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Androgen receptor phosphorylation at serine 515 by Cdk1 predicts biochemical relapse in prostate cancer patients

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Background: Prostate cancer cell growth is dependent upon androgen receptor (AR) activation, which is regulated by specific kinases. The aim of the current study is to establish if AR phosphorylation by Cdk1 or ERK1/2 is of prognostic significance.

Methods: Scansite 2.0 was utilised to predict which AR sites are phosphorylated by Cdk1 and ERK1/2. Immunohistochemistry for these sites was then performed on 90 hormone-naïve prostate cancer specimens. The interaction between Cdk1/ERK1/2 and AR phosphorylation was investigated *in vitro* using LNCaP cells.

Results: Phosphorylation of AR at serine 515 (pAR^{S515}) and PSA at diagnosis were independently associated with decreased time to biochemical relapse. Cdk1 and pCdk1¹⁶¹, but not ERK1/2, correlated with pAR^{S515}. High expression of pAR^{S515} in patients with a PSA at diagnosis of ≤ 20 ng ml⁻¹ was associated with shorter time to biochemical relapse ($P=0.019$). This translated into a reduction in disease-specific survival (10-year survival, 38.1% vs 100%, $P<0.001$). *In vitro* studies demonstrated that treatment with Roscovitine (a Cdk inhibitor) caused a reduction in pCdk1¹⁶¹ expression, pAR^{S515} expression and cellular proliferation.

Conclusion: In prostate cancer patients with PSA at diagnosis of ≤ 20 ng ml⁻¹, phosphorylation of AR at serine 515 by Cdk1 may be an independent prognostic marker.

An estimated 899 000 men were diagnosed with prostate cancer worldwide in 2008 with most cases in developed countries (Ferlay *et al*, 2010). Incidence is increasing and expected to rise 69% by 2030 in the United Kingdom (Mistry *et al*, 2011). Prostate cancer therefore represents a considerable economic burden. Treatment decision-making in prostate cancer is increasing in complexity, but in general patients with PSA < 20 ng ml⁻¹ at diagnosis are offered immediate or delayed radical treatment dependent on life expectancy, biopsy result and imaging. However, not all patients harbour disease that requires treatment. Conversely, some patients

undergo treatment delay following a period of surveillance creating an opportunity for occult aggressive disease to progress. Currently, there are no reliable diagnostic tools to differentiate indolent from aggressive cancers. This results in both over- and under-treatment problems with consequent patient-related morbidity and mortality and substantial economic cost.

The molecular mechanisms underlying the natural history of prostate cancer remain elusive. However, it is accepted that prostate cancer cell growth and survival are exquisitely dependent upon activation of the androgen receptor (AR) by androgens.

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Following ligand binding, AR undergoes further phosphorylation at serine residues (Kuijper and Brinkmann, 1995; Gioeli and Paschal, 2012). Such phosphorylation is thought to inhibit proteolytic degradation and stabilise AR homodimers (Blok *et al.*, 1998). Phosphorylation of AR may also influence transactivation of the AR since AR transcriptional activity correlates strongly with phosphorylation of specific serine residues (Blok *et al.*, 1998). Phosphorylation may be seen as a mechanism that generates changes within the specific domains that affect AR function. Although each of the major AR domains contains at least one phosphorylation site, the majority are located in the N-terminal domain (NTD), which is important for AR transactivation, including Ser-81, Ser-94, Ser-308 and Ser-515 (Gioeli and Paschal, 2012). In addition, Ser-650 is located in the hinge region, which regulates nuclear localisation, DNA binding and co-activator recruitment (Zhou *et al.*, 1995; Gioeli *et al.*, 2006; Gioeli and Paschal, 2012). Androgen receptor Ser-515 phosphorylation in particular has been linked to nuclear-cytoplasmic shuttling (Ponguta *et al.*, 2008), while Ser-81 phosphorylation has been shown to mediate chromatin binding and transcriptional activation (Chen *et al.*, 2012). It is therefore plausible that alterations in AR phosphorylation may drive prostate carcinogenesis. However, few studies have explored the significance of AR phosphorylation in prostate cancer in the clinical setting.

Several kinases have been associated with established AR phosphorylation (Zhou *et al.*, 1995; Wong *et al.*, 2004; Gioeli and Paschal, 2012). In particular, cyclin-dependent kinase 1 (Cdk1) and the mitogen-activated protein kinases also known as extracellular signal regulated kinase 1 and 2 (ERK1/2) have been associated with AR phosphorylation at various serine sites (Chen *et al.*, 2006; Shigemura *et al.*, 2009; Gioeli and Paschal, 2012). However, conflicting data exists over the responsible kinase(s) and functional consequences of phosphorylation at each site. Upregulation of the ERK1/2 pathway has been associated with development of castrate-resistant prostate cancer and reduced disease-specific survival (Mukherjee *et al.*, 2011). ERK1/2-induced phosphorylation of the AR at serine 515 has been demonstrated to hyper-sensitise LNCaP cells to low levels of synthetic androgens, increase AR transactivation, facilitate recruitment of AR cofactors and increase prostate cancer cell growth (Bakin *et al.*, 2003). Cdk1 is reported to be upregulated in prostate cancer tissue (Kallakury *et al.*, 1997) and phosphorylation of AR at serine 81 *in vitro* by Cdk1 is associated with prevention of AR degradation, thereby increasing AR stability and resulting in increased AR protein expression (Chen *et al.*, 2006). However, cell line studies that investigated mutagenesis at serine 81 indicate that phosphorylation at this site alone does not drive AR transcriptional activity or stabilisation, suggesting that Cdk1 may mediate these effects on the AR through phosphorylation at multiple sites (Zhou *et al.*, 1995; Chen *et al.*, 2006; Gioeli *et al.*, 2006).

In the current study, we aim to determine whether AR phosphorylation at Cdk1 and ERK1/2 consensus sites is associated with clinico-pathologic parameters and outcome in a cohort of hormone-naïve prostate cancer patients. It is hypothesised that AR phosphorylation at certain sites may be associated with disease progression and the knowledge of such may help to inform treatment decision-making.

MATERIALS AND METHODS

Patients. Ninety patients with hormone-naïve prostate cancer samples available were recruited at the Glasgow Royal Infirmary between 1992 and 2002. Last date of follow-up was 11/01/2012. Patients gave written consent. Clinical data included age, Gleason score, tumour lymphovascular invasion (LVI), serum PSA levels at

diagnosis, biochemical recurrence, serum PSA at biochemical recurrence and presence of metastases. Patients were considered to have biochemical recurrence dependent on treatment; radical prostatectomy serum PSA $>0.2 \text{ ng ml}^{-1}$, radical radiotherapy serum PSA of 2.0 ng ml^{-1} above the post-treatment nadir level, hormone treatment 2–3 consecutive rises in serum PSA levels above the nadir obtained at intervals of >2 weeks (Roach *et al.*, 2006; Cookson *et al.*, 2007). Study end points were biochemical relapse, survival from biochemical relapse and disease-specific survival. West of Scotland Research Ethics Committee approved the study (reference: 05/S0704/94).

Identification of kinases mediating AR phosphorylation. Scan-site 2.0 was utilised to predict which sites on the AR would be phosphorylated by Cdk1 and ERK1/2 (Obenauer *et al.*, 2003). The search was conducted using the protein ID 'ANDR_HUMAN' (accession number: P10275).

Tissue microarray construction. Three 0.6 mm^2 cores of prostate cancer tissue, identified by a uropathologist, were removed from formalin-fixed paraffin-embedded blocks. Recipient array blocks were constructed in triplicate. Control cores of normal prostate, colon, breast, pancreas, tonsil, kidney, liver and lung tissue were included in each tissue microarray (TMA).

Immunohistochemistry. Immunohistochemistry (IHC) was conducted in triplicate on aforementioned TMAs for the following proteins: Cdk1, Cdk1 activated through Thr-161 (pCdk1¹⁶¹) phosphorylation, ERK1/2 activated through Thr-202 and Tyr-204 (pERK1^{202/204}) or Thr-185 and Tyr-187 (pERK2^{185/187}) phosphorylation, Ki67 (proliferation index), AR and AR phosphorylated at Ser-81 (pAR^{S81}), Ser-94 (pAR^{S94}), Ser-308 (pAR^{S308}), Ser-515 (pAR^{S515}) and Ser-650 (pAR^{S650}). Tissue microarrays were dewaxed in xylene and rehydrated through graded alcohol. The AR antigen retrieval was performed in DakoCytomation Target Retrieval Solution (Dako UK Ltd., Ely, UK) pre-heated water bath, 99 °C, 20 min. pERK1/2 antigen retrieval was performed in pH9 Tris-EDTA buffer (10 mM Trizma Base, 0.25 mM EDTA), 96 °C, 20 min. Ki67 antigen retrieval was performed using heat treatment under pressure in citrate buffer pH 6, 5 min. Antigen retrieval for remaining proteins was performed using heat treatment under pressure in Tris-EDTA buffer (5 mM Trizma Base, 1 mM EDTA, pH 8), 5 min. Sections cooled in buffer for 20 min before washing in 3% H₂O₂. Sections blocked using 5% horse serum (10% casein for pERK1/2) in Tris-buffered saline (TBS). Antibodies for Cdk1 (#ab18; Abcam, Cambridge, UK), pAR^{S308} (#sc-26406-R; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and Ki67 (#F0788; Dako UK Ltd.) were incubated for 1 h, 25 °C diluted at 1 : 200, 1 : 75 and 1 : 150, respectively. Antibodies for pCdk1¹⁶¹ (#ab47329; Abcam), pERK1/2 (pERK1^{202/204}, pERK2^{185/187}) (#9101; Cell Signaling Technology Inc., Danvers, MA, USA), AR (#M3562; Dako UK Ltd.), pAR^{S81} (#07-1375; Merk Millipore, Billerica, MA, USA), pAR^{S94} (#ab62205; Abcam), pAR^{S515} (Protein sequence MVSVPYP-S(PO3H2)-PTCV, raised in rabbit by Eurogentec Ltd., Southampton, UK) and pAR^{S650} (#ab47563-100; Abcam) were incubated overnight at 4 °C diluted at 1 : 50, 1 : 100, 1 : 100, 1 : 1000, 1 : 25, 1 : 500 and 1 : 100, respectively. All antibodies diluted in Dako antibody diluent (Dako UK Ltd.). Bound antibody complex visualised using EnVision plus kit (#K5007; Dako UK Ltd.) followed by 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako UK Ltd.). Nuclei were counterstained with haematoxylin and Scots Tap Water Substitute, dehydrated through graded alcohol and xylene and mounted with Di-N-Butyl Phthalate in xylene.

Antibody validation. Peptide competition assays were performed to confirm antibody specificity for each AR serine phosphorylation site. pAR^{S308} (#sc-26406-P; Santa Cruz Biotechnology Inc.) peptide was incubated at a ratio of 3 : 1 for 1 h with each antibody. pAR^{S81} (Protein sequence QQQQQQET(pS)PRQQ raised in rabbit by

EZbiolab Inc., Carmel, IN, USA), pAR^{S94} (Protein sequence QQQQQGEDG(pS)PQAH raised in rabbit by EZbiolab Inc.), pAR^{S515} (Protein sequence MVSVPYP-S(pS)-PTCV raised in rabbit by Eurogentec Ltd.) and pAR^{S650} (Protein sequence EEGEASSTT(pS)PTEE raised in rabbit by EZbiolab Inc.) peptides were incubated at ratios of 1:1, 2:1, 500:1 and 100:1, respectively, with each antibody overnight at 4 °C. Immunohistochemistry was then performed as described above and results are shown in Supplementary data.

Scoring. Tissue staining intensity was scored by two blinded independent observers using a weighted histo-score (H-score) method (McCarty *et al*, 1986; Kirkegaard *et al*, 2006). H-score was calculated from the formula: (0 × % cells staining negative) + (1 × % cells staining weakly positive) + (2 × % cells staining moderately positive) + (3 × % cells staining strongly positive). The mean H-score from staining conducted in triplicate was used for analysis.

Western blotting. Cells were lysed in radioimmunoprecipitation assay buffer and Roche protease inhibitor cocktail set one. Lysates were centrifuged at 14 000 r.p.m. for 15 min at 4 °C, supernatant removed and protein concentration determined using Bradford's assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). In all, 50 µg of protein per well was resolved by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad Laboratories Ltd.). Membranes were blocked for 1 h in 5% non-fat milk in TTBS (1 × TBS and 0.1% Tween-20) and probed with primary antibodies at 4 °C overnight. Membranes were incubated with secondary antibodies (Cell Signaling Technology, Inc.) and visualised with ECL plus kit (Fisher Scientific UK Ltd., Loughborough, UK). Membranes were stripped by incubating with Re-Blot Plus stripping buffer (Merk Millipore) then incubated with ECL plus kit to ensure complete stripping. The membranes were then blocked for 1 h in 5% non-fat milk in TTBS (1 × TBS and 0.1% Tween-20) and probed with anti-Tubulin (Abcam) to confirm equal protein loading.

Cdk1 inhibition. Inhibitor studies were conducted to determine whether endogenous Cdk1 mediates AR phosphorylation in prostate cancer cells. LNCaP cells were treated with Roscovitine (20 µM), a selective inhibitor of cellular Cdk1, Cdk2 and Cdk5 for 24 h or dihydrotestosterone (DHT) (10 nM) for 3 h. Cells were trypsinised and collected by centrifugation at 1200 r.p.m. The supernatant was discarded and pellets were washed in HBSS. Cells were fixed in 4% formalin, briefly vortexed and rested at room temperature for 15 min. Cells were then centrifuged at 2500 r.p.m. for 3 min, the supernatant discarded and then washed in HBSS. Cells were set in 1% agarose at 4 °C for 1 h. Cell pellets were dehydrated through graded alcohol and xylene and embedded into paraffin blocks. Immunohistochemistry was performed as described (antigen retrieval reduced to 2.5 min) for protein expression of pCdk1¹⁶¹, pAR^{S81}, pAR^{S515} and Ki67.

Statistical analysis. Statistical analysis was performed using SPSS version 19.0 for Windows (IBM, Armonk, NY, USA). Intra-class correlation coefficients (ICCCs) confirmed histo-scoring consistency between observers. Pearson's rank correlation coefficients (CC) assessed associations between protein expression. Mann-Whitney *U*-test or Kruskal-Wallis test assessed relationships between protein expression and clinico-pathologic characteristics. Kaplan-Meier methods, using the log-rank test, compared survival between patients according to clinico-pathologic parameters and high/low protein expression. Significant univariate results were included in a backwards conditional cox-regression model to determine independence from current clinical parameters. A <0.05 significance level was used and Bonferroni correction was used where applicable.

RESULTS

Patient characteristics. Analysis was based on 90 hormone-naive prostate cancer patients. Patient characteristics are shown in Table 1. Twenty-three patients had metastases to local lymph nodes (3), bone (13) and at both sites (7).

Forty-seven patients had biochemical relapse (median time to biochemical relapse 2.7 years, interquartile range 1.5–3.8). Twenty-four patients were alive at the time of analysis, median follow-up was 11.7 years (interquartile range 9.9–14.0). Forty-six died of their disease (median time to death 4 years, interquartile range 1.9–7.2) and twenty deaths were attributed to intercurrent disease (median time to death 4.1 yr, interquartile range 0.9–5.5). Table 2 shows associations with clinical parameters (grouped data) and outcome measures using Kaplan-Meier methods.

Protein expression analysis. Expression of all proteins was observed at varying levels in the cytoplasm and nucleus of both stromal and epithelial cells (Figure 1). Protein expression was found to be heterogeneous throughout and less intense in the stromal cells. There was presence of PIN and benign tissue, adjacent to the neoplastic tissue, in some of the TMA cores. Expression of proteins in the interspersed PIN and benign tissue and the normal prostate control core was heterogeneous and less intense than the neoplastic tissue. Only protein expression observed in the tumour cells was scored. All ICC values were >0.80. Protein expression levels were subdivided into low (≤median) and high expression (>median) for analysis.

Association between protein expression and clinico-pathologic outcome measures

AR and AR phosphorylated at serine residues. High expression of pAR^{S81} (cytoplasmic), pAR^{S515} (nuclear and total) and pAR^{S650} (cytoplasmic, nuclear and total) was associated with increased age (Table 3A). High expression of nuclear pAR^{S308} was associated

Table 1. Cohort characteristics

Clinical parameter	Percentage of patients (%)
Age (years)	
<70	37.8
≥70	62.2
Gleason	
<7	31.2
=7	32.5
>7	36.4
PSA at diagnosis	
<10 ng ml ⁻¹	27.5
10–20 ng ml ⁻¹	20.3
>20 ng ml ⁻¹	52.2
Lymphovascular invasion	
Absence	93.3
Presence	6.7
Recurrence PSA	
<10 ng ml ⁻¹	77.6
10–20 ng ml ⁻¹	2.0
>20 ng ml ⁻¹	20.4

Abbreviation: PSA = prostate specific antigen.

Table 2. Relationship between clinical parameters and clinical outcome measures

	Time to biochemical relapse	Survival from biochemical relapse	Disease-specific survival
Age (<70 vs ≥70 years)	0.260	0.385	0.020
Gleason (<7 vs =7 vs >7)	0.013	0.754	0.008
Diagnosis PSA (<10 vs 10–20 vs >20 ng ml ⁻¹)	0.002	0.078	0.001
Recurrence PSA (<10 vs 10–20 vs >20 ng ml ⁻¹)		<0.001	<0.001
Lymphovascular invasion (presence vs absence)	0.001	0.612	0.114
Presence of metastases (presence vs absence)	0.001	0.008	<0.001
Ki67 (≤median vs >median)	0.730	0.279	0.033

Abbreviation: PSA = prostate specific antigen.

The clinical variables were grouped and analysed by Kaplan–Meier methods with reference to clinical outcome measures as shown.

Patients were considered to have biochemical relapse dependent on treatment; radical prostatectomy serum PSA >0.2 ng ml⁻¹, radical radiotherapy serum PSA of 2.0 ng ml⁻¹ above the post-treatment nadir level, hormone treatment 2–3 consecutive rises in serum PSA levels above the nadir obtained at intervals of >2 weeks (Roach *et al*, 2006; Cookson *et al*, 2007).

Numbers in bold denote significant associations with *P*-value <0.05.

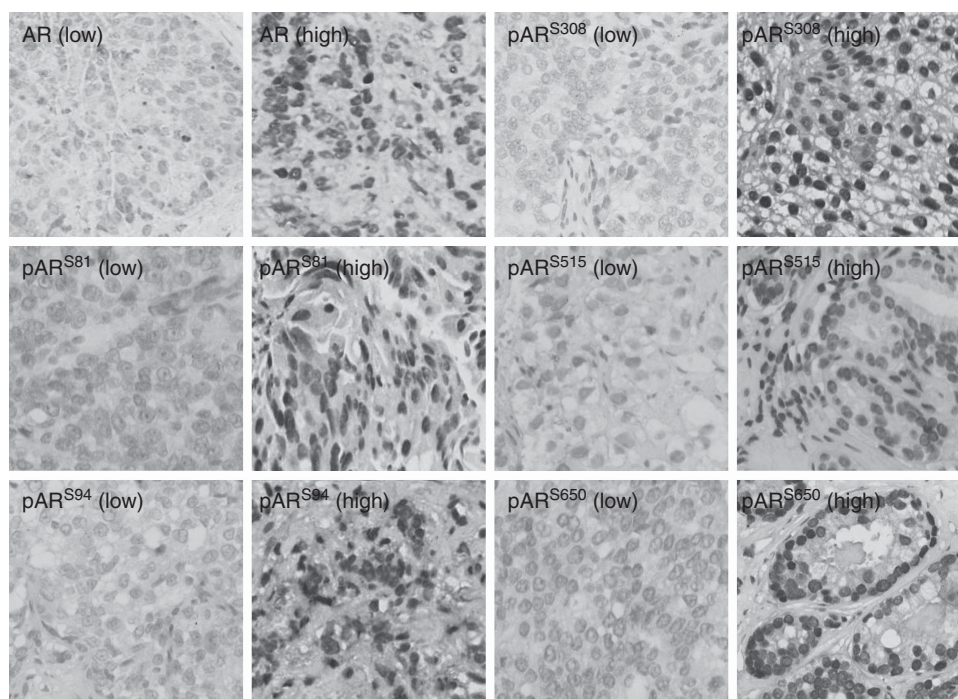


Figure 1. Image illustrating high (>median histoscore) and low (≤median histoscore) intensity immunohistochemical staining for androgen receptor (AR) and AR phosphorylated at Ser-81 (pAR^{S81}), 94 (pAR^{S94}), 308 (pAR^{S308}), 515 (pAR^{S515}) and 650 (pAR^{S650}) (×10 magnification). Intra-class correlation coefficients (ICCCs) confirmed histoscore consistency between two independent observers and all ICC values were >0.80.

with increased PSA level at diagnosis. Presence of LVI was associated with high AR (nuclear, cytoplasmic and total) and pAR^{S515} (cytoplasmic) expression. Presence of metastases was associated with high AR (total) and pAR^{S515} (cytoplasmic) expression. High expression of pAR^{S81} (nuclear and total) and low expression of pAR^{S94} (cytoplasmic) were associated with high Ki67 score (>median) (Table 3A).

Candidate kinases. High total pCdk1¹⁶¹ expression was associated with increased age (Table 3B). High expression of pCdk1¹⁶¹ (nuclear and total) was associated with increased PSA level at relapse. Conversely, high nuclear pERK1/2 expression was associated with low Ki67 score (≤median) (Table 3B).

Kinases mediating AR phosphorylation. Scansite 2.0 predicted Cdk1 and ERK1/2 as strong candidates mediating phosphorylation

of Ser-81, Ser-94, Ser-308, Ser-515 and Ser-650. In the clinical specimens, pERK1/2 expression was not associated with any of the candidate AR phosphorylation sites; however, Cdk1 and pCdk1¹⁶¹ were significantly associated with pAR^{S81} and pAR^{S515} (Table 4).

Associations observed between protein expression and time to biochemical relapse. No associations were observed between the kinases and time to biochemical relapse; however, high nuclear AR was associated with biochemical relapse (proportion of patients relapsed at 5 years 79.2% vs 46.9%) HR 2.8 (95% CI 1.5–5.3), *P* = 0.001, as was high total AR (proportion of patients relapsed at 5 years 85.1% vs 40.2%) HR 3.03 (95% CI 1.6–5.6), *P* < 0.001. High cytoplasmic pAR^{S515} was also associated with biochemical relapse (proportion of patients relapsed at 5 years 78.6% vs 56%) HR 2.15 (95% CI 1.1–4.2), *P* = 0.02 (Table 5).

Table 3. (A) Associations between clinical variables and androgen receptor phosphorylation sites. (B) Associations between clinical variables and candidate kinases

Proteins	Clinical variables						
	Age (<70 vs ≥70 years)	Gleason (<7 vs =7 vs >7)	Diagnosis PSA (<10 vs 10–20 vs >20 ng ml ⁻¹)	Recurrence PSA (<10 vs 10–20 vs >20 ng ml ⁻¹)	Lymphovascular invasion (presence vs absence)	Presence of metastases (presence vs absence)	Ki67 (≤median vs >median)
A							
AR							
Cytoplasm	0.909	0.841	0.979	0.496	0.028	0.061	0.230
Nucleus	0.284	0.450	0.301	0.380	0.006	0.107	0.154
Total	0.422	0.464	0.493	0.800	0.002	0.027	0.627
pAR^{S81}							
Cytoplasm	0.035	0.811	0.269	0.566	0.175	0.660	0.498
Nucleus	0.651	0.401	0.462	0.601	0.666	0.229	0.039
Total	0.220	0.425	0.389	0.632	0.290	0.195	0.041
pAR^{S94}							
Cytoplasm	0.447	0.714	0.437	0.820	0.234	0.165	0.040
Nucleus	0.651	0.069	0.726	0.386	0.234	0.892	0.361
Total	0.754	0.417	0.964	0.496	0.864	0.554	0.191
pAR^{S308}							
Cytoplasm	0.169	0.567	0.441	0.848	0.386	0.656	0.662
Nucleus	0.854	0.324	0.049	0.413	0.836	0.601	0.284
Total	0.515	0.651	0.109	0.375	0.769	0.906	0.531
pAR^{S515}							
Cytoplasm	0.221	0.109	0.181	0.273	0.018	0.040	0.233
Nucleus	0.042	0.336	0.052	0.116	0.673	0.948	0.156
Total	0.048	0.120	0.287	0.120	0.463	0.528	0.229
pAR^{S650}							
Cytoplasm	0.046	0.446	0.976	0.761	0.773	0.750	0.260
Nucleus	0.018	0.531	0.169	0.935	0.279	0.216	0.756
Total	0.020	0.465	0.216	0.827	0.516	0.447	0.504
B							
Cdk1							
Cytoplasm	0.320	0.259	0.741	0.129	0.232	0.690	0.436
Nucleus	0.665	0.898	0.255	0.464	0.563	0.353	0.838
Total	0.187	0.831	0.277	0.130	0.583	0.432	0.879
pCdk1¹⁶¹							
Cytoplasm	0.165	0.929	0.091	0.298	0.640	0.192	0.494
Nucleus	0.084	0.255	0.164	0.047	0.389	0.259	0.630
Total	0.044	0.263	0.108	0.017	0.621	0.204	0.918
pERK1/2							
Cytoplasm	0.293	0.175	0.854	0.639	0.471	0.180	0.530
Nucleus	0.908	0.329	0.990	0.152	0.147	0.674	0.033
Total	0.315	0.555	0.936	0.569	0.160	0.428	0.280
Abbreviations: AR = androgen receptor; PSA = prostate-specific antigen. Cytoplasmic, nuclear and total (cytoplasmic + nuclear) expression of androgen receptor and phosphorylated androgen receptor (A) and candidate kinases (Cdk1, pCdk1 ¹⁶¹ and pERK1/2) (B) were examined for significant relationships with clinical variables as shown. Protein expression scores were used and clinical variables were divided into groups. When the clinical variable consisted of two independent groups the Mann–Whitney U-test was performed, and >2 independent groups the Kruskal–Wallis test was used. Significant associations are highlighted in bold.							

Table 4. Associations between candidate kinases and androgen receptor phosphorylation sites

AR phosphorylation sites	Candidate kinases								
	Cdk1			pCdk1 ¹⁶¹			pERK1/2		
	Cytoplasmic	Nuclear	Total	Cytoplasmic	Nuclear	Total	Cytoplasmic	Nuclear	Total
Ser-81									
Cytoplasmic									
CC	0.545	0.208	0.509	0.446	0.211	0.346	-0.068	-0.158	0.218
P-value	<0.001	0.099	<0.001	0.001	0.134	0.012	0.61	0.231	0.113
Nuclear									
CC	0.057	0.569	0.439	0.185	0.278	0.305	0.019	0.003	0.014
P-value	0.657	<0.001	<0.001	0.190	0.046	0.028	0.886	0.979	0.918
Total									
CC	0.300	0.500	0.552	0.345	0.297	0.382	-0.019	-0.072	0.218
P-value	0.016	<0.001	<0.001	0.012	0.032	0.005	0.888	0.586	0.113
Ser-515									
Cytoplasmic									
CC	0.273	-0.025	0.167	0.455	0.008	0.172	0.051	0.061	0.074
P-value	0.025	0.843	0.178	<0.001	0.954	0.210	0.706	0.649	0.583
Nuclear									
CC	0.181	0.532	0.507	0.105	0.558	0.531	-0.098	0.038	-0.029
P-value	0.143	<0.001	<0.001	0.444	<0.001	<0.001	0.464	0.780	0.831
Total									
CC	0.275	0.457	0.167	0.297	0.500	0.549	-0.061	0.060	0.009
P-value	0.024	<0.001	<0.001	0.028	<0.001	<0.001	0.648	0.656	0.949

Abbreviation: AR = androgen receptor; CC = Pearson's correlation coefficients.
 Values in bold denote associations with Pearson's correlation co-efficient > 0.4 and P-value < 0.05.

Associations observed between protein expression and survival from biochemical relapse. Survival from biochemical relapse was calculated from biochemical relapse till death or last follow-up using cancer-specific deaths. High cytoplasmic Cdk1 was associated with survival from biochemical relapse (10-year survival 12.1% vs 48.9%) HR 2.9 (95% CI 1.2-6.3), P = 0.011. High nuclear pCdk1¹⁶¹ was associated with survival from biochemical relapse (10-year survival 29.2% vs 51.4%) HR 3.18 (95% CI 1.1-9.3), P = 0.026 (Table 5). High cytoplasmic pAR^{S515} was associated with survival from biochemical relapse (10-year survival 15.6% vs 55.5%) HR 2.9 (95% CI 1.2-7.1), P = 0.016 (Table 5).

Associations observed between biomarkers and disease-specific survival. Disease-specific survival was calculated from diagnosis till death or last follow-up using cancer-specific deaths. High cytoplasmic Cdk1 was associated with disease-specific survival (10-year survival 26.8% vs 57.2%) HR 2.4 (95% CI 1.2-4.5), P = 0.007. High cytoplasmic pCdk1¹⁶¹ was associated with disease-specific survival (10-year survival 29.8% vs 58.3%) HR 2.5 (95% CI 2.5-5.4), P = 0.013. High nuclear pCdk1¹⁶¹ was associated with disease-specific survival (10-year survival 27.8% vs 67.9%) HR 3.7 (95% CI 1.6-8.5), P = 0.001 (Table 5). High total pCdk1¹⁶¹ was associated with disease-specific survival (10-year survival 30.6% vs 64.4%) HR 2.9 (95% CI 1.3-6.4), P = 0.007. High nuclear pAR^{S81} was associated with disease-specific survival (10-year survival 24.4% vs 54.5%), HR 2.112 (95% CI 1.1-4.2), P = 0.031. High total pAR^{S81} was associated with disease-specific survival (10-year survival 25.6% vs 56.5%), HR 2.0 (95% CI 1.0-4.1), P = 0.039. High

cytoplasmic pAR^{S515} was associated with disease-specific survival (10-year survival 14.4% vs 65.2%) HR 4.4 (95% CI 2.1-9.1), P = 1.54 × 10⁻⁵ (Table 5). High total pAR^{S515} was associated with disease-specific survival (10-year survival 35.8% vs 57.5%), HR 2.1 (95% CI 1.0-4.2), P = 0.034.

Multivariate cox-regression analysis to determine if biomarkers are able to independently predict time to biochemical relapse. Expression of AR and pAR^{S515} was combined with Gleason score, PSA at diagnosis, LVI and presence of metastases in multivariate analysis. Cytoplasmic pAR^{S515} expression (P = 0.038, HR 4.5 (95% CI 1.1-20.6)) and PSA at diagnosis (P = 0.003, HR 7.3 (95% CI 2.0-27.5)) were independently associated with time to biochemical relapse. As these variables were deemed independent, we investigated whether pAR^{S515} expression could inform on patients with PSA ≤ 20 ng ml⁻¹ at diagnosis. These patients, within each individual clinical context, are considered as suitable for immediate or delayed radical treatment (via active surveillance). Many of these patients will have indolent disease that may never reach clinical consequence and therefore are at risk of over-treatment. In contrast, a subset have occult aggressive tumours that will progress if treatment is delayed. Therefore, biomarkers to aid treatment decision-making in this group of patients would have important clinical implications. When patients with PSA at diagnosis ≤ 20 ng ml⁻¹ were stratified by cytoplasmic pAR^{S515} expression, those tumours with high expression had significantly shorter time to biochemical relapse than those patients with low expression (P = 0.019) (Figure 2A). This translated into significantly shorter

Table 5. Relationship between kinases and androgen receptor with clinical outcome measures

	Time to biochemical relapse	Survival from biochemical relapse	Disease-specific survival
AR cytoplasm	0.466	0.922	0.517
AR nucleus	0.001	0.688	0.233
AR total	<0.001	0.711	0.580
pAR ^{S81} cytoplasm	0.166	0.578	0.057
pAR ^{S81} nucleus	0.594	0.407	0.031
pAR ^{S81} total	0.925	0.383	0.039
pAR ^{S94} cytoplasm	0.927	0.793	0.864
pAR ^{S94} nucleus	0.375	0.202	0.991
pAR ^{S94} total	0.178	0.425	0.884
pAR ^{S308} cytoplasm	0.255	0.423	0.423
pAR ^{S308} nucleus	0.974	0.634	0.628
pAR ^{S308} total	0.685	0.275	0.297
pAR ^{S515} cytoplasm	0.020	0.016	<0.001
pAR ^{S515} nucleus	0.877	0.189	0.072
pAR ^{S515} total	0.708	0.216	0.034
pAR ^{S650} cytoplasm	0.977	0.431	0.113
pAR ^{S650} nucleus	0.909	0.229	0.177
pAR ^{S650} total	0.530	0.342	0.059
Cdk1 cytoplasm	0.988	0.011	0.007
Cdk1 nucleus	0.791	0.624	0.434
Cdk1 total	0.827	0.337	0.134
pCdk1 ¹⁶¹ cytoplasm	0.396	0.356	0.013
pCdk1 ¹⁶¹ nucleus	0.889	0.026	0.001
pCdk1 ¹⁶¹ total	0.166	0.413	0.007
pERK1/2 cytoplasm	0.353	0.188	0.668
pERK1/2 nucleus	0.384	0.207	0.509
pERK1/2 total	0.525	0.487	0.973

Abbreviations: AR=androgen receptor; CC=Pearson's correlation coefficients; PSA=prostate-specific antigen.
The proteins were grouped as ≤median or >median and analysed by Kaplan–Meier methods with reference to clinical outcome measures as shown.
Patients were considered to have biochemical relapse dependent on treatment; radical prostatectomy serum PSA >0.2 ng ml⁻¹, radical radiotherapy serum PSA of 2.0 ng ml⁻¹ above the post-treatment nadir level, hormone treatment 2–3 consecutive rises in serum PSA levels above the nadir obtained at intervals of >2 weeks (Roach *et al*, 2006; Cookson *et al*, 2007).
Numbers in bold denote significant associations with *P*-value <0.05.

disease-specific survival ($P < 0.001$, 10-year survival 38.1% vs 100%) (Figure 2B).

AR agonists stimulate Cdk1 and AR phosphorylation. In the clinical specimens, pAR^{S81} and pAR^{S515} were demonstrated to correlate with pCdk1¹⁶¹. *In vitro*, it was observed by western blot analysis that the androgen DHT could induce phosphorylation of Cdk1¹⁶¹, AR^{S81} and AR^{S515}, with AR and Cdk1 expression levels remaining constant (Figure 3). Analysis of paraffin-embedded cell pellets confirmed the observation that treatment with DHT stimulates phosphorylation at pCdk1¹⁶¹, pAR^{S81} and pAR^{S515} (Figure 4). As shown in Figure 4, it was demonstrated that DHT stimulated cellular proliferation, as assessed by Ki67. In addition, treatment with a Cdk inhibitor, Roscovitine, markedly decreased

basal expression of pCdk1¹⁶¹, pAR^{S81}, pAR^{S515} and cell proliferation (Figure 4).

DISCUSSION

The current study suggests that *in vitro* activation of Cdk1 may be associated with phosphorylation of AR at Serine 81 and Serine 515, and thereby induce cellular proliferation. In addition, this association was also observed in the clinical specimens with pAR^{S515} expression observed to be a negative prognostic marker, independent of known clinical parameters.

Contrary to previous reports, pERK1/2 did not correlate with any of the AR phosphorylation sites predicted by Scansite (Anderson *et al*, 1990). In addition, pERK1/2 was not associated with any clinical outcome measures. We have previously reported that ERK1/2 is a negative prognostic marker in castrate-resistant prostate cancer; therefore, activation of the ERK1/2 pathway may be a late event and not associated with hormone-naïve disease (Mukherjee *et al*, 2011). In support of this hypothesis, it was previously reported that ERK1/2 expression was low or undetectable in the majority of prostate cancer specimens at diagnosis, however increased with stage, Gleason grade and progression to castrate-resistant disease (Gioeli *et al*, 2006). In our patient cohort, phosphorylated ERK1/2 was not associated with Gleason grade and we postulate that disease progression may occur via other indirect mechanisms such as phosphorylation of the AR co-activator steroid receptor cofactor 1, and increasing cellular proliferation through AP-1, c-MYC, and NF-κB (Bakin *et al*, 2003; Bell *et al*, 2003; Fu *et al*, 2003; Powell *et al*, 2004).

In the current study, we were surprised to observe that phosphorylated AR expression in the cytoplasm was a stronger prognostic factor than nuclear expression. However, presence of cytoplasmic AR is expected as the AR localises to the cytoplasm in the absence of ligand binding due to a ligand-regulated nuclear export signal (Tyagi *et al*, 2000; Wen *et al*, 2000). We suggest that immunohistochemical detection of cytoplasmic AR is an adverse prognostic feature as it may indicate high levels of nuclear receptor, as was the case with glucocorticoid receptors (McCarty *et al*, 1986). In support of this notion, AR expression and phosphorylation strongly correlated between the cytoplasm and nucleus in our study (results not shown) and high cytoplasmic pAR^{S515} expression was associated with worse clinical outcome. This is not the first study to observe that cytoplasmic AR expression is a stronger prognostic factor than nuclear expression, cytoplasmic AR expression in patients with negative surgical margins after radical prostatectomy was associated with worse prognosis (Diallo *et al*, 2008). Furthermore, the expression of cytoplasmic AR increased with the progression of prostate intraepithelial neoplasia to prostate cancer and from hormone-naïve to castrate-resistant cancer (Diallo *et al*, 2008). In addition, the subcellular location and activity of AR is likely to be influenced by its phosphorylation status. We propose that differential phosphorylation of the AR as it shuttles between the nucleus and cytoplasm may be a more gradual process, allowing detection of phosphorylated residues in both cellular compartments by IHC. Alternatively, the kinases mediating AR phosphorylation at these residues may be dysregulated in malignant human prostate tissue, causing their localisation and activity not to be confined to a single compartment.

Notwithstanding these differences in the reported subcellular location of the AR and phosphorylated AR, our results suggest that Cdk1 may phosphorylate multiple serine sites on the AR and demonstrate that they are of clinical significance in prostate cancer. In broad agreement with predictions by Scansite, phosphorylation of all putative Ser-Pro target sites on the AR correlated significantly with the expression of Cdk1 but not ERK1/2 in at least one cellular

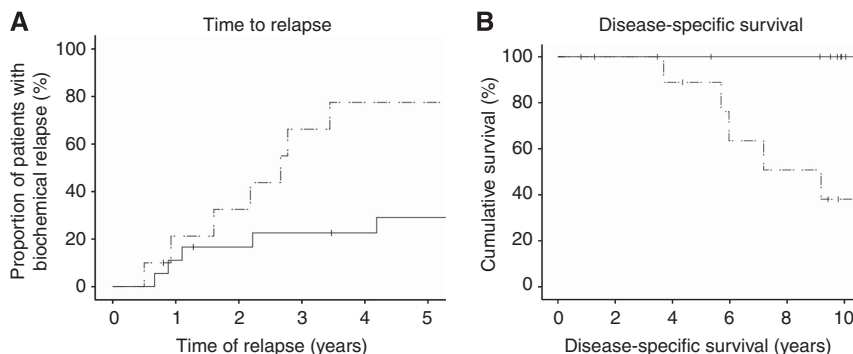


Figure 2. (A) Kaplan–Meier survival plot showing time to biochemical relapse in patients with PSA ≤ 20 ng ml $^{-1}$ at diagnosis ($n = 28$) stratified according to low (solid line) and high (dashed line) cytoplasmic pAR^{S515} expression. (B) Kaplan–Meier survival plot showing disease-specific survival in patients with PSA ≤ 20 ng ml $^{-1}$ at diagnosis ($n = 28$) stratified according to low (solid line) and high (dashed line) cytoplasmic pAR^{S515} expression.

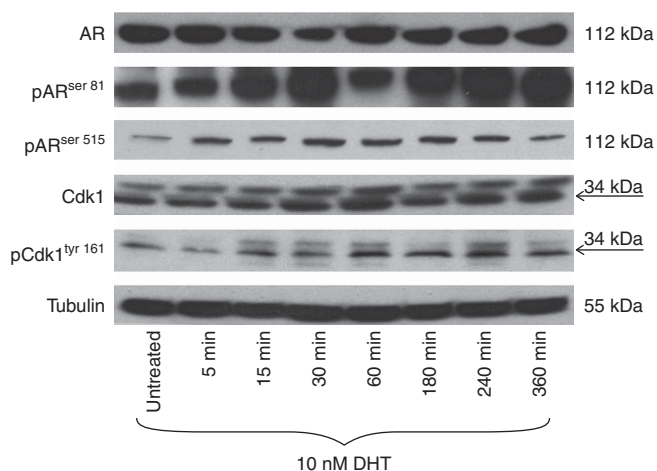


Figure 3. Western blot was performed on 50 μ g of extracts from LNCaP cells treated with 10 nM dihydrotestosterone (DHT) for various lengths of time as shown. Cdk1 is known to have an isomer called CDC2deltaT that lacks 171 nucleotides corresponding to 57 amino acids, which compose most of the T-loop (Ohta *et al*, 1998). CDC2deltaT has been identified in breast cancer tissue, and currently there is no evidence for this in prostate cancer but it may account for the duplex band seen in Cdk1 and pCdk1¹⁶¹.

location (Obenauer *et al*, 2003). Our study suggests that Cdk1 may drive phosphorylation of multiple serine sites on the AR. As Cdk1 expression correlated more strongly with certain phosphorylation sites, it may be that some sites are preferentially phosphorylated by related kinases, such as Cdk11 or Cdk7 (Zong *et al*, 2007; Chymkowitz *et al*, 2011). Not only did phosphorylation of the AR at predicted serine sites correlate with Cdk1 and pCdk1¹⁶¹, both Cdk1 and pCdk1¹⁶¹ were also strongly associated with clinical outcome measures. The association of Cdk1 expression in radical prostatectomy specimens with prostate cancer recurrence has been reported previously (Kallakury *et al*, 1997). Our study has added to this by observing that high expression of Cdk1, pCdk1¹⁶¹ and phosphorylation of the putative Cdk1-consensus site pAR^{S515} was associated with decreased survival from biochemical relapse and, in addition to pAR^{S81}, disease-specific survival. These results suggest that phosphorylation of the AR by Cdk1 may be of functional importance.

Through the use of a Cdk inhibitor, we observed that pAR^{S81} and pAR^{S515} expression is mediated, at least in part, by Cdk1. Previous work has suggested that increased Cdk1 activity is a mechanism for increasing AR expression, stability and cellular

proliferation (Chen *et al*, 2006). In the current study although we observed an increase in Cdk1 phosphorylation AR expression remained stable, however, change in expression might take place over a longer time frame than investigated in the current study. We therefore consider that another role of Cdk1, out-with cell-cycle progression, is AR phosphorylation (in particular at serine 515). Thus, Cdk1 may provide a mechanism for accelerating disease progression in hormone-naive prostate cancer and a novel point for therapeutic intervention. Future work would consist of Cdk1 knockdown via cell silencing experiments to clarify the precise nature of the Cdk1/AR axis.

Ser-81 is the most frequently phosphorylated site on AR in response to androgen binding (Chen *et al*, 2006). However, pAR^{S515} was found to be independent of current clinical parameters ahead of pAR^{S81}. This is in line with previous work which demonstrated that abolishment of pAR^{S81} in cell lines did not alter AR transcriptional activity, rapidly induced AR-regulated genes or AR stabilisation mediated by Cdk1 (Chen *et al*, 2006). In contrast, similar removal of pAR^{S515} resulted in reduction of AR transcriptional activity in response to androgens (Ponguta *et al*, 2008). Our study provides additional evidence to support the hypothesis that site-specific AR phosphorylation is of clinical importance in prostate cancer.

The phosphorylated AR antibodies were stringently validated using western blot and peptide competition assays. We acknowledge the possibility of cross-reactivity in the usage of phospho-specific antibodies particularly on a protein such as AR with multiple phosphorylation sites. To follow on from the current study, we intend to validate these findings in a larger independent patient cohort. Before commencing these studies additional antibody validation will be performed via site-directed mutagenesis followed by IHC of cell pellets to establish with absolute certainty that the results observed are due to the individual phosphorylation sites.

An obvious limitation of this study is the small sample size and as such the results should be interpreted with caution and validated in a large independent cohort. However, even with low patient numbers, we have demonstrated that AR phosphorylation by Cdk1 at serine 515 can predict time to biochemical relapse in prostate cancer patients with PSA ≤ 20 ng ml $^{-1}$. Within this group of patients, 10-year disease-specific survival was 100% in those patients with low pAR^{S515} expression compared with 38% in those with high expression. These results are striking in particular when considered that this was a hormone-naive cohort of patients who subsequently received a variety of treatments (surgery, radiotherapy and hormones) and that, due to small numbers, we were unable to unpick these groups. We now intend to pursue this work in a cohort of patients treated initially by active surveillance.

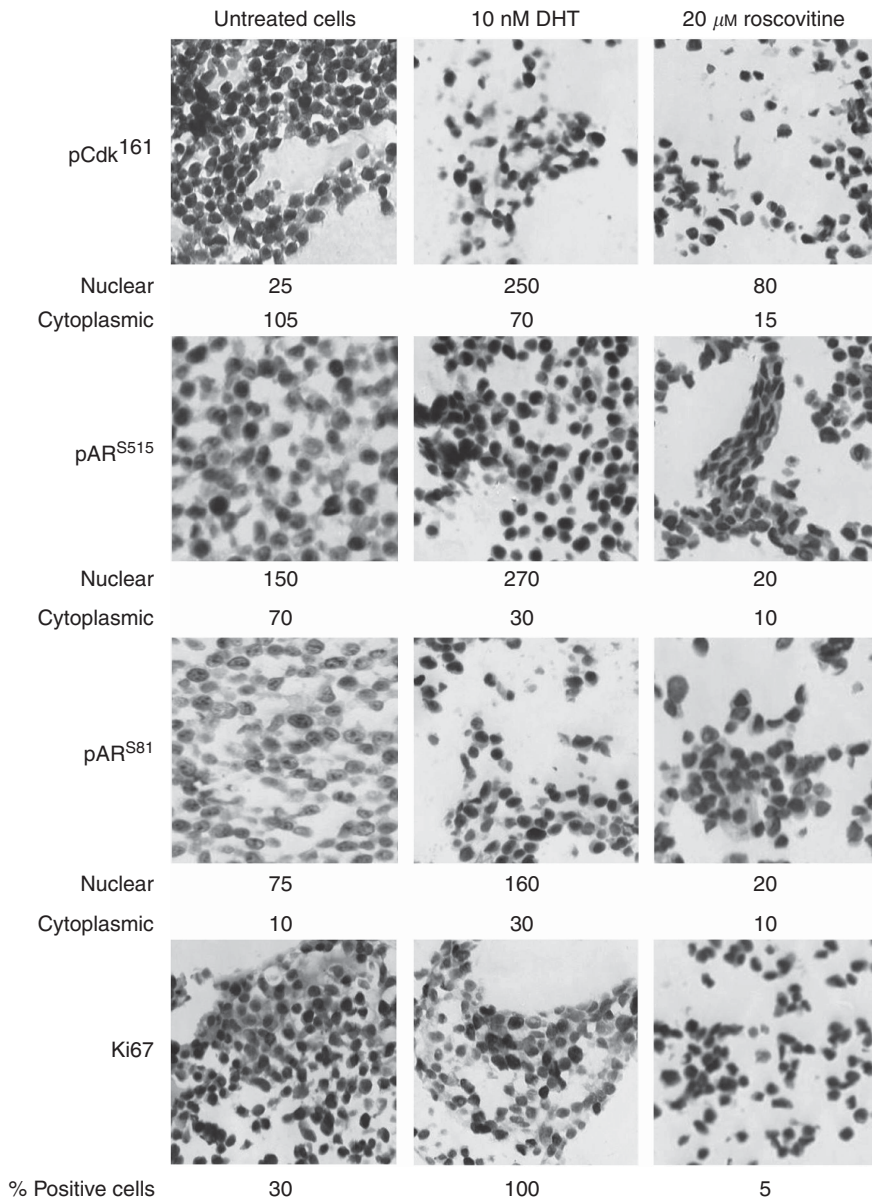


Figure 4. LNCaP cells were grown in full media and treated with either 10 nM dihydrotestosterone (DHT) (3 h) or 20 μ M Roscovitine (24 h). Pellets were stained by immunohistochemistry for expression of pCdk1¹⁶¹, pAR^{S81}, pAR^{S515} and the nuclear marker of proliferation; Ki67. Weighted histoscores for cytoplasmic and nuclear expression are shown below each image for pCdk1¹⁶¹, pAR^{S81} and pAR^{S515}. Percentage of positive cells counted is shown below the images for Ki67.

This study suggests that Cdk1 may phosphorylate AR at serine 81 and serine 515, thereby driving cellular proliferation. In combination with current diagnostic tools, pAR^{S515} could provide a desperately needed prognostic marker to aid treatment decision-making in prostate cancer patients with PSA \leq 20 ng ml⁻¹ at diagnosis. This finding has the potential to reduce over-treatment of clinically insignificant disease and prevent delay in treatment of occult aggressive disease. Overall morbidity and mortality suffered by prostate cancer patients would be drastically reduced.

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