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Edwards, J. and Traynor, P. and Munro, A. and Pirrett, C.F. and Dunne ,  
B. and Bartlett, J.M.S. (2006) *The role of HER1-HER4 and EGFRvIII in  
hormone-refractory prostate cancer*. *Clinical Cancer Research*, 12 (1).  
pp. 123-130. ISSN 1078-0432

<http://eprints.gla.ac.uk/7756/>

Deposited on: 27 October 2009

# **The Role of HER1-4 and EGFRvIII in Hormone Refractory Prostate Cancer**

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Running title: HER family in prostate cancer

Keywords: prostate, cancer, HER2, EGFR and hormone refractory

Sources of support: The Prostate Cancer Research Foundation and TENOVUS Scotland.

## **Abstract**

**Purpose:** The role of the type I receptor tyrosine kinase (HER) family in progression of prostate cancer is controversial. Breast cancer studies demonstrate that these receptors should be investigated as a family. The current study investigates expression of HER1-4 and EGFRvIII in matched hormone sensitive and hormone refractory prostate tumours.

**Experimental Design:** Immunohistochemical analysis was used to investigate protein expression of HER1-4, EGFRvIII and phosphorylated Akt in matched hormone sensitive and hormone refractory prostate tumours.

**Results:** Surprisingly, high HER2 membrane expression in the hormone sensitive tumours was associated with an increased time to biochemical relapse ( $p=0.0003$ ), and this translated into longer overall survival ( $p=0.0021$ ). Consistent with other studies HER4 membrane expression in hormone sensitive tumours was associated with longer time to biochemical relapse ( $p=0.042$ ) and EGFRvIII membrane expression was associated with shorter time to biochemical relapse ( $P=0.015$ ). An increase in phosphorylated Akt expression was associated with reduced survival ( $p=0.0098$ ). Multivariate analysis demonstrated that HER2 was an independent positive predictive marker of time to relapse in hormone sensitive prostate tumours ( $p=0.014$ ). In contrast, high HER2 expression in the hormone refractory tumours was associated with decreased time to death from biochemical relapse ( $p=0.039$ ), and EGFRvIII nuclear expression was associated with decreased time to death from biochemical relapse and decreased overall survival ( $p=0.02$  and  $p=0.005$ ).

**Conclusion:** These results suggest that the HER family may have multiple roles in prostate cancer and expression of the proteins alone is insufficient to predict the biological response that they may elicit.

## **Introduction**

The HER family of receptors comprises 4 members; - EGFR (erbB1/HER 1), HER2 (erbB2/neu), HER3 (erbB3), and HER4 (erbB4). In addition, variant receptors may be generated by alternative splicing (e.g. HER2/4) or mutations (particularly EGFR) and overexpression may occur as a result of gene amplification (especially HER2). The most frequently identified mutant form of EGFR is a constitutively active form called EGFR variant III (EGFRvIII) (1,2). The HER receptors play essential roles in development and maintenance of mammary, cardiac and neural tissues and have also been implicated in the development and progression of many cancers including breast and prostate cancer (3,4).

EGFR, HER3 and HER4 are activated via ligand binding, which results in the formation of homo or heterodimers with other family members (4). As HER2 ligands have not been identified, HER2 is believed to be activated by forming heterodimers with other family members (3,5). Formation of HER receptor homo or heterodimers results in receptor activation via tyrosine kinase mediated autophosphorylation, resulting in phosphorylation and activation of downstream pathways such as the MAP kinase cascade and the PI3K/Akt cascade (6). HER3 homodimers, however are unable to activate downstream pathways as HER3 lacks intrinsic tyrosine kinase activity and is therefore dependent upon formation of heterodimers with other member of the HER family (3,4,6).

Aberrant activation of the HER family may occur via receptor overexpression, mutational activation or increased growth factor concentrations. Increased activation of the HER family results in activation of MAP kinase and PI3K/Akt signalling cascades, culminating in increased cell proliferation and decreased cell death (3). It is therefore not surprising that modifications to the HER family are strongly associated

with tumour formation and progression. Although the role of the HER family is well characterised in breast cancer, with drugs targeting both EGFR and HER2 demonstrated to be effective in treating metastatic disease, the role of this family in the development and progression of prostate cancer remains controversial.

Data from breast and ovarian cancer suggests that since these receptors have similar function, they should be studied as a family. HER1-3 in breast cancer are associated with increased cellular proliferation and HER4 appears to have a non proliferative role (7,8). Breast cancer patients with HER1-3 positive tumours have significantly poorer prognosis than those patients with HER negative tumours or HER4 positive tumours (9). Therefore in breast cancer HER targeted agents to EGFR and HER2 may be a more effective approach than a pan HER inhibitor.

Increased expression of the EGFR, HER2, HER3, HER4 and EGFRvIII, have all been described in prostate cancer (10-14). Data suggests that there is an increase in EGFR and HER2 expression at hormone relapse (10,13,15,16). However since *EGFR* and *HER2* genes are not frequently amplified in prostate cancer (12,13), this increase was not linked to gene amplification as in other cancers. Alternatively the cell can regulate growth factor receptor expression via receptor degradation. Following ligand stimulation the receptor is internalised where it is either degraded or recycled to the cell membrane to undergo further activation (3). Disruption of this degradation process may result in increased protein expression, independent of protein synthesis (3). This may be the mechanism employed in prostate cancer to increase HER2 expression. However in the case of EGFR, loss of EGFR protein expression as the cancer progresses has been reported and this is accompanied by an increase in expression of EGFRvIII (2), resulting in deregulated growth, independent of ligand

activation. An increase in EGFRvIII expression may therefore be more common in prostate cancer than an increase in the wild type EGFR.

HER3 and HER4 are expressed in 11-20% of breast cancers (9), however few studies have investigated the role of HER3 and HER4 in prostate cancer. A recent study reported that levels of HER3 and HER4 do not change in the transition from HSPC to HRPC. However high levels of HER4 in the hormone refractory tumours was linked to improved patient survival (10), consistent with observations made in breast cancer (7,9).

To our knowledge this is the first report that investigates expression levels of all four members of the HER family and EGFRvIII in matched hormone sensitive and hormone refractory tissue. This study aims to clarify the role of the HER family in the development of clinical HRPC. We hypothesis that HER1-3 co-operatively mediate hormone relapse and early death in prostate cancer patients. This study may provide evidence to support the use of a novel pan HER inhibitor in treatment of prostate cancer or may demonstrate that specific inhibitors of one family member would be a more effective approach.

## **Materials and Methods**

### **Patient cohort**

Seventy four 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 (response was defined by PSA levels falling by at least 50%) but subsequently relapsed (2 consecutive rises in PSA greater than 10%) and had a pre and post hormone relapse sample available for analysis. Therefore tumours classed as hormone sensitive were from patients that were diagnosed with locally advanced (56) or metastatic prostate cancer (18) and received surgery, subsequently followed by hormone therapy in the form of androgen deprivation therapy (sub capsular bilateral orchidectomy and GnRH analogue) or maximum androgen blockade. In order to meet the inclusion criteria a response to this therapy had to be observed, a response to therapy was defined by PSA levels falling by at least 50% and a nadir being reached. The hormone sensitive tumour samples were obtained either from a TURP or a TRUS guided biopsy. In addition, patients were required to relapse with hormone refractory prostate cancer to meet inclusion criteria. A tumour was classified as being hormone refractory if the patients stopped responding to hormone therapy, this was defined as 2 consecutive rises in PSA concentration of greater than 10%, the patients were also required to fail to respond to any alternative hormone therapies administered. However following identification of such patients, refractory tumour samples were only available for analysis if additional surgery was required to treat clinical symptoms (bladder outflow obstruction). Therefore hormone refractory tumours samples were obtained only by TURP following failure of hormone therapy. In summary all patients in this cohort initially respond to hormone therapy but subsequently relapsed with hormone refractory disease, this provides matched hormone sensitive and hormone refractory tumours to track molecular changes associated with the transition from hormone sensitive to refractory prostate cancer. Gene amplification status has previously been investigated by fluorescent in situ hybridisation for 52 patients in this cohort for both EGFR and HER2 (13). No tumours were amplified for EGFR and

only 7% were amplified for HER2 gene. These were low level amplifications and did not correlate with protein expression, it was therefore not deemed necessary to extend these studies to the full cohort (13).

### **Immunohistochemistry**

Specificity of all antibodies used in this study were confirmed by western blotting. All immunohistochemistry (IHC) was performed on 5 µm, archival formalin fixed, paraffin embedded prostate tumour sections. EGFR and HER2 IHC was performed as previously described (13), in brief, for EGFR IHC tissue was incubated with EGFR antibody (clone 31G7, Zymed) at a 1:50 dilution for 1 hour at 25°C and for HER2 IHC the HercepTest™ (DakoCytomation, Glostrup, Denmark) and a Techmate immunostainer (DakoCytomation, Glostrup, Denmark) were used with strict adherence to kit protocol. Immunohistochemistry for EGFRvIII, HER3, HER4 and phosphorylated Akt at serine 473 (pAkt) were performed as follows: Antigen retrieval for EGFRvIII was performed using heat treatment under pressure in a Tris EDTA Buffer (10mM Trizma Base, 0.25mM EDTA) for 5 min. No Antigen retrieval was required for HER3 and HER4 and antigen retrieval for pAkt was to heat in Tris EDTA Buffer (10mM Trizma Base, 0.25mM EDTA) at 96°C for 20 minutes. HER3 and HER4 were blocked for endogenous biotin using an avidin/biotin blocking kit (Vector Labs, UK). Non-specific background staining was blocked using either 5% horse serum in TBS for 1 hour (EGFRvIII, pAkt), 2.5% horse serum in TBS for 20 minutes (HER3) or serum free blocking solution for 10 minutes (DakoCytomation, Glostrup, Denmark)(HER4). EGFRvIII (clone ZMD.82, Zymed), HER3 (clone H3.105.5, MS-303-PABX, Neomarkers), HER4 (clone HFR1, MS-637-PO, Neomarkers) and pAkt (44-622G, Biosource) antibodies were used at 1:50, 1:20, 1:50



and 1:100 dilutions, respectively. EGFRvIII, HER3 and HER4 were incubated for 2 hours at 25°C and pAkt was incubated overnight at 4°C. Staining was developed using either the LSAB plus kit (DakoCytomation, Glostrup, Denmark) for EGFRvIII and HER4, the ImmPRESS Anti-Mouse Ig (peroxidase) Kit (Vector Labs, UK) for HER3 and EnVision kit (DakoCytomation, Glostrup, Denmark) for pAkt.. Chromagen was detected using DAB (Vector Labs, UK). A positive and negative control slide was included in each IHC run, negative controls were incubated in an isotype matched control antibody at a concentration of 1mg/ml.

Tissue staining intensity was scored blind by 2 independent observers using a weighted histoscore method (17) also known as the Hscore system (18). 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 coefficients (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 EGFR, HER2, HER3, HER4 and EGFRvIII are shown in table 1.

## **Statistics**

Interclass correlation coefficients were used to confirm consistency between observers. Protein expression data was not normally distributed and is shown as median and inter quartile ranges. Wilcoxon Signed Rank Tests were used to compare

expression between pre and post hormone refractory tumours. Survival analysis was conducted using Kaplan-Meier method and curves were compared with the log-rank test. Multivariate survival analysis and hazard ratios were calculated using Cox Regression analysis. Correlations between HER family members was performed using a Spearman's rank test.

## **Results**

### **Patient characteristics**

A total of 74 prostate cancer patients (diagnosed between 1984-2000) were included in this study with matched hormone sensitive and hormone refractory prostate tumours available for analysis (148 tumours in total). Patients in this cohort were diagnosed with locally advanced (56) or metastatic prostate cancer (18) and subsequently received surgery and androgen deprivation therapy (28 sub capsular bilateral orchidectomy, 48 GnRH analogue, 2 had both). Fifty eight of the 74 patients also received anti androgens therapy in the form of maximum androgen blockade and this included all those who received GnRH analogues. At initial diagnosis the median age was 70(67-74) years and 18% of patients had metastasis. The median time to biochemical relapse was 2.35 (1.48-4.40) years, the median PSA at relapse was 17 (6-41) ng/ml and the percentage of patients with metastasis had increased to 35%. Sixty-seven patients died during follow-up and median survival for these patients was 4.39(3.03-6.86). Seven patients were alive at last follow-up, the median time of follow-up for all 74 patients was 4.38(2.75-6.93) years.

## **Immunostaining**

Membrane protein expression and cytoplasmic protein expression was observed for all family members (although expression was very low for EGFR and HER2) (figure 1, table 1). Nuclear expression was observed for HER3, HER4 and EGFRvIII (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, all ICC values in this study were greater  $>0.7$  (which is classed as excellent) (table 1). The level of protein expression observed for EGFR and HER2 was lower than that observed for HER3, HER4 and EGFRvIII (table 1). No overall significant increase was observed in median protein expression levels for any of the proteins investigated in the transition from hormone sensitive to hormone refractory disease (table 1). However a significant decrease was observed for HER3 cytoplasmic expression in the transition from hormone sensitive to hormone refractory disease ( $p=0.004$ , table 1). As described in the materials and methods section, a change in protein expression between matched hormone sensitive and hormone refractory tumour pairs is defined as the mean difference between the histoscores that each observer assigns for the protein expression plus 2 standard deviations. The number of histoscore units that represents a change in protein expression between tumour pairs for each protein is given in table 1. Using this definition it was noted that for each protein there were sub groups of patients whose tumours exhibited either a fall or rise in protein expression (table 1).

## **Time to biochemical relapse and patient overall survival**

In order to determine if protein expression in the hormone sensitive tumours was linked to time to biochemical relapse, Kaplan-Meier graphs of tumours expressing

low levels of protein (< 3<sup>rd</sup> quartile) versus high levels of protein (> 3<sup>rd</sup> quartile) were plotted and compared using the log rank test. The 3<sup>rd</sup> quartile was determined as the cut off as the medians was not deemed as an appropriate cut-off for EGFR and HER2 as the medians were very low for both (0 and 2.5 respectively). However when the histograms of score distribution for both proteins were plotted the 3<sup>rd</sup> quartile appeared as the natural cut-off to use. Therefore in order to keep the cut-offs consistent for all proteins the 3<sup>rd</sup> quartile was employed as the cut-off for all proteins in this study. Using the 3<sup>rd</sup> quartile as the cut-off neither EGFR nor HER3 expression, at any cellular location, was associated with time to biochemical relapse. However those patients whose tumours expressed low levels of HER2 or HER4 in the membrane relapsed significantly earlier than those patients whose tumours expressed high levels of HER2 or HER4 in the membrane (figure 2a and 2b, p=0.0001 and p=0.042, respectively) and this effect translated into a difference in overall survival. The median survival for patients whose tumours had low HER2 membrane expression is 4.2(2.59-5.81) years compared to 7.27(4.48-10.06) years for patients whose tumours expressed high levels (p=0.0021; hazard ratio 0.31(95% C.I. 0.14-0.67)). Similarly, the median survival for patients whose tumours expressed low levels of HER4 membrane expression is 4.92(3.34-6.50) years compared to 6.44(4.96-7.93) years for patients whose tumours had high HER4 expression (p=0.027; hazard ratio 0.48 (95% C.I. 0.24-0.991)). HER2 and HER4 expression in the hormone sensitive tumour at any other cellular location did not influence time to biochemical relapse or overall survival.

Conversely, patients with tumours that express high levels of membrane EGFRvIII relapse significantly earlier than those patients whose tumours expressed low levels of membrane EGFRvIII (figure 3, p=0.015). Again this effect translated into overall

survival, those patients with tumours expressing high levels of membrane EGFRvIII have a median survival period of 3.92(3.02-4.82) years compared to 6.04(4.56-7.52) years for those with tumours expressing low membrane EGFRvIII ( $p=0.037$ ; hazard ratio 1.98 (95% C.I. 1.07-3.68)). Expression of EGFRvIII in the cytoplasm or nucleus was not related to time to biochemical relapse, however those patients whose tumours expressed high levels of EGFRvIII in the cytoplasm had a significantly shorter survival period (4.36(2.68-6.04) years) compared to those patients whose tumours expressed low levels of EGFRvIII in the cytoplasm (6.50 (5.48-7.52) years,  $p=0.012$ ; hazard ratio 2.1(95% C.I. 1.15-3.91)).

Multivariate analysis demonstrated that when expression of EGFR, HER2, HER3, HER4 and EGFRvIII is combined with Gleason and presence of metastases at diagnosis, only HER2 membrane expression was independently positively associated with time to relapse, suggesting that HER2 might be a positive independent predictive marker of time to relapse in hormone sensitive tumours ( $p=0.015$ ). Gleason and presence of metastases at diagnosis were negatively associated with time to relapse and are known negative prognostic factors in prostate cancer ( $p=0.020$  and  $0.031$ , respectively, table 2). When the same parameters were combined for overall survival, only Gleason and presence of metastases at diagnosis were independently associated with overall survival ( $p=0.002$  and  $p=0.045$ , respectively, table 2).

### **Time to death from biochemical relapse and patient overall survival**

In order to determine if protein expression in the hormone refractory tumours were linked to time to death from biochemical relapse, Kaplan-Meier graphs of tumours expressing low levels of protein and high levels of protein were plotted and compared using the log rank test. EGFR, HER3 and HER4 were not significantly associated

with time to death from biochemical relapse at any cellular location. However those patients whose tumours expressed high levels of HER2 in the membrane, of the hormone refractory tumour died significantly earlier than those patients whose tumours expressed low levels of HER2 in the membrane (Figure 4a,  $p=0.0394$ ; hazard ratio 1.6(95% C.I. 0.80-3.19)), this did not however translate into overall survival. In addition, those patients whose tumours expressed high levels of EGFRvIII in the nucleus, of the hormone refractory tumour died significantly earlier than those patients whose tumours expressed low levels of EGFRvIII in the nucleus ( $p=0.02$ ; hazard ratio 1.4(95% C.I. 0.74-2.65) this effect also translated into overall survival ( $p=0.0059$ ; hazard ratio 2.6 (95% C.I. 1.29-5.49)).

### **Changes in protein expression with the development of hormone refractory disease are associated with patient survival.**

When time to death from biochemical relapse was investigated in relation to an increase in protein expression in the transition from hormone sensitive to hormone refractory disease it was noted that only an increase in HER2 membrane expression was associated with decreased time to death from biochemical relapse (Figure 4b,  $p=0.012$ ; hazard ratio 2.62(95% C.I. 1.2-5.75)). This effect did not translate into reduced overall patient survival and was not observed for any other protein at any other location.

### **Correlation between protein expression of HER family members**

In the hormone sensitive tumours when protein expression levels (expressed as histoscore units) were correlated, EGFR protein expression weakly correlates with HER2 protein expression (membrane: correlation coefficient=0.342 and  $p=0.013$ ,

cytoplasmic: correlation coefficient=0.397 and  $p=0.004$ ). EGFR membrane and cytoplasmic expression also weakly negative correlates with EGFRvIII cytoplasmic expression (membrane: correlation coefficient=-0.285 and  $p=0.031$ , cytoplasmic: correlation coefficient=-0.286 and  $p=0.031$ ). In the hormone refractory tumours the positive correlation between EGFR and HER2 and the negative correlation between EGFR and EGFRvIII are lost. No other correlations were observed.

### **Downstream signalling**

In order to establish if the downstream PI3K/Akt pathway is activated in this patient cohort, protein expression of activated Akt phosphorylated at serine 473 (pAkt) was assessed in those patients with sufficient tumour material remaining for analysis (56 patients). Those patients with high pAkt expression in their primary tumours have a shorter time to death compared to those patients with low pAkt expression (Figure 5a, median overall survival is 3.50(2.52-4.48) years versus 6.04(4.29-7.79) years,  $p=0.058$ ). Although this did not reach significance, possibly due to the small patient number a hazard ratio of 1.7 (95% C.I. 0.97-2.29) was observed. In addition an increase in pAkt expression in the transition from hormone sensitive to hormone refractory disease was observed in approximately  $\frac{1}{4}$  of (13/56) patients, suggesting that this pathway is up-regulated in a sub group of patients in the transition from hormone sensitive to hormone refractory disease. This increase in expression was demonstrated to be associated with reduced patient survival. Patients with an increase in pAkt expression compared to patients with a decrease or no change in expression had a reduced survival period from biochemical relapse (median survival falling from 1.58 to 0.74 years,  $p=0.050$ , hazard ratio 1.9 (95% C.I. 0.98-3.68)) and a reduced

overall survival (Figure 5b, median survival falling from 5.82 to 3.36 years,  $p=0.0098$ , hazard ratio 2.3 (95% C.I. 1.2-4.5 )).

## **Discussion**

Although HER2 has considerable clinical importance in advanced breast cancer (7), its role in prostate cancer remains controversial. In the current study we observed that those patients whose tumours had an increase in HER2 expression with the development of hormone refractory disease survived for a significantly shorter period following biochemical relapse than those patients whose tumours had no change or a decrease in HER2 expression, consistent with our previous findings (13). We also observed that those patients whose hormone refractory tumour expressed high levels of HER2 die significantly earlier than those with low levels of HER2 expression, consistent with Hernes et al. 2004, who also investigated HER2 expression in matched prostate cancer patient samples (10). These results fit with previous breast cancer studies that demonstrate that HER2 is linked to increased proliferation and decreased apoptosis providing possible mechanisms for disease progression (7,9,19). HER2 expression in prostate cancers is also linked with development of metastatic prostate cancer and it is possible that patients with high HER2 in their hormone refractory tumour represents a sub group of patients at high risk of developing metastatic disease, this is also one of the roles demonstrated for HER2 in breast cancer (20). HER2 has also been demonstrated to be required for optimum AR activity, therefore inhibition of HER2 may in hormone refractory tumours may be one method of inhibiting activation of the AR in the absence of androgens (21) as high HER2 expression in hormone refractory prostate cancer tumours appears to be a



negative predictive factor for response to therapy, as it is associated with a shorter survival period from biochemical relapse (21).

In contrast to our previous report we found no link between patient outcome and an increase in EGFR expression in the transition from hormone sensitive to hormone refractory disease (13). A similar percentage of patients' tumours were noted to have an increase in EGFR protein expression with the development of hormone refractory disease (6/74, 8.1% in current study compared to 4/48, 8.3% in previous study), however the follow-up is more mature in the current study. It is difficult to make any firm conclusions with so few numbers but it appears that an increase in EGFR expression is not linked to decreased patient survival in prostate cancer or impacts on a sufficiently small cohort (<10%) that is unlikely to be a valuable target in this context. However high EGFRvIII expression in the hormone sensitive tumours is associated with shorter time to biochemical relapse and also shorter overall survival. EGFRvIII has a constitutively active tyrosine kinase that signals most frequently via the PI3K cascade (22). Constitutive activation of the PI3K/Akt cascade in combination with loss of PTEN commonly observed in prostate cancer results in uncontrolled cell proliferation and reduced apoptosis (23). In the current study an increase in expression of phosphorylated and hence activated Akt is associated with a significant reduction in overall patient survival ( $p=0.0098$ ). Therefore the current study suggests a role of EGFRvIII and PI3K/Akt cascade in progression of prostate cancer, this pathway is currently being investigated in this patient cohort.

Both high HER4 and HER2 protein expression in the HSPC tumours were associated with increased time to biochemical relapse and increase overall survival. In the normal human prostate epithelium HER4 expression is high and is reported to be coupled to differentiation, growth arrest and tumour suppression (14,24). It is

therefore not surprising that when this receptor is expressed in tumour cells, the tumour appears to be less aggressive. When the androgen insensitive prostate cancer cell lines DU145 and PC-3 are transfected with HER4, the cells undergo growth arrest (24), similar observations are made in breast cancer studies. HER4 transfection in breast cells results in reduced proliferation and increased apoptosis (25,26). In breast cancer cells the anti-proliferative role of HER4 correlates with heregulin induced HER4 tyrosine phosphorylation (27). Following degradation of HER4 in breast cancer cells by tumor necrosis factor  $\alpha$  converting enzyme and presenilin dependent gamma secretase the intercellular domain (ICD) of HER4 is released (28). The ICD of HER4 is then able to enter the cytoplasm and accumulate in the mitochondria resulting in induction of apoptosis (28), HER4 may function similarly in prostate cancer.

HER2 may also signal for apoptosis following degradation in hormone sensitive prostate cancer cells (29). However, it is more likely that HER2 itself is not responsible for the mechanism underlying the effect we are observing, as 86% of the group of hormone sensitive tumours that express high HER2 levels, express high levels of 2 or more family members and 46% have high expression levels of 3 or more family members. In contrast only 10% of low HER2 expressing hormone sensitive tumours express high levels of 2 other family members. HER2 may act as a surrogate for a subset of tumours with markedly different biology and may not be solely related to the function of HER2 itself.

Different ligands and ligand concentrations can activate the HERs to signal via different pathways and induce different biological responses (27). Specifically, low concentrations of heregulin are mitogenic whereas higher concentrations lead to differentiation and inhibition of cell growth (27). This may reflect the most likely

explanation of why overexpression of HER2(multiple HERs) is a positive predictive factor in hormone sensitive tumours but a negative one in hormone refractory tumours. Heregulin is present in hormone naïve prostate cancer specimens, however in prostate cancer specimens from patients that have undergone androgen withdrawal heregulin expression is no longer detectable (30). Heregulin treatment of androgen sensitive LNCaP cells which express HER2 and HER3 results in activation of the mHOG/p38 pathway, resulting in a significant reduction in cell proliferation and morphological changes (cell clustering and increase cell to cell membrane contact) consistent with a more differentiated phenotype (14,30). In contrast treatment of the hormone resistant cell line CWR-R1 with heregulin, results in activation of HER2 and HER3 which signal via the MAP kinase and PI3K cascades resulting in increase AR transactivation and increased proliferation (31).

In summary, this rigorously controlled study identified EGFRvIII and an increase in HER2 expression as prostate cancer risk factors. In contrast high HER4 and HER2(multiple HERs) expression in hormone sensitive tumours appeared to have a protective role.

## **Acknowledgments**

I would like to acknowledge The Prostate Cancer Research Foundation and TENOVUS Scotland for their financial support and Dr Wilson Angerson for statistical advice.

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

	<b>HSPC(IQR)</b>	<b>HRPC(IQR)</b>	<b>P value</b>	<b>ICCC</b>	<b>Change</b>	<b>Fallers</b>	<b>Risers</b>
<b>EGFRm</b>	0(0-9)	0(0-18)	0.48	0.89	28	7%	8%
<b>EGFRc</b>	0(0-0)	0(0-0)	0.365	0.87	26	1%	2%
<b>HER2m</b>	2.5(0-30)	5(0-22)	0.22	0.91	26	10%	15%
<b>HER2c</b>	0(0-2.5)	0(0-0)	0.985	0.85	24	6%	6%
<b>HER3m</b>	85(26-107)	75(0-100)	0.06	0.95	48	32%	14%
<b>HER3c</b>	100(78-136)	80(60-100)	0.004	0.93	49	28%	10%
<b>HER3n</b>	20(0-79)	55(1-91)	0.349	0.95	34	24%	42%
<b>HER4m</b>	63(5-100)	40(75-200)	0.10	0.90	48	27%	15%
<b>HER4c</b>	75(50-100)	50(10-160)	0.094	0.90	47	29%	14%
<b>HER4n</b>	0(0-20)	0(0-18)	0.501	0.91	32	15%	11%
<b>EGFRvIII<sub>m</sub></b>	200(159-255)	200(150-225)	0.09	0.82	75	26%	16%
<b>EGFRvIII<sub>c</sub></b>	120(100-150)	115(100-150)	0.116	0.83	41	33%	44%
<b>EGFRvIII<sub>n</sub></b>	130(50-170)	70(7.5-150)	0.133	0.98	31	20%	31%

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 these values 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 mean difference in observer scores plus 2 standard deviations is also shown as the number of histoscore units that is defined as a change in protein expression (change). 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.

Table 2 Multivariate analysis

	<b>Relative Hazard Ratio (95% C.I.)</b>	<b>P value Time to Relapse</b>	<b>Relative Hazard Ratio (95% C.I.)</b>	<b>P value Overall survival</b>
<b>EGFR</b>	1.37(0.32-5.88)	0.667	1.33(0.18-10)	0.779
<b>HER2</b>	0.18(0.04-0.76)	0.0155*	0.58(0.12-2.7)	0.482
<b>HER3</b>	2.5(0.64-9.20)	0.191	1.67(0.34-6.17)	0.604
<b>HER4</b>	0.75(0.28-2.11)	0.594	0.92(0.32-2.68)	0.880
<b>EGFRvIII</b>	0.78(0.25-2.50)	0.677	0.68(0.19-2.38)	0.547
<b>pAkt</b>	1.81(0.63-5.26)	0.269	1.17(0.44-3.12)	0.741
<b>Gleason</b>	4.68(1.28-12.21)	0.020*	13.2(2.56-69.44)	0.045*
<b>Mets</b>	4.41(1.15-17.01)	0.031*	4.5(0.99-19.01)	0.002*

Table 2 shows the p values gained for both time to relapse and overall survival when expression levels of EGFR, HER2, HER3 HER4, EGFRvIII and phosphorylated Akt were combined with Gleason sum and presence of metastasises at diagnosis. The significant values are marked with an \*.

## Figure Legends

**Figure 1** Images of hormone refractory tumours stained by standard Immunohistochemistry for protein expression of EGFR(A), HER2(B), HER3(C), HER4(D) EGFRvIII(E) and pAkt(F). Positive staining is brown in colour, membrane (M) and cytoplasmic (C) staining for all family members (excluding pAkt) is indicated by an arrow and nuclear (N) protein expression for HER3 and HER4 is also marked (Magnification x 400).

**Figure 2a** shows a Kaplan Meier curve plotted for high (grey) HER2 protein expression verses low (black) HER2 protein expression. Those patients who express low levels of HER2 relapse significantly quicker than those who express high levels of HER2 ( $p=0.0003$ ).

**Figure 2b** shows a Kaplan Meier curve plotted for high (grey) HER4 protein expression verses low (black) HER4 protein expression. Those patients who express low levels of HER4 relapse significantly quicker than those who express high levels of HER4 ( $p=0.042$ ).

**Figure 3** shows a Kaplan Meier curve plotted for high (grey) EGFRvIII protein expression verses low (black) EGFRvIII protein expression. Those patients who express high levels of EGFRvIII relapse significantly quicker than those who express low levels of EGFRvIII ( $p=0.042$ ).

**Figure 4a** shows a Kaplan Meier plot of hormone refractory tumours expressing low levels of HER2 (black) versus hormone refractory tumours expression high levels of HER2 (grey). Those patients that expressed high levels of HER2, died significantly quicker those patients who expressed low levels of HER2 ( $p=0.0394$ ).

**Figure 4b** shows a Kaplan Meier plot for an increase in HER2 protein expression (black) versus no change or a decrease in HER2 protein expression (grey) in the transition from hormone sensitive to hormone refractory disease. Those patients with an increase in HER2 expression had significantly shorter time to death from biochemical relapse compared to those with no change or a decrease in HER2 expression ( $p=0.012$ ).

**Figure 5a** shows a Kaplan Meier curve plotted for high (grey) pAkt protein expression verses low (black) pAkt protein expression. Those patients who express high levels of pAkt have shorter overall survival than those who express low levels of pAkt ( $p=0.058$ ).

**Figure 5b** shows a Kaplan Meier plot for an increase in pAkt protein expression (black) versus no change or a decrease in pAkt protein expression (grey) in the transition from hormone sensitive to hormone refractory disease. Those patients with an increase in pAkt expression had significantly shorter time to death compared to those with no change or a decrease in pAkt expression ( $p=0.0098$ ).