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The Detection of Androgen Receptor Splice Variant 7 in Plasma-derived Exosomal RNA Strongly Predicts Resistance to Hormonal Therapy in Metastatic Prostate Cancer Patients

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Abstract

Background: The androgen receptor splice variant 7 (AR-V7) is associated with resistance to hormonal therapy in castration-resistant prostate cancer (CRPC). Due to limitations of the methods available for AR-V7 analysis, the identification of a reliable detection method may facilitate the use of this biomarker in clinical practice.
Objective: To confirm AR-V7 as a predictor of resistance to hormonal therapy and develop a new approach to assess AR-V7 by highly sensitive digital droplet polymerase chain reaction (ddPCR) in plasma-derived exosomal RNA.
Design, setting, and participants: Plasma samples were collected from 36 CRPC patients before they began second-line hormonal treatment. Exosomes were isolated and RNA extracted for analysis of AR-V7 by ddPCR.
Outcome measurements and statistical analysis: The absolute target gene concentration as copies per milliliter (copies/ml) was determined by ddPCR. Statistical analyses were performed with SPSS software (IBM Corp., Armonk, NY, USA).
Results and limitations: A total of 26 patients received abiraterone and 10 enzalutamide; 39% of patients were found to be AR-V7 positive (AR-V7$^+$). Median progression-free survival was significantly longer in AR-V7 negative (AR-V7$^-$) versus AR-V7$^+$ patients (20 vs 3 mo; $p < 0.001$). Overall survival was significantly shorter in AR-V7$^+$ participants at baseline compared with AR-V7$^-$ participants (8 mo vs not reached; $p < 0.001$).
Conclusions: This study demonstrates that plasma-derived exosomal RNA is a reliable source of AR-V7 that can be detected sensitively by ddPCR assay. We also showed that resistance to hormonal therapy may be predicted by AR-V7, making it a clinically relevant biomarker.
Patient summary: We report a first study on a method for androgen receptor splice variant 7 (AR-V7) detection in RNA extracted from cancer cell vesicles released in blood. Results confirmed the role of AR-V7 as a predictive biomarker of resistance to hormonal therapy. Our assay showed that vesicles are a reliable source of AR-V7 RNA and that the method is fast, highly sensitive, and affordable.

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1. Introduction

In prostate cancer the only predictive biomarkers of resistance to therapy are the androgen receptor (AR) splice variants and DNA-repair deficiency, relative to hormonal therapy (HT) and poly(adenosine diphosphate-ribose) polymerase inhibitors, respectively [1,2]. The clinical condition of patients and radiographic or symptomatic progression are still the key parameters for therapeutic intervention [3], whereas prostate-specific antigen (PSA) is a poor predictor of clinical response to HT or chemotherapy. Castration-resistant prostate cancer (CRPC) is managed by taxane-based chemotherapy (docetaxel, cabazitaxel), anti-AR therapies (abiraterone acetate, enzalutamide), immunotherapies (sipuleucel-T6), and radium-223. Unfortunately, a validated biomarker for predicting the outcome of second-line HT in CRPC is still elusive [4]. Antonarakis et al [1] found in circulating tumor cells (CTCs) that one splicing variant, androgen receptor splice variant 7 (AR-V7), is associated with resistance to enzalutamide and abiraterone. AR-splice variants are truncated receptor isoforms lacking the C-terminal ligand-binding domain (LBD) that is a key regulator region of the full-length AR (AR-FL). The LBD is responsible for androgen-dependent receptor activity and the target of flutamide, bicalutamide, and enzalutamide [5,6]. Therefore, LBD deletion results in loss of the antiandrogen binding site and constitutive activation of AR-V7 [6]. AR-V7 is the hallmark of biological disease progression, and its detection can be of strategic importance for treatment management. To implement this test into clinical practice, it should be highly sensitive, specific and easy to perform, and cost effective. AR-V7 may be detected in tumor tissue [7], CTCs [1,8], or in messenger RNA (mRNA) extracted from whole blood [9]. Unfortunately, these methods have substantial limitations: a biopsy to assess the molecular tumor evolution and heterogeneity is invasive and not always feasible, and the cost and complexity of isolating CTCs and the low sensitivity of mRNA extracted from whole blood are relevant drawbacks. Even though it has been demonstrated in vitro and in a few studies in patients that AR-V7 is a biomarker of resistance, its role needs to be confirmed. The present study aimed at confirming the role of AR-V7 to predict resistance to HT and developing a new methodological approach based on digital droplet polymerase chain reaction (ddPCR) to assess this marker reliably. Plasma-derived exosomal RNA was used as the source material, and the study provided new data to address the correlation between exosomal AR-V7 and therapy resistance, given that the translation of available data on CTCs to exosomes is not obvious.

2. Materials and methods

2.1. Extraction of VCaP AR-V7 RNA and its detection by digital droplet polymerase chain reaction

VCaP cells (ATCC CRL-2876) were used to set up the ddPCR method because they are known carriers of AR-V7. RNA was extracted from VCaP using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), transcribed into complementary DNA (cDNA) and amplified using the Duplex One-Step RT-ddPCR Kit (Bio-Rad, Hercules, CA, USA). Primers and probes for both AR-FL and AR-V7 were designed in our laboratory by Primer3 software (ThermoFisher Scientific, Waltham, MA, USA). FAM/HEX labeling and primer synthesis were done by Bio-Rad: AR-FL forward primer: 5′-CATCAAGGAACTCGATCGT-3′; AR-FL reverse primer: 5′-GAACTGATGCAGCTCTCTC-3′; AR-FL probe: 5′-ACATCCTGCTCAAGACGCTCCT-3′; AR-V7 forward primer: 5′-CTGTGCGCCAGCAGAAAT-3′; AR-V7 reverse primer: 5′-TCAGGGTCTGGTCATTTTGA-3′; AR-V7 probe: 5′-TGTCCATCTTGTCGTCTTCG-3′.

PCR reactions were assembled into individual wells according to the following protocol: 1 ng RNA template (5 μl), 1 μl 20X AR-V7 primer/probe assay (FAM), 1 μl 20X AR-FL primer/probe assay (HEX), 5 μl 1X ddPCR Super Mix, 2 μl RT 20 U/μl, 1 μl 300 mM DTT, 5 μl DNase/RNase-free water (total volume: 20 μl). Droplet generation oil (70 μl) was added, and the eight-well cartridge was placed into the droplet generator; 40 μl of the droplet solution was then transferred into a 96-well PCR plate. The following conditions were used for the reverse transcriptase PCR reaction: 50 °C × 60 min, 95 °C × 10 min, 95 °C × 30 s and 55 °C × 60 s (40 cycles), 98 °C × 10 min, 4 °C hold. The droplet reader was used for fluorescence signal quantification. The QuantaSoft software (Bio-Rad) measures the number of positive versus negative droplets for both fluorophores (FAM/HEX); their ratio is then fitted to a Poisson distribution to determine the copy number of the target molecule, as copies per milliliter (copies/ml), in the input reaction.

To evaluate the sensitivity of the ddPCR AR-V7 assay, cDNA obtained from the RNA of VCaP cells was diluted in water at 250, 100, 50, 25, 15, 10, 5, 1, 0.5, and 0.1 ng/μl. The number of copies/ml was measured by ddPCR.

2.2. Patient selection

A total of 36 participants with metastatic CRPC treated with enzalutamide or abiraterone as per approved label were enrolled. Patients were required to have histologically confirmed prostate adenocarcinoma, progressive disease despite castration levels of serum testosterone (<500 ng/l) while on stable androgen-deprivation therapy, and documented metastases, confirmed by computed tomography or technetium-99 bone scans. Patients must have had at least three increasing serum PSA values taken at least 2 wk before the last value of at least 2.0 ng/ml, consistent with the Prostate Cancer Working Group-2 guidelines. Prior taxane-based chemotherapy was permitted. The analysis of AR-V7 in primary tumors was not performed because this is not part of clinical practice. The study was approved by the Ethics Committee of Pisa University Hospital and conducted in accordance with the principles of the Declaration of Helsinki. All patients gave their signed informed consent before blood collection and data analysis.

2.3. Plasma collection and RNA extraction from exosomes

Overall, 3 ml of blood were collected before the start of abiraterone or enzalutamide; in seven participants, a sample was also available at disease progression. Blood was transferred in ethylenediaminetetraacetic acid tubes and centrifuged at 1900 *g* for 10 min at 4 °C within 2 h after drawing. Plasma was stored at −80 °C until analysis. Plasma

Table 1 – Characteristics of patients and androgen receptor splice variant 7 status

Baseline characteristic	All patients *n* = 36	AR-V7$^-$ *n* = 22	AR-V7$^+$ *n* = 14
Age, median (range), y	66 (51–81)	67 (51–81)	66 (57–73)
Race, *n* (%)			
White	36 (100)	22 (100)	14 (100)
Nonwhite	0 (0)	0 (0)	0 (0)
ECOG performance status, *n* (%)			
0	26 (72)	17 (77)	9 (64)
1 or 2	10 (28)	5 (23)	5 (36)
Time since diagnosis, median (range), yr	4.86 (0.32–17.03)	5.64 (0.91–17.03)	3.60 (0.32–12.80)
Tumor stage at diagnosis, *n* (%)			
T1/2	3 (8)	2 (9)	1(7)
T3/4	13 (36)	9 (41)	4 (29)
Unknown	20 (56)	11 (50)	9 (64)
Gleason sum at diagnosis, *n* (%)			
≤7	16 (44)	12 (55)	4 (29)
≥8	19 (53)	9 (41)	10 (71)
Unknown	1 (3)	1 (4)	0 (0)
Type of local treatment, *n* (%)			
Surgery	17 (47)	11 (50)	6 (43)
Radiation therapy	10 (28)	8 (36)	2 (14)
None	9 (25)	3 (14)	6 (43)
No. of prior hormonal therapies, median	2	2	2
Current treatment, *n* (%)			
Abiraterone	26 (72)	18 (82)	8 (57)
Enzalutamide	10 (28)	4 (18)	6 (43)
Docetaxel	0 (0)	0 (0)	0 (0)
Cabazitaxel	0 (0)	0 (0)	0 (0)
Prior use of abiraterone, *n* (%)			
Yes	4 (11)	0 (0)	4 (28)
No	32 (89)	22 (100)	10 (72)
Prior use of enzalutamide, *n* (%)			
Yes	0 (0)	0 (0)	0 (0)
No	36 (100)	22 (100)	14 (100)
Prior use of docetaxel, *n* (%)			
Yes	24 (67)	11 (50)	13 (93)
No	12 (33)	11 (50)	1 (7)
Prior use of cabazitaxel, *n* (%)			
Yes	5 (14)	1 (5)	4 (29)
No	31(86)	21(95)	10 (71%)
Presence of bone metastases, *n* (%)			
Yes	30 (83)	16 (73)	14 (100)
No	6 (17)	6 (27)	0 (0)
Presence of lymph node metastases, *n* (%)			
Yes	23 (64)	13 (59)	10 (71)
No	13 (36)	9 (41)	4 (29)
Presence of visceral metastases, *n* (%)			
Yes	7 (19)	1 (5)	6 (43)
No	29 (81)	21 (95)	8 (57)
Baseline PSA level, ng/ml, median (range)	26.3 (0.63–4581)	22.3 (0.78–4581)	99.6 (0.63–521)
Baseline alkaline phosphatase level, U/l, median (range)	180 (49–917)	152 (49–917)	258 (53–575)
Baseline lactase dehydrogenase level, U/l, median (range)	220 (110–1723)	220 (110–1723)	266 (150–1720)
Baseline Hb level, g/dl, median (range)	12.3 (7.9–14.9)	13.25 (9.9–14.9)	10 (7.9–12)
Use of opioid, *n* (%)			
Yes	14 (39)	5 (23)	9 (75)
No	22 (61)	17 (77)	5 (25)

AR-V7 = androgen receptor splice variant 7; ECOG = Eastern Cooperative Oncology Group; Hb = hemoglobin; PSA = prostate-specific antigen.

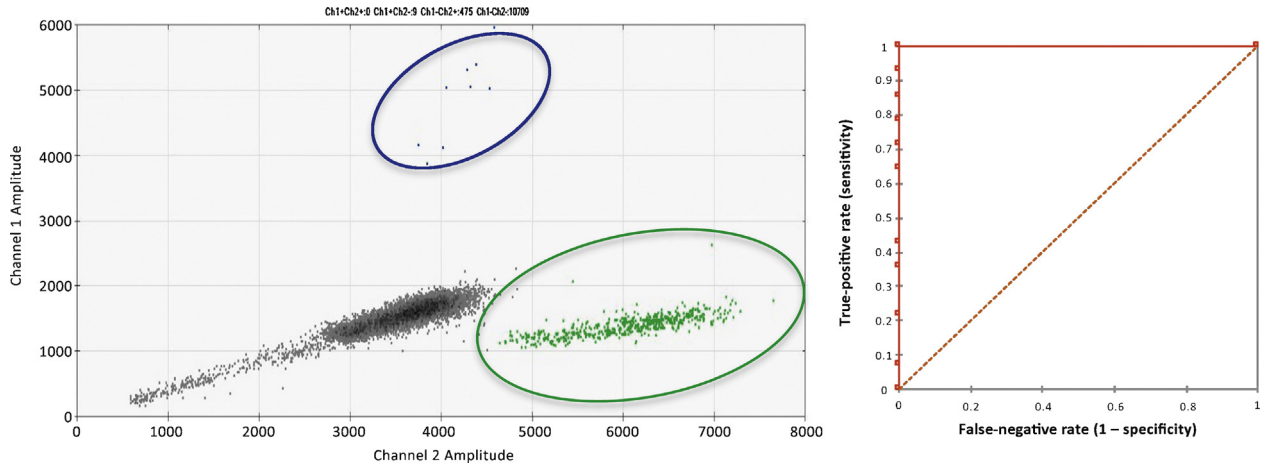

Fig. 1 – Androgen receptor splice variant 7 (AR-V7) amplification by digital droplet polymerase chain reaction. The blue dots represent positives droplets for the AR-V7 amplification. The green dots represent positive droplets for the AR wild-type amplification. The gray dots are empty droplets or droplets containing primers dimers. The insert represents the receiver operating characteristic curve generated by the data of the present study. AR-V7 = androgen receptor splice variant 7; ddPCR = digital droplet polymerase chain reaction.

Table 2 – Fractional abundance (percentage) and numbers of copies per milliliter in androgen receptor splice variant 7 samples

Sample	Baseline AR-V7, %	Baseline AR-V7, copies/ml	PD AR-V7, %	PD AR-V7, copies/ml
1	3.4	360	NA	NA
2	5.4	390	NA	NA
3	6.5	530	NA	NA
4	2.1	100	NA	NA
7	6	600	3	410
9	8	700	3.5	600
10	8	500	3	500
11	6	500	4.1	1200
12	2.2	300	8	2400
13	10	600	NA	NA
14	5	500	6	600
15	8	700	NA	NA
16	2.8	400	3	500
17	1.8	900	NA	NA
5	0	0	NA	NA
6	0	0	NA	NA
8	0	0	NA	NA
18	0	0	NA	NA
19	0	0	NA	NA
20	0	0	NA	NA
21	0	0	NA	NA
22	0	0	NA	NA
23	0	0	NA	NA
24	0	0	NA	NA
25	0	0	NA	NA
26	0	0	NA	NA
27	0	0	NA	NA
28	0	0	NA	NA
29	0	0	NA	NA
30	0	0	NA	NA
31	0	0	NA	NA
32	0	0	NA	NA
33	0	0	NA	NA
34	0	0	NA	NA
35	0	0	NA	NA
36	0	0	NA	NA

AR-V7 = androgen receptor splice variant 7; copies/ml = copies per milliliter; NA = not available; PD = progression of disease.

samples were then thawed and centrifuged at 1900 *g* for 15 min to remove cellular debris. Exosome isolation from plasma was performed using the exoRNeasy kit (Qiagen, Valencia, CA, USA) on exoEasy spin columns, and RNA was extracted from the vesicles bound to the silica membrane using the QIAzol phenol/guanidine-based lysis solution. Chloroform was then added to QIAzol-samples and centrifuged at 12 000 *g* for 15 min at 4 °C. The aqueous phase containing RNA was recovered and applied to the RNeasy MinElute spin column, in which the RNA binds to the membrane and contaminants are discarded. The RNA was finally eluted in 20 μl of the elution buffer.

2.4. Analysis of AR-V7 on plasma-derived exosomal RNA

The investigational part of this study included the assessment of AR-V7 in CRPC patients. Other mutations of the androgen-related pathway were not examined because of the limited amount of RNA available and lack of strong scientific evidence concerning their role in resistance. The analysis of AR-V7 in RNA was performed by ddPCR using the One-Step RT-ddPCR kit, as described earlier. VCaP RNA was used to spike clinical samples and blank specimens as the control. Results were reported as copies of mutant allele per milliliter of plasma.

2.5. Data analysis

The ddPCR QuantaSoft software determined the absolute target concentration as copies/ml in the samples. Statistical analyses were performed separately in the AR-V7-positive (AR-V7$^+$) and AR-V7-negative (AR-V7$^-$) cohorts. PSA response rates (RRs) were compared with the Fisher exact test. Time-to-event outcomes (ie, clinical or radiographic progression-free survival [PFS] and overall survival [OS]) were evaluated by the Kaplan-Meier method, and survival-time differences were compared by the log-rank test. Additional statistics were performed by paired *t* test. All tests were two sided, and *p* values $\leq$0.05 were considered statistically significant. The performance of the test was assessed by the performance of a binary classifier system because its discrimination threshold is varied. The receiver operating characteristic curve (ROC curve) was created by plotting the true-positive rate against the false-positive rate at various threshold settings. SPSS software v2.0 (IBM Corp., Armonk, NY, USA) was used for calculations.

3. Results

Using ddPCR we were able to detect the AR-V7 transcript, even at low concentrations (ie, 2 copies/ml) (Supplementary Fig. 1). No AR-V7 signal was detected in the unspiked control plasma or in blank wells, confirming the specificity of the assay. Supplementary Table 1 reports the median number of copies spiked per unit volume of plasma and measured by ddPCR, 95% confidence intervals (CIs), and coefficients of variation.

3.1. Patient characteristics and AR-V7 expression

Overall, 36 patients were enrolled, of whom 26 received abiraterone and 10 enzalutamide; median follow-up time was 9 mo (range: 2.0–31.0). Baseline characteristics for the study population are reported in Table 1. A total of 14 of 36 patients (38.8%) were AR-V7$^+$ (100–2400 copies) (Fig. 1) before the start of HT. Seven of 14 AR-V7$^+$ patients had a plasma sample at baseline (median: 500 copies/ml) and at progression (median: 887 copies/ml); although there was an increase, the difference was not statistically significant (paired *t* test, $p = 0.25$) (Table 2). The AR-FL was used as an internal control to validate the extraction process; AR-V7$^-$ samples were true negative because the AR-FL signal was detectable. The starting volume of plasma samples (1 vs 2 ml)

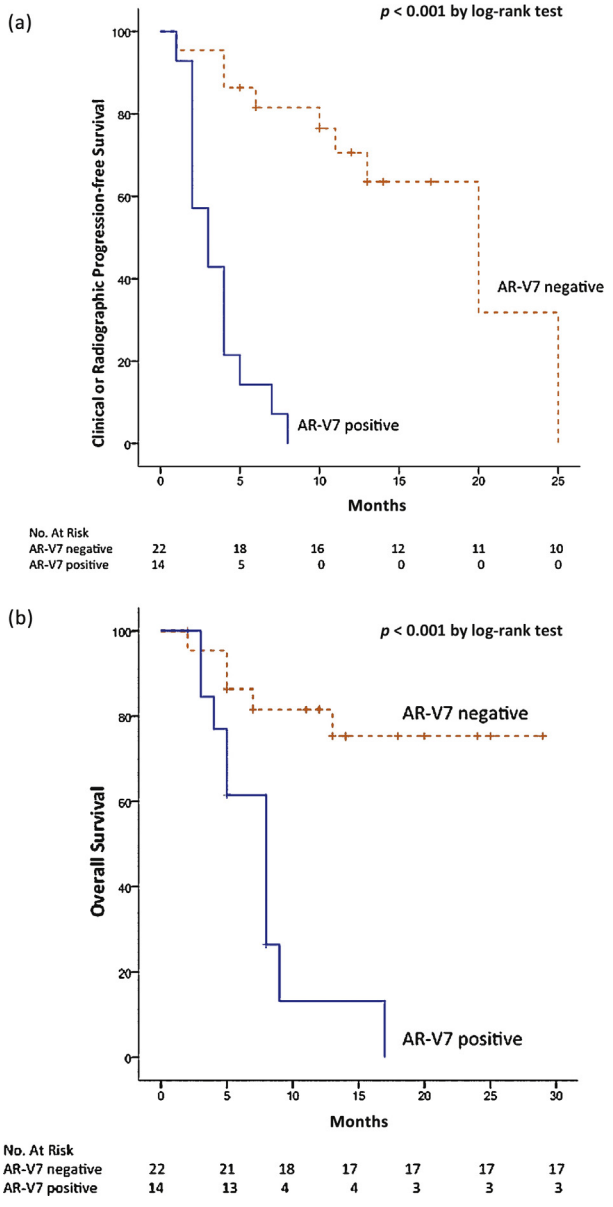

Fig. 2 – (a) Progression-free survival of androgen receptor splice variant 7–positive (AR-V7$^+$) versus AR-V7-negative (AR-V7$^-$) patients; (b) overall survival of AR-V7$^+$ versus AR-V7$^-$ patients.
AR-V7 = androgen receptor splice variant 7.

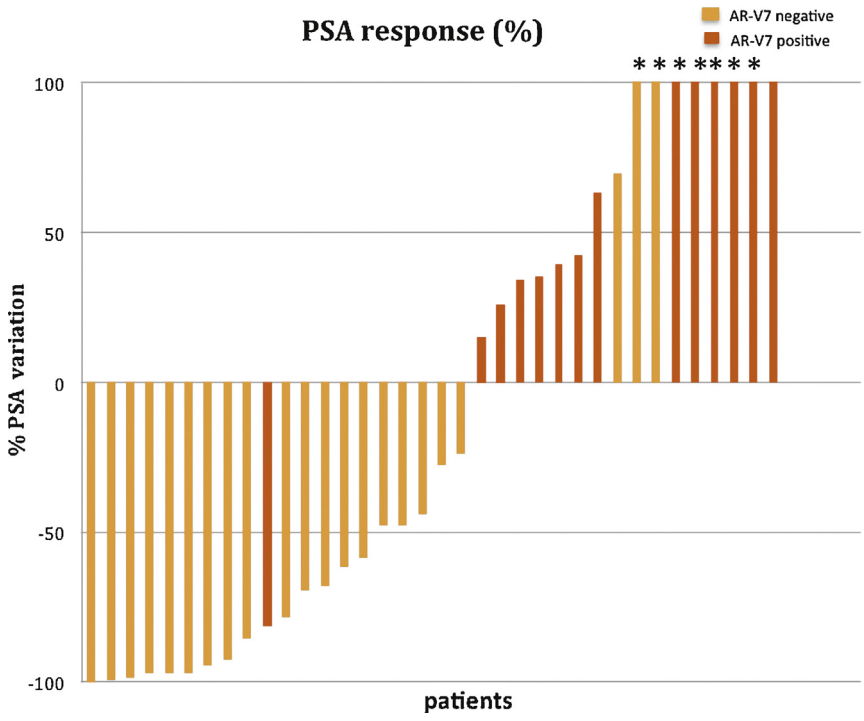

Fig. 3 – Waterfall plot depicting prostate-specific antigen (PSA) responses, according to androgen receptor splice variant 7 status. The dotted line shows the threshold for defining a PSA response (≥50% reduction in PSA level from baseline). Asterisks indicate an increase of >100% in PSA response. AR-V7 = androgen receptor splice variant 7; PSA = prostate-specific antigen.

did not significantly affect the ratio of total versus positive droplets. The median number of total droplets generated was 11 000. The ROC curve is reported in Figure 1; with the curve very close to the upper left corner, the overall accuracy of the test was very high.

3.2. *Clinical outcomes according to the AR-V7 analysis*

Table 1 reports the characteristics of patients and their AR-V7 status. The median clinic or radiographic PFS was significantly longer (20 mo) in AR-V7$^-$ patients compared with AR-V7$^+$ patients (3 mo; 95% CI, 1.526–14.474; $p < 0.001$ log-rank test) (Fig. 2a). The OS was shorter in AR-V7$^+$ patients at baseline compared with AR-V7$^-$ patients (median 8 mo vs not reached) (95% CI, 3.751–30.285; $p < 0.001$ log-rank test) (Fig. 2b), making AR-V7 detection in exosomes a valuable marker of resistance to HT. The overall proportion of patients who achieved a PSA response during HT was 42% (15 of 36). In the AR-V7$^+$ patients, the PSA RR was 7% (1 of 14 men); in the AR-V7$^-$ patients, the RR was 64% (14 of 22 men). PSA responses are shown in Figure 3. The AR-V7$^+$ participants were more likely to be younger, with a Gleason score of at least 8, visceral metastases, higher PSA levels, and prior docetaxel treatment than AR-V7$^-$ patients. Twenty-six of 36 patients were treated with taxanes before HT, of whom 11 patients were AR-V7$^-$ and 13 were AR-V7$^+$. Clinical or radiographic PFS did not differ significantly depending on AR-V7 status. Median PFS was 11 mo in AR-V7$^+$ patients (95% CI, 7.3–14.3 mo, $p = 0.6$) and 11 mo in AR-V7$^-$ patients (95% CI, 9.9–12.0 mo, $p = 0.6$) (Fig. 4). Univariate and multivariable Cox proportional hazard ratios were used to assess the effect of AR-V7 status on the prediction of time-to-event outcomes. In the univariate model, we analyzed known risk factors for

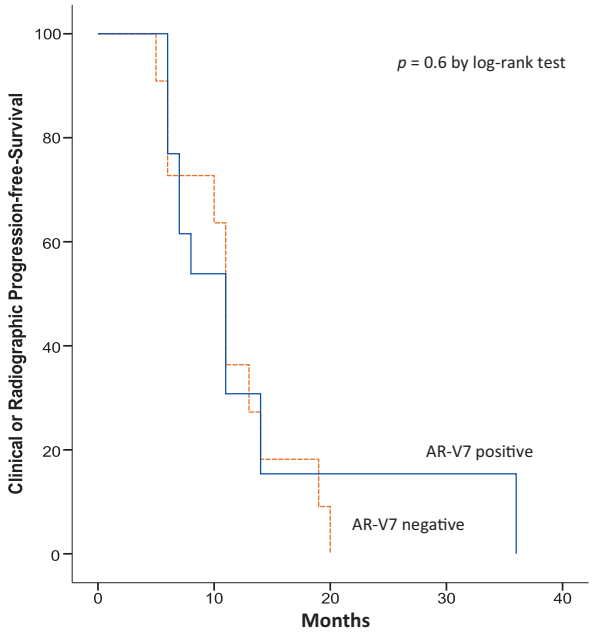

Fig. 4 – Kaplan-Meier curves showing clinical and/or radiographic progression-free survival in taxane-treated patients, according to androgen receptor splice variant 7 status.
AR-V7 = androgen receptor splice variant 7.

progression such as serum alkaline phosphatase level (<30 vs ≥130 U/l), lactate hydrogenase (<250 vs ≥250 U/l), and hemoglobin (Hb) levels (<12 vs ≥12 g/dl). This latter was the only one that correlated with a better PFS (25 mo vs 3 mo; $p < 0.05$). Owing to the small sample size and the limited number of events, the multivariable model included only AR-V7 status, AR-V7 expression levels, prior use of taxanes, and Hb levels. However, probably due to the small population, the multivariable model did not confirm the role of AR-V7 as an independent prognostic factor (hazard ratio: 3.6; $p = 0.18$).

4. Discussion

Vesicles released by cancer cells are loaded with nucleic acids and proteins [10,11] and represent tumor heterogeneity. As an alternative, CTCs may be used to interrogate tumor biology [12–14]; however, the extraction and processing of exosomes is considerably less expensive and labor intensive than CTCs. Additional limitations of the use of CTCs are morphologic and immune-phenotypic heterogeneity (ie, epithelial markers may significantly vary) [15] and the fragility of CTCs, causing inaccurate detection of AR-V7. The comparison of CTCs [1] versus plasma-derived exosomes as a source of RNA (present study) demonstrates that CTC AR-V7$^+$ patients have a median OS of 8 mo versus not reached for AR-V7$^-$ patients [1], a result similar to the present study (8 mo vs not reached, respectively, AR-V7$^+$ vs AR-V7$^-$ patients). However, PFS of patients stratified as AR-V7$^+$ versus AR-V7$^-$ by the two methods was different: 2.2 versus 6.2 mo for CTCs and 3 versus 20 mo for exosomes. The higher PFS of AR-V7$^-$ patients (exosomal RNA as a source) may depend on false negatives in the cohort of participants whose RNA was extracted from CTCs, thus reflecting a higher sensitivity of the exosome approach over CTC. RNA for AR-V7 analysis may also be extracted from whole blood [9], but this approach may be adversely affected by the instability of free RNA in blood and the large amount of contaminating RNA from leucocytes. This is possibly the reason why the analysis of AR-V7 mRNA in whole blood failed to predict the PSA response/resistance in patients treated with HT [9]. However, comparisons between methods performed on different samples should be made with great caution.

Although promising, the clinical role of the AR-V7 as a predictive biomarker of resistance to HT in CRPC and its inclusion in clinical practice is still being debated because of the small number of enrolled patients in published studies. Several reports demonstrated and validated its role not only in vitro, but also in vivo [1,8,16,17]. The studies on CTCs [1,4,8], tissue samples from biopsies [7], and our study on exosomal RNA clearly confirm that AR-V7$^+$ patients have both lower PSA RR and shorter PSA PFS compared with AR-V7$^-$ participants. In a recent study on AR-V7 analysis on CTCs, it was shown that the incidence and amount of AR-V7 increase by line of therapy, reconfirming the role of AR-V7 as an acquired mechanism of resistance to systemic therapy [18]. An immunohistochemical assay was recently validated for AR-V7 detection in tissue biopsy [7]. The study demonstrated a clear effect of AR-V7 on survival parameters and confirmed the predictive role of this biomarker on OS in patients stratified in tertiles depending on AR-V7 (15.6 vs 9.6 vs 8.8 mo, respectively) [7]. However, the use of this approach to monitor the development of resistance is limited by the invasiveness of the procedure. In addition, a single tissue sample is not representative of tumor heterogeneity, and molecular evolution over time cannot be monitored, making this approach scientifically sound but practically less attractive. This study first demonstrates that plasma-derived exosomes provide a viable source of RNA for AR-V7 analysis and confirms its role as a strong biomarker of resistance to HT in CRPC patients.

Although in previous studies [1,18] AR-V7 levels increased over time, in our study only four of seven samples had increasing AR-V7 copies/ml. An explanation is that additional mechanisms of resistance may have been developed.

5. Conclusions

The present study provides evidence that RNA recovered from plasma-derived exosomes may offer obvious advantages, streamlining the diagnostic workflow by reducing the costs, eliminating the invasiveness of tissue sampling, and minimizing the negative effect of tumor heterogeneity.

Author contributions: Marzia Del Re had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Del Re, Danesi.
Acquisition of data: Del Re, Biasco, Derosa, Galli.
Analysis and interpretation of data: Del Re, Biasco, Rofi, Crucitta, Derosa, Danesi.
Drafting of the manuscript: Del Re, Danesi, Biasco.
Critical revision of the manuscript for important intellectual content: Danesi, Jenster, van Schaik, Galli, Falcone.
Statistical analysis: Del Re, Biasco, Orlandini, Miccoli.
Obtaining funding: Danesi.
Administrative, technical, or material support: Biasco, Del Re.
Supervision: Danesi, Galli.
Other (specify): None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2016.08.012.

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