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Phosphorylation of Androgen Receptor Serine 81 is Associated with its Reactivation in Castration-Resistant Prostate Cancer

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Abstract

Phosphorylation of serine 81 (pS81) in the N-terminal transactivation domain of the androgen receptor (AR) has been linked to its transcriptional activation in prostate cancer (PCa) cell lines, but in vivo studies have been limited. Moreover, the role of pS81 in the reactivation of AR when tumors relapse after androgen deprivation therapy (castration-resistant prostate cancer, CRPC) has not been determined. In this study we validate a pS81 antibody for immunohistochemistry (IHC) and show it yields strong nuclear staining in primary PCa clinical samples and in the VCaP PCa xenograft model. Moreover, this staining was decreased at 7 days post-castration in VCaP xenografts, coinciding with markedly decreased AR transcriptional activity. Staining with the pS81 antibody then was restored when the VCaP xenografts relapsed, which was associated with restoration of AR transcriptional activity. Significantly, analysis of CRPC clinical samples, including tumors that had progressed during treatment with abiraterone, showed strong nuclear staining with the pS81 antibody. Together these findings indicate that AR reactivation in CRPC is associated with S81 phosphorylation, and suggest that IHC for pS81 may be useful as a biomarker of AR activity in CRPC.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

prostate cancer; androgen receptor; phosphorylation; immunohistochemistry; transcription

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1. Introduction

The androgen receptor (AR) pathway is the central axis in prostate cancer (PCa) development and therapy. Upon ligand induction, AR binds to specific genes and recruits transcription cofactors that mediate chromatin remodeling and transcriptional activation. Most metastatic PCa patients initially respond to androgen deprivation therapy (ADT), but they generally relapse within several years and develop castration-resistant prostate cancer (CRPC). AR is highly expressed in the majority of CRPC and many of these castration-resistant tumors respond to second-line ADT agents such as the CYP17A1 inhibitor abiraterone (which further suppresses androgen synthesis) and the competitive AR antagonist enzalutamide which directly binds and blocks AR activation. However, responses to these second-line therapies are not durable and mechanisms mediating resistance to these agents and driving AR activity in advanced CRPC remain to be established [1; 2; 3].

The AR undergoes phosphorylation at multiple sites in response to androgen [4; 5; 6; 7]. AR serine 81 (S81) is followed by a proline and is structurally embedded in a long polyglutamine (Poly-Q) stretch. In response to androgen, S81 is the most highly phosphorylated site on AR, while AR antagonists stimulate only low levels of phosphorylated S81 that correlate with their partial agonist activities [6; 8; 9; 10]. S81 phosphorylation occurs over a prolonged time course and its induction is dependent on chromatin binding, indicating that phospho-S81 (pS81) is coupled to transcriptional activation [6; 10; 11; 12; 13]. S81 is targeted for phosphorylation by the kinases CDK1 and CDK9, and together these two kinases co-sustain AR S81 phosphorylation [8; 13; 14; 15]. CDK9 (presumably in combination with cyclin T in the P-TEFb complex) phosphorylates S81 when it becomes bound to chromatin and interacts with the promoter, while CDK1 mediates S81 phosphorylation in the absence of androgen [8; 10; 13; 15].

Phosphorylation of serine 81 (pS81) in the N-terminal transactivation domain of the AR has been linked to its transcriptional activation in PCa cell lines, as mutation of this site impairs AR mediated activation of at least a subset of genes [10; 13; 15]. The in vivo significance of S81 phosphorylation is also supported by several studies that have used a pS81 antibody for immunohistochemistry (IHC), although the antibody specificity was not necessarily fully addressed [16; 17; 18; 19; 20]. More importantly, while it has become clear that AR transcriptional activity is substantially restored in CRPC, the role of S81 phosphorylation in driving this AR activity is not clear. In this study we have addressed this question by validating the sensitivity and specificity of an AR pS81 antibody for IHC, and then examining pS81 in a CRPC xenograft model and in clinical samples.

2. Materials and methods

2.1 Antibodies

The sources for the antibodies and control IgGs were as following: pS81 (Cat. 07–1375, EMD Millipore); AR (PG21, EMD Millipore, Cat. 06–680; N20, Cat. sc-816, Santa Cruz; AR-441, Cat. sc-7305, Santa Cruz); Histone 3 (H3, Cat. ab1791, Abcam); β -Tubulin (Cat. MAB3408, EMD Millipore); PSA (Meridian Life Science, Cat. K92110R); and normal rabbit IgG (Cat. sc-2027, Santa Cruz). The Dual-link PLA kit was from Sigma.

2.2 Peptide Competition Assay

The indicated peptides (AR-S81-P: Q Q Q E T [pSER] P R Q Q Q; AR-S81-C: Q Q Q E T S P R Q Q Q; AR-S650-P: A S S T T [pSER] P T E E T) were from Genscript USA. The peptides were suspended in solvent A (99.8% Water, 0.1% Acetonitrile and 0.1% TFA) and aliquots were stored at -80°C . For blocking, indicated folds (in molar concentration as compared to the primary antibody) of peptides were added to the primary antibody suspension, and then incubated by rotation at room temperature for 2 hours, followed by proceeding to IHC staining.

2.3 Immunohistochemistry (IHC)

FFPE sections were deparaffinization by baking at 60°C for 1 hr, followed by processing the slides sequentially in xylene $\rightarrow$ xylene $\rightarrow$ 100% ethanol $\rightarrow$ 95% ethanol $\rightarrow$ 80% ethanol $\rightarrow$ 70% ethanol $\rightarrow$ 50% ethanol, each for 3min; and then rinsing with tap water 2 $\times$ 5min. For nonenzymatic antigen retrieval and epitope recovery, slides were next boiled in 1xDivia Decloaker buffer (Biocare Medical #DV2004LX, MX) for 30 min, cooled at room temperature, and then rinse the slide with tap water. Endogenous peroxidase activity was then quenched by putting slides in 3% H_2O_2 in methanol for 10 min, followed by rinsing in tap water and blocking buffer (TBS/0.025% Triton X-100/5% BSA/1% normal goat serum) (Vector EliteABC kit) for 2 hrs. The pS81 antibody is preincubated in TBS/0.025% Triton X-100/5% BSA/ 5% non-fat milk/ 1% normal goat serum) (Vector EliteABC kit) for 2hrs prior to applying to slides. Slides are then incubated with primary antibody (1:2500 dilution, $\sim 25\text{ ng/ml}$) overnight at 4°C . Following incubation, slides are washed in TBS with 0.025% Triton X-100, then incubated with biotin-linked goat anti-rabbit secondary antibody (1:400) in TBS with 0.025% Triton X-100, 5% BSA, 5% non-fat milk, and 15 normal goat serum (Vector EliteABC kit) at room temperature for 2 hrs. After then washing the slides in TBS with 0.025% Triton X-100, they are incubated with the ABC reagent (Vector EliteABC kit) for 30–45 min, following by washing the slides in TBS buffer. Slides are then developed using the DAB kit (Cat. SK-4100, Vector) for up to 1–10 min, and then immersed in tap water to stop the reaction. Counter-staining was then conducted with 10% Hematoxylin (Sigma MHS32–1L) solution for 2min. Finally, slides were processed sequentially in 50% ethanol $\rightarrow$ 70% ethanol $\rightarrow$ 80% ethanol $\rightarrow$ 95% ethanol $\rightarrow$ 100% ethanol $\rightarrow$ xylene $\rightarrow$ xylene, each for 3 min, followed by mounting with nonaqueous mounting reagent (PERMASLIP; Alban Scientific).

pS81 and AR immunostaining were defined by moderate to strong, predominantly nuclear staining. PSA was defined by weak to strong cytoplasmic and membranous staining.

Immunointensity for all the above were scored as negative (0), weak (1), moderate (2), and strong (3), based on the most predominant intensity pattern. Percentage was scored on the basis of percentage of tumor cells demonstrating the most predominant intensity pattern or stronger as: 0 (negative), 1 (1%–9%), 2 (10%–49%), and 3 (≥ 50%). The immunoscore was based on the immunointensity score multiplied by the percentage score. Rapid autopsy specimens were obtained from an IRB approved protocol (#14–411) at Beth Israel Deaconess Medical Center.

2.4 Proximity Ligation Assay (PLA)

Proximity ligation assay (PLA) was performed following the manufacturer's instructions using the Duolink In Situ Detection Reagents Brightfield Kit (DUO92012–100RXN, Millipore Sigma), Duolink In Situ PLA Probe Anti-Rabbit PLUS (DUO92002–100RXN), and Duolink In Situ PLA Probe Anti-Mouse MINUS (DUO92004–100RXN). For PLA of pS81, the anti-pS81 antibody was used at 1:50 dilution and anti-AR (AR441) antibody (ab9474, Abcam) was used at 1:10 dilution. For PLA of total AR, anti-AR (PG-21) antibody (#06–680, Millipore Sigma) was used at 1:50 dilution and anti-AR (AR441) antibody (ab9474, Abcam) was used at 1:10 dilution.

2.5 Cell culture and qRT-PCR analysis

LNCaP were obtained from ATCC and grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Cell identity was validated by STR analysis, and were negative for Mycoplasma. For androgen-starving conditions, cells were grown in medium containing 5% charcoal-dextran treated FBS (CDS). RNA isolation was carried out using the TriZOL reagent (Ambion) and the qRT-PCR analysis on gene expression was performed with the TaqMan One-Step RT-PCR Master Mix Reagents (Cat. 4309169; Applied Biosystems). The TaqMan primer-probe sets for KLK3/PSA (FAM labelled, Cat. PN4351370), KLK2 (FAM labelled, Cat. Hs00428384_g1), and the internal control GAPDH (VIC-TAMRA labelled, Cat. 4310884E) transcripts were purchased as inventoried mix from Applied Biosystems.

2.6 Cytoplasmic and nuclear fractionation

The assay was performed with the Subcellular Protein Fractionation Kit (Cat. 78840, Pierce), following the manufacturer's directions.

2.7 Animal xenografts

VCaP cells were from ATCC (Manassas, VA) and used for subcutaneous xenograft injections within 4 passages. To generate VCaP xenografts, 6 week old male ICR scid mice (Taconic Biosciences) were injected subcutaneously with 5 million VCaP cells in 100% Matrigel. Xenografts were grown until 1000 mm³, then mice were castrated (Cx). Tumors were serially biopsied prior to Cx (Pre-Cx), ~7 days after castration, and at tumor relapse (CRPC). All animal experiments were approved by Beth Israel Deaconess Medical Center (BIDMC) Institutional Animal Care and Use Committee (IACUC) and were performed in accordance with institutional and national guidelines.

3. Results

3.1 S81 phosphorylation is linked to AR functional activation

The serine 81 residue of AR is a proline-directed phosphorylation site surrounded by poly-glutamine stretches that has pronounced surface accessibility (Fig. 1A). Although most AR residues become maximally phosphorylated within 2 hours of androgen stimulation, Ser-81 phosphorylation (pS81) occurs over a much longer duration [6; 7; 8]. Androgen-dependent LNCaP cells exhibit very low levels of basal pS81 in the absence of exogenous androgens (cultured in androgen-depleted medium containing charcoal/dextran stripped serum, CDS), and show a dose-dependent increase in pS81 expression upon androgen stimulation (Fig. 1B). Cell fractionation assays further reveal that 1 nM DHT induces nuclear localization and chromatin binding of pS81, and that there is enrichment of pS81 AR versus total AR in the chromatin bound fraction (Fig. 1C). The dose-dependent induction of pS81 was well-correlated with AR transactivation as assessed by increased PSA (KLK3) mRNA (Fig. 1D).

3.2 pS81 antibody shows specific nuclear immunostaining in primary PCa clinical samples

The affinity-purified polyclonal antibody against pS81 has been previously shown to specifically bind the phosphorylated form of Ser81 in AR via western blotting of LNCaP cell lysates, but its specificity for IHC has not been addressed [15]. Therefore, to assess the expression and cellular localization of pS81 in PCa clinical samples, we initially focused on validation of the pS81 antibody against pS81 for IHC. To assess the specificity of the antibody in the IHC setting, we used peptide competition assays with the pS81-specific peptide versus several control peptides. As shown in Figure 2, the pS81 peptide attenuated the pS81 antibody immunostaining in a dose-dependent manner both in normal prostate (upper) and PCa (lower) clinical samples. In contrast, control peptides (S81-C-P that mimics the unphosphorylated Ser81 site and pS650-P that corresponds to a distinct AR pSer-Pro site in the AR hinge region) failed to block staining even at the highest concentration used. In addition, compared to IHC with an anti-AR antibody against total AR, the pS81 antibody also gave a more specific nuclear distribution pattern in primary PCa. These findings are consistent with the observation that Ser81 phosphorylated AR is preferentially distributed into the nucleus (see Fig. 1C) [12]. Together, these studies support the specificity of the pS81 antibody for IHC.

3.3 Nuclear pS81 expression by IHC correlates with AR transactivation in VCaP xenografts

While peptide the peptide blocking supported the antibody specificity, it did not rule out cross-reactivity with other nuclear proteins. Therefore, we next took steps to further assess the specificity of this pS81 antibody by immunostaining PCa samples under androgen deprivation conditions. We examined total and pS81 AR by IHC in VCaP xenografts (which express high levels of AR) prior to castration, 7 days following castration (when AR transcriptional activity is reduced), at relapse at ~6 weeks after castration when AR activity is restored (CRPC), and following the development of resistance to dual treatment with the androgen deprivation agents abiraterone and enzalutamide when AR activity is also restored [21; 22]. In the pre-castration xenografts, the AR and pS81 antibodies showed predominant

nuclear staining, consistent with high levels of androgen driving AR nuclear localization and AR transactivation (Fig. 3A, top panels). In xenografts harvested 7 days after castration where androgens levels have significantly decreased, there is a shift towards cytoplasmic distribution for total AR, and decreased intensity and positivity of nuclear pS81 (Fig. 3A, 2nd panels and Fig. 3B, left and middle panels), consistent with a decrease in AR signaling. This was associated with a significant decrease in expression of the AR regulated PSA (KLK3) protein (Fig. 3B, right panel). When the VCaP xenografts relapse (CRPC), there is an increase in nuclear pS81 staining that is associated with substantial restoration of PSA protein expression (Fig. 3A, 3rd panels). When these CRPC tumors become resistant to dual abiraterone/enzalutamide treatment they also express significant nuclear pS81 staining and PSA (KLK3) protein (Fig. 3A, bottom panels).

To further assess the specificity of the polyclonal antibody against pS81 used in the above studies, we performed pS81 IHC on sections of VCaP xenografts and compared them to xenografts generated from PC3, a PCa cell line that is AR-deficient. As shown in Figure 4, the pSer81 antibody showed nuclear positivity in the VCaP xenografts. However, the pSer81 antibody also gave rise to weak nuclear positivity in the PC3 xenografts. Significantly, in contrast to the diffuse nuclear staining in VCaP cells, the nonspecific staining in the PC3 xenografts is observed in the condensed chromatin found in mitotic figures. There are multiple proteins that become phosphorylated during M-phase, and we hypothesize that this is the basis for the apparent weak cross-reactivity. However, this can be clearly distinguished from the diffuse nuclear staining in VCaP.

3.4 Specificity of pS81 immunostaining can be further enhanced by proximity ligation assays

We next examined whether the proximity ligation assay (PLA, which is based on dual-antigen recognition) could be used to improve the specificity of the rabbit polyclonal pS81 AR antibody by combining it with the AR441 mouse monoclonal antibody (which recognizes another site in the AR N-terminal domain). We first used AR441 in combination with the PG21 rabbit polyclonal antibody (generated against the extreme AR N-terminus), which resulted in a clear PLA signal of total AR in VCaP and not in PC3 xenografts (Fig. 5A, left panels). Combining AR441 with the pS81 antibody also generated a PLA signal specifically in the VCaP xenograft, although it was weaker than with the PG21 antibody (consistent with only a small fraction of AR being S81 phosphorylated). Significantly, the PLA signal for pS81 is negative in the PC3 xenografts (Fig. 5A, middle panels). In addition, treatment with the AR441 or pS81 antibodies alone did not give a PLA signal (Fig. 5A, right panels). Figure 5B shows a larger section of the VCaP xenograft analyzed by PLA with AR441 in combination with PG21 (left panel) or the pS81 antibody (right panel).

3.5 Nuclear expression of S81 phosphorylated AR in CRPC

We next used the pS81 antibody to examine AR in clinical CRPC tumor biopsies. AR IHC showed diffuse staining in the cytoplasm and nucleus, while the pS81 antibody showed intense nuclear staining (Fig. 6A, upper panels). Examination of tumor biopsies from 3 patients who were progressing on treatment with abiraterone plus dutasteride ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01393730) identifier NCT01393730) also showed intense nuclear pS81 (Fig. 6A,

lower panels). This result is consistent with our previous transcriptome analysis from patients on this trial showing persistent AR transcriptional activity [23]. The nuclear localization of the Ser81 phosphorylated form of AR in clinical specimens is consistent with a role for Ser81 phosphorylation in AR transactivation and extends this role to the castration-resistant clinical setting.

Similar results were seen in multiple metastatic sites from two rapid autopsy cases of heavily-treated, advanced CRPC (#11 and #12) (Fig. 6B). Interestingly, while total AR immunostaining was lower in case 11, pS81 relative to total AR immunostaining was higher, which may indicate increased AR activity and is consistent with similar PSA immunostaining in the two cases (Fig. 6B). These results indicate that pS81 immunostaining might be a more sensitive indicator of AR activity than nuclear AR immunostaining. Overall these results show that nuclear pS81 is highly expressed in PCa clinical samples, that it correlates with AR transcriptional activity, and is a potential biomarker of AR activity in tumors that are resistant to castration and second generation AR targeted therapies.

Discussion

S81 is the highest stoichiometric phosphorylation site on the AR in response to hormone and its phosphorylation correlates with AR transcription of the PSA gene [6; 7; 8]. Previous studies have shown that S81 undergoes phosphorylation in response to androgen-stimulated chromatin binding, and that pS81 enhances AR chromatin binding and transcriptional activity [4; 6; 8; 10; 11]. S81 phosphorylation may increase binding of Pin1, a peptidylprolyl cis/trans isomerase that is thought to broadly alter AR conformation [24]. Previous reports also indicated pS81 mediates an AR interaction with the coactivator p300, and thereby induces the interaction of AR with BRD4 and the P-TEFb complex on the chromatin [15; 25]. These observations support a mechanism in which pSer81 is a marker and mediator of AR nuclear activation, but its role in advanced CRPC has not been established.

In this report, we used a pS81 antibody to assess for phosphorylation of this site in clinical advanced CRPC samples. This affinity-purified pS81 AR antibody has been used previously for western blotting and chromatin immunoprecipitation (ChIP) assays [8; 10; 15], and has been shown to react with pAR-Ser81 specific peptide in western blotting based on peptide competition [15]. Here we established the specificity of the antibody for pS81 in the context of IHC by several criteria including peptide competition and by showing a decrease in immunostaining after castration in VCaP xenografts. Moreover, pS81 immunostaining was subsequently increased when these xenografts recurred as CRPC, and this was associated with restoration of AR transcriptional activity.

We then found intense nuclear staining for pS81 in CRPC clinical samples. This included samples from three patients who were progressing on a clinical trial of abiraterone plus dutasteride, where we showed previously by RNA-seq that AR was transcriptionally activated [23]. Using the PLA technique that is based on dual recognition of total AR and pS81-AR, we confirmed pS81 staining in the VCaP xenograft. However, while we could obtain a PLA signal in VCaP cells, which have an amplified AR gene and express very high levels of AR, we have not yet had adequate sensitivity to detect pS81 by this method in

clinical samples. Therefore, we cannot yet entirely rule out cross-reactive staining as we could detect weak nonspecific nuclear staining in PC3 xenografts. However, the pattern of this nonspecific nuclear staining is distinct and weak compared to the specific staining when the antibody is appropriately titrated.

Taken together, our results indicate that S81 phosphorylation is associated with AR reactivation in CRPC, and that pS81 IHC may be a useful biomarker of AR activity in PCa. However, the application of pS81 IHC alone as a surrogate marker of AR transcriptional activity should be interpreted with caution and include appropriate titrations and controls as background staining can occur and may vary based on the precise experimental conditions. The specificity of pS81AR IHC can be validated by PLA, but further improvements may be needed to enhance sensitivity in clinical samples. An independent pS81 monoclonal antibody may be able to provide further sensitivity and specificity, but is not yet available.

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Highlights

Ser81 phosphorylated (pS81) AR is nuclear by IHC in primary prostate cancer (PCa)

pS81 is decreased after castration concurrent with loss of AR activity

pS81 is highly expressed in castration-resistant PCa model and clinical samples

AR reactivation in castration-resistance is associated with S81 phosphorylation

Figure 1. Expression of AR S81 phosphorylation is correlated with androgen levels and AR functional activities.

(A), AR protein surface accessibility prediction by the scansite program (<http://scansite3.mit.edu>). Highlighted are the amino acids in the vicinity of S81 – Proline 82) site that is embedded in a polyglutamine (Poly-Q) stretch, which exhibits outstanding surface accessibility. (B–C) LNCaP cells in androgen-depleted medium were treated with various doses of DHT for 24hrs and harvested for blotting (B) and subjected to cellular fractionation assay (C), respectively. Ct: cytosolic fraction; Nu: nuclear soluble fraction; Ch: chromatin-bound fraction. (D) LNCaP cells in androgen-depleted medium were treated with indicated doses of DHT for 24 hours and then subjected to RNA isolation and real-time qRT-PCR analysis of PSA expression.

Figure 2. Peptide competition assay to validate pS81 AR antibody in immunohistochemistry (IHC) assay in primary PCa.
IHC staining of normal prostate (above) or primary PCa (below) with anti-AR (N20) and anti-pS81 antibodies (at 1:2,000 dilution), without or with peptide inhibition (1–20-fold molar excess). pS81-P: phosphorylated Ser81 AR peptide; S81-C-P: non-phosphorylated Ser81 AR control peptide; pS650-P: phosphorylated Ser650 AR peptide.

Figure 3. pS81 staining intensity positively correlates with AR transactivation as determined by PSA expression.

VCaP xenografts were serially biopsied prior to castration (Pre-Cx, n=7), 7 days Post-Cx (7 Days Post-Cx, n=3), when tumors progressed (CRPC, n=4), and when tumors became resistant to dual therapy with abiraterone and enzalutamide (Abi/Enza Resistant, n=4). (A) Immunohistochemistry of pS81, full-length AR, and PSA protein in the serial biopsies of representative tumors are shown. (B) Immunoscoring was performed as described in materials and methods. * = $P < 0.03$, Mann-Whitney U.

pS81

Pre-Cx VCaP

PC3

1:2,500

Figure 4. pS81 antibody shows weak non-specific nuclear staining in PC3 xenografts. IHC with pS81 antibody was performed on sections from VCaP or PC3 xenografts. Pre-Cx VCaP and PC3 sections were on the same slide and thus were internally controlled for antibody and DAB development exposure times. High power images of pS81 immunohistochemistry shows a predominantly diffuse nuclear pattern in VCaP versus staining in the condensed chromatin structures of mitotic figures in PC3.

Figure 5. Comparison of pS81 IHC staining and AR-pS81 proximity ligation assay (PLA) in PCa xenografts.

(A), PLA of VCaP and PC3 (AR-deficient, as negative control) xenografts. For PLA the antibodies were: PG21 (1:50), a rabbit polyclonal antibody recognizing the AR N-terminus; pS81 (1:50); AR441 (1:10), a mouse monoclonal antibody recognizing aa 299–315 of AR.

(B), a low power field of a larger area as compared to (A) of the PLA staining on VCaP xenografts.

Figure 6. Nuclear expression of pS81 in clinical CRPC.

(A) AR(N20) and pS81 staining of biopsy cores from patients with metastatic CRPC prior to abiraterone intervention (upper panels) or from patients treated with abiraterone plus dutasteride who were biopsied at progression ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01393730) identifier NCT01393730) (lower panels). (B) AR(N20) and pS81 staining of multiple metastatic sites from two rapid autopsy cases of heavily-treated CRPC (Left). Immunoscore was performed as described in Materials and Methods (Right). * = $P < 0.05$, Mann Whitney U.