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Clinical value of circulating splicing factors in prostate cancer: SRRM1 as a novel predictive biomarker and therapeutic target

Antonio J. Montero-Hidalgo,[1,2,3,4] Enrique Gómez-Gómez,[1,3,5] Manuel Galán-Cañete,[1,2,3,4] Francisco Porcel-Pastrana,[1,2,3,4] Jesús M. Pérez-Gómez,[1,2,3,4] María Ortega-Bellido,[1,2,3,4] Julia Carrasco-Valiente,[1,3,5] Laura Chamorro-Castillo,[1,3,5] Juan P. Campos-Hernández,[1,3,5] Oriol A. Rangel-Zuñiga,[1,3,6] Teresa González-Serrano,[1,3,7] Rafael Sánchez-Sánchez,[1,3,7] André Sarmento-Cabral,[1,2,3,4] Manuel D. Gahete,[1,2,3,4] Juan M. Jiménez-Vacas,[1,2,3,4] and Raúl M. Luque[1,2,3,4]

[1]Maimonides Institute for Biomedical Research of Cordoba (IMIBIC), 14004 Cordoba, Spain; [2]Department of Cell Biology, Physiology, and Immunology, University of Córdoba, 14004 Cordoba, Spain; [3]Hospital Universitario Reina Sofía (HURS), 14004 Cordoba, Spain; [4]Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERobn), 14004 Cordoba, Spain; [5]Urology Service, HURS/IMIBIC, 14004 Cordoba, Spain; [6]Internal Medicine Unit, HURS/IMIBIC, 14004 Cordoba, Spain; [7]Anatomical Pathology Service, HURS, 14004 Cordoba, Spain

Prostate cancer (PCa) is the second most common cancer among men worldwide. The main screening tool remains the prostate-specific antigen (PSA), which shows significant limitations, including poor sensitivity/specificity. Therefore, establishing accurate non-invasive diagnostic biomarkers remains an unmet clinical need in PCa. In this context, the splicing process dysregulation represents a PCa hallmark. Here, plasma SRRM1, SNRNP200, and SRSF3 levels, previously identified to play a pathophysiological role in PCa, were determined in control individuals (*n* = 40) and PCa patients (*n* = 166). We found that plasma SRRM1 and SNRNP200 levels were elevated in PCa patients and discriminated between control individuals and PCa patients. High plasma SRRM1 levels were associated with a shorter castration-resistant PCa-free survival and correlated with androgen-receptor (AR)/AR-splicing variant 7 (AR-V7) expression levels and activity in PCa tissues. Therefore, the functional and molecular effects of *in vivo* SRRM1 silencing were then tested in 22Rv1-derived xenograft tumors. *In vivo* SRRM1 silencing reduced aggressiveness features and altered AR/AR-V7 activity. Our data reveal that SRRM1 holds potential as a non-invasive diagnostic and prognostic biomarker and novel therapeutic target in PCa, offering a clinically relevant opportunity worth exploring in humans.

INTRODUCTION

Prostate cancer (PCa) is the second cancer type in terms of incidence and the fifth leading cause of cancer-related death among men population worldwide.[1] Nonetheless, the mortality associated with this pathology has been tempered by the early detection of PCa, fostered by implementing the serum determination of the prostate-specific antigen (PSA) as a screening tool for PCa.[2] However, this biomarker is associated with numerous false positive cases, leading to unnecessary biopsies, and it demonstrates low sensitivity in specific subsets of patients (e.g., patients with obesity).[3] Additionally, although PSA determination is useful for following up patients after treatment, its prognostic value to predict the progression from localized hormone-sensitive prostate cancer (HSPC) to the aggressive castration-resistant prostate cancer (CRPC), mainly responsible for the mortality associated with this pathology,[4] is certainly compromised as shown in the CHAARTED trial, where 25% of patients progressed even in the absence of confirmed PSA progression (<2 ng/mL).[5] This phenotypical progression is mainly characterized by a persistent androgen receptor (AR) signaling, even when patients are treated with androgen-deprivation therapy (ADT) and/or second-generation AR signaling inhibitors (e.g., abiraterone, enzalutamide).[6]

In this scenario, it has been suggested that the AR splicing variant 7 (AR-V7), which is generated by an alternative splicing process of the *AR* pre-mRNA, drives resistance to AR targeting therapies.[7,8] Also, a wide variety of splicing variants have been reported to play a key role in PCa progression, and consequently, the dysregulation of the splicing process has emerged as a hallmark of PCa.[9–13] Indeed, we and others have recently shown that the components of the cellular machinery that catalyzes and regulates the splicing process (i.e., spliceosome components and splicing factors) are deeply dysregulated in PCa, and can control pivotal PCa-related pathways,

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Correspondence: Juan M. Jiménez-Vacas, Maimonides Institute for Biomedical Research of Cordoba (IMIBIC), 14004 Cordoba, Spain.
E-mail: juanmanueljimenezvacas@gmail.com
Correspondence: Raúl M. Luque, Maimonides Institute for Biomedical Research of Cordoba (IMIBIC), 14004 Cordoba, Spain.
E-mail: raul.luque@uco.es

Table 1. Demographic, biochemical, and clinical parameters of patients included in our internal cohort[a] of patients for plasma levels analyses

	Control	PCa
Patients, *n*	40	166
Age, y, median (IQR)	62 (42–78)	67.5 (48–95)
PSA levels, ng/mL, median (IQR)	1.0 (0.1–6.0)	6.4 (1.7–805.0)
SRRM1 levels, ng/mL, median (IQR)	13.6 (5.9–33.5)	18.6 (2.5–49.7)
SNRNP200 levels, pg/mL, median (IQR)	77.6 (17.7–221.9)	126.5 (20.7–311.9)
SRSF3 levels, pg/mL, median (IQR)	977.1 (479.1–2,114.1)	1048.8 (214.2–2,312.8)
Recurrence, *n* (%)	–	39 (23.5)
Metastasis, *n* (%)	–	16 (9.6)
CRPC, *n* (%)	–	11 (6.6)

CRPC, castration-resistant prostate cancer; IQR, interquartile range; PCa, prostate cancer; PSA, prostate-specific antigen.
[a]Patients with PCa and healthy control individuals.

including AR activity.[14–18] Interestingly, although poorly explored, some splicing factors have been shown to be present in extracellular fluids from patients with specific cancer types, therefore, holding potential as non-invasive biomarkers.[19–21] However, whether splicing factors are detectable in body fluids from patients with PCa remains unknown.

In this context, we recently demonstrated that the Serine And Arginine Repetitive Matrix 1 (SRRM1), the Small Nuclear Ribonucleoprotein U5 Subunit 200 (SNRNP200), and the Serine And Arginine Rich Splicing Factor 3 (SRSF3) are upregulated in PCa tissues (at mRNA and protein levels) and directly associated with tumor progression, while their silencing showed antitumor effects and sensitized CRPC-derived cells to enzalutamide treatment *in vitro*.[14] Nonetheless, despite the clear dysregulation of the expression of these key elements of the splicing machinery in PCa, their clinical potential remains to be fully elucidated. Therefore, the present study was aimed at determining, for the first time, whether SRRM1, SNRNP200, and SRSF3 could be detected in plasma from PCa patients and evaluating their potential role as non-invasive diagnostic and prognostic biomarkers, as well as therapeutic targets for PCa.

RESULTS

SRRM1, SNRNP200, and SRSF3 are detected in plasma samples, and their levels are elevated in patients with PCa compared with healthy control subjects

We determined the levels of SNRNP200, SRRM1, and SRSF3 in a cohort of plasma samples derived from healthy control subjects (n = 40) and PCa patients (n = 166). Demographic, biochemical, and clinical parameters of this human cohort are summarized in Table 1. Interestingly, we found that SRRM1 and SNRNP200 (Figures 1A and 1B), but not SRSF3 (Figure 1C), plasma levels were elevated in samples derived from patients with PCa vs. control subjects. Additionally, receiver operating characteristic (ROC) curve analyses revealed that SRRM1 and SNRNP200, but not SRSF3, plasma levels were able to significantly discriminate between PCa patients and controls (Figures 1D–1F, respectively). The presence of these splicing factors in human plasma samples was corroborated in a multicentric discovery cohort of 313 samples derived from healthy volunteers obtained from the PeptideAtlas database (2023-04-27).[22] Specifically, SRSF3 and SRRM1 were the splicing factors with the highest and lowest levels, respectively (Figure S1A).

Plasma levels of SRRM1, SNRNP200, and SRSF3 in PCa patients with adverse metabolic conditions

Certain adverse metabolic conditions such as diabetes and obesity have been reported to drastically influence the clinical utility of some biomarkers, including PSA.[3] For that reason, we investigated whether plasma SRRM1, SNRNP200, or SRSF3 levels might be influenced by concomitant metabolic conditions in PCa patients. Interestingly, we identified that plasma SRSF3 levels tended (p = 0.06) to be higher in diabetic compared to non-diabetic patients (Figure S2A), but not in obesity compared to normal-weight patients (Figure S2B), while no associations were found for SRRM1 or SNRNP200 levels when analyzing both metabolic conditions (Figures S2A and S2B).

SRRM1, SNRNP200, and SRSF3 are highly expressed and secreted by PCa cells

SRRM1, *SNRNP200*, and *SRSF3* mRNA levels were found to be highly expressed in PCa tissue from patients from The Cancer Genome Atlas (TCGA) (Figure S3A) and Stand Up to Cancer (SU2C) (Figure S3B) cohorts. Likewise, mRNA levels of *SRRM1*, *SNRNP200*, and *SRSF3* were prominent in our internal cohort of PCa tissues, being *SNRNP200* levels higher than those of *SRRM1* and *SRSF3* (Figure S3C). Interestingly, SRRM1, SNRNP200, and SRSF3 were also detectable in the secretion media of the PCa-derived LNCaP and 22Rv1 cells, being SRRM1 the splicing factor predominantly secreted by both PCa cell models (LNCaP and 22Rv1: SRRM1 > SNRNP200 > SRSF3; Figure 1G). Supporting these results, we found that SRRM1 exhibited the highest plasma/tissue level ratio (calculated by dividing plasma protein levels [pg/mL] by their patient-matched mRNA tissue expression normalized by a normalization factor [calculated from *ACTB* and *GAPDH* expression levels]), followed by SRSF3 and SNRNP200, in our internal human cohort

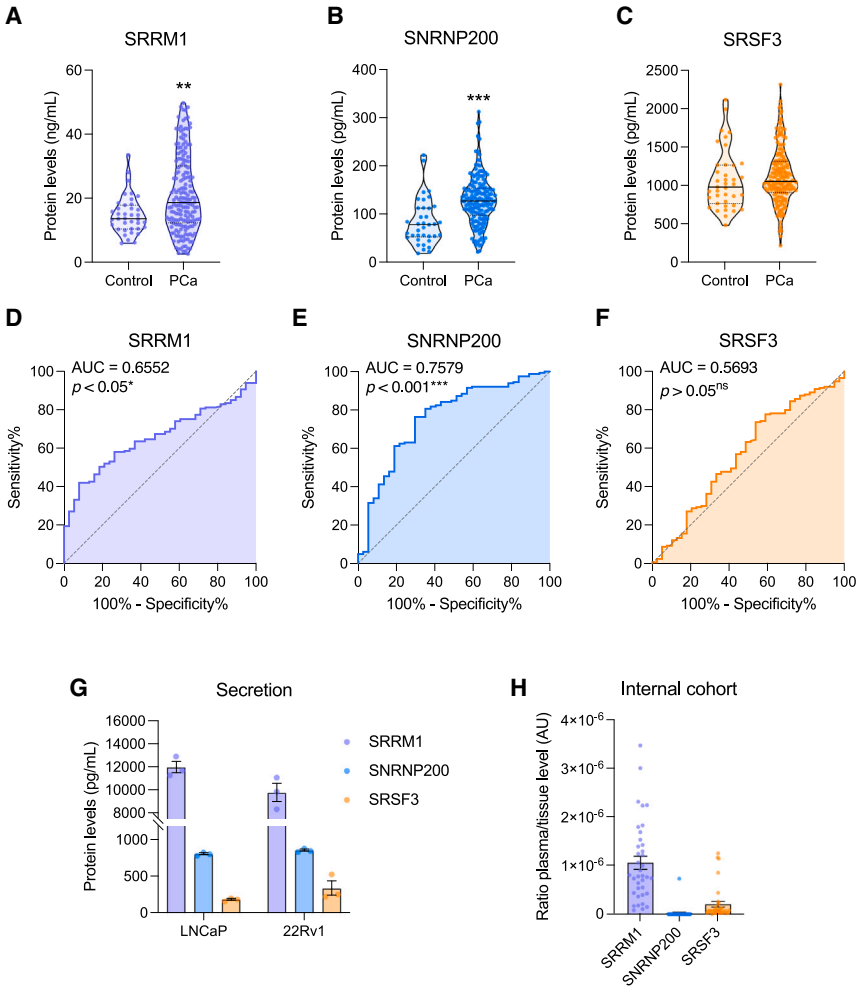

Figure 1. Protein levels of SRRM1, SNRNP200, and SRSF3 in plasma and extracellular media

(A–C) Comparison of plasma SRRM1 (A), SNRNP200 (B), and SRSF3 (C) protein levels between samples derived from control individuals (n = 40) vs. PCa patients (n = 166). Data represent the minimum to maximum boxplot, with median, of plasma protein levels. (D–F) ROC curves of plasma SRRM1 (D), SNRNP200 (E), and SRSF3 (F) protein levels to distinguish between PCa- and control-derived samples. Area under the curve (AUC) and p value are depicted in the plots. (G) Comparison of SRRM1, SNRNP200, and SRSF3 protein levels in LNCaP- and 22Rv1-derived secreted media. (H) Comparison of the ratio between plasma protein and mRNA tissue level of SRRM1, SNRNP200, and SRSF3 (n = 38). mRNA levels were adjusted by normalization factor (calculated from *ACTB* and *GAPDH* expression levels). Asterisks (**p < 0.01; ***p < 0.001) indicate statistically significant differences between groups, while ns indicates not significant associations between variables.

of samples (Figure 1H). Additionally, we found that SRRM1, SNRNP200, and SRSF3 were also detected in urine samples derived from PCa patients (internal cohort of samples), being SRRM1 the one to exhibit the highest protein levels (Figure S3D) and urine/tissue level ratio (Figure S3E; ratio calculated by dividing urine protein levels [pg/mL] by their patient-matched mRNA tissue expression [normalized by a normalization factor calculated from *ACTB* and *GAPDH* expression levels]), followed by SRSF3 and SNRNP200.

SRRM1 plasma levels are associated with ADT and AR activity in PCa patients

First, to further characterize these splicing factors in PCa, we analyzed their potential association with some relevant genomic aberrations commonly linked to splicing dysregulation in cancer.[9,23–27] Interestingly, tissue SRRM1 levels were consistently associated with *AR* gene copy-number alteration in TCGA and SU2C cohorts (Figures S4A and S4B), while *SRSF3* mRNA levels were associated with *MYC* gene amplification in TCGA cohort but not the SU2C cohort (Figures S4C and S4D). Despite that, data derived from the LNCaP-ADT repository showed no significant differences regarding *SRRM1* mRNA levels in response to AR modulation in LNCaP cells (Figure S4E).[28]

Second, we used treatment with systemic ADT for more advanced disease as a surrogate marker of PCa aggressiveness in comparison with patients with localized disease treated with prostatectomy. Thus, patients treated with systemic ADT tended to have higher plasma SRRM1 levels than those treated with prostatectomy (p = 0.06; Figure 2A, top). Conversely, patients with high plasma SRSF3 levels were less frequently treated with ADT, while no significant associations were observed for SNRNP200 plasma levels (Figure 2A, bottom and center, respectively).

Molecularly, SRRM1 plasma levels were positively correlated with matched tissue *AR* mRNA levels and AR activity (defined by a set of AR-regulated genes)[29] in our internal cohort of human samples (Figures 2B and 2C, top), while no correlation was found with SNRNP200 or SRSF3 plasma levels (Figures 2B and 2C, bottom and center, respectively). Furthermore, tissue *SRRM1* and *SNRNP200* mRNA levels were weak but positively correlated with AR activity in both TCGA cohort (Figure 2D, top and center) and the SU2C cohort (Figure S5A, left and center). Consistently, *SRRM1* and *SNRNP200* mRNA levels were also positively correlated with a set of 49 genes associated with resistance to hormonal blockade (ADT resistance score)[30] in both the TCGA cohort (Figure 2E, left and center) and the SU2C cohort (Figure S5B, left and center). However, the analysis of tissue *SRSF3* mRNA levels reported inconsistent molecular associations, showing a negative correlation with AR activity and ADT resistance in TCGA cohort

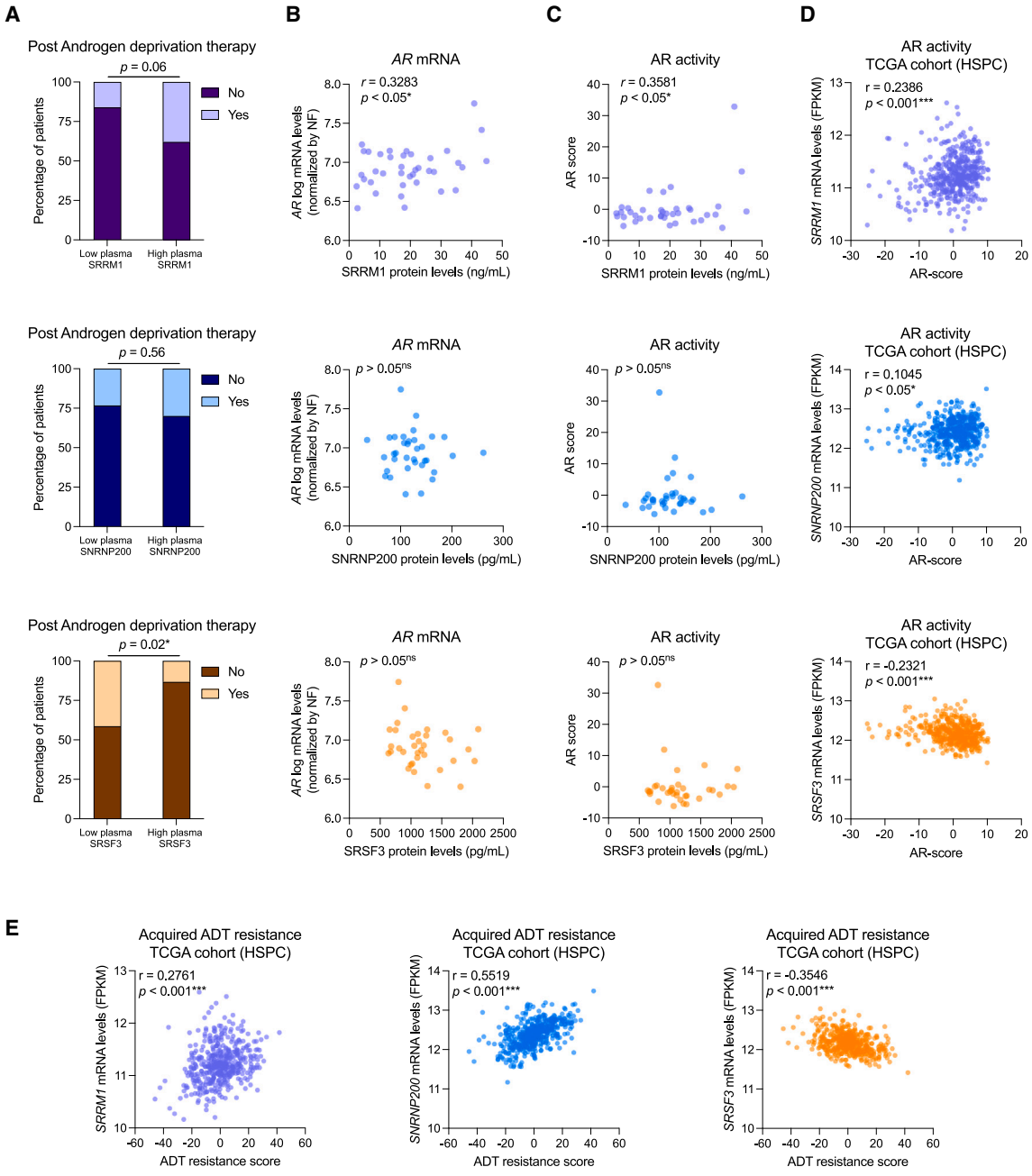

Figure 2. Associations of *SRRM1*, *SNRNP200*, and *SRSF3* levels with AR-related features of PCa progression

(A) Proportion of patients treated with ADT in patients with high/low plasma SRRM1 (top), SNRNP200 (center), and SRSF3 (bottom) protein levels. High/low groups were obtained by comparing Q4 vs. Q1 patients, respectively, according to plasma SRRM1, SNRNP200, or SRSF3 protein levels. (B and C) Correlation of *AR* mRNA tissue levels (B) and AR activity (C) with plasma SRRM1 (top), SNRNP200 (center), and SRSF3 (bottom) protein levels. Data were obtained from matched patients from whom we collected both plasma and tissue samples. mRNA levels were adjusted by normalization factor (calculated from *ACTB* and *GAPDH* expression levels). (D) Correlation of AR activity with tissue *SRRM1* (top), *SNRNP200* (center), and *SRSF3* (bottom) mRNA levels from TCGA cohort. (E) Correlation of ADT resistance score with tissue *SRRM1* (left), *SNRNP200* (center), and *SRSF3* (right) mRNA levels from TCGA cohort. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate statistically significant differences between groups, while ns indicates not significant associations between variables. ADT, androgen-deprivation therapy; HSPC, hormone-sensitive prostate cancer.

(Figures 2D and 2E, bottom and right, respectively), while a weak positive or non-significant correlation was found in the SU2C cohort (Figures S5A and S5B, right).

High SRRM1 plasma levels are associated with progression to CRPC

We then evaluated whether the levels of these elements of the splicing machinery were associated with the pathological progression from HSPC to CRPC. Notably, we first found that the expression levels of *SRRM1*, *SNRNP200*, and *SRSF3* were significantly elevated in CRPC vs. HSPC samples from Roudier cohort[31] (Figure 3A). Moreover, data derived from the liquid biopsy cohort showed that patients with higher SRRM1 plasma levels progressed earlier to CRPC (Figure 3B, top), while non-significant or inverse associations were observed for SNRNP200 and SRSF3 plasma levels, respectively (Figure 3B, center and bottom). Furthermore, we explored the potential association between the expression of these splicing machinery elements and the expression/activity of the oncogenic splicing variant AR-V7, tightly implicated in the resistance to ADT and progression to CRPC.[8] Specifically, when analyzing the SU2C cohort (cohort with available CRPC cases), we found that *SRRM1* and *SNRNP200* mRNA levels were positively correlated with the expression of *AR-FL* (canonical *AR* full-length variant; Figure S6A, left and center) and *AR-V7* (Figure 3C, top and center) and, most important, with the ratio of *AR-V7*/*AR-FL* (Figure 3D, top and center) and with AR-V7 activity (defined by a set of 59 genes associated with AR-V7 protein expression)[8] (Figure 3E, top and center). In the case of *SRSF3* mRNA levels, although a weak positive correlation was observed with AR-V7 activity (Figure 3E, bottom), no correlations were found with the *AR-V7*/*AR-FL* ratio (Figure 3D, bottom) or with *AR-FL* (Figure S6A, right), or *AR-V7* expression (Figure 3C, bottom). Hence, although both tissue *SRRM1* and *SNRNP200* mRNA levels were robustly associated with AR-V7 expression/activity and highly expressed in CRPC-derived samples, only SRRM1 plasma levels were associated with shorter CRPC-free survival, highlighting its role as a predictive biomarker in PCa.

Silencing of SRRM1 *in vivo* reduces the aggressiveness of PCa xenograft models

Based on the previous results, we decided to explore the potential role of SRRM1 as a therapeutic target for advanced PCa. To that aim, we generated 22Rv1-derived xenograft tumors in immunocompromised mice and treated them with *SRRM1*-targeting small interfering RNA (siRNA) *in vivo* once already formed (Figure 4A). A single injection of SRRM1 siRNA resulted in a significant reduction in tumor growth within 2 weeks of administration (Figure 4B). Validation of *SRRM1* silencing is shown in Figure 4C. The treatment with SRRM1 siRNA tended to reduce the number of mitosis and Ki67 index, although the differences did not reach statistical significance (Figures 4D and 4E). We next evaluated the consequences of SRRM1 modulation over AR signaling pathway activity, revealing that the silencing of SRRM1 significantly reduced *AR* and *AR-V7* expression levels (Figure 4F). Consequently, we also found that SRRM1 silencing altered the activity of both AR and AR-V7 by reducing the expression of relevant AR- and AR-V7-regulated genes, respectively (Figures 4G and 4H, respectively). Hence, these results reinforce the role that SRRM1 might play in the pathological progression from HSPC to CRPC, potentially by regulating the AR signaling axis.

DISCUSSION

PCa is a global health concern, ranking as the second most common cancer among men worldwide. While PSA is the gold standard diagnostic biomarker for PCa, there are decisive limitations for this diagnosis, including its low specificity.[32] To address these challenges, researchers are exploring innovative approaches to identify more reliable non-invasive biomarkers in the PCa field. In this context, PCa is one of the tumor pathologies whose development and progression is mostly influenced by the alteration of the normal gene expression pattern and the aberrant presence of oncogenic splicing variants (e.g., *AR-V7*, *In1-ghrelin*, *SST5TMD4*).[8–11] In fact, we recently demonstrated that the dysregulation of the molecular components belonging to the cellular machinery involved in the control of the splicing process (i.e., spliceosome components and splicing factors) might be responsible for the broad presence of oncogenic splicing variants observed in PCa (and other tumor pathologies), and linked to their development and progression,[14,33] suggesting that these components could represent a novel source for the identification of diagnostic, prognostic, and therapeutic targets in highly prevalent tumor pathologies.[34]

In this sense, SRRM1, SNRNP200, and SRSF3 are three elements of the splicing machinery reported to be implicated in the regulation of various biological processes, and they play a key role in several pathological conditions.[14,33,35] Specifically, SNRNP200 is part of the U5 small nuclear ribonucleoprotein particle (snRNP), integrated in the core of the spliceosome and participating in catalyzing the splicing process. However, SRRM1 and SRSF3 act as *trans*-acting splicing factors whose function is to recognize sequences within the pre-mRNA to finely regulate the recruitment and action of the spliceosome.[34] Indeed, our group has recently reported that SRRM1, SNRNP200, and SRSF3 are drastically upregulated in PCa tissue vs. non-tumor regions, which may position these factors as promising diagnostic biomarkers. Indeed, some studies have confirmed that the cellular localization of the elements of the splicing machinery might not be limited to intracellular regions but also extend to the cell membrane,[36] and even to be actively secreted by cancer cells to modulate in some cases the surrounding cell behavior into a pro-oncogenic state through an alteration of their splicing pattern.[19–21] Nonetheless, despite its dramatic relevance in a pathological context, this "secretory event" of key spliceosome components has been poorly described. Indeed, to date, there is no information regarding the presence or clinical value of circulating splicing factors in PCa patients. Therefore, in this study, we have identified for the first time that the splicing factors SRRM1, SNRNP200, and SRSF3 are detectable in plasma, being SRRM1 and SNRNP200 levels higher in samples derived from patients with PCa vs. healthy control individuals. Furthermore, we have demonstrated here that these factors are secreted by PCa cell lines, suggesting that prostate tumor tissues may be a source of

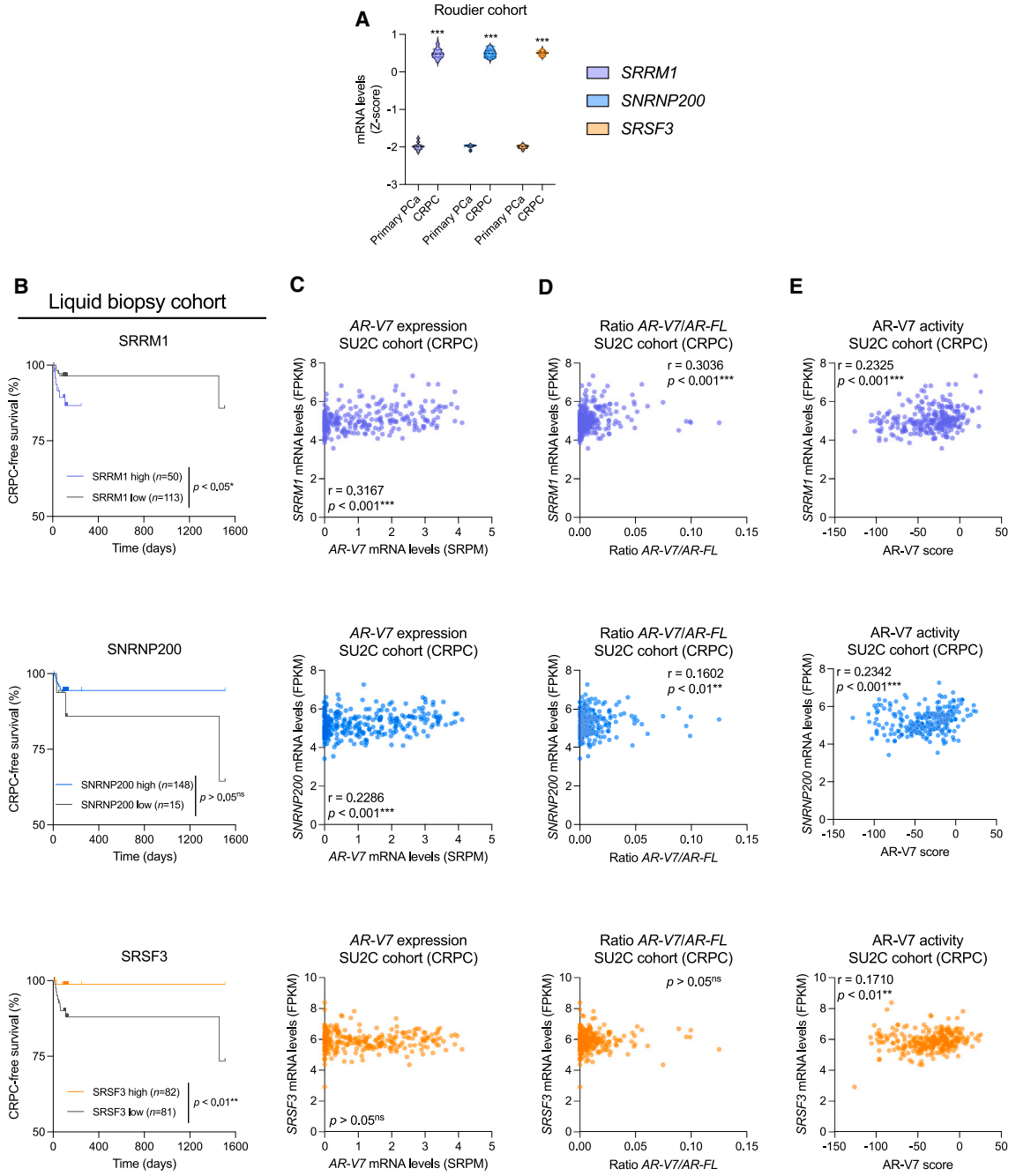

Figure 3. Association of SRRM1, SNRNP200, and SRSF3 levels with CRPC development

(A) Comparison of *SRRM1*, *SNRNP200*, and *SRSF3* mRNA levels between primary PCa and CRPC samples from Roudier cohort. (B) Association between CRPC-free survival and plasma SRRM1 (top), SNRNP200 (center), and SRSF3 (bottom) protein levels. High/low groups were obtained by Survminer package. (C–E) Correlation of tissue *AR-V7* expression levels (C), *AR-V7/AR-FL* mRNA ratio (D), and AR-V7 activity (E) with tissue *SRRM1* (top), *SNRNP200* (center), and *SRSF3* (bottom) mRNA levels from SU2C cohort. *AR-V7/AR-FL* ratio was obtained by dividing *AR-V7* SRPM counts to total AR full-length (*AR-FL*) fragments per kilobase million counts. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate statistically significant differences between groups, while ns indicates not significant associations between variables.

increased levels of SRRM1 and SNRNP200 observed in the plasma of patients with PCa, being also reinforced by the presence of these proteins in the urine of patients with PCa.

Apart from this overt diagnostic value, we have also reported that SRRM1 plasma levels are positively associated with the proportion of ADT-treated patients. Additionally, the circulating and mRNA

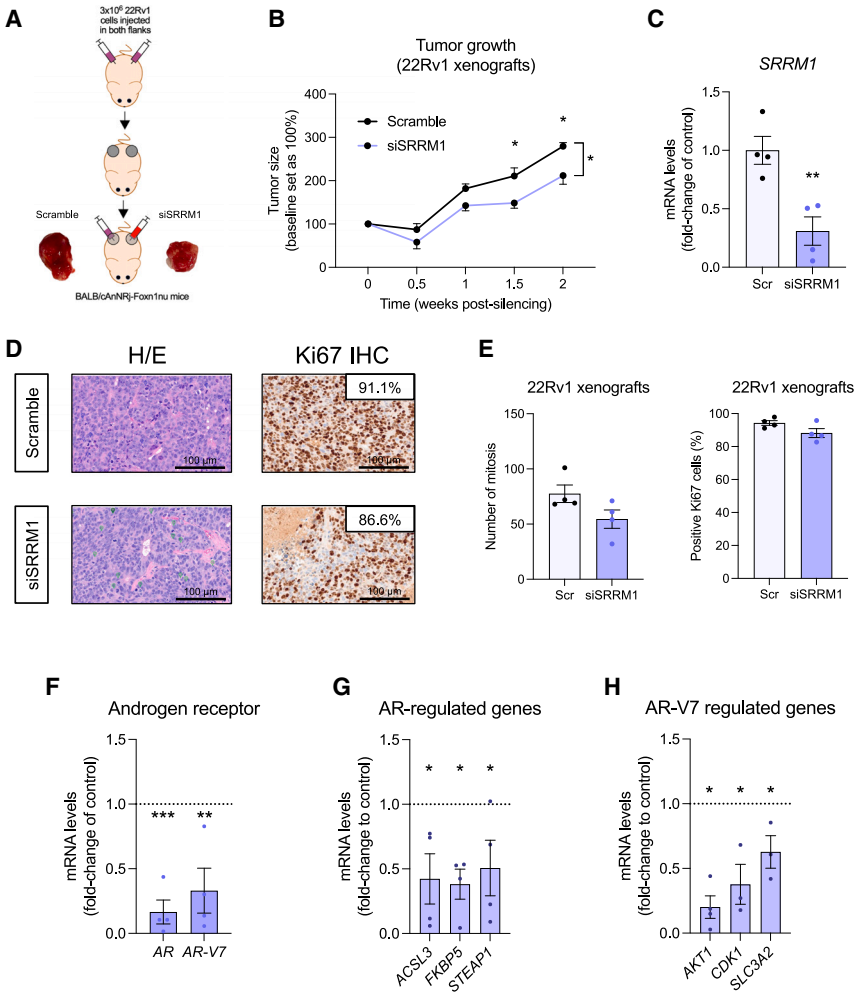

Figure 4. *In vivo* consequences of SRRM1 silencing

(A) Schematic representation of the *in vivo* tumor growth experiment in response to SRRM1 silencing. Representative images of endpoint tumors are depicted. (B and C) Comparison between the growth over time (B), *SRRM1* mRNA levels (C), and number of mitoses and %Ki67 index (D and E) of scramble- or siSRRM1-transfected 22Rv1-xenografted tumors. Representative images of H&E and Ki67 staining are depicted (D). (F–H) Comparison of the expression levels of *AR*, *AR-V7* (F), and AR- (G) and AR-V7- (H) regulated genes in response to *SRRM1* silencing *in vivo*. mRNA levels were adjusted by *ACTB* expression levels. Data are represented as mean ± SEM. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate statistically significant differences between groups.

levels of SRRM1 were positively correlated to the AR activity of PCa tissues, a key molecular determinant of PCa aggressiveness, and the clinical progression from localized HSPC to the highly aggressive CRPC.[37–39] Furthermore, the expression of *SRRM1* was positively correlated with a set of 49 genes (ADT resistance score) differentially expressed in response to hormone deprivation *in vitro* and was jointly associated with shorter time to biochemical recurrence in human PCa patients.[30] Thus, these results suggest that SRRM1 might be implicated in the phenotypical progression from HSPC to CRPC. Consistent with this, we identified that patients with higher levels of SRRM1 progressed earlier to a CRPC phenotype, and that CRPC patients express more *SRRM1* mRNA levels than HSPC patients. These results point to the promising value of plasma SRRM1 levels, not only as a diagnostic biomarker but also as a prognostic and predictive tool for ADT response. In this scenario, it is important to remark that the predictive value of PSA is inadequate to forecast the clinical response to hormonal blockade as shown by the CHAARTED trial, where 25% of the patients experienced clinical progression without PSA progression.[4,5] In this regard, several alternative non-invasive biomarkers and multivariate nomograms have been proposed to fill this clinical gap, such as cell-free DNA determination,[40] circulating tumor cells,[7,41] or microRNA determination,[42,43] among others. However, their impact on the patients has been limited due to their high cost and cumbersome translation to clinic in some cases, which may increase the relevance of plasma SRRM1's prognostic value for PCa patients as a cost-affordable alternative.

Apart from that, the expression levels of *SRRM1* were found to be significantly correlated with the expression and activity of the AR-V7, fundamentally involved in the progression to CRPC and the resistance to AR-targeting therapies (e.g., enzalutamide).[7,8,18] Notably, other members of the SRRM gene family (i.e., SRRM3 and SRRM4) have been described to be involved in the tumor progression to CRPC by promoting the generation of the neural isoform *REST4* from the *REST* gene,[13,44] as our group similarly reported for SRRM1.[14] Nonetheless, while SRRM3 and SRRM4 have been postulated to drive the cellular differentiation to a neuroendocrine-like AR^- PCa phenotype, we herein propose that SRRM1 may have a divergent role in promoting AR-persistent CRPC.

In a previous publication, we reported that SRRM1 silencing impacts the *AR* splicing process to reduce the *AR-V7*/*AR-FL* mRNA ratio, resensitizing CRPC cells to enzalutamide *in vitro*.[14] In this study, we expand these analyses using a more clinically relevant *in vivo* model and demonstrate that SRRM1 silencing diminishes the growth of PCa tumors *in vivo*, parallel to the downregulation of the expression of *AR*, *AR-V7*, and well-known AR- and AR-V7-regulated genes, which reinforce the potential of SRRM1 as a worth-to-explore therapeutic target for PCa.

Our results indicate that SRRM1, SNRNP200, and SRSF3 are detectable in human plasma samples and are secreted by PCa cells. Additionally, SRRM1 is consistently associated with the molecular

Table 2. Demographic, biochemical, and clinical parameters of patients with PCa used for tissue expression analyses

	PCa
Patients, *n*	38
Age, y, median (IQR)	60.5 (48–69)
PSA levels, ng/mL, median (IQR)	5.0 (2.6–15.3)
SRRM1 levels, ng/mL, median (IQR)	18.0 (2.5–44.8)
SNRNP200 levels, pg/mL, median (IQR)	116.1 (34.8–261.3)
SRSF3 levels, pg/mL, median (IQR)	1106.8 (637.1–2,092.6)
Recurrence, *n* (%)	12 (31.6)
Metastasis, *n* (%)	1 (2.6)
CRPC, *n* (%)	0 (0)

CRPC, castration-resistant prostate cancer; IQR, interquartile range; PCa, prostate cancer; PSA, prostate-specific antigen.

parameters of CRPC development such as AR and AR-V7 activity, which were abrogated by SRRM1 silencing *in vivo*. Therefore, SRRM1 could represent a promising non-invasive diagnostic, prognostic, and predictive biomarker, as well as an exploitable therapeutic target for PCa. Consequently, these data provide valuable new avenues to develop novel strategies to tackle this terrible tumor pathology. Additionally, this study may set the groundwork to further comprehensively screen the secretion of additional splicing factors by PCa cells as an attractive new dimension in the study of tumor biology with potential clinical implications.

MATERIALS AND METHODS

Human cohorts and samples

The present study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Reina Sofia University Hospital Ethics Committee. The regional biobank coordinated the collection, processing, management, and assignment of biological samples according to standard procedures. All patients provided written informed consent. Our study implicates two cohorts of human samples:

(1) Liquid biopsy cohort: healthy volunteers (n = 40) who donated blood samples and patients diagnosed with PCa (biopsy proven, n = 166), who also donated urine samples. Clinical and biochemical data are summarized in Table 1.
(2) Tissue cohort: consisting of formalin-fixed paraffin-embedded (FFPE) PCa tissues (n = 38), collected from matched patients from the liquid biopsy cohort diagnosed with clinically localized PCa who underwent radical prostatectomies. Clinical and biochemical data are summarized in Table 2.

Patients were collected between 2013 and 2015 by consecutive recruitment of individuals with suspicion of PCa who underwent a transrectal ultrasound-guided prostate biopsy according to clinical practice in the Urology Department of the Reina Sofia Hospital (Cordoba, Spain), and were clinically followed up until 2023. Blood and plasma samples were collected early in the morning after an overnight fast and just before the prostate biopsy. Tumor regions from the FFPE samples were identified by expert urologic pathologists as previously reported[11,16] and used to isolate RNA and perform gene expression analyses.

Clinical parameters were obtained in collaboration with the Urology Department of the Reina Sofia University Hospital. Progression to CRPC was defined as follows: (1) for patients treated with hormonal blockade, CRPC was considered for those who presented with three consecutive weekly interspaced increased PSA levels, two of those increments being 50% of nadir PSA and a PSA >2 ng/dL, with low testosterone levels (<50 ng/dL); and/or (2) two or more new bone metastases or radiologic progression according to Response Evaluation Criteria in Solid Tumors criteria.[45]

In addition, genomic and/or transcriptomic data from three additional human cohorts of PCa patients were acquired. Specifically, cBioPortal[46,47] was used to obtain data from TCGA (n = 545)[48] and from the SU2C/Prostate Cancer Foundation (n = 266)[37] cohorts, while GEO[49] was used to obtain data from Roudier (GEO: GSE74367; n = 56)[31] cohort. Moreover, proteomic data of human plasma samples were also obtained from the PeptideAtlas database (2023-04 build; n = 313).[22]

Cell culture

Cell lines derived from PCa (LNCaP and 22Rv1) were obtained from the American Type Culture Collection following the manufacturer's recommendations. Specifically, cells were cultured with RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and glutamine (2 mM) and were maintained in a humidified incubator with 5% CO_2 at 37°C. Cell line identity was validated by short tandem repeat sequences analysis. All cell lines were tested for mycoplasma contamination by PCR, as previously reported.[50]

Determination of protein levels by ELISA

Commercial ELISA kits were used to determine the plasma levels of SRRM1 (MBS7249770, MyBioSource), SNRNP200 (MBS1607214, MyBioSource), and SRSF3 (MBS762996, MyBioSource), following the manufacturer's indications. The sensitivity of these assays is 0.1 ng/mL, 7.5 pg/mL, and 0.1 ng/mL, respectively, while the detection ranges are between 0.2 and 10 ng/mL, 15 and 3,000 pg/mL, and 0.2 and 10 ng/mL, respectively. The intra-/inter-assay accuracy showed coefficients of variations lower than 8% and 10%, respectively. As previously mentioned, plasma samples were obtained through the regional biobank of the Andalusian Public Health System. Moreover, secreted media from serum-starved LNCaP and 22Rv1 cells was obtained as reported elsewhere.[51] Specifically, cultured cells were washed with PBS and serum-free media was added. After 24 h, secreted media was collected, centrifuged for 10 min at 850 × g, aliquoted, and stored at −20°C for future use. Before assaying, plasma and secreted media were thawed and centrifuged for 15 min at 1,000 × g, and supernatant was immediately used in the different ELISAs. Colorimetric values were measured following

manufacturer's indications using the FlexStation III system (Molecular Devices).

Preclinical models of PCa

Experiments with mice were carried out according to the European Regulations for Animal Care under the approval of the university/regional government research ethics committees. As previously reported,[33,51] to evaluate *in vivo* tumor growth in response to SRRM1 silencing, 10-week-old male athymic BALB/cAnNRj-Foxn1nu mice were subcutaneously grafted in both flanks with 3×10^6 viable naive 22Rv1 cells (n = 4 mice; n = 8 tumors) that were resuspended in 100 μL basement membrane extract and RPMI 1640 complemented with 10% FBS (1:1 ratio). Once the tumors reached 100 mm^3, each flank was transfected with scramble-control (AM4611, Thermo Fisher Scientific) or SRRM1-targeting siRNA (siSRRM1; s20018, Thermo Fisher Scientific) by using the AteloGene reagent (Koken), following the manufacturer's recommendations. Tumor size was determined twice per week using a digital caliper, as previously reported.[52] Two weeks after transfection, the animals were euthanized, and each tumor was processed and divided into specular fragments for (1) formalin fixation followed by paraffin inclusion and (2) storage at −80°C for later RNA extraction using TRIzol reagent (Thermo Fisher Scientific).

RNA extraction, retrotranscription, and real-time qPCR

RNA was isolated from FFPE samples, fresh tissues, and cell lines as previously described.[14,16] Briefly, RNA was extracted from FFPE samples by using the Maxwell 16 LEVRNA FFPE Kit (Promega) and the Maxwell MDx 16 Instrument (Promega), while TRIzol Reagent (Thermo Fisher Scientific) was used to isolate RNA from fresh tissues. RNA was treated with RNase-Free DNase Kit (Qiagen) to remove DNA. The Nanodrop One Spectrophotometer (Thermo Fisher Scientific) was used to determine the total RNA concentration and purity. cDNA was synthesized from total RNA using the cDNA First Strand Synthesis Kit (Thermo Fisher Scientific) and random hexamer primers. Real-time qPCR was performed using the Stratagene Mx3000p device with the Brilliant III SYBR Green Master Mix (Stratagene) as previously described.[53] Normalization was done using a normalization factor calculated with GeNorm 3.3 software[54] using *ACTB* and/or *GAPDH* expression levels, as previously reported.[14] The specific primers used in this study were designed as previously described,[14,16] and sequences can be found in Table S1.

Immunohistochemistry

As previously described,[33,51] the percentage of positive cells for Ki67 staining and the number of mitoses were examinated on samples from xenograft 22Rv1-derived tumors (scramble vs. siSRRM1 samples, n = 4 per group). Briefly, deparaffinized sections were incubated overnight (4°C) with anti-Ki67 antibody (PA0118; Leica Biosystems), followed by incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody. Finally, sections were developed with 3,3-diaminobenzidine (EnVision system, Agilent) and contrasted with H&E.

AR score, ADT resistance score, and AR-V7 score

Based on its function as a transcription factor, AR activity can be inferred by analyzing the expression of its downstream targets.[55] Thus, AR signaling activity (AR score) was determined as a sum of the ranked expression levels of nine canonical AR-regulated genes (*ACSL3*, *FKBP5*, *KLK2*, *KLK3*, *NKX3-1*, *PLPP1*, *RAB3B*, *STEAP1*, and *STEAP2*), as previously described.[29,56] The ADT resistance score was defined as a set of genes reported to be involved in the cellular resistance to ADT.[30] The AR-V7 score comprised 59 genes associated with AR-V7 protein expression.[8] A complete list of gene sets used in this study is included in Table S2.

Bioinformatic and statistical data analysis

At least three independent experiments were performed for all analyses ($n \geq 3$). Statistical differences between two groups were calculated using unpaired parametric t test and non-parametric Mann-Whitney U test, depending on normality, which was assessed by the Kolmogorov-Smirnov test. For differences among three groups, one-way ANOVA analysis was used. The chi-squared test was used to calculate differences between group proportions. For correlations, the Spearman coefficient was calculated. Statistical significance was considered when $p < 0.05$. A trend for significance was indicated when p values ranged between >0.05 and <0.1. All analyses were assessed using GraphPad Prism 8 (GraphPad Software) or Rstudio (version 2023.12.0 + 369, R version 4.3.2). Survminer (version 0.4.9) and survival (version 3.5-8) packages were used to identify the best cutoff values for Kaplan-Meier analyses.

DATA AND CODE AVAILABILITY

All data are available in the main text or the supplemental information.

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AUTHOR CONTRIBUTIONS

A.J.M.-H., J.M.J.-V., and R.M.L. were major contributors to the conception of the study and they designed the work. A.J.M.-H., E.G.-G., M.G.-C., F.P.-P., J.M.P.-G., M.O.-B., J.C.-V., L.C.-C., J.P.C.-H., T.G.-S., R.S.-S., and A.S.-C. acquired and/or analyzed the data. A.J.M.-H., E.G.-G., M.G.-C., L.C.-C., M.D.G., J.M.J.-V., and R.M.L. interpreted the data. A.J.M.-H., J.M.J.-V., and R.M.L. were major contributors to drafting and revising the work. All the authors have read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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