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An increase in N-Ras expression is associated with development of hormone refractory prostate cancer in a subset of patients

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Abstract. Protein expression of H, K and N-Ras was assessed in hormone sensitive and hormone refractory prostate tumour pairs from 61 patients by immunohistochemistry. Expression of H-Ras and K- Ras was not associated with any known clinical parameters. In contrast an increase in N-Ras membrane expression in the transition from hormone sensitive to hormone refractory prostate cancer was associated with shorter time to relapse ($p = 0.01$) and shorter disease specific survival ($p = 0.008$). In addition, patients with an increase in N-Ras membrane expression had lower levels of PSA at relapse ($p = 0.02$) and expression correlated with phosphorylated MAP kinase ($p = 0.010$) and proliferation index (Ki67, $p = 0.02$). These results suggest that in a subgroup patients N-Ras expression is associated with development of hormone refractory prostate cancer via activation of the MAP kinase cascade.

Keywords: PSA, MAP kinase and proliferation

1. Introduction

Prostate cancer is the second leading cause of cancer related deaths in the Western World, contributing to approximately 14% of overall cancer mortality [1]. It is extremely prevalent and 30,100 new cases are diagnosed in the UK each year, making it the most common male malignancy [1]. In the normal functioning prostate the rate of cell death is 1–2% per day which is balanced by a proliferation rate of 1–2% [2,5].

The majority of prostate cancers diagnosed are dependent on the presence of androgens for growth and survival. Androgens, acting through the androgen receptor, modify transcription of androgen-regulated genes e.g. prostatic specific antigen (PSA) to promote the survival and proliferation of secretary epithelia. The main stay therapy for locally advanced or metastatic prostate cancer is therefore androgen ablation therapies, resulting in a reduction of androgens in the circulation and inhibition of tumour growth. Although 60–80% of patients initially respond to androgen ablation therapy, patients tend to relapse within a median time of 18–24 months. Up-regulation of growth factor signal transduction cascades provide one possible mechanism for the development of hormone refractory prostate cancer that appears to have escaped androgen control [8,9,29].

The mechanisms of resistance to androgen ablation therapy are not fully understood although *in vitro* studies have demonstrated that activation of signal transduction cascades such as Raf/MAPK and PI3K/Akt

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pathways may be involved, both these pathways may be activated directly by the 21kDa Ras family of proteins, key mediators of growth factor pathway activity [13]. The *RAS* gene family encode 21 Kdalton proteins of which there are four isoforms; H-Ras, N-Ras and two isoforms of K-Ras (A and B). A hyper-variable region of 25 amino acids in the C-terminal accounts for the differences in function and properties of these Ras isoforms [16]. Inactive Ras is located in the cytosol; activation requires a series of post-translational modifications and translocation to the membrane. Post-translational modifications of Ras slightly differ between the four isoforms. All are farnesylated and H-Ras and N-Ras then become palmitoylated and transit through the Golgi to the plasma membrane (PM) whereas K-Ras does not undergo palmitoylation and follows a yet unknown route to the plasma membrane. Once located at the inner face of the plasma membrane Ras isoforms function as GTPases interacting with downstream signalling cascades. Gene mutations in the *RAS* family are associated with multiple cancers including breast, colorectal [7], and prostate [26,28,31]. Ras proteins are over-expressed and activated in a high frequency of prostate cancer tumours and are associated with the development of hormone refractory disease, possibly due to gene amplification [10]. Ras over-expression correlates with prostate cancer progression and is associated with the formation of metastases [4]. When LNCaP cells are serially passaged in low steroid medium H-Ras expression increases, as LNCaP cells become hormone resistant [30]. In addition when mutated *hRAS* is transfected into a hormone sensitive cell line, MAP kinase activity, a down stream target of Ras, is elevated and hormone dependence decreases [30]. It is hypothesised that wild-type Ras is chronically activated by autocrine and paracrine growth factor stimulation, resulting in activation of down stream pathways associated with the development of hormone refractory disease e.g. MAP kinase pathways. In human prostate tumours, phosphorylated MAP kinase expression increases with tumour stage and grade and is over-expressed in hormone refractory disease [30] and down regulates androgen receptor mediated promoter activity [25]. We have therefore hypothesized that Ras promotes the development of hormone refractory prostate cancer via the MAP kinase-signalling cascade.

2. Materials and methods

2.1. Patient cohort

Ethical approval for this study was obtained from the multi-centre Research Ethics Committee (MREC Scotland) and relevant Local Research Committees (LRECs). All tumours had patient identification removed, including block number and hospital number, and were coded to anonymise the database. Sixty-one prostate cancer patients were selected for study, each patient had both hormone sensitive and hormone refractory tumour available for analysis. All patients in the cohort were newly diagnosed and were not identified from a watchful waiting programme. Therefore each patient in the cohort had received no prior treatment before receiving androgen deprivation therapy (25 patients had sub capsular orchidectomy, 36 patients received GnRH analogues, 3 had both), in addition all patients that received GnRH also received anti androgens. All patients in the cohort responded to androgen deprivation therapy and tumours were defined as hormone sensitive or responsive to androgen deprivation therapy if patient serum PSA levels decreased by at least 50% whilst undergoing androgen deprivation therapy. Patients were then defined as having biochemical relapse if PSA subsequently rose by 10% or more, during hormone ablation therapy. The hormone sensitive tumour samples were obtained by either a TURP or a TRUS guided biopsy and hormone refractory tumour samples were obtained by TURP following failure of hormone deprivation therapy (and to treat clinical symptoms of bladder outflow obstruction). PSA profile and full clinical follow up was available for each patient, in addition expression of phosphorylated Akt (serine 473) and Ki67 were available for each tumour from a previous study [11]. Akt was scored using the weighted histoscore method [11] and Ki67 scored using the proliferation index method [17].

2.2. Western blot

Before commencing immuno staining, western blotting was carried out to confirm antibody specificity (Fig. 1). Cell lines were lysed for 5 minutes at 4°C using cell lysis buffer (cell signalling technology Beverly, USA) with 1mM PMSF added immediately before use. Protein samples (50 ug) were resolved by SDS-PAGE and transferred to nitrocellulose membrane at 100V for one hour. After transfer membranes were blocked in 5% Marvel in Tween-TBS (TTBS) for one hour and incubated with Hras, KRas, NRas (IgG_1 Ab, F155, Santa Cruz Biotechnology, CA, USA, 2 μg/ml) or phosphorylated MAP kinase antibody 1 μg/ml (cell signalling technology Beverly, USA) diluted in 10 ml 5% marvel in TTBS over night at 4°C. This was followed by incubating in horseradish peroxidase-labelled anti-mouse

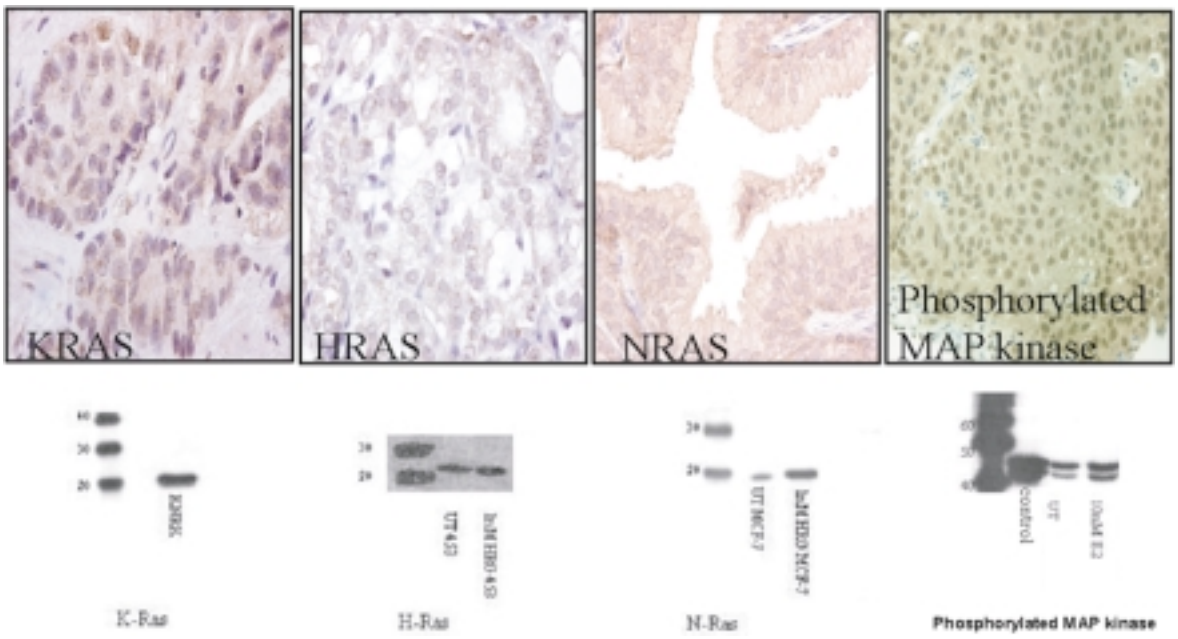

Fig. 1. Photographs of H, K and N-Ras and phosphorylated MAP kinase protein expression in hormone refractory prostate cancer tumours. Western blots for the appropriate antibodies are also included to confirm specificity.
K-Ras specificity was assessed using cell lysates from KRNK cells, which are a normal rat kidney [NRK] **cell** line transformed by Kirsten murine sarcoma virus.
H-Ras specificity was assessed using cell lysates from MDAMB 453 breast cancer cell line, which is known to exhibit an increase in H-Ras expression in response to heregulin treatment, the band labelled UT453 are untreated MDAMB 453 cells and the band labelled 1nMHRG453 are MDAMB 453 cells treated with 1 nM heregulin. A small increase in expression of H-Ras is noted in response to heregulin stimulation.
N-Ras specificity was assessed using cell lysates from MCF-7 breast cancer cell lines, which are known to exhibit an increase in N-Ras expression in response to heregulin treatment, the band labelled UTMCF-7 are untreated MCF-7 cells and the band labelled 1nMHRGMCF-7 are MCF-7 cells treated with 1 nM heregulin. A small increase in expression of N-Ras is noted in response to heregulin stimulation.
Phosphorylated MAP kinase specificity was assessed using cell lysates from MCF-7 breast cancer cell lines, which are known to exhibit an increase in N-Ras expression in response to oestrogen treatment, the band labelled control is purified phosphorylated MAP kinase protein, the band labelled UT are untreated MCF-7 cells and the band labelled $10nME_2$ are MCF-7 cells treated with 10 nM oestrogen. A small increase in expression of phosphorylated MAP kinase is noted in response to oestrogen stimulation.

Ig 0.5 μg/ml (cell signalling technology Beverly, USA) diluted in 10 ml 5% marvel in TTBS for one hour at room temperature. Detection was performed using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.3. Immunohistochemistry

Tumour expression of H, K, N-Ras and phosphorylated MAP kinase (Threonine 202/Tyrosine 204) was determined in paraffin embedded tissue sections (5 μm) 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 TE Buffer (1 mM EDTA, 5 mM Tris, pH 8.0) for five minutes in a microwave. Sections were blocked and incubated in K-Ras (IgG2 Ab, 234-4.2, Sigma, Missouri, USA, 20 μg/ml), H-Ras (IgG_1 Ab, F235, Santa Cruz Biotechnology, CA, USA, 20 μg/ml), N-Ras (IgG_1 Ab, F155, Santa Cruz Biotechnology, CA, USA, 20 μg/ml) and phosphorylated MAP kinase (human p44 Map Kinase, Cell Signaling Technology, USA, 4 μg/ml) primary antibody overnight at 4°C. In each run a negative isotype matched control was included. H, N-Ras and phosphorylated MAP kinase was visualised using Dako LSAB+ Kit (Dako) and the chromagen 3,3'-diaminobenzidine (DAB, Vector Laboratories, CA, USA) and K-Ras was detected using the Super Sensitive Non-Biotin HRP Detection System (BioGenex, CA, USA).

2.4. Histoscore method

Protein expression level was determined using the weighted histoscore method (Hscore method) by two

independent observers [19,21]. Histoscores were calculated from the sum of (1 × the % of cells staining weakly positive) + (2 × the % of cells staining moderately positive) + (3 × the % of cells staining strongly positive) with a maximum histoscore of 300. The inter-class correlation coefficient (ICCC) between each observer for each of the proteins was confirmed to measure consistency between observers. All ICCC values were > 0.7, which is classed as excellent as; an ICCC of 1 indicates identical scores. The mean of two observers scores were used for analysis. Changes in 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 2SD).

2.5. Statistical analysis

All statistical analysis was performed using the SPSS version 9.0 for Windows. Protein expression data was not normally distributed and is shown as median and interquartile ranges. Wilcoxon signed Rank tests were used to compare Ras protein expression between hormone sensitive prostate cancer (HSPC) and hormone refractory prostate cancer (HRPC) tumours. Survival analysis was conducted using the Kaplan-Meier method and curves were compared with the log rank test. Hazard ratios (HR) were calculated using Cox regression analysis. Correlations for continuous variables were calculated using Spearman Rank Correlation Coefficients, correlations for categorized variable were calculated using Chi square test and non parametric tests were conducted using Mann Whitney U test. A value of $p < 0.05$ was considered statistically significant.

3. Results

Sixty one patients were diagnosed with locally advanced (42) or metastatic prostate cancer (19). The median age at diagnosis was 70 (inter quartile range (IQR) 66–74) and the median PSA at diagnosis was 19 μg/ml (IQR 6–42 μg/ml), median time to relapse was 24 months (IQR 12–60 months) and median time to death from relapse was 12 months (IQR 12–36 months).

Gleason sum at diagnosis ranged from 4 to 10 and high Gleason sum was associated with shorter time to relapse ($p = 0.05$). In addition high Gleason sum at diagnosis ($p = 0.017$), presence of metastases at diagnosis ($p = 0.005$) and presence of metastases at relapse ($p = 0.001$) were associated with shorter disease specific survival. In contrast, low PSA (< 4 ng/ml) at relapse was associated with shorter time to relapse ($p< 0.0001$) and shorter disease specific survival ($p = 0.0002$), suggesting patients with low PSA levels at relapse have poorer prognosis, possibly due to having escaped androgen control (Fig. 2).

Although membrane and cytoplasmic expression was observed for all Ras isoforms, cytoplasmic expression was stronger and more commonly observed than membrane expression. However, as membrane localisation can be employed as a surrogate marker of Ras activation, membrane and cytoplasmic expression were individually assessed. Cytoplasmic expression levels of any Ras isoforms in this cohort was not associated with time to biochemical relapse and only high N-Ras membrane expression levels correlated with shorter disease specific survival ($p = 0.01$). Twenty five percent of patients (15/61) were classified as having high N-Ras expression in their hormone sensitive tumours, their median survival period was 5.5 years compared to 12.5 years for those with low N-Ras expression, the hazard ratio for high N-Ras expression was calculated to be 3.12 (95% C.I. 1.19–8.18). N-Ras expression was not associated with Gleason at diagnosis ($p = 0.91$) or presence of metastases at diagnosis ($p = 0.30$).

However, the strength of this cohort was that matched hormone refractory tumours were also available for each patient in addition to their hormone sensitive tumour. We were therefore able to establish if expression levels significantly changed in the transition from hormone sensitive to hormone refractory disease. When median protein expression levels in the hormone sensitive and hormone refractory tissue were compared no statistically significant increase was observed for any Ras isoforms at any location with the exception of N-Ras located at the membrane ($p = 0.04$). The median expression of N-Ras located at the membrane in hormone sensitive tumours was 0 (IQR 0–20) increasing to 20 (IQR 0–80) in the hormone refractory tumours. This increase in median expression was modest, however the nature of the cohort enabled us to investigate the increase on an individual patient basis, patients were then subdivided into those whose matched tumours exhibited an increase compared to those whose tumours exhibited a decrease or no change in expression in the transition from hormone sensitive to hormone refractory disease. Eleven percent (7/61) of patients tumours exhibited an increase in N-Ras membrane expression

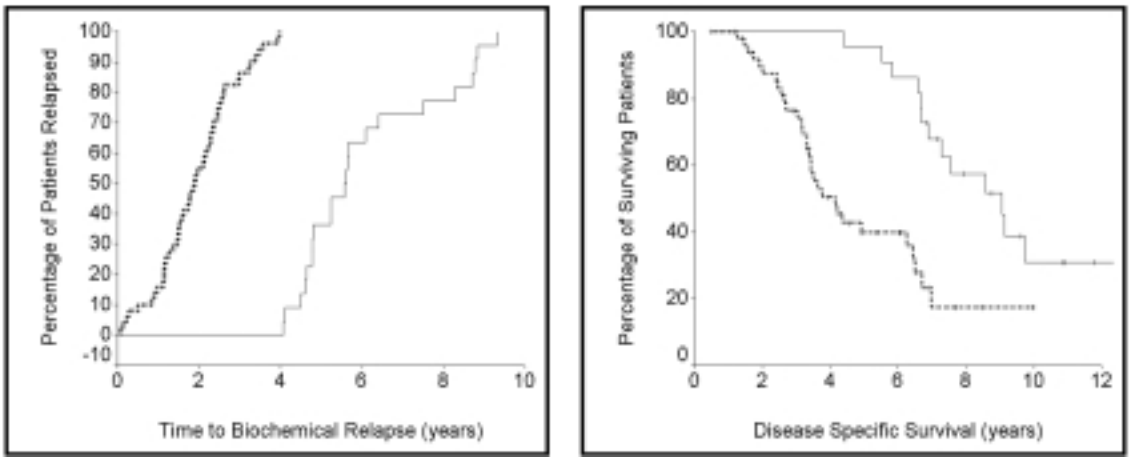

Fig. 2. Kaplan Meier plots demonstrating that those patients with low PSA ($<$ 4 ng/ml) at relapse (dotted line) have (a) shorter time to relapse ($p < 0.0001$) and (b) shorter disease specific survival ($p = 0.0002$) than those patients with high PSA at relapse (solid line).

and when you consider only those patients with an increase in N-Ras expression at the membrane, the median histoscore for membrane expression rose from 0 (0–17.5) to 75 (57.5–87.5) histoscore units (Fig. 3, $p = 0.018$)).

Subgroup analysis revealed that changes in expression levels of H-Ras or K-Ras in the transition from hormone sensitive to hormone refractory disease were not associated with any clinical parameters. However, an increase in N-Ras membrane expression with the development of hormone refractory disease was significantly associated with shorter time to biochemical relapse (Fig. 4a, $p = 0.01$). The median time to relapse for those patients who had an increase in N-Ras membrane expression was 1.97 years (IQR 0.2–3.7), compared to 2.47 years (IQR 1.35–3.59) for those patients who had a decrease/no change in N-ras membrane expression. The hazard ratio for patients who had a significant increase in N-Ras membrane expression was 2.8 (95% C.I. 1.2–6.3). The significant association with increased N-Ras membrane expression also translated into shorter disease specific survival (Fig. 4b, $p = 0.008$). The median survival period for those patients who had an increase in N-Ras membrane expression was 3.37 years (IQR 2.18–4.56), compared to 6.57 years (IQR 5.63–7.51) for those patients who had a decrease/no change in N-Ras membrane expression. Therefore those patients with an increase in membrane N-Ras expression had a median survival disadvantage of more than 3 years, the hazard ratio for patients who had a significant increase in N-Ras membrane expression was 3.2 (95% C.I. 1.3–7.9). Those patients with an increase in N-Ras membrane expression had significantly lower PSA levels at relapse than those patients with no change/decrease in N-Ras membrane expression (Fig. 4c, $p = 0.02$) and significantly higher levels of phosphorylated MAP kinase expression in the hormone refractory tissue (Fig. 4d, $p = 0.04$). Median phosphorylated MAP kinase expression was 81 (IQR 50–106) for the tumours with decrease/no change in N-Ras expression compared to 117 (IQR 107–145) for the tumours with an increase in N-Ras expression.

When protein expression levels of the Ras family were correlated with expression levels of phosphorylated Akt^{473} (marker of PI3K pathway activation), phosphorylated MAP $kinase^{202/204}$ (marker of MAP kinase pathway activation), androgen receptor and Ki67 (proliferation index), N-Ras at the membrane in the hormone refractory tumours correlated with phosphorylated MAP kinase and Ki67 but not with phosphorylated Akt or androgen receptor (Table 1). These results suggest that in hormone refractory prostate cancer N-Ras may activate the MAP kinase cascade to induce proliferation independent of androgens and the androgen receptor.

4. Discussion

In the current study we have demonstrated that low PSA at relapse is associated with poorer outcome both in terms of length of time to biochemical relapse and also with disease specific survival. This suggests that when there is clear evidence of symptomatic relapse (bladder outflow obstruction and presence of metastases), but low PSA levels, prostate cancer tumour

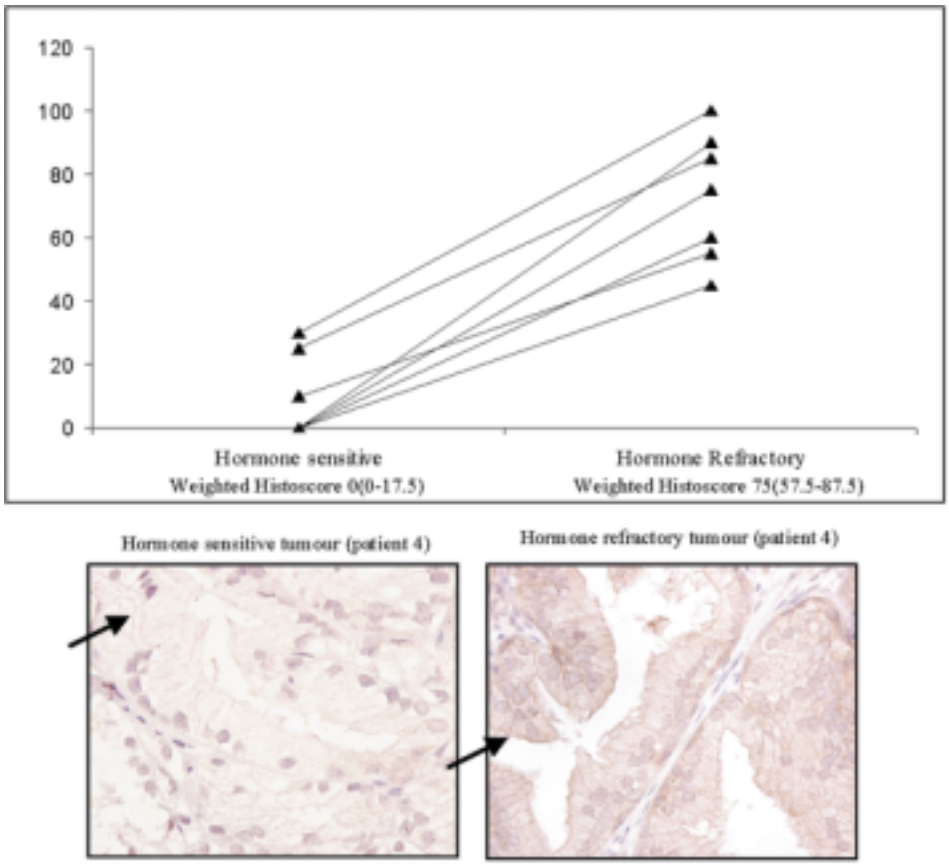

Fig. 3. Patients who demonstrated a significant rise ($>$ 43 histoscore units) in N-Ras membrane expression with the development of hormone refractory prostate cancer. Membrane expression is highlighted using an arrow. The median histoscore for the hormone sensitive samples is 0 (IQR 0–17.5) compared to 75 (IQR 57.5–87.5) in the hormone refractory tumours ($p = 0.018$).

growth is controlled independent of the androgen receptor. Activation of Ras has recently been demonstrated to stimulate androgen independent prostate cancer cell line growth and inhibit PSA expression via Ras-responsive element binding protein-1 (RREB-1) [20, 22,25], therefore suggesting that up-regulation of Ras may be involved in progression from the hormone sensitive to hormone refractory state.

The aim of the current project is to establish if Ras expression is up-regulated in clinical hormone refractory prostate cancer and therefore determine if it has a role as a possible therapeutic target and/or biomarker in hormone refractory prostate cancer. In the current cohort cytoplasmic expression of H, K and N Ras was observed in both hormone sensitive and hormone refractory tumours, however membrane expression (which may be used as a surrogate marker of activation) was expressed at lower levels and most commonly observed for N-Ras only. Previous studies report that Ras proteins are activated in prostate cancer tumours and are associated with the development of hormone refractory disease, however these studies did not investigate expression at an isoform specific level [4,15]. In the current study, although cytoplasmic expression was observed for all 3 isoforms, it was not associated with any known clinical parameters or patient outcome measures for K or H-Ras. In contrast, a significant increase in N-Ras membrane expression (activation) was observed with development of hormone refractory disease and in a sub-cohort of patients was associated with shorter time to relapse and shorter disease specific survival. In addition, N-Ras membrane expression was associated with low levels of PSA at relapse, high phosphorylated MAP kinase expression and high proliferation index. Suggesting that activation of N-Ras may stimulate prostate cancer proliferation via the MAP kinase cascade, independent of androgen receptor activation.

The difference in the function of the Ras isoforms may be due to their ability to activate different downstream effectors [16]. K-Ras has shown to be more

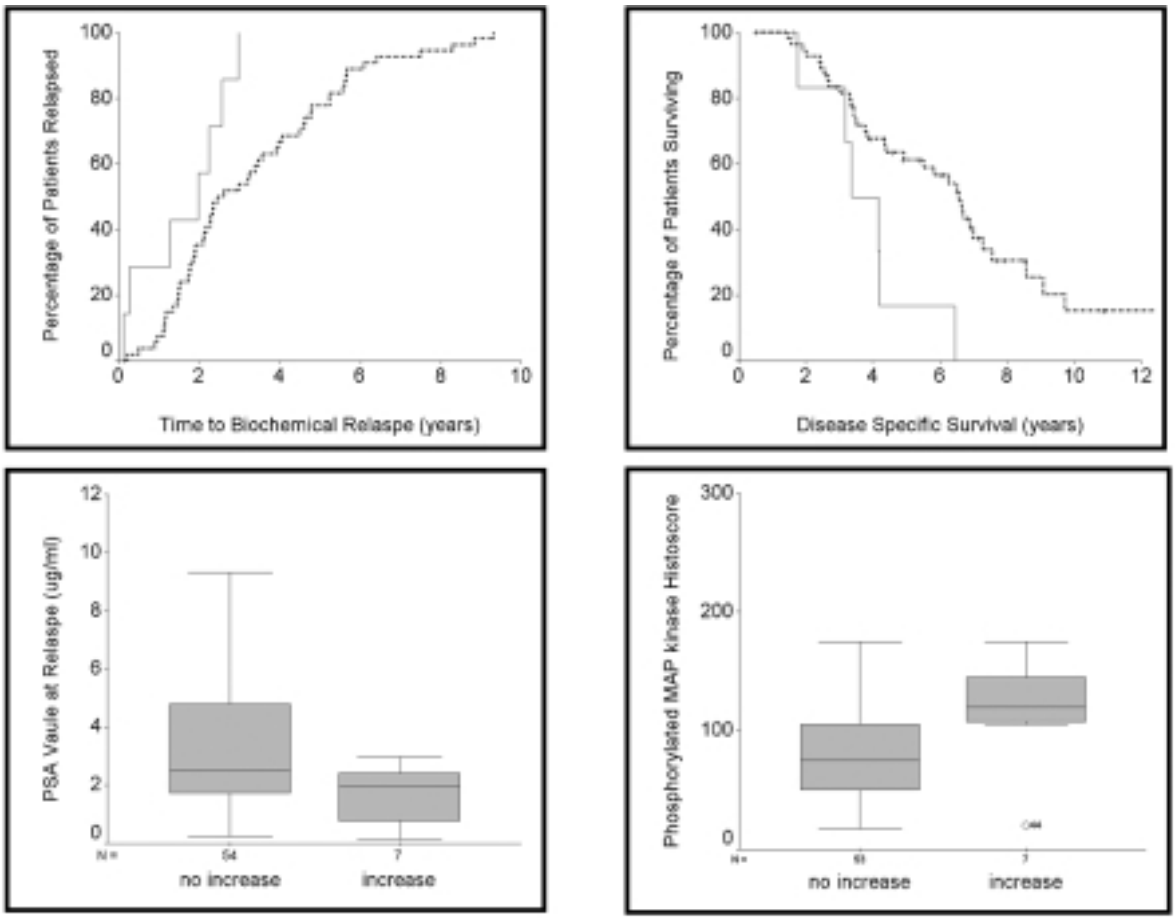

Fig. 4. Kaplan Meier plots demonstrating that those patients with an increase in N-Ras membrane expression (dotted line) have (a) shorter time to relapse ($p = 0.01$) and (b) shorter disease specific survival ($p = 0.008$) than those patients with a decrease/no change in N-Ras membrane expression (solid line). (c) Box plot showing PSA levels at relapse of patients with a decrease/no change in N-Ras expression compared to those with an increase in N-Ras expression ($p = 0.02$). (d) Box plot showing phosphorylated MAP kinase expression levels in hormone refractory tumours of patients with a decrease/no change in N-Ras expression compared to those with an increase in N-Ras expression ($p = 0.04$).

effective in activating Raf-1 whereas H-Ras is more effective in activating PI3K [3,18]. H-Ras has recently been reported to stimulate ARE-gene transcription in prostate cancer lines, resulting in an increase in PSA expression and cellular proliferation [6], however in the current cohort N-Ras expression is associated with low PSA expression. This is consistent with a recent study that reports that Ras can down regulate androgen receptor mediated promoter activity and suppress expression of PSA protein via RREB-1 interaction with the androgen receptor [25]. We therefore hypothesise that N-Ras may be influencing down-regulation of PSA via RREB-1 and stimulating prostate cancer growth in an androgen receptor independent manner via activation of the MAP kinase cascade. *In vitro* studies demonstrate that N-Ras stimulates prostate cancer cell proliferation (LNCaP cells) via the MAP kinase cascade [14] and we have previously linked activation of the MAP kinase cascade with development of hormone refractory disease using the current patient cohort [23,24]. Both an increase in Raf-1 and MAP kinase expression are significantly associated with a shorter time to biochemical relapse and MAP kinase expression was also associated with reduced overall survival.

Therefore Ras, in particular N-Ras may be a potential target to inhibit signal transduction cascades driving hormone refractory prostate cancer. Interestingly, previous studies have been devoted to the design of Ras inhibitors, including the design of farnesyltransferase inhibitors (FTIs) to block Ras processing and membrane anchorage and also the design of compounds that resemble the farnesylcystine of Ras to inhibit Ras- membrane association [27]. Recent studies demonstrate that Ras inhibition results in growth arrest and death of

Table 1
Positive correlations between Ras family members and downstream proteins

	Akt	MAP kinase	Androgen Receptor	Ki67
Hormone sensitive				
H-Ras (membrane)	X	X	X	X
p value				
r^2				
H-Ras (cytoplasmic)	X	X	X	X
p value				
r^2				
K-Ras (membrane)	0.009	X	X	X
p value	0.367			
r^2				
K-Ras (cytoplasmic)	X	X	X	X
p value				
r^2				
N-Ras (membrane)	0.038	0.033	X	X
p value	0.281	0.288		
r^2				
N-Ras (cytoplasmic)	X	X	X	X
p value				
r^2				
Hormone insensitive				
H-Ras (membrane)	X	X	X	0.014
p value				0.375
r^2				
H-Ras (cytoplasmic)	X	X	X	X
p value				
r^2				
K-Ras (membrane)	X	X	X	X
p value				
r^2				
K-Ras (cytoplasmic)	X	X	X	X
p value				
r^2				
N-Ras (membrane)	X	0.01	X	0.02
p value		0.381		0.390
r^2				
N-Ras (cytoplasmic)	X	X	X	X
p value				
r^2				

Table 1 shows significant correlations between Ras family member expression (as determined by histoscore) and phosphorylated Akt, phosphorylated MAP kinase, androgen receptor and Ki67 (proliferation index) assessed by Spearman's Rank Correlation test.

androgen independent prostate cancer cells therefore suggesting that Ras may be employed as a therapeutic target for hormone refractory prostate cancer [12,22].

In summary this study provides evidence that N-Ras, but not K- or H-Ras, may have a role in the development of hormone refractory prostate cancer.

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