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# **Is PTEN loss associated with clinical outcome measures in human prostate cancer?**

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Running title: PTEN in prostate cancer.

Inactivating PTEN mutations are commonly found in prostate cancer, resulting in increased activation of Akt. In the current study we investigate the role of PTEN deletion and protein expression in the development of hormone refractory prostate cancer using matched hormone sensitive and refractory tumours.

Fluorescent insitu hybridisation and immunohistochemistry was carried out to investigate *PTEN* gene deletion and PTEN protein expression in the transition from hormone sensitive to hormone refractory prostate cancer utilising 68 matched hormone sensitive and hormone refractory tumour pairs (one before and one after hormone relapse).

Heterogeneous *PTEN* gene deletion was observed in 23% of hormone sensitive tumours, this increased significantly to 52% in hormone refractory tumours ( $p=0.044$ ).

PTEN protein expression was observed in the membrane, cytoplasm and the nucleus.

In hormone sensitive tumours, low levels of cytoplasmic PTEN was independently associated with shorter time to relapse compared to high levels of PTEN ( $p=0.028$ , hazard ratio 0.51 (95%CI 0.27-0.93). Loss of PTEN expression in the nucleus of hormone sensitive tumours was independently associated with disease specific survival ( $p=0.031$ , hazard ratio 0.52 (95%CI 0.29-0.95).

The results from this study demonstrate a role for both cytoplasmic and nuclear PTEN in progression of prostate cancer to the hormone refractory state.

**Keywords:** PTEN, Akt, prostate, hormone refractory.

## INTRODUCTION

Every year in the UK almost 32,000 cases of prostate cancer are diagnosed and approximately 10,000 die of the disease (Cancer Research UK, 2004). The underlying molecular mechanisms of prostate cancer initiation and progression are largely unknown due to extensive tumour heterogeneity. Patients diagnosed with locally advanced or metastatic prostate cancer may be treated by androgen ablation therapy, resulting in a reduction of androgens in the circulation and inhibition of tumour growth. This treatment is initially successful, but patients tend to relapse within 18-24 months with disease progression refractory to therapy (Arnold and Isaacs, 2002). Hormone refractory prostate cancer has a poor prognosis, with median survival period reported to be 24 months. Loss of androgen dependence is often correlated with overexpression of anti-apoptotic and cell survival signalling pathways (Johnson and Hamdy, 1998; Karan *et al*, 2003). Components of these pathways are mutated or abnormally expressed in many tumour types and mutations of upstream receptors such as EGFR transmit their signals through these cascades. Loss of tumour suppressor function is also a common event in the progression to hormone refractory prostate cancer and is associated with gain of oncogenic signalling (Isaacs and Kainu, 2001). P53, Retinoblastoma (Rb), NKX3.1 and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) have all been well documented to have a loss of function during the progression to hormone refractory prostate cancer (Bookstein *et al*, 1990; Davies *et al*, 1999; He *et al*, 1997; McMenamin *et al*, 1999; Phillips *et al*, 1994).

PTEN functions as a lipid phosphatase that dephosphorylates phosphatidylinositol (3, 4, 5) triphosphate (PIP3), a second messenger of PI3K at the plasma membrane. PIP3 recruits phosphatidylinositol dependent kinase-1 (PDK1) and Akt to the membrane. Here PDK1 phosphorylates Akt, which leads to its activation. Akt mediates signals down stream that promote cell survival and proliferation. PTEN mutants that retain protein tyrosine phosphatase activity but lose the ability to

dephosphorylate PIP3 are found in many tumours, indicating that the lipid phosphatase activity is needed for tumour suppression (Myers *et al*, 1998). To function in this manner PTEN must be located in the cytoplasm, however it has recently emerged that there is also a role for PTEN in the nucleus (Chung and Eng, 2005; Eng, 2003; Lian and Di Cristofano, 2005).

Prostate cancer cell lines that have been cultured from metastatic sites such as the lymph nodes (LNCaP) or brain metastasis (PC3) have highly active PI3K/Akt signalling (Davies *et al*., 1999; Murillo *et al*., 2001). *PTEN* mutation has been associated with 5-27% of localised and 30-60% of metastatic prostate tumours (Feilotter *et al*, 1998; Suzuki *et al*, 1998; Wang *et al*, 1998). In addition, loss of PTEN expression is associated with disease progression and increased risk of recurrence (Burton *et al*, 2000; Fenci and Woenckhaus, 2002; Gray *et al*, 1998; McMenamin *et al*, 1999; Reiss *et al*, 2000), although substantial heterogeneity has been observed between different metastatic sites within the same patients (Suzuki *et al*, 1998). Here we examined both deletion of the *PTEN* gene and expression of PTEN protein at individual cellular locations in a cohort of matched hormone sensitive and hormone refractory prostate tumours, with the aim of clarifying the prognostic significance of PTEN loss in prostate cancer. Using the same cohort we have previously demonstrated that up-regulation of Akt activation is significantly associated with decreased survival, therefore PTEN loss is one possible route by which this may occur (McCall *et al*, 2008).

## **MATERIALS AND METHODS**

### **Patients**

Sixty eight patients with matched hormone sensitive and hormone refractory tumour pairs were retrospectively selected for analysis. All tumours had patient identification removed, and the clinical information database was anonymised. 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. Hormone refractory prostate cancer specimens were obtained by TURP when patients required further surgical to treat clinical symptoms such as bladder outflow obstruction. All these samples were obtained with 8 weeks of biochemical relapse being diagnosed (2 consecutive rises in PSA greater than 10%). Phosphorylated Akt expression is already available for this patient cohort (McCall *et al*, 2008).

### **Fluorescent In Situ Hybridisation**

Fluorescent in situ hybridisation (FISH) was performed on 5 µm, archival formalin fixed, paraffin embedded prostate tumour tissue arrays. Slides were incubated for 1 hour at 56°C, dewaxed and re-hydrated through graded alcohols. Tissue was then pre-treated using histology FISH accessory kit (Dako A/S, Glostrup, Denmark). In brief, slides were rinsed twice in wash buffer and then incubated for 10 min in pre-treatment buffer at 95°C, followed by 3 min incubation at room temperature in wash buffer. Slides were then incubated in Pepsin for 26 mins at 25°C, followed by 3 min incubation at room temperature in wash buffer. Dako A/S provided *PTEN* probe, 10 µl of probe was applied to each slide and incubated at 82°C for 22 min followed by an overnight incubation at 45°C. Slides were then washed in stringent wash buffer for 10 min at 65°C, followed by two 3 min washes in wash buffer. Slides were dehydrated through graded alcohols, mounted in DAPI Vectashield (Vector Laboratories, CA, USA) and viewed using a Leica DMLB microscope at x400 magnification (Fig. 1a). FISH was scored by two independent observers; the number of signals for each chromosome (green) and gene (red) were counted in 20 non-overlapping nuclei. The

gene to chromosome ratio was then calculated. *PTEN* loss was classified as a gene to chromosome ratio of  $\leq 0.8$  (Watters *et al*, 1998).

### **Immunohistochemistry**

Tumour expression of PTEN was determined in archival formalin fixed, paraffin embedded prostate tissue sections (5 $\mu$ m) by Immunohistochemistry (IHC). Sections were dewaxed in xylene and rehydrated through graded alcohols prior to blocking endogenous peroxidase in 3% hydrogen peroxide. Antigen retrieval was performed by heating tissue sections under pressure in citrate buffer (pH6) for 5 minutes. Sections were blocked using 1.5% horse serum, PTEN antibody (Cell Signalling Technology), was used at 1 $\mu$ g/ml, and incubated overnight at 4°C. Staining was developed using Envision plus kit (Dako A/S). Nuclei were counterstained with haematoxylin before mounting. An example of the staining is shown in figure 1 (Fig. 1b). A positive and negative control slide was included in each IHC run; negative controls were incubated with an isotype matched control antibody at a concentration of 1mg/ml. Cell pellets known to express or not express PTEN were also included in each run. Antibody specificity was confirmed by western blot analysis (Fig. 1c).

Staining was scored blind by two independent observers using a weighted histoscore method (Kirkegaard *et al*, 2006) also known as the Hscore system (McCarty *et al*, 1986). Histoscores were calculated from the sum of (1 x the % of cells staining weakly positive) + (2x the % of cells staining moderately positive) + (3 x the % of cells staining strongly positive) with a maximum histoscore of 300. The inter-class correlation coefficient (ICCC) between each observer was confirmed to measure consistency. The ICC value was  $> 0.7$ , which is classed as excellent as; an ICC of 1 indicates identical score (Kirkegaard *et al*, 2006). The mean of the two observer's histoscores was used for analysis. Changes in protein expression staining between hormone sensitive and 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 histoscore that each observer assigns for protein expression plus or minus 2 standard deviations) (Edwards *et al*, 2003).

All statistical analysis was performed using the SPSS version 9.0 for Windows. Protein expression data was not normally distributed and is given as median and interquartile ranges. Wilcoxon signed Rank tests were used to compare protein expression between hormone sensitive prostate cancer and hormone refractory prostate cancer tumours. Survival analysis was conducted using the Kaplan–Meier method and curves were compared with the log rank test. Multivariate survival analysis and hazard ratios (HR) were calculated using Cox regression analysis. A value of  $p < 0.05$  was considered statistically significant.

## **RESULTS**

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.



## **Factors Associated With Time To Biochemical Relapse, Time To Death From Biochemical Relapse And Disease Specific Survival**

When serum PSA level, age, metastasis and Gleason grade at diagnosis were analysed for this patient cohort using the Kaplan-Meier log rank method, PSA at diagnosis ( $p=0.036$ ) and Gleason score at diagnosis ( $p=0.010$ ) were associated with shorter time to biochemical relapse.

Death from time of biochemical relapse was associated with PSA level at relapse ( $p=0.016$ ). Overall survival was associated with presence of metastases at relapse ( $p=0.0019$ ) and Gleason score at diagnosis ( $p=0.049$ ).

## **Fluorescent In Situ Hybridisation**

### **PTEN deletions**

Of the 68 prostate carcinomas investigated, *PTEN*/chromosome 10 ratio was successfully evaluated in 57 (84%) cases. The remaining cases were excluded from the study because of insufficient tumour material in the cores. The mean *PTEN*/chromosome 10 ratio for the hormone sensitive tumours was 0.98 (range 0.71-1.11) and for the hormone refractory tumours was 0.92 (range 0.39-2.16). Gene deletion as identified by FISH was observed in 23% of hormone sensitive tumours, this increased significantly to 52% in hormone refractory tumours ( $p=0.044$ ). Loss of one copy of *PTEN* was commonly observed, and this was heterogeneous in nature, being frequently observed in only one area of tumour. Loss of *PTEN* was correlated with prostate cancer progression, however, no correlation was observed between loss of *PTEN* and Gleason score at diagnosis, loss of *PTEN* and presence of metastasis at diagnosis or loss of *PTEN* and PSA at diagnosis. When loss of *PTEN* was correlated with survival, a trend between loss of *PTEN* and poorer disease specific survival was noted ( $p=0.086$ ), this was not independently significant by Cox regression analysis.

### **Gene number and chromosomal aneusomy**

In the informative cases, the mean *PTEN* gene copy number per counted cancer cell for the hormone sensitive cases was 1.90 (range 1.4-2.5) and for the hormone refractory cases was 1.71 (0.80-3.45). When assessing the frequency of chromosome aneusomy, the mean chromosomal copy number for chromosome 10 for the hormone sensitive tumours was 1.93 (range 1.75-2.25) and for the hormone refractory tumours was 1.87 (range 1.60-2.05). Normal range for chromosomal copy number is 1.35-2.01, in the current cohort none of the hormone sensitive or hormone refractory tumours appeared to have lost chromosome 10 as 0% had chromosome 10 copy number per counted cancer cell of less than 1.35 which is the lower limit of the normal range, in contrast 12% of the hormone sensitive tumours and 21% of the hormone refractory tumours had chromosome 10 copy numbers per counted cancer cell higher than that of the normal range. It was noted that this was a different subgroup of patients to those exhibiting loss of the *PTEN* gene.

### **Immunohistochemistry**

Membrane, cytoplasmic, and nuclear *PTEN* expression was observed in prostate tissue. In the hormone sensitive tumours, membrane and nuclear expression were less frequently observed than cytoplasmic expression, 41% and 46% of patients respectively had membrane and nuclear expression compared to 95% expressing *PTEN* in the cytoplasm. The inter quartile range of expression for each location is shown in table 1. This rate of expression did not significantly change in the hormone refractory tissue. When median protein expression levels in the hormone sensitive and hormone refractory tissue were compared no statistically significant change was observed at any cellular location. Loss of *PTEN* expression was heterogeneous.

## Hormone Sensitive Tumours

To determine if protein expression was linked to time to biochemical relapse, Kaplan-Meier graphs were plotted for the hormone sensitive tumours expressing low levels of protein (< median histoscore) versus high levels of protein (> median histoscore) and compared using the log rank test. The patients whose tumours expressed low levels of PTEN in the cytoplasm were shown to have relapsed significantly earlier than those patients whose tumours expressed high levels of PTEN in the cytoplasm (Fig. 2a,  $p=0.027$ ). Cox regression analysis indicates that cytoplasmic PTEN expression is independent of known clinical prognostic factors ( $p=0.028$ , hazard ratio 0.51 (95%CI 0.27-0.93)). It was noted, however, that the Kaplan Meier curves did not separate until approximately 2.5 years after diagnosis. Therefore PTEN loss appeared to be influencing relapse in those patients who took more than 30 months to relapse. If patients that relapsed within 30 months were excluded from analysis the median time to relapse for those with low PTEN expression was 3.9 (IQR 2.98-4.92) years compared to 5.6 (4.36-6.84) years for those with high PTEN expression ( $p=0.0035$ )(Fig. 2b). In addition, those patients with high levels of cytoplasmic PTEN expression in their hormone sensitive tumours were observed to have longer median overall survival (6.1 years (IQR 2.8-9.4)) compared to those with low PTEN expression (4.4 years (IQR 3.3-5.4)), although this did not reach significance ( $p=0.072$ )(Fig. 2c). Again the curves first separate approximately 30 months after diagnosis.

Expression of phosphorylated Akt at serine 473 (activated Akt) for this cohort of patients had already been established for a previous study (McCall *et al*,2008). Levels of phosphorylated Akt expression in tumours that expressed low levels of PTEN was higher compared to tumours that expressed high levels of PTEN ( $p=0.047$ ).

The median for both PTEN membrane and nuclear expression in the hormone sensitive tumours was 0; therefore patients were divided into those patients whose

tumours did not express PTEN at these locations and those that did. Patients with PTEN membrane expression in their hormone sensitive tumour had significantly longer overall survival than those patients without (Fig. 2d,  $p=0.002$ ). The median time to relapse for patients whose tumours did not express PTEN in the membrane was 3.8 years (IQR 2.7-4.85) compared to 6.5 (IQR 5.8-7.2) years for patients with membrane expression.

Patients with no nuclear PTEN expression relapsed earlier than those with nuclear PTEN expression, although this did not reach significance (Fig. 2e,  $p=0.078$ ). PTEN nuclear expression was associated with overall survival, those patients whose tumours had no nuclear PTEN expression had a significantly shorter overall survival compared to those patients with PTEN nuclear expression (Fig. 2f,  $p=0.003$ ). Median overall survival was 3.4 years (IQR 2.6-4.2) compared to 6.5 years (IQR 5.1-7.8) which confers a survival advantage of 3 years for those patients whose tumours express nuclear PTEN. Nuclear PTEN expression was demonstrated to be an independent prognostic marker by Cox regression analysis when compared with known clinical prognostic parameters ( $p=0.031$ , hazard ratio 0.52 (95%CI 0.29-0.95)).

As observed with membrane expression, phosphorylated Akt expression was lower in the nucleus of tumours with high levels of nuclear PTEN than those with low levels but this did not reach significance ( $p=0.132$ ). In contrast, PTEN membrane expression correlated strongly with nuclear PTEN expression ( $p<0.001$ ,  $R_s$  0.66).

## Hormone Refractory Tumours

PTEN expression levels in the hormone refractory tumours were not associated with time to death from relapse, disease specific death from relapse or with presence of metastasis.

## DISCUSSION

The PTEN tumour suppressor has emerged as a critical regulator of cellular processes, which is frequently mutated or deleted in a number of human cancers, including prostate. FISH and IHC have demonstrated that a significant proportion of patients have heterogeneous *PTEN* deletion and loss of PTEN protein expression, which is, associated with clinical outcome measures.

The frequency and mode of PTEN inactivation reported at various stages of clinical prostate cancer are variable (Verhagen *et al*,2006). In the current study we investigated the level of *PTEN* loss by FISH in matched hormone sensitive and hormone refractory tumours. Loss was noted in 23% hormone sensitive tumours compared to 52% hormone refractory tumours, these rates of loss are similar to those previously reported by FISH analysis (Verhagen *et al*,2006). FISH depending on the region that the probe binds to does not always detect small deletions and in the case of our study the probe covers the whole of the *PTEN* gene, therefore in the current study loss of the whole gene is being measured. It was observed that very few tumours had homogeneous *PTEN* deletion and complete loss of PTEN expression (2%), but almost all have heterogeneous loss of expression, this is consistent with previous reports (Verhagen *et al*,2006). In the current study, PTEN loss does not correlate with *PTEN* gene deletion, although all tumours with PTEN deletion have low PTEN expression. An explanation for low PTEN expression in tumours that appear not to have *PTEN* deletion is hypermethylation of the *PTEN* promoter region. Evidence for promoter hypermethylation has been reported in prostate cancer xenografts (Whang *et al*,1998).

Although the mechanism of PTEN inactivation is currently controversial and possibly due to different mechanisms in different tumours, it is widely accepted that PTEN loss is one of the most common events associated with prostate cancer (Majumder and Sellers, 2005). Consistent with these findings, the current study observed a higher rate of PTEN deletion by FISH, in hormone refractory tumours compared to hormone sensitive tumours, suggesting that *PTEN* loss is associated with tumour progression. Although this was not significantly associated with clinical outcome measures a trend was observed that demonstrated that those patients with *PTEN* loss had shorter overall survival. If the FISH studies were expanded to a larger dataset these results may have reached significance.

As predicted, loss or low cytoplasmic PTEN expression was independently associated with time to relapse and linked with increased Akt activation. Surprisingly, however this was observed to be a late event with curves separating 30 months following diagnosis, suggesting that other factors such as PI3K expression may also contribute to Akt activation and disease progression. Cytoplasmic PTEN expression was only weakly associated with overall survival and this did not reach significance in the current study.

However, in addition to cytoplasmic PTEN expression, nuclear PTEN expression was also observed. Unlike cytoplasmic PTEN expression, loss of nuclear PTEN expression was weakly associated with time to relapse and this did not reach significance. Nuclear PTEN expression was however independently strongly associated with overall survival and the curves on the Kaplan Meier plot began to separate almost immediately after diagnosis. These results in combination with the lack of correlation with Akt activation suggest that the role of PTEN in the nucleus is independent of cytoplasmic PTEN. It is now recognised that PTEN has a function in the nucleus, and reports of PTEN nuclear localisation have begun to multiply over the past few years in tumour and non tumour cells (Gimm *et al*, 2000). Chung *et al* have

demonstrated that PTEN has dual nuclear localisation signal like sequences, that mediate nuclear import (Chung *et al*, 2005) and have since shown that nuclear PTEN is required for PTEN mediated cell cycle arrest and growth inhibition via down-regulation of cyclin D1. Reports in the literature also suggest that PTEN localises to the nucleus during the G<sub>0</sub>-G<sub>1</sub> phase of cell cycle and mediates growth suppression via inhibition of MAP kinase phosphorylation independent of Akt activation (Chung *et al*, 2006; Chung and Eng, 2005). Results from our study support the hypothesis that PTEN has a distinct function in the nucleus independent of its cytoplasmic role.

It is however a possibility that nuclear PTEN is simply a surrogate marker of PTEN activation, as *in vitro* studies demonstrate that following phosphorylation, PTEN is released from the membrane bound scaffolding proteins and enters the nucleus. In support of this we report a correlation between membrane and nuclear PTEN expression ( $p < 0.001$ ,  $R_s$  0.66) and PTEN membrane expression is also linked to survival. However, in contrast to cytoplasmic PTEN expression, no correlations were observed between nuclear PTEN expression and Akt activation, therefore the evidence to support nuclear PTEN as a surrogate marker of PTEN cytoplasmic activation is not convincing in the current study.

In summary, the rate of loss of *PTEN* as measured by FISH increased with disease progression and a trend was noted between *PTEN* loss and poorer disease specific survival suggesting that this arm of the study should be expanded to larger cohort. In addition, both cytoplasmic and nuclear PTEN are independently associated with good outcome measures in hormone sensitive prostate cancer, but appear to have independent roles.

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

	<b>HSPC</b> <b>(IQR)</b>	<b>HRPC</b> <b>(IQR)</b>	<b><i>P</i></b> <b>value</b>	<b>ICCC</b>	<b>Fallers</b> <b>(%)</b>	<b>Risers</b> <b>(%)</b>
<b>PTEN</b> membrane	0-67.5	0-40	0.086	0.84	33	15
<b>PTEN</b> cytoplasm	80-150	80-107	0.104	0.90	33	23
<b>PTEN</b> nucleus	0-50	0-80	0.588	0.82	28	25

Table 1 shows the interquartile range (IQR) for hormone sensitive tumours (HSPC) and hormone refractory tumours (HRPC) and the *p* value of these compared using a Wilcoxon sign rank test. 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 percentage of tumours that was defined as having a rise or fall in protein expression (calculated using the number of histoscore units that is defined as a change in expression) is also shown.

## Figure Legends

### Figure 1:

Figure 1a, shows an example of Fluorescent in situ hybridisation for chromosome 10 (red signal) and *PTEN* (green signal).

Figure 1b, shows an example of immunohistochemistry for PTEN protein expression; in hormone sensitive prostate cancer, both cytoplasmic expression and nuclear expression are present.

Figure 1c western blot analysis for PTEN protein to confirm antibody specificity, LNCaP cells do not express PTEN protein, where as DU145 cells do express PTEN protein. Lane 1, hormone sensitive LNCaP cell lysates lane 2 hormone refractory LNCaP cell lysates, lane 3 DU145 cell lysates.

### Figure 2:

Figure 2a, shows a Kaplan Meier Plot for high (above the median, solid line) and low (below the median, dotted line) PTEN cytoplasmic expression and time to biochemical relapse ( $p=0.027$ ).

Figure 2b, shows a Kaplan Meier Plot for high (above the median, solid line) and low (below the median, dotted line) PTEN cytoplasmic expression for patients that took longer than 30 months to relapse ( $p=0.0035$ ).

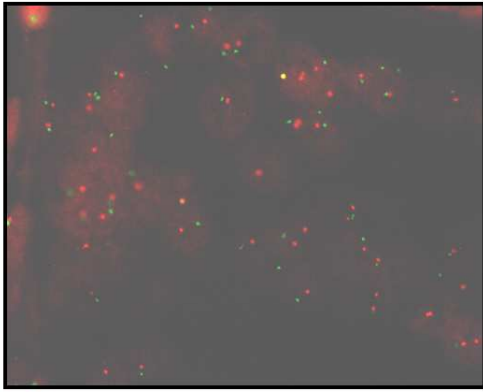
Figure 2c, shows a Kaplan Meier Plot for high (above the median, solid line) and low (below the median, dotted line) PTEN cytoplasmic expression and disease specific survival (labelled overall survival) ( $p=0.072$ ).

Figure 2d, shows a Kaplan Meier Plot for patients with tumours that have membrane PTEN expression (solid line) compared to patients that have tumours that do not have membrane PTEN expression (dotted line) and disease specific survival (labelled overall survival) ( $p=0.002$ ).

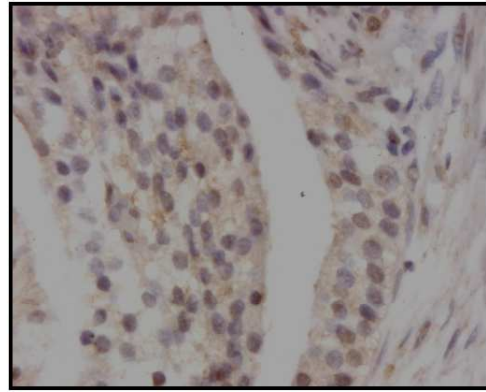
Figure 2e, shows a Kaplan Meier Plot for patients with tumours that have nuclear PTEN expression (dotted line) compared to patients whose tumours do not have nuclear PTEN expression (solid line) and time to biochemical relapse ( $p=0.078$ ).

Figure 2f, shows a Kaplan Meier Plot for patients with tumours that have nuclear PTEN expression (solid line) compared to patients whose tumours do not have nuclear PTEN expression (dotted line) and disease specific survival (labelled overall survival) ( $p=0.003$ ).

A



B



C

