 Clinical Significance of Abnormal Extracellular Signal–Regulated Kinase 5 (ERK5) Expression in Human Prostate Cancer.

Running Title: ERK5 Expression in Human Prostate Cancer

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Abstract:

The MEK5/ERK5 pathway has recently been implicated in prostate and breast carcinogenesis. Over-expression of MEK5 is associated with aggressive prostate cancer. In this study, we examined the role of ERK5 (a MAPK and specific substrate for MEK5) in prostate cancer. ERK5 immunoreactivity is significantly upregulated in prostate cancer (PC) (n=81) when compared to BPH (n=20) (P<0.0001). Increased levels of ERK5 cytoplasmic signals correlates closely with Gleason sum score (P<0.0001), bony metastases (P=0.0044), and locally advanced disease at diagnosis (P=0.0023), with a weak association with shorter disease-specific survival (P=0.036). A subgroup of 15 (of 81) patients showed strong nuclear ERK5 localisation, which correlates with poor disease-specific survival and, on multi-variant analysis, was an independent prognostic factor (P<0.0001). Analysis of ERK5 expression in matched tumour pairs (before and after hormone relapse, n=26) revealed that the presence of ERK5 nuclear expression is significantly associated with hormone insensitive disease (P=0.0078), a trend not seen for ERK5 cytoplasmic expression. Similarly, ERK5 protein expression was found to be increased in the androgen independent LNCaP-DCC cell line. We obtained the following *in vitro* evidence to support the above expressional data: (i) ERK5 overexpressing PC3 cells have enhanced proliferative capability (P<0.0001); (ii) the MEK1 inhibitor, PD184352, blocking ERK1/2 activation at low dose, did not suppress proliferation but did significantly decrease proliferation at a higher dose required to inhibit ERK5 activation; (iii) overexpression of ERK5 upregulated MMP-1, -2 and -9 promotor activity; (iv) co-transfection of ERK5*wt* and MEK5D constructs in PC3 cells results in predominant ERK5 nuclear localisation, similar to that observed in aggressive clinical disease. Taken together, our results establish the importance of ERK5 in aggressive prostate cancer.

Keywords: Prostate Cancer; ERK5; MEK5

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second commonest cause of cancer related death in men in the western world [1]. Prostate cancer is usually androgen dependent and the majority will regress upon treatment with androgen ablation. Unfortunately, about 20% of patients do not show favourable response, and even among the responders, there is an overall risk of 80% to develop relapsed cancer at a median period of 24 months with hormone manipulation. Therefore, better prognostic markers will facilitate stratification of patients to different treatment protocols aimed at improving survival outcome.

The clinical picture in the advanced stages of prostate cancer is dominated by the problem of bony metastases, which occur in 85% of patients. They represent the most important cause of morbidity, causing pain and complications such as pathological fractures and spinal cord compression. The development of bone metastases in patients with advanced prostate cancer occurs at a constant rate of approximately 8% per year, reaching 40% at 5 years. There is a remarkable affinity between prostate cancer cells and bone, the predominant site of metastasis, which is frequently osteoblastic in nature.

The family of mitogen-activated protein kinases (MAPKs) plays an essential role in the transduction of extracellular signals to cytoplasmic and nuclear effectors. MAP kinase kinases (MEKs/MAPKKs) represent protein kinases upstream of MAP kinases (MAPKs), critically controlling cellular proliferation and apoptosis. Mitogen/extracellular signal regulated kinase kinase-5 (MEK5) is the most recently identified MAPKK and specifically activates ERK5, also known as Big MAP kinase 1 (BMK1) [2, 3]. The

ability of inhibitors of the classical MAPK (ERK1/2) cascade to block ERK5 activation suggested that ERK5 might regulate some cellular functions originally attributed to ERK1/2. The MEK5/ERK5 pathway is potently activated by EGF and is required for EGF induced proliferation [4] and connexin 43 gap junction communication [5]. ERK5 is almost twice the size of the other MAPKs (815 amino acids). Its unique COOH-terminal tail contains a myocyte enhancer factor 2 (MEF2)-interacting domain and a potent transcriptional activation domain [6]. The activity of several transcriptional factors have been shown to be regulated by ERK5, including MEF2, c-Fos and Fra-1, Sap-1, c-Myc and NF-κB [7-11]. It plays key role in embryogenesis: ERK5 knock-out embryos die between day 9.5 and 11.5 due to severe cardiovascular defects [12, 13] whereas mice lacking ERK1 develop normally [14, 15]. Interestingly, a recent study has shown that MEK5 knock-out mice also die, at approximately embryonic day 10.5 [16].

We have previously shown that abnormal MEK5/ERK5 signalling is important in prostate cancer [17]. Strong MEK5 expression correlates with aggressive disease. Activation of MEK5 induced signalling induced proliferation ($p<0.0001$), motility ($p=0.0001$) and invasion in LNCaP prostate cancer cells ($p<0.0001$). In this study, we further explore the role of ERK5, the specific substrate for MEK5, in prostate cancer.

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Materials and methods

Human prostate tissue samples

For immunohistochemical studies of ERK5 expression, Ethics Committee approval and the consent of patients with cancer of the prostate (CaP) and benign prostatic hypertrophy (BPH), undergoing transurethral prostatectomy (TURP), were obtained. Diagnosis was confirmed by histopathological examination. Specimens containing greater than 75% tumour involvement were selected for analysis. The primary immunohistochemical analysis included 81 patients with prostate cancer, median age 73 years (range 55 – 90) and median Gleason sum score 8 (range 5 – 10). Twenty cases of BPH specimens were studied as controls. Formalin-fixed and paraffin waxed-embedded specimens were obtained at the time of diagnosis, following TURP, prior to any anti-androgen therapy. The secondary immunohistochemical analysis included 26 patients (52 matched tumours), all of which had received conventional androgen deprivation therapy (orchidectomy, antiandrogens or androgen ablation therapy). Patients were selected for treatment if they initially responded to treatment (response was defined by prostate-specific antigen (PSA) levels falling by at least 50%), but subsequently relapsed. Patients were classed as having hormone escaped cancer when sustained rising PSA levels were noted and were selected for study if a post-hormone relapse sample was available [18].

Immunohistochemical analysis

Polyclonal anti-ERK5, raised in sheep as previously described [19] and polyclonal anti-MEK5 (Stressgen, USA) were optimised for immunohistochemical analysis (data not

shown). Archive paraffin wax-embedded serial sections (4μm) taken from clinical prostate samples were prepared and incubated with the desired antibody overnight at 4°C and immunohistochemical analysis was performed as described previously [20]. The staining was developed with diaminobenzidine tetrahydrochoride (DAB; Sigma, UK), with haematoxylin used as a counterstain. Heart muscle was used as a positive control; no primary antibody as a negative control. The slides were assessed by two independent observers (MM and SMcC) with inter-observer agreement on the grading system confirmed in the first 20 cases. Both observers were blinded to the clinical details for these cases prior to evaluation. The cytoplasmic immunoreactivity signals were correlated with ERK5 expression and scored as either absent (0), weak (1), moderate (2), or strong (3) as previously published [21, 22]. Nuclear immunoreactivity signals were scored as either positive or negative.

Cells and Cell Culture

The PC-3 cell line (passage 27) was purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK) and was maintained in growth medium [RPMI 1640 (Gibco BRL, Invitrogen, Paisley, UK), containing HEPES buffer (25mM) and L-Glutamine (20mM)], supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich, Dorset, U.K.), 100units/ml of penicillin, and 100μg/ml of streptomycin (Gibco BRL); this was referred to as full medium. Androgen-independent LNCaP (LNCaP-DCC) cells were derived in-house by Prof. Craig Robson from LNCaP cells, following continuous passaging in an environment depleted of androgens for a period of over 9 months. The LNCaP-DCC cell line did not require androgen to proliferate, however the

cells were still responsive to androgen stimulation. Passage number matched parental LNCaP cells were also maintained.

Plasmids and generation of stable clones

The EGFP-C1 ERK5 and pCMV HA-ERK5*wild type*(*wt*) plasmids were provided by Prof. P Cohen. A constitutively active mutant of MEK5 (MEK5D-HA) was obtained from Dr JD Lee (Department of Immunology, The Scripps Research Institute, USA) and inducible 293 cells expressing MEK5D was generated as previously described [17]. PC3 cells overexpressing ERK5 were generated by transfection with EGFP-ERK5 using Superfect® Transfection Reagent (Qiagen, Crawley, UK) according to the manufacturer's recommendations. Following selection with geneticin (G418, 1mg/ml; Sigma), colonies were left to form for 2-3 weeks. Control stable cell lines were similarly established with the vector (EGFP-C1) alone. Individual colonies were sub-cultured and screened for ERK5 expression by Western analysis. Presence and subcellular localisation of ERK5 was also confirmed by fluorescent microscopy.

Fluorescent Microscopy

PC3 cells were plated on sterile coverslips placed in 6-well culture dishes and cultured in the desired condition for 48 hours. Cells were washed in PBS, fixed with methanol for 20 minutes at -20ºC and allowed to air dry. The coverslips were mounted on microscope slides using an anti-photobleaching solution, Vectashield (Vector Laboratories, Peterborough, UK), which contains the nuclear stain Diamino-2-phenylinodole (DAPI). The slides were then viewed using a Leica DMR/HCS microscope (100x magnification;

Leica Microsystems Ltd., Milton Keynes, UK). Photographs were taken with a SPOT digital camera and software (version 3.5.2 for Windows; Diagnostic Instruments Inc., Michigan, USA).

MMP promoter studies

At 48 h prior to transfection, $2x10^4$ PC3 cells were seeded per well in a 48-well plate. MMP-1 (-3803), MMP-2 (-1659) and MMP-9 (-670) constructs containing 5'- flanking fragments upstream to the transcription initiation start site linked to a luciferase reporter gene (MMP-luc) were co-transfected with pCMV HA-ERK5*wt* (wild type) or empty plasmid (pCMV) using Superfect reagent (Qiagen), according to manufacturer's recommendation. After 24 h, cells were serum starved and left for another 16-24 h before luciferase activities were determined using an automated optical plate reader (Dynatech MR 5000). For all transfections, cells were co-transfected with a β-gal-CMV plasmid to allow normalization of transfections. A total of 450ng DNA was transfected, which contained 300ng ERK5*wt* DNA, 100ng MMP-luc and 50ng β-galactosidase reporter.

Western blotting

Cultured cells were lysed directly on plates with 6 x SDS sample buffer containing 10% β-mercaptoethanol. Samples were denatured and analysed by SDS-PAGE, followed by transfer to nitrocellulose. Antibodies were used at the following dilutions: anti-ERK5, 1:1000; and anti-α tubulin, 1:2000 (Santa Cruz, USA). Horse-radish peroxidase-conjugated secondary antibodies (DAKO, High Wycombe, U.K.) were applied at 1:500

dilution and detected using enhanced chemiluminescence detection kit (ECL, Amersham).

WST1 proliferation assay

WST1 assays were carried out according to the manufacturer's instructions (Roche, UK). PC3 cells were cultured in 96-well, flat-bottom tissue culture plates at a density of 5,000 cells/well in a final volume of 100µl/well culture medium at 37°C, 5% CO_2. After 48h, WST-1 reagent was added at a volume of 10µl/well and the cells incubated for up to 3 h. Absorbance at 450nm was measured using a microplate reader (Bio-Rad Laboratories Ltd., Model 680). Each assay was conducted in sets of ten and the experiment was repeated three times. Control wells containing culture medium and WST-1 reagent but no cells were also included in order to determine background fluorescence.

Statistical analysis

The statistical significance of trends between ERK5 expression and standard prognostic variables was assessed using the Mann-Whitney U and Kruskal-Wallis tests (Table 1) Linear regression analysis was employed to evaluate correlation of ERK5 and MEK5 expression. Correlation of ERK5 expression and patient survival was analysed using the Kaplan-Meier method with log rank testing. Multivariate analysis was performed using the Cox regression model for survival data. Wilcoxon matched pairs test was used for matched tumour samples. All these tests were undertaken using SPSS, version 11.0, computer software (SPSS, Inc). A *p*-value of <0.05 was taken to indicate statistical significance.

Results

ERK5 protein expression in resected human prostate cancer

Normal and hyperplastic glands in the BPH group demonstrated either negative (n=2) or weak cytoplasmic staining (n=18). Analysis of staining patterns of ERK5 showed that, in the benign gland, expression was mainly localized to the glandular epithelium with patchy areas of weak stromal immunoreactivity (Figure 1A). Within the benign epithelium, weak ERK5 cytoplasmic immunoreactivity was seen in 18/20 cases. In contrast, when compared with BPH, cytoplasmic ERK5 expression was significantly upregulated in the malignant epithelium (Mann-Whitney U-test $p<0.0001$) (Figure 1B, 1C). Increasing ERK5 expression is associated with less differentiated tumours: (1) The Kruskal-Wallis test demonstrated a significant association of ERK5 cytoplasmic immunoreactivity with Gleason sum score ($p<0.0001$) (Figure 2A); (2) Linear regression analysis revealed a strong significant correlation between ERK5 cytoplasmic protein expression and increasing Gleason sum score ($p<0.0001$; $\rho=0.998$). We then compared ERK5 cytoplasmic expression with our previous data on MEK5 cytoplasmic expression (matched cases, n=75)[17]. Linear regression demonstrated a strong significant correlation between ERK5 and MEK5 cytoplasmic expression (two sided $p=0.0053$; $\rho=0.91$) (Figure 2B). There was also a significant correlation between ERK5 cytoplasmic expression and the presence of bone metastases ($p=0.0044$) and extra-capsular (locally advanced) disease ($p=0.0023$). In our clinical series, with median follow-up of 42 months (range 0.5-126), increasing cytoplasmic ERK5 immunoreactivity was

significantly associated with an overall poor patient prognosis, comparing low and intermediate expression groups with the high expression group (p=0.036, Kaplan-Meier and log rank analysis) (Figure 2C). Further statistical analysis revealed that ERK5 cytoplasmic expression did not relate to prostate-specific antigen levels (p=0.295).

ERK5 nuclear staining is an independent prognostic marker in prostate cancer.

Of particular interest, strong nuclear ERK5 signals was noted in 15 of the 81 PC cases (Figure 1D). This was in direct contrast to absent or scanty nuclear staining in the other 66 cases of CaP and BPH controls (n=20). The presence of nuclear ERK5 over-expression is strongly associated with poor patient prognosis (p<0.0001, Kaplan-Meier and log rank analysis) (Figure 2D). Further stratification revealed that strong nuclear expression retains its significant association with poorer patient prognosis, irrespective of Gleason grade (p=0.0008, Kaplan-Meier and log rank analysis) (Figure 2E) or presence of strong ERK5 cytoplasmic intensity (p=0.0052, Kaplan-Meier and log rank analysis) (Figure 2F). Multivariate analysis was carried out using Cox regression for survival data. For ERK5 cytoplasmic expression, this did not provide any further independent prognostic information, while ERK5 nuclear expression was shown to be an independent prognostic marker in prostate cancer (p<0.0001).

ERK5 is more strongly expressed in androgen independent prostate cancer

Analysis of ERK5 expression in matched tumour pairs revealed that the presence of ERK5 nuclear expression is significantly associated with the transition from hormone sensitive to hormone insensitive disease (p=0.0078) (Figure 1E, 1F). Non-significant

trends are seen with stronger ERK5 cytoplasmic expression in hormone relapsed disease (p=0.064). This was further investigated by performing western blot analysis for ERK5 protein expression on lysates from the androgen dependent prostate cancer cell line, LNCaP, and its androgen independent derivative, LNCaP-DCC. The androgen independent LNCaP-DCC cells have increased ERK5 protein expression compared to the parental cells (Figure 3A).

Localisation of ERK5 expression in human prostate cancer

To evaluate localisation of ERK5 expression in human prostate cancer cell lines we transfected PC3 cells grown on a coverslip with EGFP-C1 ERK5 +/- pcMEK5D and viewed the cells under fluorescent light. Transfection with the GFP-ERK5 fusion protein alone resulted in strong cytoplasmic or perinuclear fluorescence, though ERK5 appeared to be excluded from the nucleus. However, co-transfection of the GFP-ERK5 expression vector with the pcMEK5D expression vector resulted in appearance of ERK5 in the nucleus with absence of cytoplasmic signalling. Both controls showed pan-cellular fluorescence (Figure 3B).

ERK5 enhances MMP-1, MMP-2 and MMP-9 expression

We identified a significant correlation between ERK5 overexpression and the presence of bony metastases in our clinical series (Figure 3C). We hypothesise that ERK5 may act as a modulator of genes involved in metastasis. The matrix metalloproteins (MMPs) constitute a broad family of zinc-binding endopeptidases that play a key role in the degradation of the extracellular matrix and basement membrane. To determine if ERK5

overexpression may modulate MMP expression, we transiently transfected ERK5 *wild type* DNA into PC3 cells and subsequent reporter gene assays were carried out with three separate MMP/Luciferase constructs (MMP-1, MMP-2 and MMP-9), corrected for β-galactosidase, and empty vector as control. Upon ERK5 *wild type* transfection, MMP-1 was found to have an approximate 4x fold induction relative to empty vector control. MMP-2 was found to be induced approximately 5.5x relative to control. MMP-9 was induced 4x relative to control. All three inductions were found to be statistically different to the empty vector control, (p value <0.001) (Figure 3D).

Overexpression of ERK5 increases while inhibition of ERK5 activation suppresses proliferation in prostate cancer cells

To test if proliferation is driven by overexpression of ERK5 in human prostate cancer, WST-1 assays were performed on PC3 cells overexpressing ERK5 (PC3-ERK5 cells). The proliferative index of ERK5 overexpressing cells was 2.7 fold greater than that of PC3 cells stably transfected with the vector alone (P<0.0001) (Figure 4A). Pre-incubation of the EcR293 MEK5D stable clone with the small molecule inhibitor PD184352 prior to PonA induction lead to inhibition of activation of ERK1/2 and ERK5. However, whereas 1-3μM was sufficient to block the classical MAPK cascade, as judged by suppression of phosphorylation of ERK1/2, it was necessary to increase PD184352 to 10μM to suppress ERK5 activation (Figure 4B). A similar result was seen in PC3 cells where 0.3μM PD184352 was sufficient to block ERK1/2 activation and 3μM PD184352 was required to supress ERK5 activity (data not shown). Further proliferation analyses were performed in untransfected PC3 cells, pre-incubated at low dose 0.3μM PD184352,

sufficient to block ERK1/2 activation, and high dose 3μM PD184352 required to block ERK1/2 and ERK5 activation. Blockade of activation of the classic MAPK pathway was found to cause an increase in cellular proliferation, but this increase did not quite reach statistical significance (P=0.0728). However, pre-incubation at the higher dose of the inhibitor caused a statistical decrease in proliferation (P<0.0001) (Figure 4C).

Discussion

In keeping with our previous findings on MEK5, here we report our original findings of ERK5 overexpression in resected human prostate cancer. Importantly, we demonstrated a strong, linear correlation between MEK5 and ERK5 protein expression in matched cases (P=0.0053). This close relationship ties in with current knowledge of ERK5 as the only known substrate of MEK5, and phosphorylated MEK5 as the only known activating molecule of ERK5 [2, 23]. We have demonstrated a significant correlation between ERK5 cytoplasmic overexpression and Gleason sum score (P<0.0001); the inference being that the higher the Gleason grade of the disease, the higher the ERK5 protein expression in the tumour cells. This correlation was not observed between MEK5 overexpression and Gleason sum score [17]. In keeping with the notion that ERK5 has a more important role in carcinogenesis, a previous report studying the *in vitro* phosphorylation and activation status of ERK5 revealed evidence for ERK5 auto-phosphorylation, as well as its ability to phosphorylate MEK5 extensively at residues 129, 137, 142 and 149 [23]. Taken together, these results suggest that ERK5 is the driving molecule in this pathway and perhaps, not surprisingly, has a closer correlation with clinical parameters than MEK5.

We also discovered a significant correlation between ERK5 cytoplasmic expression and the presence of bone metastases (p=0.002). The clinical picture in the advanced stages of prostate cancer is dominated by the problem of bony metastases, which occur in approximately 90% of patients, representing the most important cause of morbidity, causing bone pain and complications such as pathological fracture, immobility,

haemopoietic compromises and spinal cord compression [24, 25]. A number of cytokines have been implicated in bony metastasis, including BMP-6 [26], TGFβ1 [27], interleukin 6 (IL-6) and IL-6 receptor [28, 29]. It is therefore of interest that a recent paper has identified IL-6 as an activator of ERK5 in multiple myeloma cells [30].

In normal prostate development, the interaction of prostate epithelial cells with surrounding stroma influences their growth, survival and differential potential. Components of the surrounding stroma include numerous cell types, such as fibroblast, endothelial, neuroendocrine and inflammatory cells, soluble growth factors and insoluble laminin-rich extracellular matrix (ECM). Many of the steps in cancer metastasis involve the production of ECM degrading proteases and compromised cell adhesion [31]. The matrix metalloproteinases (MMPs) are a key family of zinc-dependent enzymes involved in metastatic development that are known to be regulated by MAPK family members. MMPs play an important role in ECM remodelling, and a positive correlation between MMP expression and tumour progression has been demonstrated in both human and animal studies [32]. We have previously observed that activation of MEK5 results in the upregulation of MMP-9 transcription, demonstrating a moderate increase in promoter activity and confirming this induction at the mRNA level. Consistent with this here we have found that MMP family members -1, -2 and -9 are upregulated following ERK5 activation. Better understanding of the mechanisms involved in the formation of metastatic bony lesions in prostate cancer will facilitate the development of new therapeutic agents.

In addition, this study shows that increased cytoplasmic ERK5 expression correlates with a less favourable disease-specific survival, comparing low and intermediate expression

groups with the high expression group (P=0.036, Kaplan-Meier and log rank analysis). This result is in accord with that seen previously with MEK5 expression and survival [17]. Another important finding is revealed in the second immunohistochemical analysis, examining ERK5 expression in matched tumour pairs, revealing that the presence of ERK5 nuclear expression is significantly associated with the transition from hormone sensitive to hormone insensitive disease (P=0.0078). Non-significant trends are seen with stronger ERK5 cytoplasmic expression in hormone relapsed disease (P=0.064). Further evidence of increased ERK5 expression in androgen independent disease is evident from western blot analysis comparing androgen dependent LNCaP cells, and its androgen independent derivative LNCaP-DCC cells. The androgen independent derived cells, LNCaP-DCC, have increased ERK5 protein expression.

Of great interest in this paper is the finding that the presence of strong nuclear ERK5 expression was shown to have a strong significant association with poorer patient prognosis (P<0.0001). The strong nuclear expression retains this significant association with survival, irrespective of Gleason grade (P=0.0008) or presence of strong ERK5 cytoplasmic intensity (P=0.0052). A major novel finding of this paper is that a multivariate analysis, by Cox regression, demonstrated that ERK5 nuclear expression is an *independent* prognostic marker in prostate cancer (P<0.0001). In light of this, it is also interesting to note that in this paper we find that co-transfection of an ERK5 wild type with a MEK5D expression plasmid lead to nuclear accumulation of ERK5 in the prostate cancer cell line, PC3. This has previously been observed in CHO-K1 cells [7]. The duration and magnitude of MAPK activation on one hand, and the localization of MAPKs on the other hand have been identified as critical parameters that define specific

cellular responses [33, 34]. Nucleo-cytoplasmic transport of signalling molecules is essential for the regulation of eukaryotic cellular processes such as cell cycle progression, differentiation and circadian clocks [35, 36]. In the context of MAPK signalling, relocalisation of MAPKs from the cytosol to the nucleus is essential for phosphorylating and activating those transcription factors within the nuclear compartment [34, 37]. Buschbeck and Ullrich (2005) have analysed several cell lines and shown that, unsurprisingly, endogenous ERK5 is localised to cytoplasmic as well as nuclear compartments. They proposed a simplified model of ERK5 in which we can consider ERK5 to exist in a closed or open conformation. Under steady-state conditions, and in the absence of any factor, the majority of ERK5 is assumed to adopt the closed formation, under which ERK5 activation by MEK5 is hampered resulting in a dominant cytosolic localisation. The open conformation, on the other hand, facilitates activation and leads to nuclear accumulation [38].

In this study we have identified a subgroup of patients with strong nuclear ERK5 expression and very poor survival. These observations, including those of Buschbeck and Ullrich (2005), lead us to believe that, in this subgroup, the ability of the cell to dephosphorylate or shuttle activated ERK5 out of the nucleus has been lost, leading to increased transcription and, in turn, more aggressive disease culminating in decreased survival. The exact mechanism of how this abnormal, unsteady state arises is of great interest both biochemically and clinically as we have demonstrated that ERK5 nuclear expression is an independent prognostic factor in prostate cancer and, in tandem with PSA, may have a future in predicting disease prognosis.

Another possible mechanism explaining the increased nuclear expression of ERK5 stems from the recently published study establishing that in the mouse fibroblast cell line, NIH3T3, treatment with 1μM PD184352 (a MEK1/2 inhibitor) enhances nuclear accumulation of ERK5, suggesting that when ERK1/2 is suppressed, ERK5 activation is enhanced [39]. This is of interest in the light of the observations of Uzgare *et al.* (2003), who established that activation of ERK1/2 occurs in the early progression of prostate cancer but subsequent inactivation of ERK1/2 is contemporaneous with the emergence of the poorly differentiated, metastatic phenotype. Evidence for cross-talk between the ERK1/2 pathway and ERK5 has previously been suggested by the observation of Mody and co-workers [19], in that 1μM PD184352 led to a more sustained activation of ERK5 following growth factor stimulation. Our proliferation assays revealed firstly that ERK5 overexpression in human prostate cancer leads to increased proliferation, and secondly that pre-incubation of prostate cells with low dose PD184352, sufficient to block ERK1/2 activation, does not cause a decrease in cell proliferation; in fact cell proliferation is actually increased, though not quite significantly (P=0.0728), in these experiments. PD184352 (CI-1040) showed promise in a phase I trial of 77 heavily pre-treated patients achieving 28% stable disease in this population, but no objective responses were noted in a Phase II setting tested against various solid neoplasms [40, 41]. We now know PD184352 also blocks ERK5 activation and it is not clear, as it was not considered, if the given dose was high enough to block ERK5 activation in these studies.

In this paper, we have discovered a close association between ERK5 expression and adverse clinical parameters in human prostate cancer. The finding of strong nuclear ERK5 staining and its strong correlation with less favourable disease survival, and

androgen independent disease, opens up another field in the elucidation of our understanding of the role of the MEK5/ERK5 pathway in prostate cancer. Further studies are required to clarify the mechanism and consequences of ERK5 nuclear localisation. Of clinical importance, ERK5 nuclear staining has here been shown to be an independent marker of prognosis in prostate cancer, and may prove useful as a stratification tool to identify a more aggressive/ metastatic phenotype.