q, Wakefield LM, Goldstein DJ. Capillary nano-immunoassays: advancing quantitative proteomics analysis, biomarker assessment, and molecular diagnostics. *J Transl Med*. 2015;13:182.

8. Knuuttila M, Yarkin E, Kallio J, Savolainen S, Laajala TD, Aittokallio T, Oksala R, Häkkinen M, Keski-Rahkonen P, Auriola S, Poutanen M, Mäkelä S. Castration induces up-regulation of intratumoral androgen biosynthesis and androgen receptor expression in an orthotopic VCaP human prostate cancer xenograft model. *Am J Pathol*. 2014;184(8):2163–73.

9. Mooney SM, Parsana P, Hernandez JR, Liu X, Verdone JE, Torga G, Harberg CA, Pienta KJ. The presence of androgen receptor elements regulates ZEB1 expression in the absence of androgen receptor. *J Cell Biochem*. 2015;116 (1):115–23.

10. Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Nakazawa M, Nadal R, Paller CJ, Denmeade SR, Carducci MA, Eisenberger MA, Luo J. Androgen ReceptorSplice Variant 7 and Efficacy of Taxane Chemotherapy in Patients With Metastatic Castration-Resistant Prostate Cancer. *JAMA Oncol*. 2015;1(5):582–91.

11. Mostaghel EA, Plymate SR, Montgomery B. Molecular pathways: targeting resistance in the androgen receptor for therapeutic benefit. *Clin Cancer Res Off J Am Assoc Cancer Res*. 2014;20(4):791–8.

12. Liu G, Sprenger C, Sun S, Epilepsia KS, Haugk K, Zhang X, Coleman I, Nelson PS, Plymate S. AR variant ARv567es induces carcinogenesis in a novel transgenic mouse model of prostate cancer. *Neoplasia N Y N*. 2013;15(9):1009–17.

13. Qu Y, Dai B, Ye D, Kong Y, Chang K, Jia Z, Yang X, Zhang H, Zhu Y, Shi G. Constitutively active AR-V7 plays an essential role in the development and progression of castration-resistant prostate cancer. *Sci Rep*. 2015;5:7654.
14. Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, Kim K, Sawyers CL. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. *Proc Natl Acad Sci U S A*. 2010;107(39):16759–65.
15. Cao B, Qi Y, Zhang G, Xu D, Zhan Y, Alvarez X, Guo Z, Fu X, Plymate SR, Sartor O, Zhang H, Dong Y. Androgen receptor splice variants activating the fulllength receptor in mediating resistance to androgen-directed therapy. *Oncotarget*. 2014;5(6):1646–56.
16. Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Chen H, Kong X, Melamed J, Tepper CG, Kung H-J, Brodie AMH, Edwards J, Qiu Y. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer Res*. 2009; 69(6):2305–13.
17. Sun S, Sprenger CCT, Vessella RL, Haugk K, Soriano K, Mostaghel EA, Page ST, Coleman IM, Nguyen HM, Sun H, Nelson PS, Plymate SR. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *J Clin Invest*. 2010; 120(8):2715–30.
18. Zhang X, Morrissey C, Sun S, Ketchandji M, Nelson PS, True LD, Vakar-Lopez F, Vessella RL, Plymate SR. Androgen receptor variants occur frequently in castration resistant prostate cancer metastases. *PloS One*. 2011;6(11):e27970.

19. Scher HI, Lu D, Schreiber NA, Louw J, Graf RP, Vargas HA, Johnson A, Jendrisak A, Bambury R, Danila D, McLaughlin B, Wahl J, Greene SB, Heller G, Marrinucci D, Fleisher M, Dittamore R. Association of AR-V7 on Circulating Tumor Cells as a Treatment-Specific Biomarker With Outcomes and Survival in Castration-Resistant Prostate Cancer. *JAMA Oncol.* 2016;2(11):1441-1449
20. Pal SK, He M, Wilson T, Liu X, Zhang K, Carmichael C, Torres A, Hernandez S, Lau C, Agarwal N, Kawachi M, Yen Y, Jones JO. Detection and phenotyping of circulating tumor cells in high-risk localized prostate cancer. *Clin Genitourin Cancer.* 2015; 13(2):130–6.
21. Kong D, Sethi S, Li Y, Chen W, Sakr WA, Heath E, Sarkar FH. Androgen receptor splice variants contribute to prostate cancer aggressiveness through induction of EMT and expression of stem cell marker genes. *The Prostate.* 2015; 75(2):161–74.

TABLES AND FIGURES

Figures

Figure 1. AR-V7 and AR detection by Western Blot in PC3 and VCaP cell lines (A) Results of AR-V7 and AR Western Blot

Figure 2. Size-CNIA assay using anti-AR-V7 antibody. (A)Pseudo-blot view of AR-V7 with serial dilution of antibody for PC3 and VCap cell lines. (B)Peaks of AR-V7 signal in VaCp cell line. (C) Linearity of AR-V7 signal detection using serial dilution of sample. On Y-axis presented the chemiluminescence signal intensity in arbitrary units (AU) normalized to signal detected for System Control antibody.

Figure 3. Sample result of AR-V7 detection in plasma samples, five positive and five negative sample results are represented as pseudoblots

Tables

Table 1. Clinical characteristics of the patients and CD133 by AR-V7 form.

Table 2. Signals for CEPX/AR and CEP10/PTEN and AR-V7

Table 3. Patterns of AR copy number