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Phosphorylation of the Androgen Receptor is associated with reduced survival in hormone refractory prostate cancer patients.

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Short title: Phosphorylation of androgen receptor in prostate cancer

Cell line studies demonstrate that the PI3K/Akt pathway is up-regulated in hormone refractory prostate cancer and can result in phosphorylation of the androgen receptor (AR). The current study therefore aims to establish if this has relevance to the development of clinical hormone refractory prostate cancer.

Immunohistochemistry was employed to investigate the expression and phosphorylation status of Akt and AR in matched hormone sensitive and refractory prostate cancer tumours from 68 patients.

In the hormone refractory tissue only phosphorylated AR (pAR) was associated with shorter time to death from relapse ($p=0.003$). However when an increase in expression in the transition from hormone sensitive to hormone refractory prostate cancer was investigated an increase in expression of PI3K was associated with decreased time to biochemical relapse ($p=0.014$) and an increase in expression of pAkt⁴⁷³ and pAR²¹⁰ were associated with decreased disease specific survival ($p=0.0019$, and $p=0.0015$, respectively). Protein expression of pAkt⁴⁷³ and pAR²¹⁰ also strongly correlated ($p<0.001$ c.c. 0.711) in the hormone refractory prostate tumours.

These results provide evidence using clinical specimens, that up-regulation of the PI3K/Akt pathway is associated with phosphorylation of the AR during development of hormone refractory prostate cancer, suggesting this pathway could be a potential therapeutic target.

Key words, Akt, androgen receptor, hormone refractory prostate cancer.

Introduction

Prostate cancer is an increasing health care problem, 1 in 6 UK men are diagnosed with this disease which ranks 2nd behind lung cancer as a cause of male cancer specific mortality (Cancer Research UK web site, 2004). Advanced prostate cancer treatment has relied on hormone deprivation therapy for the past 50 years. Response rates are initially high (70-80%), however almost all patients relapse and develop hormone refractory prostate cancer, resulting in increased morbidity and death (Cleeve and Goad, 1995).

Amplification of the androgen receptor (AR) may explain development of hormone refractory prostate cancer in 20-30% of patients (Edwards et al., 2001). *In vitro* studies demonstrate that Akt/protein kinase B, phosphorylates AR at serine residues (Ser²¹⁰ and Ser⁷⁹⁰) resulting in modulation of AR transcriptional activity (Lin et al., 2001; Lin et al., 2003) suggesting that AR phosphorylation might promote development of hormone refractory prostate cancer. It has also been reported that activation of PI3K/Akt pathway can induce expression of AR at the protein and mRNA level, again suggesting that this pathway may be involved with hormone refractory disease (Yang et al., 2005). Akt is a downstream member of the phosphatidylinositol 3-OH kinase (PI3K) cascade, which plays an important role in cell growth, death, adhesion and migration and is frequently activated in cancer cells (Lin et al., 2003). Akt is activated when phosphorylated at threonine 308 and subsequently serine 473. There are 3 members of the Akt family, Akt 1, 2 and 3, and evidence suggests that the 3 isoforms of Akt have different roles in the development of hormone resistant breast cancer either via interactions with the oestrogen receptor or via proteins involved in proliferation and apoptosis e.g. mTOR and BAD (Kirkegaard et al., 2005). Akt may play a similar role in the development of hormone refractory prostate cancer (Liao et al., 2003). Cell line studies demonstrate that low

passage LNCaP cells (hormone sensitive prostate cancer cells) have low basal Akt activity, possibly due to early stage prostate cancer relying on hormones for growth and survival. However during hormone ablation or anti hormone treatment LNCaP cells undergo growth arrest and apoptosis, and Akt activity is up-regulated (more than 20 fold higher), resulting in stimulation of cell growth, compensating for the effects of androgen withdrawal (Gao et al., 2003). Data from these experiments suggests that Akt signals for cell growth and survival at low levels of androgen and therefore may promote development of hormone refractory prostate cancer (Ghosh et al., 2003;Lin et al., 2003). This is supported by a report that demonstrates that up-regulation of the PI3K cascade allows cells to grow in the presence of anti androgens and contributes to failure of endocrine therapy (Murillo et al., 2001).

A recent review suggests that the PI3K/Akt pathway is a possible therapeutic target for treatment of prostate cancer (Pommery and Henichart, 2005). Prostate tumours are reported to have significantly higher Akt expression than BPH (Liao et al., 2003) and only 10% of well-differentiated prostate tumours strongly express phosphorylated Akt compared to 92% of poorly differentiated tumours (Ayala et al., 2003;Ghosh et al., 2003;Malik et al., 2002). Akt-1 and Akt-2 expression in hormone sensitive tumours have been associated with shorter time to biochemical relapse, however no association was reported with the activated forms or with survival (Le Page et al., 2006). However, to our knowledge this is the first study to conduct a comprehensive investigation into the changes that occur to multiple members of the pathway in the transition from clinical hormone sensitive to hormone refractory prostate cancer, and in particular to test if phosphorylation of AR is associated with patient outcome measures.

Materials and methods

Patient

Sixty eight patients with matched hormone sensitive and hormone refractory tumour pairs were retrospectively selected for analysis. All tumours had patient identification removed, including block number and hospital number and were coded to make the database anonymous. Ethical approval was obtained from the Multicentre Research Ethics Committee for Scotland (MREC/01/0/36) and Local Research and Ethical Committees. Patients were only selected for analysis if they initially responded to hormone treatment (in the form of sub capsular bilateral orchidectomy or maximum androgen blockade) but subsequently relapsed (2 consecutive rises in PSA greater than 10%) and had a pre and post hormone relapse tissue sample available for analysis.

Immunohistochemistry

All IHC was performed on 5 μ m, archival formalin fixed, paraffin embedded prostate tumour sections on separate slides. Immunohistochemistry for PI3K (p110 catalytic subunit), Akt1-3, phosphorylated Akt at threonine 473 (pAkt⁴⁷³), mTOR, phosphorylated mTOR at serine 2448 (mTOR2⁴⁴⁸) and phosphorylated AR at serine 210 (pAR²¹⁰) were performed as follows: Antigen retrieval was performed using heat treatment under pressure in a Tris EDTA Buffer (5mM Trizma Base, 1mM EDTA, pH 8: Akt 1-3 and pAR²¹⁰) or citrate buffer (PI3K, and mTOR) for 5 min or by heating to 96°C for 20 minutes in citrate buffer (pAkt³⁰⁸, pAkt³⁰⁸ and pmTOR²⁴⁴⁸). Non-specific background staining was blocked using either 2.5% horse serum in TBS for 20 minutes (PI3K, pAR²¹⁰), in 1% casein for 10 min (Akt1-3, mTOR, pmTOR²⁴⁴⁸) or in Serum Free Block (Dako A/S) for 10 min (pAkt⁴⁷³). PI3K (Cell Signalling Technology, Beverly, USA), Akt1-3 (Santa Cruz Biotechnology Inc, Santa Cruz, USA), pAkt⁴⁷³ (Cell Signalling Technology), mTOR (Santa Cruz Biotechnology Inc), pmTOR²⁴⁴⁸ (Cell Signalling Technology) and pAR²¹⁰ (Imgenex, San Diego, USA) antibodies were used at the following concentrations (1 μ g/ml, 1 μ g/ml, 2 μ g/ml,

2µg/ml, 4µg/ml, 5µg/ml, 2µg/ml and 50µg/ml). pAR²¹⁰ was incubated for 1 hour at 25°C and all other antibodies were incubated overnight at 4°C. For the mTOR antibody only, incubation with rabbit anti-goat antibody (Dako A/S) (1:4000) for 1 h at room temperature was also required. Staining for PI3K, pAkt³⁰⁸, pAkt⁴⁷³, mTOR and pmTOR2⁴⁴⁸ was developed using EnVision plus kit (Dako A/S) and staining for Akt 1-3 and pAR²¹⁰ was developed using LSAB kit (Dako A/S) and 3,3-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories). Nuclei were counterstained with haematoxylin before mounting. A positive and negative control was included in each IHC run, negative controls were incubated with an isotype matched control antibody at a concentration of 1mg/ml. Positive control slides were breast tissue known to express PI3K, Akt and mTor, BPH tissue known to express AR and MCF-7 and LNCaP cell pellets. Antibody specificity was confirmed by western blotting. In addition, phosphorylated antibodies were confirmed to detect only the phosphorylated forms using Calf Intestinal Alkaline Phosphatase to destroy phosphorylated proteins. Two identical slides had IHC performed on them, the only difference being that one was previously treated with Calf Intestinal Alkaline Phosphatase, the untreated slide expressed the phosphorylate protein and the treated slide did not, this technique confirms that the antibody is only detecting the phosphorylated form of the protein and not the unphosphorylated form.

Tissue staining intensity was scored blind by 2 independent observers using a weighted histoscore method (Fraser et al., 2003) also known as the Hscore system (McCarty, Jr. et al., 1986). Histoscores were calculated from the sum of (1 x % cells staining weakly positive) + (2 x % cell staining moderately positive) + (3 x % cells staining strongly positive) with a maximum of 300. The inter-class correlation coefficient (ICCC) for each protein was calculated to confirm consistency between observers and the mean of the two observers' scores were used for analysis. Changes in staining between pre and post hormone refractory cases were defined as an increase

or decrease out with the 95% confidence interval for the difference in inter-observer variation i.e. the mean difference between the histoscores that each observer assigns for protein expression plus 2 standard deviations. Change in expression of PI3K, Akt1, Akt2, Akt3, mTOR, pmTOR²⁴⁴⁸ and pAR²¹⁰ are shown in table 1.

Statistical Analysis

Interclass correlation coefficients were used to confirm consistency between observers. Protein expression data were not normally distributed and are shown as median and inter quartile ranges. Wilcoxon Signed Rank Tests were used to compare expression between pre and post hormone refractory tumours. Survival analyses were conducted using Kaplan-Meier method and curves were compared with the log-rank test. Hazard ratios (HR) were calculated using Cox Regression analysis. Correlations between members of the pathway were performed using a Spearman's rank test.

Results

Patients

A total of 68 prostate cancer patients (diagnosed between 1984-2000) were included in this study with matched hormone sensitive (hormone sensitive tissue was obtained from 26 patients by TRUS guided biopsy and the remaining 42 by TURP) and refractory prostate tumours (all obtained by TURP) available for analysis (136 tumours in total). Patients in this cohort were diagnosed with locally advanced (50) or metastatic prostate cancer (18) and subsequently received surgery and androgen deprivation therapy (26 sub capsular bilateral orchidectomy, 44 GnRH analogue, 2 had both). Forty five of the 68 patients also received anti androgen therapy and this included all those who received GnRH analogues. At initial diagnosis the median age was 70 (66-74) years and 26% of patients had metastatic disease. The median time to biochemical relapse was 2.32 (1.48-4.00) years and the percentage of patients with

metastatic disease had increased to 57%. Sixty-one patients (89.7%) died during follow-up and median survival for these patients was 4.34 (2.94-6.63). Seven patients were alive at last follow-up, the median time of follow-up for all 68 patients was 4.34 (2.86-6.74) years.

Protein expression patterns

Akt 1, pAkt⁴⁷³ and pmTOR²⁴⁴⁸ protein expression was observed in the cell membrane, cytoplasm or nucleus. mTOR expression was observed at the membrane and cytoplasm and PI3K, Akt2 and Akt3 expression was observed only in the cytoplasm. Nuclear expression was observed for pAR²¹⁰, (figure 1, table 1). In order to assess the level of agreement between observers, interclass correlation coefficients (ICCC) were calculated for each antibody at each location using SPSS, all ICCC values in this study were greater >0.7 (which is classed as excellent) (table 1).

Protein expression levels and changes in protein expression.

The median expression levels for all proteins investigated were calculated for hormone sensitive and hormone refractory tumours (table 1). The Wilcoxon Signed Rank test was used to compare expression levels in the hormone sensitive tumours compared to hormone refractory tumours. Using this method only pAR²¹⁰ significantly increased with the development of hormone refractory disease. The median expression of pAR²¹⁰ in hormone sensitive tumours was 35(0-85) increasing to 103(50-169) in hormone refractory tumours (p<0.0001) (table 1). This demonstrates an increase in AR phosphorylation at the Akt consensus site in the transition from hormone sensitive to hormone refractory disease.

The nature of our cohort however (matched hormone sensitive and hormone refractory tumours for each patient) allowed us to establish if there was a change in protein expression levels in the transition from hormone sensitive to hormone

refractory disease for each individual patient. By examining the change in protein expression for each patient we were able to create sub groups of patients whose tumours exhibited either a fall or rise in protein expression for all proteins investigated (table 1). Using this technique it was observed that 42% of patients investigated in this study had an increase in pAR²¹⁰ expression in the transition from hormone sensitive to hormone refractory disease (figure 1).

Are protein expression levels in hormone sensitive or hormone refractory tumours associated with relapse or survival?

When expression levels of each protein investigated in the hormone sensitive tumours were divided into to high or low expression (levels above or below the median) none of the proteins investigated were associated with time to relapse or disease specific survival. The histoscores used as a cut off for each analysis was the median histoscore, the median histoscore for each protein investigated is given in table 1.

When expression levels of each protein investigated in the hormone refractory tumours were divided into to high or low expression (levels above or below the median) only pAR²¹⁰ was associated with quicker time to death from relapse (figure 2a, p=0.003, HR 2.85 (95% C.I., 1.38-5.87)) and quicker disease specific survival (figure 2b, p=0.0136, HR 2.33 (95% C.I., 1.16-4.66)). Median survival from time of relapse for those patients with tumours that expressed low levels of pAR²¹⁰ was 3.42 (IQR, 2.82-4.02) years compared to 1.40 (IQR, 0.85-1.95) years for those who had tumours that expressed high levels of pAR²¹⁰ and the median disease specific survival for those patients with tumours that expressed low levels of pAR²¹⁰ was 8.57 (IQR, 5.41-11.73) years compared to 5.82 (IQR, 3.18-8.46) years for those who had tumours that expressed high levels of pAR²¹⁰. This represents a survival difference of almost 3 years for patients expressing high levels of pAR²¹⁰ in their hormone refractory tumour.

Are changes in protein expression in the transition from hormone sensitive to hormone refractory disease associated with relapse or survival?

When expression levels in hormone sensitive or hormone refractory tumours were used to investigate a link between activation of the PI3K pathway and development of hormone refractory disease, only AR phosphorylated at the Akt consensus site was associated with survival. However due to the nature of the current cohort we were also able to investigate if those patients whose tumours exhibit an increase or rise in expression of members of the pathway in the transition from hormone sensitive to hormone refractory disease were more likely to relapse or die quicker. The cut-off histoscore selected to separate subgroups of patients is displayed in table 1. Using this method an increase in PI3K (Figure 3a, $p=0.014$, HR 2.11 (95% C.I., 1.14-3.91)) was associated with quicker time to relapse. The median time to relapse for those patients whose tumours have a decrease or no change in PI3K expression was 2.57 (IQR, 1.74-3.40) years compared to 1.36 (IQR, 1.20-2.72) years for those patients whose tumours had an increase in PI3K expression.

A rise in pAR²¹⁰ (Figure 3b, $p<0.0001$, HR 4.18 (95% C.I., 1.99-8.74)) was associated with quicker time to death from relapse, the median survival from biochemical relapse for those patients whose tumours had a decrease or no change in pAR²¹⁰ expression was 3.46 (IQR, 1.39-5.53) years compare to 1.25 (IQR, 0.83-1.67) years for those patients whose tumours had an increase in pAR²¹⁰ expression.

A rise in pAkt⁴⁷³ (Figure 3c, $p=0.0019$, HR 2.89(95% C.I., 1.43-5.8)) and pAR²¹⁰ (Figure 3d, $p=0.0015$, HR 2.86 (95% C.I., 1.45-5.67)) were associated with shorter disease specific survival. The median survival from diagnosis for those patients whose tumours had no change or decrease in pAkt⁴⁷³ was 6.68 (IQR, 6.22-7.14) years compare to 4.15 (IQR, 2.65-65) years for those patients whose tumours had an increase in pAkt⁴⁷³ expression and the median survival from diagnosis for those

patients whose tumours had a decrease or no change in expression of pAR²¹⁰ was 6.95 (IQR, 4.07-9.83) years compare to 4.36 (IQR, 1.67-7.10) years for those patients whose tumours had an increase in pAR²¹⁰ expression. Therefore an increase in expression in the transition from hormone sensitive to hormone refractory disease of phosphorylated members of this pathway was associated with a reduction in median survival of 2.5-3 years.

Correlations between active members of the pathway

In hormone sensitive tumours expression levels of the phosphorylated proteins did not correlate, no correlation was made between Akt activation and AR phosphorylation (Figure 4a). However in the hormone refractory tumours pAkt⁴⁷³ correlated with pAR²¹⁰ ($r_s=0.711$, $p<0.001$) (Figure 4b) and pmTOR2⁴⁴⁸ ($r_s=-0.489$, $p=0.003$). Table 2 shows the individual histoscores for pAkt and PI3K in patients with and without pAR²¹⁰ increases, no changes in median expression levels was observed between the groups.

Discussion

Cell line studies demonstrate that the PI3K cascade may influence the development of hormone refractory prostate cancer (HRPC), suggesting this pathway may provide a novel therapeutic target for prostate cancer. However in order to translate this into the clinic we are required to provide evidence that this pathway is up-regulated in the development of clinical hormone refractory prostate cancer and also identify what proteins would make the best targets and most effectively identify patients suitable for therapy.

It is well established in the literature that there is a link between Akt activation and development of hormone refractory prostate cancer. Cell line work demonstrates that Akt activity increases during androgen ablation to stimulate cell growth and survival when androgen reliance is weaker and therefore promote development of hormone refractory prostate cancer (Gao et al., 2003; Ghosh et al., 2003; Lin et al., 2003). Work

using human prostate tissue confirms that pAkt⁴⁷³ is expressed in PIN and invasive prostate cancer and staining intensity positively correlated with PSA levels and Gleason (Altomare and Testa, 2005; Ghosh et al., 2003; Majumder and Sellers, 2005). In addition a large study of 640 radical prostatectomy specimens demonstrated that high levels of phosphorylated Akt was predictive of biochemical recurrence (Ayala et al., 2003). Although in our current cohort we did not observe a significant association with phosphorylated Akt 473 and biochemical recurrence (pAkt473 p= 0.151, results not shown), the Kaplan Meier curves did separate and the difference in the median time to biochemical recurrence in the 2 groups was 1.8 year compared to 2.4 years, suggesting that significance might have been met if a larger cohort had been used. As the previous study was conducted on a cohort of 640 patient samples and our cohort was only contained 68 patients, we performed a power calculation to assess what cohort size would have been required, this calculation suggested that a cohort of 200 patients would have been sufficient to reach significance. It is therefore not surprising that a significant result was achieved on a cohort of 640 samples. However the design of our study was to mirror the cell line experiments and investigate if changes in protein expression of members of the PI3K/Akt cascade were involved in the transition from hormone sensitive disease to hormone refractory disease, therefore we felt that it was not necessary to increase that cohort size. The strength of this study was the use of the paired samples, by doing so we observed that an increase in expression of multiple members of the pathway was linked to time to recurrence and disease specific survival. In addition, if primary tumours only had been used we would not have observed that pAR²¹⁰ was linked to survival, as marked expression was only observed in hormone refractory tumours. In the current study we observe that an increase pAkt⁴⁷³ expression, fully activated Akt and an increase in pAR²¹⁰ was associated with decreased survival. Providing further evidence that activation of the

PI3K/Akt cascade is associated with development of hormone refractory prostate cancer.

Cell line data suggests that phosphorylation of AR by Akt at serine 210 results in an increase in AR translational activity when androgen levels are low and in addition activated Akt, over a longer period, has been demonstrated to up-regulated AR expression in LNCaP cells (Manin et al., 2002; Yang et al., 2005). The impact of both these actions of Akt on the AR will serve to increase the sensitivity of the AR to androgens, enabling transcription of AR regulated genes even when androgens levels are very low, similar to those experienced during chemical or surgical castration. This therefore provides a mechanism for the development of hormone refractory prostate cancer. In the current cohort, hormone refractory prostate tumours express significantly higher levels of pAR²¹⁰ compared to hormone sensitive tumours, the median histoscore of refractory tumours is 103 histoscore units compared to 35 histoscore units for sensitive tumours, $p < 0.0001$. Approximately 42% of patients have an increase in pAR²¹⁰ expression and these patients have a significantly shorter survival period than those with no increase in expression. In addition, expression levels of pAR²¹⁰ and AR strongly correlate with pAkt⁴⁷³ expression levels in the refractory tumours only, suggesting that it is only when androgen levels are low that this pathway is activated. These results provide additional evidence that the PI3K/Akt pathway is up-regulated during development of hormone refractory prostate cancer, resulting in phosphorylation of the AR and sensitisation to circulating adrenal androgens. Cell line studies demonstrate that this occurs *in vitro*, however the current data demonstrates for the first time that this may be one possible mechanism allowing development of hormone refractory prostate cancer in the clinical setting (Figure 5).

The protein mTOR is also down stream of Akt and was also investigated in this study as it has previously been to hormone refractory prostate cancer (Bubley et al., 2003). Akt can phosphorylate mTOR directly at threonine 2446 and serine 2448, but

can also activate mTOR indirectly via phosphorylation of TSC2 (Majumder and Sellers, 2005). In the current cohort pAkt⁴⁷³ expression correlates with pmTOR²⁴⁴⁸ expression, however expression levels or changes in expression levels of pmTOR²⁴⁴⁸ do not correlate with any clinical parameters in our cohort. This suggests that mTOR may not be involved in the development of hormone refractory prostate cancer. This was surprising as stimulation of mTOR ultimately results in increase protein synthesis and enhances translation of proteins involved in growth control via turning off 4EBP and activating S6Kinase. Although Akt has been demonstrated to phosphorylate mTOR directly, the role of these phosphorylation sites remains unclear. Phosphorylation of mTOR by Akt at serine 2448 might not correlate with mTOR activation, therefore pmTOR²⁴⁴⁸ may not necessary be involved with the development of HRPC and a more appropriate marker of mTOR activation could be S6Kinase (Ruggero and Sonenberg, 2005).

In summary, the current study demonstrates a role for the PI3K/Akt/AR pathway in the development of hormone refractory prostate cancer, however now that drugs are being developed to target specific components of pathways it is necessary to identify the proteins in the individual pathways that would make the most appropriate targets. Results from the current study suggest that a larger portion of tumours would respond to drugs targeting Akt activation in contrast to PI3K or PTEN. Methods currently being explored for inhibition of Akt activation include Akt antibodies, similar to humanized trastuzumab (Herceptin), ATP- competitive inhibitors and PDK1 inhibitors (Cheng et al., 2005). In addition to Akt being the appropriate target for therapy, Akt in combination with pAR²¹⁰ might be the appropriate marker for identifying patients most likely to respond to drugs that target Akt activation. Phosphorylation of Akt and AR are clearly linked with decreased survival, those patients who have an increase in pAkt⁴⁷³ and/or pAR²¹⁰ have median overall survival period of 3.98 years compared to 6.68 years, this is a survival difference of almost 3

years and approximately 50% of the patients in our study have an increase in pAkt⁴⁷³ and/or pAR²¹⁰ expression suggesting that the majority of patients likely to respond to Akt inhibition will be identified by these markers. This study emphasises the need for a rational approach for new drug design and the progress already made in breast cancer demonstrates the effectiveness of such an approach.

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Table 1 Histoscore variation and comparison of staining intensity for hormone sensitive and hormone refractory tumours.

| | HSPC (IQR) | HRPC (IQR) | P value | ICCC | Histoscore units | Fallers | Risers |
|------------------------------|-----------------------|-----------------------|-------------------|-------------|-----------------------------|----------------|---------------|
| PI3Kc | 100(58-140) | 100(79-134) | 0.875 | 0.85 | 60 | 23 | 23 |
| Akt1m | 0(0-11) | 0(0-90) | 0.798 | 0.88 | 30 | 14 | 13 |
| Akt1c | 75(20-100) | 70(0-90) | 0.488 | 0.82 | 55 | 20 | 13 |
| Akt1n | 0(0-18) | 0(0-0) | 0.110 | 0.95 | 21 | 19 | 9 |
| Akt2 | 125(100-185) | 120(98-165) | 0.551 | 0.81 | 61 | 22 | 13 |
| Akt3 | 50(0-100) | 60(0-95) | 0.619 | 0.84 | 48 | 20 | 30 |
| pAkt⁴⁷³m | 40(0-90) | 33(0-90) | 0.988 | 0.90 | 58 | 22 | 23 |
| pAkt⁴⁷³c | 88(54-110) | 80(40-105) | 0.671 | 0.89 | 49 | 25 | 23 |
| pAkt⁴⁷³n | 0(0-25) | 0(0-35) | 0.465 | 0.93 | 40 | 15 | 18 |
| pAR²¹⁰n | 35(0-85) | 103(50-169) | <0.0001 | 0.93 | 52 | 8 | 42 |
| mTORM | 0(0-20) | 0(0-10) | 0.134 | 0.92 | 47 | 10 | 3 |
| mTORc | 43(15-87) | 40(10-62) | 0.123 | 0.83 | 31 | 35 | 23 |
| pmTOR²⁴⁴⁸m | 0(0-22) | 0(0-15) | 0.330 | 0.95 | 33 | 14 | 8 |
| pmTOR²⁴⁴⁸c | 61(20-100) | 40(8-70) | 0.044 | 0.91 | 45 | 33 | 23 |
| pmTOR²⁴⁴⁸n | 0(0-10) | 0(0-0) | 0.575 | 0.90 | 19 | 3 | 6 |

Table 1 shows the median histoscore and interquartile range (IQR) for hormone sensitive tumours (HSPC) and hormone refractory tumours (HRPC) and the p value of HSPC histoscores compared to HRPC histoscores using a Wilcoxon sign rank test. The median histoscore was the cut off histoscore used to select for the separate subgroups when defining high and low expressers. The interclass correlation coefficient (ICCC) which measures consistence between observers for each protein is consistently higher than 0.7 which is classed as excellent. The mean difference in observer scores plus 2 standard deviations is also shown as the number of histoscore units, the is the cut-off in histoscore units used to select for the separate subgroups ie defined as a change in protein expression. The percentage of tumours that were defined as having a fall or rise in protein expression (calculated using the number of histoscore units that is defined as a change in expression) are also shown. m, c and n

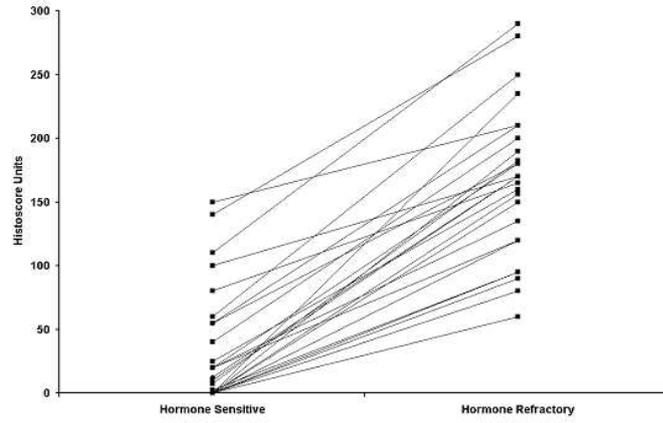
relates to protein location, m = membrane, c= cytoplasm and n = nucleus. P before a protein indicates that the antibody detects phosphorylated protein and the number following the protein represents the site of phosphorylation.

Table 2 Histoscores of pAkt and PI3K and comparison of staining intensity between patients with or without an increase in pAR210 expression.

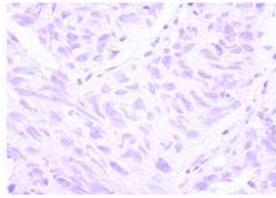
| Group 1 | | | | Group 2 | | | |
|---------|---------|---------|---------|---------|---------|---------|---------|
| HS pAkt | HS PI3K | HR pAkt | HR PI3K | HS pAkt | HS PI3K | HR pAkt | HR PI3K |
| 0 | 80 | 0 | 70 | 0 | 30 | 110 | 95 |
| 0 | 100 | 105 | 90 | 70 | 110 | . | 180 |
| 90 | 160 | 0 | 60 | 50 | 80 | . | 105 |
| 0 | 260 | 0 | 100 | 55 | 50 | 100 | 40 |
| . | | . | | 105 | 105 | . | 160 |
| 130 | 140 | 55 | 60 | 90 | 105 | 180 | 80 |
| 90 | 20 | 125 | 90 | 0 | 130 | 40 | 90 |
| | | 120 | 160 | 25 | 30 | 140 | 100 |
| 50 | 15 | . | 100 | 75 | 190 | 2.5 | 280 |
| 130 | 70 | 105 | 0 | 110 | 200 | 55 | 35 |
| 50 | 53.75 | 60 | 100 | 85 | 25 | 150 | 10 |
| 110 | 200 | 0 | 132.5 | 100 | 40 | 5 | 180 |
| . | | . | | 100 | 80 | 117.5 | 130 |
| 135 | 130 | 40 | 140 | 80 | 25 | 90 | 105 |
| 20 | 100 | 102.5 | 170 | 95 | 140 | 0 | 62.5 |
| 130 | 105 | 50 | 120 | 97.5 | 100 | 75 | 90 |
| . | 100 | . | 30 | 72.5 | 115 | 105 | 100 |
| 100 | 0 | 5 | 50 | 0 | 0 | 110 | 70 |
| 110 | 180 | 80 | 195 | 100 | 0 | . | |
| 100 | 100 | 20 | 200 | 75 | 110 | 120 | 115 |
| 100 | 195 | 100 | 165 | 65 | 80 | 185 | 100 |
| 162.5 | 190 | 90 | 100 | 0 | 15 | 90 | 100 |
| 180 | 210 | 100 | 160 | . | | . | |
| . | 90 | . | 120 | 137.5 | 170 | 35 | 105 |
| 165 | 0 | 40 | 100 | 0 | 90 | 75 | 75 |
| 60 | 40 | 5 | 25 | . | 200 | . | 137.5 |
| 85 | 190 | 80 | 0 | 160 | 200 | 135 | 150 |
| 25 | 115 | 100 | 100 | 125 | 170 | 0 | 190 |
| 90 | 100 | 155 | 80 | 220 | 200 | 0 | 150 |
| 35 | 100 | 70 | 25 | 120 | 120 | . | 160 |
| 70 | 120 | 40 | 100 | 72.5 | 100 | 100 | 80 |
| 85 | | 60 | | 75 | 40 | 130 | 0 |
| . | 200 | . | 100 | 120 | 80 | 150 | 0 |
| 90 | 100 | 60 | 100 | | | | |

Table 2 shows the histoscore for cytoplasmic phosphorylated Akt and PI3K in hormone sensitive tumours (HS) and hormone refractory tumours (HR). Group 1 represents those patients with no increase in phosphorylated AR expression and group 2 represents those patients with an increase in phosphorylated AR expression in the transition from hormone sensitive to hormone refractory disease.

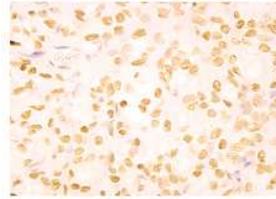
A



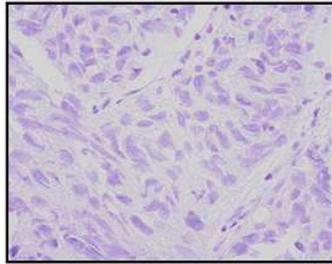
B



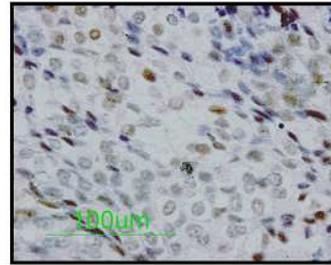
C



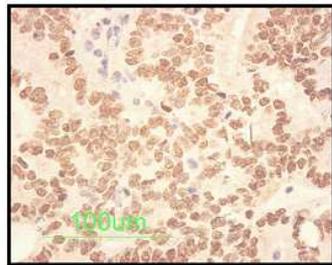
D



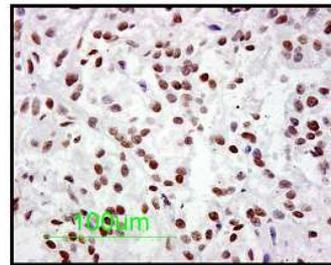
Histoscore 0



Histoscore 1-100

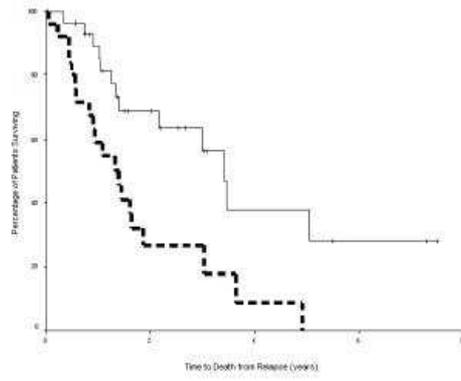


Histoscore 101-200

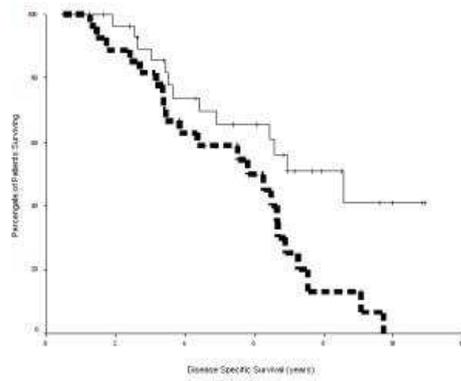


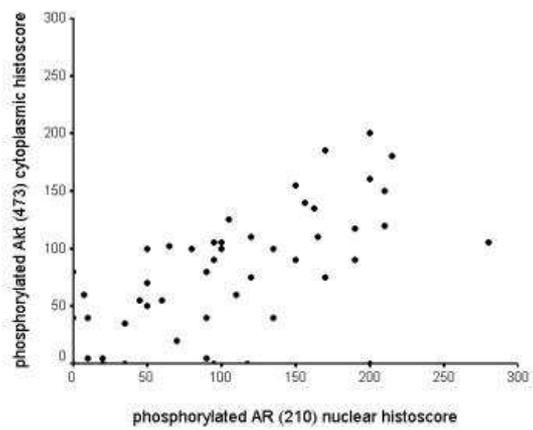
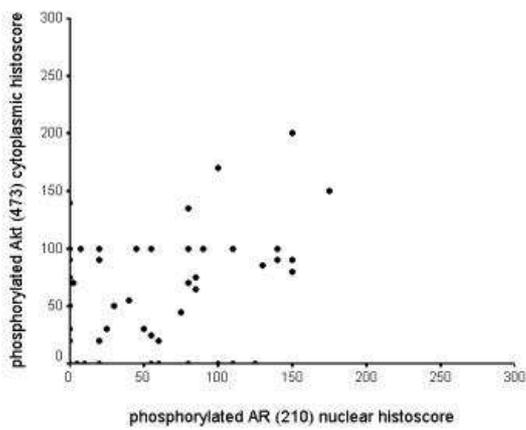
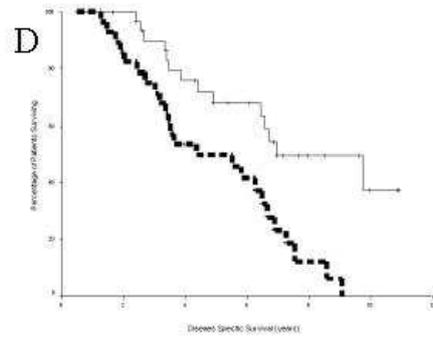
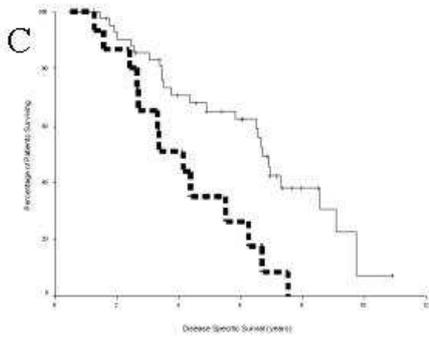
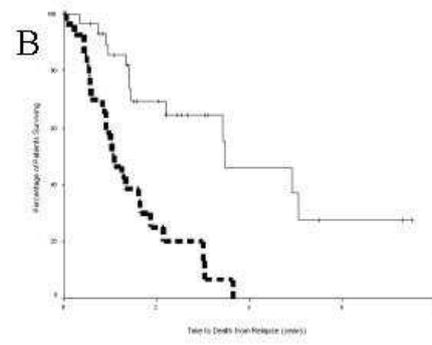
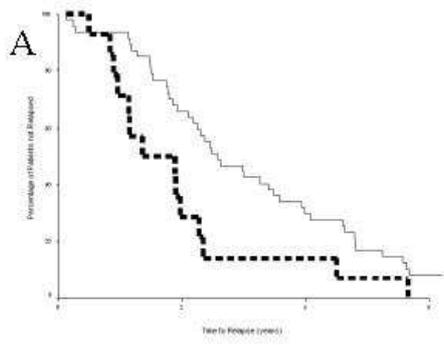
Histoscore 201-300

A



B





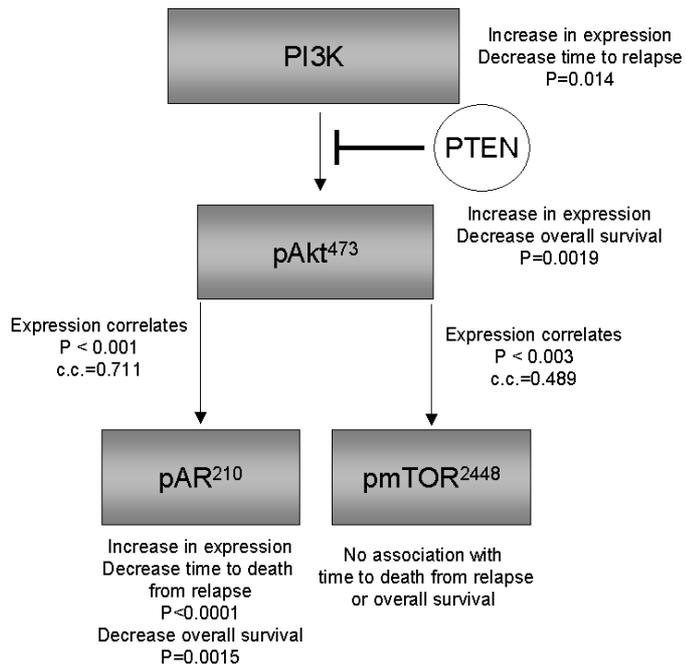


Figure Legends

Figure 1a shows the actual increase in histoscore units of the 42% of patients whose tumours exhibited an increase in pAR²¹⁰ expression in the transition from hormone sensitive to hormone refractory disease.

Figure 1b and 1c These images are of an actual pair of hormone sensitive and hormone refractory tumours whose expression increased by 150 histoscore units. Brown nuclear staining denotes pAR²¹⁰ expression. Magnification x400.

Figure 1d. These images show examples of a negative control, a tumour that was score with a histoscore in the range of 1-100, 101-200 or 201-300.

Figure 2a. Kaplan Meier plot demonstrates that those patients whose hormone refractory tumour has high pAR²¹⁰ nuclear expression (broken line) have shorter time to disease specific death from time of biochemical relapse than those patients whose hormone refractory tumour has low pAR²¹⁰ nuclear expression (solid line)(p=0.003).

Figure 2b. Kaplan Meier plot demonstrates that those patients whose hormone refractory tumour has high pAR²¹⁰ nuclear expression (broken line) have shorter disease specific survival than those patients whose hormone refractory tumour has low pAR²¹⁰ nuclear expression (solid line)(p=0.014).

Figure 3a Kaplan Meier plot demonstrates that those patients whose tumours exhibit a rise in PI3K expression (broken line) relapse quicker than those patients whose tumours exhibit no change or a fall in PI3K expression (solid line).

Figure 3b Kaplan Meier plot demonstrates that those patients whose tumours exhibit a rise in pAR²¹⁰ expression (broken line) have shorter time to disease specific death from time of biochemical relapse than those patients whose tumours exhibit no change or a fall in pAR²¹⁰ expression (solid line). whose tumours had an increase in pAR²¹⁰ expression.

Figure 3c Kaplan Meier plot demonstrates that those patients whose tumours exhibit a rise in pAkt⁴⁷³ cytoplasmic expression (broken line) have shorter time to disease specific death than those patients whose tumours exhibit no change or a fall in pAkt⁴⁷³ expression (solid line).

Figure 3d Kaplan Meier plot demonstrates that those patients whose tumours exhibit a rise in pAR²¹⁰ expression (broken line) have shorter time to disease specific death than those patients whose tumours exhibit no change or a fall in pAR²¹⁰ expression (solid line).

Figure 4 Scatter plots of phosphorylated Akt 473 histoscore compared to phosphorylated AR 210 histoscore, figure 4a is in the hormone sensitive tissue and no significant correlation was observed ($p=0.061$, correlation coefficient 0.251), however figure 4b is in the hormone refractory tissue where a significant correlation was observed ($p<0.001$ and correlation coefficient 0.711).

Figure 5 A simplified cartoon of the PI3K/Akt pathway; the p values and correlation coefficients (r_s) represent those found when protein expression/activation were correlated in hormone refractory prostate cancer tissue specimens.

