

Application for Clinical Research Fellowship

The role of Cdk1 in the development and progression of prostatic diseases

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Introduction

Prostate cancer (Pca) is the most common cancer in men in the UK. Approximately 37,051 men were diagnosed in 2008, of which 10,170 died (1). Although Pca can be readily diagnosed, prediction of likely outcome remains problematical, many will have an asymptomatic course and will not die of their disease while some patients progress rapidly to castrate resistant prostate cancer (CRPC) and die within 18-24 months (1). Research has demonstrated that activation of the androgen receptor (AR) stimulates proliferation and differentiation in the normal prostate, and has also been linked to development and progression of Pca. Anti androgens are initially effective in controlling Pca. However resistance associated with the loss of androgen regulated cell cycle control is a major problem.

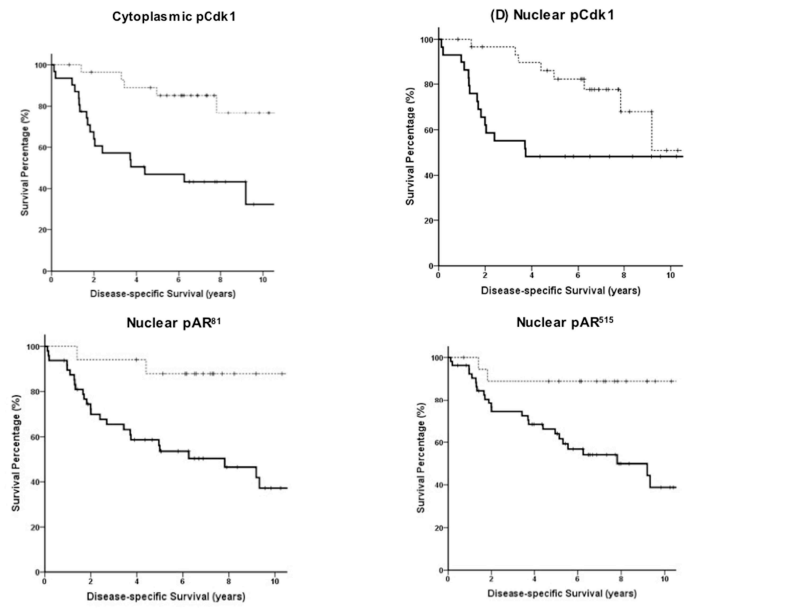
Cellular proliferation is driven by Cyclin dependent kinases (Cdks) and their partner Cyclins which in turn are controlled by Cyclin dependent kinase inhibitors (Cdkis). These proteins play a crucial role in mediating cell cycle arrest, DNA repair and apoptosis in cells with damaged genomes. Following DNA damage the action of Cdks are inhibited and the cells arrest at G1, S or G2/M phase of the cell division cycle. In mammalian cells, Cdk 1, with its partners cyclin A2 and B1, alone can drive the cell cycle. Thus given its essential role in cell cycle progression, Cdk1 is highly regulated. Cdk1 is regulated by the binding with its cyclin partners and by phosphorylation at Thr 161 which increases the complex activity. Cyclin-Cdk complexes phosphorylate substrates appropriate for the particular cell cycle phase. Cdk inhibitory phosphorylation is also vital for regulation of the cell cycle. A conserved tyrosine (Tyr15) leads to inhibition of Cdk1. Wee1 phosphorylates Tyr15, whereas members of the Cdc25 family are phosphatases, counteracting this activity.

Cancer is described as an uncontrolled cell division process. Cancer cells proliferate in a disordered way, and carry out cycles of cellular division unaware of the signals of alarm. Irregular activation of cyclins and Cdks may potentiate the growth of certain cancers including Pca (2). Cyclins are a family of proteins whose levels vary during the cell cycle to activate specific Cdks necessary for the correct progression through the cell cycle. Cyclin B1 is essential for cell cycle progression through mitosis and is overexpressed in a variety of cancers compared with normal cells and tissues (3). Increasing evidence suggests that AR is required for growth and survival of Pca. In androgen sensitive LNCaP cells, androgen withdrawal has been shown to induce G1 arrest and, the reintroduction of androgen displayed an increase in the expression of cyclins A and B and Cdk1 and Cdk2 (4). Phosphorylation of AR at Serine 81 in vitro by Cdk1 is associated with prevention of AR degradation, therefore increasing AR stability and resulting in increased AR protein expression (5). Inhibition of Cdk 1 has been shown to induce G2 check point arrest and consequent in apoptosis in Pca

cells(6). A number of groups have shown that Cdk1 expression is increased in Pca relative to normal prostatic tissue (7;8). Moreover Cdc25C which directs dephosphorylation of cyclin B-bound Cdk1 and triggers entry into mitosis has also been reported to be upregulated in its active form in Pca (9). Furthermore, the loss of function mutations of the *chk 2* gene which is an upstream regulator of Cdk1 has previously been reported in Pca patients (10). In addition cyclins B1 and B2 are observed to be overexpressed in the progression of Pca(11). Due to the overexpression of cyclins and their associated kinases correlating with increased proliferation of cancer cells, small-molecule inhibitors of CDK activity have been identified and are being investigated in multiple clinical trials as potential chemotherapeutic agents(12).

The forkhead box O (FOXO) proteins transcriptionally regulate expression of an array of genes which encode proteins that function at various cell cycle check points. FOXO proteins induce cell cycle arrest at G1 by upregulating the cyclin dependent kinase inhibitors p27^{KIP1}, p21^{WAF1} and the retinoblastoma related protein p130, and also by down regulating cyclins D1 and D2 (13;14). The FOXO proteins often function out with their regulatory control due to aberrant signalling cascades. It has been well documented that loss of PTEN activation or aberrant Akt activation leads to FOXO protein inhibition through phosphorylation and cytoplasmic localisation (14). In addition it has been established that FOXO 1 mediates inhibition of AR transcriptional activity but requires the nuclear localisation of FOXO1 (15). We have previously demonstrated the PTEN/Akt signalling cascade is involved in regulating AR activity (16;17) in Pca patients. Lui et al have previously demonstrated that loss of PTEN or activation of Akt promotes nuclear exclusion of FOXO 1 and in so doing abolishes the AR inhibitory function of FOXO 1(15). Cdk1 specifically phosphorylates FOXO1 at serine 249 in vitro and in vivo and overexpression of Cdk1 has been shown to inhibit the transcriptional activity of FOXO1 in Pca cells by phosphorylation at this site (18). Our previous results suggest that high expression of Cdk1 (cytoplasmic p=0.001 & nuclear p=0.03) and phosphorylation of AR at two putative Cdk1-consensus sites (pAR⁸¹ p=0.008 and pAR⁵¹⁵ p=0.001) were associated with decreased disease-specific survival and/or overall survival in hormone naïve Pca patients.





One of the many challenges in the effective management of prostate cancer is the identification of molecular markers capable of predicting disease progression. Key functions of cyclin/Cdk complexes are cell cycle progression and neoplastic transformation, consequently, to stop or to slow down cell cycle progression would result in inhibiting cell proliferation and thus fighting against cancer. It is evident that multiple signal transduction cascades are critical to prostate cancer progression and resistance to therapy. Cdk 1 has been implicated in Pca survival via FOXO 1 inhibition and AR phosphorylation and stabilisation.

We hypothesize that Cdk1 influences Pca development and progression by modulating the proliferation, migration and invasion of Pca cells via phosphorylation of the AR and/or inhibition of FOXO1.

Therefore, the aims of this project are to establish if Cdk1 expression and activation is involved in Pca development and progression and identify the mechanisms by which this is achieved.

a) Is Cdk1 expression and activation clinically important in the development and/or progression of Pca?

We will assess if expression levels and/or phosphorylation status of Cdk1 are associated with outcome in 3 different clinical cohorts.

b) How does Cdk1 act to drive the development and progression of Pca?

The 3 patient cohorts will be utilised to investigate correlations between Cdk1 expression/phosphorylation status with AR expression/phosphorylation status and FOXO1 expression/phosphorylation status.

In addition, we will assess the effect of inhibition and silencing of Cdk1 on cellular proliferation, apoptosis, invasion and migration on PNT2 normal prostate cell line, BPH-1 Benign prostate hyperplasia human cell line (AR negative), BPH-1-AR

Benign prostate hyperplasia human cell line (AR positive), prostate cancer PC3 (AR negative) and prostate cancer LNCaP cells (AR positive).

Experimental Plan

The following three clinical cohorts will be utilised in the current project:

1. Benign Prostate Hyperplasia

Benign prostate hyperplasia (BPH) is a highly prevalent disease. Fifty percent of men over the age of fifty have histological evidence of BPH, increasing to over ninety percent of men in their eighties(19). Similarly almost fifty percent of men over sixty years old will have clinical symptoms relating to BPH(20;21). Currently BPH costs the NHS an estimated £175million each year and with an aging population this cost is set to rise exponentially.

BPH causes an array of urinary voiding problems such as urinary frequency, urgency and nocturia, which can range from “bothersome” to seriously impacting upon a patients’ quality of life(22). In addition, complications of BPH such as acute urinary retention (AUR) and renal failure cause serious morbidity. This high morbidity as a result of BPH combined with the associated financial burden on the health service is a serious issue.

Although the molecular mechanisms underlying the development of BPH are not well understood, BPH is known to be characterised by an increase in epithelial and stromal cells in the periurethral region of the prostate. This causes a narrowing of the prostatic urethra leading to a bladder outlet obstruction and urinary voiding symptoms. The formation of new epithelial glands is ordinarily seen only during fetal development and it is thought that the prostatic stroma may play a role in the regulation of prostatic epithelial cell growth.

Androgens have been shown to be important in the maintenance of BPH, as chemical or surgical castration results in disease regression. 5 alpha reductase inhibitors comprise part of the medical treatment of BPH. These drugs block the conversion of testosterone to the more potent dihydrotestosterone (DHT) resulting in a reduction in prostate size. However BPH incidence increases with age, whilst circulating serum androgen levels decrease. This suggests some change in the way in which the androgens are recognised or processed. Activation and/or phosphorylation of AR and FOXO1 via Cdk1 may be responsible for disease progression.

Currently there is no way to predict which patients will go on to develop severe symptoms and complications from BPH. Through the investigation of Cdk1 expression levels and phosphorylation status and its effects on AR and FOXO1 we hope to identify those patients who are more likely to have symptoms and/or serious complications from BPH and may benefit from prompt surgical or medical intervention.

We have an expanding cohort of 371 patients with full clinical information; demographics, comorbidities, international prostate symptom scores (IPSS), flow test results, serial serum PSA levels, complications, reoperation and survival status. The study of Cdk1, AR and FOXO1 in a benign cohort may change patient management and in addition will provide an invaluable comparison to the continuation of this work in prostate cancer.

2. Localised Prostate Cancer: Active Surveillance

Since the early 1990s prostate cancer incidence has increased dramatically, whilst mortality rates have remained fairly stable (1). These statistics reflect the recent stage and grade migration of prostate cancer and the subsequent over detection of early stage, low risk disease by the widespread use of PSA testing and prostate biopsies. The over detection of prostate cancer leads to the risks of over treatment such as urinary incontinence, erectile dysfunction, and a small risk of surgery or radiotherapy related death. Active surveillance (AS) provides a potential solution to the problem of over treatment of clinically insignificant disease.

AS is a deferred treatment approach for prostate cancer. Carefully selected patients with low risk disease who are deemed suitable for radical therapy enter a formal programme of regular repeat biopsies and serum PSA checks. Radical intervention is initiated only when biochemical, histological or clinical progression is demonstrated. The national institute for clinical excellence (NICE) recommends AS as the preferred treatment approach for men with low risk disease (23). Unfortunately inherent sampling errors in prostate biopsies and the poor predictive value of serum PSA testing can lead to under-staging of disease and therefore progression is likely (24;25). Living with prostate cancer has a significant emotional and psychological burden and can have a major impact on the lives of patients on AS. The identification of a biomarker for clinically significant disease would avoid biopsy related morbidity and unnecessary delays in treatment, increase the success of radical therapy and may result in novel therapeutic targets. In addition a prognostic biomarker would give patients and clinicians confidence in choosing AS as a treatment option for prostate cancer. We have an expanding cohort of 115 AS patients with full clinical information; demographics, comorbidities, primary pathology, serial serum PSA levels, evidence of disease progression, intervention and survival.

3.Pre and Post CRPC Tissue Matched Cohort

80-90% of all prostate cancers are androgen dependent at diagnosis and endocrine therapy is aimed at reducing the concentration of circulating androgens and inhibiting AR(26). Initial response rates to androgen deprivation therapy (ADT) are high but inevitably treatment fails within 18-24 months and biochemical relapse signifies androgen independent disease. The development of androgen independence (also called castrate resistant prostate cancer CRPC) is thought to be the most significant event in the progression of prostate cancer. The median survival of patients following a diagnosis of CRPC is 12 months (27). The treatment of CRPC prostate cancer has remained essentially unchanged for over 50 years. Available therapy is almost entirely focused on symptom control and comprises docetaxel chemotherapy and palliative radiotherapy. Androgen biosynthesis inhibitors, such as Abiraterone, have been shown to extend survival in metastatic prostate cancer but only by 4 months (28). Despite these treatments CRPC patients suffer significant morbidity in the form of urinary tract obstruction and bone pain from metastases. Further progression can only be made by elucidation of the mechanisms by which prostate cancer escapes hormonal control. To this end the functionality of AR has been shown to be critical, even in CRPC. Evidence suggests that prostate cancer progression may occur through dysregulation of AR activity via changes in AR coregulator expression, signal transduction cascades and receptor mutations allowing AR to be activated by ligands other than androgens (29). Phosphorylation of the androgen receptor has been shown to be associated with a shorter time to death and reduced disease specific survival in CRPC (17). We postulate that Cdk1 expression levels and phosphorylation status, and

its effects on AR and FOXO1 may contribute to the development of CRPC and the investigation of such may result in novel therapeutic targets. We have a unique patient cohort consisting of 44 patients with matched hormone naïve and castrate resistant tissue. We have full clinical information and outcome data.

The above clinical cohorts will be utilised in a series of immunohistochemical (IHC) studies. The Scansite 2.0 program (30), available via <http://scansite.mit.edu>, was utilised to identify serine residues on FOXO1 and AR most likely to be phosphorylated by Cdk1. Using this method FOXO1²⁴⁹, AR⁸¹ and AR⁵¹⁵ were identified. Therefore in the current proposal protein expression of pCdk1¹⁶¹, Cdk1, FOXO1 and FOXO1²⁴⁹, AR, AR⁸¹ and AR⁵¹⁵ and proliferation and apoptotic index will be established. IHC will be performed using specific antibodies for each site, and expression will be assessed using the weighted Histoscore method by 2 independent observers (30).

Antibody specificity will be stringently tested before use in IHC, by western blot (WB), IHC of paraffin embedded cell pellets of control and silenced cells and IHC in combination with blocking peptides for specific phosphorylation sites. .

Results will determine if expression of the proteins investigated are associated with development and or progression of prostate cancer, Gleason grade, PSA levels, time to recurrence, and disease specific survival. In addition, protein expression of pCdk1¹⁶¹ and Cdk1 will be correlated with AR and FOXO1 expression and phosphorylation status, cellular proliferation (Ki-67 MIB1) and apoptotic (TUNEL assay) indexes.

Proteins established as being linked with clinical outcome data and involved with tumour development, tumour growth and apoptosis will also be assessed by the mechanisms outlined below.

In vitro experiments will be performed using normal, benign and cancer prostate cell lines as outlined above. The effects of Cdk1 stimulation (EGF/DHT) and inhibition (Roscovitine) on FOXO1 and AR expression/ phosphorylation will be ascertain by western blotting and in parallel by IHC on paraffin embedded cell pellets.

The functional consequences of Cdk1 stimulation/inhibition will be assessed. Cell growth will be determined directly by cell counts and cleavage of WST-1 reagent. Apoptosis will be measured by Cell Death Detection ELISA^{PLUS} (Roche, UK). Cell migration will be assessed using Bio-coat cell migration chambers (BD, UK), and for cell invasion the filters will be coated with the basement membrane Matrigel, to present a barrier for the cells to migrate through. Cell motility will also be assayed by a wound healing assay using time lapse microscopy.

In addition, these experiments will be repeated by transiently transfecting the cells with Cdk1 and/or AR and FOXO1 specific siRNA smartPools (Dharmacon, UK) using Lipofectamine2000 (Invitrogen, UK) based on manufacturers guidelines. Best knockdown will be established post-transfection as measured by western blotting and RT-PCR.

The use of siRNA as a tool to study the functional role of proteins is often associated with off-target effects. In order to ensure that any measured effect in our proliferation, apoptosis, migration and invasion assays are associated with our silenced proteins and are not an artefact, we are planning to validate our results by using different siRNA

sequences which we have already identified from publications that have been used successfully on different cell lines. Furthermore, appropriate rescue experiments will also be performed to ensure the specificity of the siRNA used.

Statistical analysis

All statistical analyses shall be carried out using SPSS. Kaplan-Meier survival plots and log rank method will be employed to establish the significance of Cdk1/AR/FOXO1 expression and phosphorylation on time to relapse and disease specific survival and any significant results will then be entered into a multi-variate cox regression model to assess if they are independent of known clinical parameters. Correlations between continuous variables will be established using the Spearman Rank test, and correlations between continuous variables divided into 2 groups will be established using Mann Whitney U test or multiple groups Kruskal-Wallis H test. Significant changes to cell growth, apoptosis, migration and invasion will be assessed using the Dunnett's and T-Tests.

Dissemination plans:

It is anticipated that the results arising from this study will be of such quality and impact to be submitted for presentation at international/ national cancer meetings. Results will be further disseminated through publication in high impact factor (> 6) oncology journals.

Value of this project to the prostatic disease research community:

A more complete knowledge of the role of key signalling pathways is a prerequisite for addressing the current problems associated with active surveillance and progression to castrate-resistant disease. This project aims to provide further understanding of the mechanisms underlying the development and progression of prostate cancer utilising unique clinical cohorts and parallel cell line studies, as such the information gained from this project could significantly move forward our knowledge in this area.

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