Ras/Raf-1/MAPK pathway mediates response to tamoxifen but not chemotherapy in breast cancer patients.

Liane M. McGlynn[1], Tove Kirkegaard[1], Joanne Edwards[1], Sian Tovey[1], David Cameron[2], Chris Twelves[3], John M.S. Bartlett[1,4*] & Timothy G. Cooke[1]

[1]Endocrine Cancer Group, Division of Cancer Studies and Molecular Pathology, University Department of Surgery, Glasgow Royal Infirmary, Glasgow, G31 2ER, UK

[2] Department of Medical Oncology, Western General Hospital, Edinburgh, EH4 2XR.

[3] Cancer Research UK Clinical Centre, University of Leeds, St James Hospital, Leeds, LS9 7TF

[4] Present address: Endocrine Cancer Group, Edinburgh Cancer Research Centre, Western General Hospital, Edinburgh, EH4 2XR, UK

***Corresponding author**

Dr John MS Bartlett
Endocrine Cancer Group
Cancer Research Centre,
University of Edinburgh
Western General Hospital,
Crewe Road South,
Edinburgh EH4 2XR
E-mail: John.Bartlett@ed.ac.uk
Tel: +44 131 777 3584; Fax: +44 131 777 3520

Statement of Translational Relevance

Tamoxifen and chemotherapy are key treatments for breast cancer patients. Tamoxifen, an oestrogen antagonist, is a non-steroidal that acts as a selective oestrogen receptor modulator (SERM). It competitively inhibits the interaction of oestrogen with the oestrogen receptor, blocking the effects of E2 and inhibiting receptor activity. Chemotherapy uses cytotoxic drugs to kill cancer cells, by preventing them from multiplying, invading and metastasing. Despite the extensive use of both treatments, failure to respond to them is a major clinical problem and this is the cause of significant morbidity and mortality. To overcome this and to improve patients' treatment options, we need to understand the mechanisms regulating the development of resistance. This study suggests that activation of the Ras pathway predicts for poor outcome on tamoxifen but not chemotherapy, and identifies pRaf(ser338) as a potential marker of resistance to ER-targeted therapy. In addition, it suggests that expression of pRaf(ser338) could identify patients for whom tamoxifen alone is insufficient adjuvant systemic therapy, but for whom the addition of chemotherapy may be of benefit.

Abstract

Purpose: Expression and activation of the Ras/Raf-1/MAPK pathway plays an important role in the development and progression of cancer, and may influence response to treatments such as tamoxifen and chemotherapy. In this study we investigated whether expression and activation of the key components of this pathway influenced clinical outcome, to test the hypothesis that activation of the MAPK pathway drives resistance to tamoxifen and chemotherapy in women with breast cancer.

Experimental Design: Breast tumours from patients at Glasgow Royal Infirmary and others treated within the BR9601 trial were analysed for expression of the three Ras isoforms, total Raf-1, active and inactive forms of Raf-1 (pRaf(ser338) and pRaf(ser259), respectively), MAPK and phospho-MAPK using an immunohistochemical approach. Analyses were performed with respect to disease free-survival and overall survival.

Results: Expression and activation of the Ras pathway was associated with loss of benefit from treatment with tamoxifen but not chemotherapy. Overexpression of pRaf(ser338) was associated with shortened disease-free and overall survival time in univariate analyses. Multivariate analysis suggested pRaf(ser338) was independent of known prognostic markers in predicting outcome following tamoxifen treatment ($p=0.03$).

Conclusion: This study suggests that activation of the Ras pathway predicts for poor outcome on tamoxifen but not chemotherapy, and identifies pRaf(ser338) as a potential marker of resistance to ER-targeted therapy. In addition, it suggests that expression of pRaf(ser338) could identify patients for whom tamoxifen alone is insufficient adjuvant systemic therapy, but for whom the addition of chemotherapy may be of benefit.

Keywords: Breast cancer; Ras/Raf-1/MAPK cascade, Oestrogen Receptor, Tamoxifen Resistance, Chemotherapy

Abbreviations: Oestrogen Receptor (ER), Cyclophosphamide, Methotrexate and 5-Flourouracil (CMF), Oestrogen Response Elements (ERE), Overall Survival (OS), Disease Free Survival (DFS)

Introduction

The Ras/Raf-1/MAPK pathway controls multiple cellular processes, including proliferation, differentiation, senescence and apoptosis (1). Activation follows membrane tyrosine kinase receptors (EGFR, HER2-4) binding appropriate ligands, dimerising and undergoing autophosphorylation (2,3). This activates Ras, which translocates to the plasma membrane and promotes Raf-1 phosphorylation and activation (4-13). Raf-1 phosphorylates and activates MEK, which activates MAPK, a serine/threonine kinase responsible for phosphorylating and activating substrates, including c-fos and c-myc, which regulate proliferation (14,15). MAPK also phosphorylates and activates the oestrogen receptor (ERα) (16-19).

Expression and activation of the Ras/Raf-1/MAPK pathway plays an important role in the development and progression of cancer, and may additionally influence response to treatments targeted against ERα and proliferation. Chemotherapy and endocrine therapy are thought to target proliferating cells, and whilst it remains unclear if highly proliferating tumours are more sensitive to treatment, we hypothesised that activation of this pathway would be an indicator of responsiveness to endocrine and cytotoxic therapies.

The majority of chemotherapeutic agents are thought to function most effectively against proliferating tumour cells in growth phase. Recently the National Epirubicin Adjuvant Trial (NEAT) and BR9601 trial confirmed that the anthracycline, epirubicin, plus CMF, is superior to CMF alone as adjuvant treatment for patients with early breast cancer (22). Since these agents are more effective against rapidly cycling cells (23), it is feasible that tumours with low proliferative indices may be less sensitive to these therapeutic agents. However, proliferative indices alone are poor predictors of chemotherapeutic response (24).

Several studies suggest that the Ras/Raf-1/MAPK pathway is linked to response to anthracycline treatment. In vitro Raf-1 (25,26) and MAPK (27) expression are associated with increased proliferation and anthracycline resistance. Conversely, increased MAPK activation has been linked to enhanced apoptosis and anthracycline sensitivity (28). The role of the Ras pathway in determining chemosensitivity therefore requires further investigation.

Activation of Ras/Raf-1/MAPK is also linked to tamoxifen resistance through phosphorylation of ERα. The classic "genomic" action of ERα requires ligand binding, which induces phosphorylation, dissociation from heat shock proteins (Hsps), conformational changes, homodimerisation and nuclear translocation. Nuclear ERα binds to ERE sequences in the promoter region of oestogen regulated genes (29) and recruits co-activators, co-repressors and transcription machinery (16,30,31). ERα can, however, be activated in a ligand-independent manner via signalling pathways. The Ras/Raf-1/MAPK pathway phosphorylates Serine 118, within the AF-1 domain of the receptor, (16,18,19,32) resulting in its activation and transcription of oestrogen-regulated genes and cell proliferation. Consequently, the Ras pathway is thought to increase ERα sensitivity to low concentrations of oestrogen, resulting in tamoxifen resistance (33-35). Tamoxifen-resistant cells have been shown to have increased levels of activated MAPK, phosphorylated ERα and transcription of oestrogen-regulated genes (36,37).

This study investigated whether expression and activation of the key components of the Ras/Raf-1/MAPK pathway influenced clinical outcome, to test the hypothesis that activation of the pathway drives resistance to tamoxifen and chemotherapy in clinical breast cancer. In order to achieve this two different patient groups were used, a tamoxifen treated and a chemotherapy treated cohort. The study

population included ER+ve patients who received tamoxifen treatment alone and in combination with chemotherapy, and patients from the Scottish BR9601 trial who received either CMF or sequential epirubicin followed by CMF.

Materials & Methods

Patient Cohorts

Two patient cohorts were studied, after obtaining ethical approval for each study separately. The first comprised 402 patients with ERα positive tumours who were treated at Glasgow Royal Infirmary between 1980-1999 (Steroid Resistant Tumour Bank – STB). These patients received adjuvant tamoxifen for a median of 5 years (range 0.6-18 years) and follow-up data was available for a median of 6.45 years (range 0.64-18.42 years). In addition, 99 (24.8%) of these patients received adjuvant chemotherapy and 110 (27.5%) received adjuvant radiotherapy. ERα status was defined as previously described (38). Clinical/pathological characteristics for these patients are shown in Table 1. In this study there were 74 breast cancer specific deaths and 100 breast cancer relapses, 78 of which occurred during tamoxifen treatment.

An additional 318 patients were studied, from the BR9601 adjuvant chemotherapy trial designed to test the possible benefit of 4 cycles of epirubicin followed by 4 cycles of CMF over 8 cycles of CMF chemotherapy in women with early breast cancer. There was a median follow up of 4.95 years (range 0.27-8.52 years), with 84 breast cancer related deaths and 111 breast cancer recurrences (both local and distant). Clinical/pathological characteristics are again shown in Table 1.

Antibodies

Ras protein expression was investigated using three isoform specific antibodies: H-Ras (IgG_1 Ab, F235, Santa Cruz, CA, USA); K-Ras (Sigma, Dorset, UK) and N-Ras (IgG1 Ab, F155, Santa Cruz). Raf-1 protein expression was measured using a Raf-1 antibody (IgG1 Ab, E-10, Santa Cruz) and two phospho-specific antibodies recognising active and inactive Raf-1: phospho-Raf(ser338) (Upstate, CA, USA) and

phospho-Raf(ser259) (Cell Signalling Technology, MA, USA; CST), respectively. MAPK expression was investigated using a p44/42MAPK antibody (CST) and a phospho-specific p44/42 MAPK(Thr202/Tyr204) antibody (CST). All Ras antibodies were used at a concentration of 20μg/ml; the Raf-1 antibody was used at 5μg/ml, the phospho-Raf-1 antibodies at 4μg/ml, and both MAPK antibodies at 0.5μg/ml in antibody diluent (DAKO, Glostrup, Denmark) for immunohistochemistry. All antibodies were used to investigate protein expression in the STB study but only pRaf(ser259), pRaf(ser338), MAPK and pMAPK antibodies were used in the BR9601 study. The specificity of all antibodies was confirmed by western blotting.

Western Blotting

Proteins from unstimulated and 10nM Heregulin (HRG) stimulated MCF-7 and MDA-MB-453 cells were resolved by 10% SDS-PAGE at 40mA for 1 hour and transferred to PVDF membrane overnight at 10V. The membrane was treated with 5% BSA in TTBS (Tris Buffered Saline-Tween) for 1 hour and incubated with primary antibody (H-Ras, N-Ras, Raf-1, pRaf(ser338) = 0.4μg/ml, K-Ras, pRaf(ser259), MAPK, pMAPK = 0.2μg/ml) overnight at 4^{o}C. Membranes were incubated in appropriate secondary antibody for 1 hour, anti-mouse IgG (CST, 1:10000) for H-Ras, K-Ras, N-Ras and Raf-1 and anti-Rabbit IgG (CST, 1:5000) for pRaf(ser259), pRaf(ser338), MAPK and pMAPK, and visualised using chemiluminescence (Western blotting detection reagent; Amersham Biosciences, Buckinghamshire, UK).

TMA Construction

Tissue microarrays (TMA) were constructed for 402 ERα positive STB tumours and the 318 BR9601 breast tumours (39). TMA construction allows rapid tumour processing under standardised conditions and has been extensively validated in breast cancer. Up to 100-200 (0.6 diameter) individual tumour cores were placed into a

single recipient block enabling simultaneous multiple tumour analysis. A consultant breast pathologist was responsible for marking tumour areas on haematoxylin and eosin stained tumour slides prior to coring. To account for tumour heterogeneity, 3* 0.6 mm cores were removed from the marked areas in each tumour block and transferred to recipient paraffin blocks to form the TMA.

Immunohistochemistry

IHC for H-Ras, N-Ras and Raf-1 was performed as previously described (40). For pRaf(ser338) and K-Ras, antigen retrieval was performed by heating under pressure in TE buffer (1mM EDTA, 5mM Tris, pH 8.0) for five minutes in a microwave. For pRaf(ser259), p44/42 MAPK and phospho-p44/42 MAPK, slides were incubated in 10mM Citrate Buffer at 96^{o}C for twenty minutes. Endogenous peroxide was blocked by incubation in 0.3% hydrogen peroxide (H_2O_2) (except for K-Ras, where 3% H_2O_2 was used). Blocking was performed using 1.5% normal horse serum (Vector Laboratories, CA, USA; H-Ras, N-Ras, Raf-1, p44/42 MAPK and phospho-p44/42 MAPK) or Casein solution (Vector Laboratories; K-Ras, pRaf(ser259) and pRaf(ser338)). Antibody incubations were performed overnight at 4^{o}C, with the exception of phospho-p44/42 MAPK (six hours at room temperature). In each run a negative isotype matched and a positive control, using breast tumour tissue known to express the protein of interest, were included. Signal was visualised using Envision (DAKO) and 3,3'-diaminobenzidine (DAB, Vector Laboratories). For the K-Ras antibody, the Super Sensitive Non-Biotin HRP Detection System (BioGenex, CA, USA) was used.

Histoscore Method

Two observers (LM & TK) trained by a pathologist, independently scored tumour cores, as selected by a pathologist, using a weighted histoscore method(41,42). The intensity of cytoplasmic and nuclear staining was categorised as negative (0), weak (1), moderate (2) and strong (3) and the percentage of tumour cells within each category estimated. The histoscore was calculated using the following formula: Histoscore = 0 x % negative tumour cells + 1 x % weakly stained tumour cells + 2 x % moderately stained tumour cells + 3 x % tumour cells stained strongly. The histoscore ranged from a minimum of zero to a maximum of 300. Agreement between the two observers was monitored. Cases with discordant results between observers were re-evaluated. Agreement between observers was excellent with Interclass correlation coefficients (ICCC) scores between 0.74-0.97.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical package (version 9.0 for Windows). Correlations between proteins were calculated using the Spearman Rank Test. Pearsons chi-square test was used to correlate protein expression with known prognostic factors. Kaplan-Meier life-table analysis and Cox's multiple regression (including known prognostic factors tumour size, grade and nodal status) were performed to estimate differences in breast cancer related survival, in terms of disease-free survival (DFS) and overall survival (OS), using breast cancer recurrences and breast-cancer specific deaths as the respective end-points. To establish the relative risk of a patient relapsing or dying as a result of either high or low levels of a particular protein in their breast tumour, hazard ratio analysis was calculated by Cox's multiple regression using only the protein of interest as a variable. For survival analysis and chi-square tests, patients were split into two groups, those that expressed

high levels of protein and those that expressed low levels. For all proteins analysed, high levels were defined as IHC scores equal to or above the upper quartile value, whilst low levels were defined as IHC scores less than the upper quartile value. A value of $p<0.05$ was deemed statistically significant.

Results

Patient cohort & treatment

Of 402 STB patients, 303 individuals received tamoxifen alone, whilst the remaining 99 received both tamoxifen and chemotherapy. Survival analysis was performed on the entire cohort but also on the subgroup of patients who received only tamoxifen, thus addressing the potential confounding effects of both endocrine and chemotherapy treatments. Patients from the BR9601 trial were randomly allocated to receive either CMF alone or epirubicin followed by CMF. Three hundred and eighty four patients were randomised in BR9601 and tissue samples retrieved from 318 cases (84%). One hundred and fifty five (49%) patients received treatment with epirubicin followed by CMF while the remaining 163 (51%) patients received only CMF. Additionally, 165 of the BR9601 patients received tamoxifen. A survival analysis of those patients whose samples contributed to this sub-study confirmed the statistical advantage of Epi-CMF over CMF observed in the main BR9601 and NEAT studies (43) (data not shown).

Protein Expression

H-, K- and N-Ras and Raf-1 expression was investigated in the STB cohort, whilst the inactivated and activated form of Raf-1, pRaf(ser259) and pRaf(ser338) respectively, and MAPK and pMAPK, were analysed in both the STB and BR9601 cohort of patients. With the exception of pRaf(ser259), which was localised primarily to the cytoplasm, all proteins were expressed in both the cytoplasm and nuclei of tumour cells (Figure 1, Table 2). Despite the high frequency of patients expressing both cytoplasmic and nuclear H- K- and N-Ras, there was no significant relationship between the expression levels of Ras in the two locations. However, a strong positive

correlation was evident between the cytoplasmic and nuclear localisation of Raf-1, pRaf(ser338), MAPK and pMAPK.

Activation of the Ras/Raf-1/MAPK pathway in breast tumours

Tumours from the STB and NEAT cohorts with elevated activated Raf, pRaf(ser338), also expressed increased levels of cytoplasmic and nuclear pMAPK (Spearman's rank test; Table 3). In the STB study, overexpression of Ras isoforms was associated with increased activated Raf expression; N-Ras was most markedly correlated with pRaf ($p<0.0005$, CC = 0.274, Table 3).

Protein expression and association with known prognostic markers

In the STB cases nuclear pRaf(ser338) expression was positively correlated with node positivity ($p=0.009$), elevated expression of cytoplasmic pRaf(ser338) was associated with increased tumour grade ($p=0.001$). Cytoplasmic MAPK expression was positively associated with tumour grade ($p=0.025$), size ($p=0.002$) and nodal status ($p<0.0005$). No correlations were observed between nuclear MAPK and known prognostic markers.

Conversely, in the BR9601 chemotherapy treated patients no significant correlations were observed between pRaf(ser338) expression and node positivity, tumour size or grade. Increased nuclear MAPK and cytoplasmic pMAPK expression was related to lower (grade 1 or 2) grade of tumour ($p=0.01$ and $p=0.0017$ respectively).

Steroid tumour bank (STB) survival analysis

Raf-1 activation was associated with shortened disease-free survival (DFS) in the 402 STB patients. High cytoplasmic pRaf(ser338) expression in tumours was associated with a reduced time to recurrence (p=0.002) (Figure 2a). Patients with tumours expressing increased levels of nuclear pRaf(ser338) also exhibited reduced DFS (p=0.006). Hazard ratios (HR) were 1.84 (95% CI 1.24 - 2.75, p=0.0026) and 1.78 (95% CI 1.17 – 2.71, p=0.007) respectively. (Figure 2b). No association was observed between Ras, pRaf(ser259), MAPK or pMAPK and DFS in this series.

Overall survival (OS) for the 402 patients treated in this series was not associated with increased expression of the Ras isoforms, pRaf(ser259), MAPK or pMAPK. Patients whose tumours expressed increased levels of cytoplasmic or nuclear pRaf(ser338) exhibited a reduced OS following tamoxifen treatment (p=0.0229) and (p=0.0006) respectively (Figure 2c,d). Hazard ratios were 1.74 (95% CI 1.07-2.81, p=0.0247) and 2.29 (95% CI 1.41-3.74, p=0.0009) respectively.

BR9601 survival analysis

Survival analysis revealed no significant association between tumour expression of pRaf(ser259), pRaf(ser338), MAPK or pMAPK and risk of breast cancer recurrence or death in BR9601 patients receiving chemotherapy (Epi/CMF or CMF) either alone or with tamoxifen.

ER positive tamoxifen only treated cases (303 STB cases) survival analysis

In the 303 STB cases treated only with tamoxifen, patients with tumours overexpressing cytoplasmic (p=0.0023, 10.2 years versus 13.3 years) or nuclear pRaf(ser338) (p=0.002, 10.3 years versus 12.8 years) had worse DFS (Figure 3a,b).

The relative risks for relapse associated with increased expression of cytoplasmic or nuclear pRaf(ser338) were 2.02 (95% CI 1.27-3.21, p=0.0028) and 2.08 (95% CI 1.29-3.33, p=0.0025) respectively. Increased cytoplasmic or nuclear pMAPK expression was associated with a shorter DFS in patients treated with tamoxifen alone (p=0.01, 8.5 years versus 12.9 years, Figure 3c, HR 2.04) and (p=0.04, 11.4 years versus 12.5 years, HR1.61) respectively. Multivariate Cox-Regression analysis revealed that only nuclear pRaf(ser338) expression was independent of tumour size, grade or nodal status in influencing relapse (p=0.03, Table 4).

Activation of Raf-1 was also linked to a poor outcome in this sub-set of patients. Elevated cytoplasmic or nuclear pRaf(ser338) expression was associated with shortened OS time (p=0.015, 13.6 years versus 15.06 years) and (p=0.0008, 11.8 years versus 15.72 years) respectively (Figure 4a,b). Increased expression of cytoplasmic or nuclear pRaf(ser338) raised the risk of death by 1.96 (95% CI 1.13-3.42, p=0.017) and 2.52 (95% CI 1.44-4.41, p=0.0012) times respectively.

Increased expression of MAPK and pMAPK were also associated with a significant reduction in OS time in patients treated only with tamoxifen. Patients whose tumours expressed high levels of cytoplasmic and nuclear MAPK were more likely to die sooner than those with low levels (p=0.033, 13.2 years versus 15.6 years) and (p=0.039, 13.11 years versus 15.60 years) respectively (Figure 4c,d). The relative risk for these patients was 1.84 (95% CI 1.04-3.26) and 1.78 (95 CI% 1.02-31.2) for cytoplasmic and nuclear MAPK respectively. Likewise patients with increased tumour levels of nuclear pMAPK exhibited a shortened OS time (p=0.0336, 13.50 years versus 16.10 years) (Figure 4e). Increased expression of nuclear pMAPK increased the risk of death in patients treated with tamoxifen by 1.83 (95% CI 1.04-3.24) times. Multivariate analysis excluded pRaf(ser338), MAPK or pMAPK as being

independent of known predictive factors (tumour size, grade and nodal status) for overall survival.

ER positive tamoxifen and chemotherapy treated cases (STB and BR9601)

For the 264 ER positive patients in the combined STB and BR9601 populations who received both tamoxifen and chemotherapy there was no significant association between tumour expression of pRaf(ser259), pRaf(ser338), MAPK, or pMAPK and shortened DFS or OS time (data not shown).

Discussion

This study demonstrates that the Ras/Raf-1/MAPK pathway is activated in clinical breast tumours and suggests that activation is associated with poor outcome, particularly in patients treated with tamoxifen alone.

Analysis of 402 cases treated with tamoxifen suggested that poor outcome was associated with the expression and activation of the Ras cascade, in particular activation of Raf-1 (Figure 1). In contrast, activation of this pathway was not associated with outcome in a chemotherapy and tamoxifen treated population from the BR9601 study. This contrasting result from two populations of breast cancer patients, analysed within the same laboratory, but with differing treatment regimens, led us to hypothesise that activation of Raf-1 may be associated with poor outcome in ERα positive patients treated with adjuvant tamoxifen. We therefore performed two exploratory analyses, one on the 303 ERα positive cases from the STB cohort which received only tamoxifen and on the 264 ERα positive cases (99 from the STB and 165 from the BR9601 study) who received chemotherapy and tamoxifen.

Survival analysis demonstrated that increased expression of cytoplasmic and nuclear pRaf(ser338) was associated with increased risk of relapse and death in the 303 tamoxifen only treated patients and on multivariate analysis nuclear pRaf(ser338) expression was independent of nodal status, tumour size and grade ($p=0.031$). In addition, elevated levels of cytoplasmic and nuclear pMAPK were associated with an increased risk of recurrence in this patient cohort. Conversely, in the 264 ERα positive patients treated with both chemotherapy and tamoxifen, no association with phosphorylated Raf-1 or MAPK was observed with patient outcome measures.

These results suggest that increased Raf-1/MAPK phosphorylation, or activation is associated with early relapse on adjuvant tamoxifen and that nuclear

pRaf(ser338) is a candidate for identifying ERα positive patients at risk of relapse if treated with tamoxifen alone. We cannot at present, rule out the possibility that pRaf(ser338) functions as a prognostic marker, as opposed to predictive marker, since all cases analysed received adjuvant therapy, however it appears to function at least additionally as a predictive factor for improved benefit from chemotherapy. In the 99 STB patients who received both adjuvant tamoxifen and chemotherapy, it seemed that the addition of chemotherapy increased the time to relapse in those patients expressing high levels of pRaf(ser338). Interestingly, pRaf(ser338) was not an independent predictor of tamoxifen resistance when the 303 tamoxifen only and 99 tamoxifen and chemotherapy treated patients were combined. This suggests that chemotherapy partially overrides the negative effects of increased expression of nuclear pRaf(ser338). Increased tumour levels of pRaf(Ser338) are perhaps indicative of decreased benefits from tamoxifen but enhanced response to chemotherapy. This supports previous findings that increased expression of Raf-1 in cell lines makes them more responsive to chemotherapeutic agents (44). To confirm this hypothesis, ideally analysis of an untreated patient cohort would be undertaken to address any potential prognostic role and to determine if pRaf(ser338)is also a predictive marker in the context of benefit associated with tamoxifen therapy.

Raf-1 is a serine-threonine kinase that plays a role in cell proliferation, differentiation and apoptosis, and is prominent in controlling tumour angiogenesis and metastasis (45,46). We show that increased Raf-1 activity in tumours was related to poorly differentiated tumours (high grade) and tumour spread (node positivity). A recent study demonstrated that targeting Raf-1 inhibits tumour growth and that this represents an important therapeutic strategy (47).

The ligand-independent phosphorylation of ERα at serine 118 by MAPK is believed to be a major contributor to the development of tamoxifen resistance and this relationship between the Ras/Raf-1/MAPK pathway and ERα has been well documented. It is hypothesised that phosphorylation of ERα contributes to tamoxifen resistance by promoting tumour growth in the presence of low levels of oestrogen (48,49). However in the current study nuclear pRaf(ser338) appears to be dominant over MAPK, which implies that whilst MAPK driven phosphorylation of ERα may be important, it is not the only contributing factor in the development of tamoxifen resistance. It also suggests that the mechanism by which nuclear pRaf(ser338) influences tamoxifen resistance is independent of MAPK.

In summary, this study demonstrates that expression and activation of the Ras/Raf-1/MAPK pathway in breast tumours is associated with increased risk of relapse and death with tamoxifen treatment but not when chemotherapy is also given to ERα positive cases. These results suggest that activated Raf-1 is a potential predictive marker for identifying patients who are least likely to benefit from tamoxifen and for whom additional therapy may be required.

Acknowledgements

Sadly during the preparation of this manuscript our colleague and friend Professor Timothy Cooke died suddenly. We wish to acknowledge his support through the development and execution of this study. This research was supported by the White Lily Breast Cancer Charity, the Scottish Breast Cancer Trials Group, Cancer Research UK & the Glasgow Royal Infirmary Endowment Fund.

Formatted: Font: Not Bold

Reference List

1. Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 2000;351 Pt 2:289-305.

2. Reese DM and Slamon D. J. HER-2/neu signal transduction in human breast and ovarian cancer. Stem Cells 1997;15:1-8.

3. Yarden Y and Sliwkowski M. X. Untangling the ErbB Signalling Network. Nature Reviews:- Molecular Cell Biology 2001;2:127-37.

4. Adjei AA. Blocking Oncogenic Ras Signaling for Cancer Therapy. Journal of the National Cancer Institute 2001;93:1062-74.

5. Macaluso M, Russo G., Cinti C., Bazan V., Gebbia N., and Russo A. Ras family genes: an interesting link between cell cycle and cancer. J Cell Physiol 2002;192:125-30.

6. Pruitt K and Der C. J. Ras and Rho regulation of the cell cycle and oncogenesis. Cancer Lett 2001;171:1-10.

7. Barbacid M. ras genes. Annu Rev Biochem 1987;56:779-827.

8. Burgering BM and Bos J. L. Regulation of Ras-mediated signalling: more than one way to skin a cat. Trends Biochem Sci 1995;20:18-22.

9. Roskoski R, Jr. Protein prenylation: a pivotal posttranslational process. Biochem Biophys Res Commun 2003;303:1-7.

10. Dhillon AS and Kolch W. Untying the regulation of the Raf-1 kinase. Archives of Biochemistry and Biophysics 2002;404:3-9.

11. Xing H, Kornfeld K., and Muslin A. J. The protein kinase KSR interacts with 14-3-3 protein and Raf. Current Biology 1997;7:294-300.

12. Dhillon AS, Meikle S., Yazici Z., Eulitz M., and Kolch W. Regulation of Raf-1 activation and signalling by dephosphorylation. EMBO J 2002;21:64-71.

13. Therrien M, Michaud N. R., Rubin G. M., and Morrison D. K. KSR modulates signal propagation within the MAPK cascade. Genes Dev 1996;10:2684-95.

14. Santen RJ, Song Robert Xinde, McPherson Robertet al. The role of mitogen-activated protein (MAP) kinase in breast cancer. The Journal of Steroid Biochemistry and Molecular Biology 2002;80:239-56.

15. Silvius JR. Mechanisms of Ras Protein Targeting in Mammalian Cells. The Journal of Membrane Biology 2002;190:83-92.

16. Chen D, Washbrook E., Sarwar N.et al. Phosphorylation of human estrogen receptor α at serine 118 by two distinct signal transduction pathways revealed by phosphorylation specific antisera. Oncogene 2002;21:4921-31.

17. Kato S, Endoh H., Masuhiro Y.et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 1995;270:1491-4.

18. Driggers PH and Segars J. H. Estrogen action and cytoplasmic signaling pathways. Part II: the role of growth factors and phosphorylation in estrogen signaling. Trends In Endocrinology And Metabolism 2002;13:422-8.

19. Lannigan DA. Estrogen receptor phosphorylation. Steroids 2003;68:1-9.

20. Shiau AK, Barstad D., Loria P. M.et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927-37.

21. Kong EH, Pike A. C. W., and Hubbard R. E. Structure and mechanism of the oestrogen receptor. Biochemical Society Transactions 2003;31:56-9.

22. Poole CJ, Earl H. M., Hiller L.et al. Epirubicin and cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy for early breast cancer. N Engl J Med 2006;355:1851-62.

23. Amadori D, Volpi A., Maltoni R.et al. Cell proliferation as a predictor of response to chemotherapy in metastatic breast cancer: a prospective study. Breast Cancer Res Treat 1997;43:7-14.

24. Rees M, Stahl M., Klump B., Willers R., Gabbert H. E., and Sarbia M. The prognostic significance of proliferative activity, apoptosis and expression of DNA topoisomerase II alpha in multimodally-treated oesophageal squamous cell carcinoma. Anticancer Res 2001;21:3637-42.

25. Weinstein-Oppenheimer CR, Henriquez-Roldan C. F., Davis J. M.et al. Role of the Raf signal transduction cascade in the in vitro resistance to the anticancer drug doxorubicin. Clin Cancer Res 2001;7:2898-907.

26. Davis JM, Navolanic P. M., Weinstein-Oppenheimer C. R.et al. Raf-1 and Bcl-2 induce distinct and common pathways that contribute to breast cancer drug resistance. Clin Cancer Res 2003;9:1161-70.

Formatted: Spanish (Spain-Modern Sort)

27. Zhao Y, You H., Yang Y.et al. Distinctive regulation and function of PI 3K/Akt and MAPKs in doxorubicin-induced apoptosis of human lung adenocarcinoma cells. J Cell Biochem 2004;91:621-32.

28. Guise S, Braguer D., Carles G., Delacourte A., and Briand C. Hyperphosphorylation of tau is mediated by ERK activation during anticancer drug-induced apoptosis in neuroblastoma cells. J Neurosci Res 2001;63:257-67.

29. Hall JM, Couse J. F., and Korach K. S. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 2001;276:36869-72.

30. Ali S and Coombes R. Charles. ENDOCRINE-RESPONSIVE BREAST CANCER AND STRATEGIES FOR COMBATING RESISTANCE. Nat Rev Cancer 2002;2:101-12.

31. Kato S, Endoh H., Masuhiro Y.et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 1995;270:1491-4.

32. Kato S, Endoh H., Masuhiro Y.et al. Activation of the Estrogen Receptor Through Phosphorylation by Mitogen-Activated Protein Kinase. Science 1995;270(5241):1491-4.

33. Chung YL, Sheu M. L., Yang S. C., Lin C. H., and Yen S. H. Resistance to tamoxifen-induced apoptosis is associated with direct interaction between Her2/neu and cell membrane estrogen receptor in breast cancer. Int J Cancer 2002;97:306-12.

34. Osborne CK and Schiff R. Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. Breast 2003;12:362-7.

35. Kurokawa H, Lenferink A. E., Simpson J. F.et al. Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. Cancer Res 2000;60:5887-94.

36. Nicholson RI, Hutcheson I. R., Britton D.et al. Growth factor signalling networks in breast cancer and resistance to endocrine agents: new therapeutic strategies. The Journal of Steroid Biochemistry and Molecular Biology 2005;93:257-62.

37. Britton DJ, Hutcheson I. R., Knowlden J. M.et al. Bidirectional cross talk between ERalpha and EGFR signalling pathways regulates tamoxifen-resistant growth. Breast Cancer Res Treat 2006;96:131-46.

38. Tovey S, Dunne B., Witton C. J., Forsyth A., Cooke T. G., and Bartlett J. M. Can molecular markers predict when to implement treatment with aromatase inhibitors in invasive breast cancer? Clin Cancer Res 2005;11:4835-42.

39. Tovey S, Dunne B., Witton C. J., Forsyth A., Cooke T. G., and Bartlett J. M. Can molecular markers predict when to implement treatment with aromatase inhibitors in invasive breast cancer? Clin Cancer Res 2005;11:4835-42.

40. Mukherjee R, Bartlett J. M., Krishna N. S., Underwood M. A., and Edwards J. Raf-1 expression may influence progression to androgen insensitive prostate cancer. Prostate 2005;64:101-7.

41. Kirkegaard T, Edwards J., Tovey S.et al. Observer variation in immunohistochemical analysis of protein expression, time for a change? Histopathology 2006;48:787-94.

42. Mukherjee R, Bartlett J. M., Krishna N. S., Underwood M. A., and Edwards J. Raf-1 expression may influence progression to androgen insensitive prostate cancer. Prostate 2005;64:101-7.

43. Poole CJ, Earl H. M., Hiller L.et al. Epirubicin and cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy for early breast cancer. N Engl J Med 2006;355:1851-62.

44. Lee M, Koh W. S., and Han S. S. Down-regulation of Raf-1 kinase is associated with paclitaxel resistance in human breast cancer MCF-7/Adr cells. Cancer Lett 2003;193:57-64.

45. El-Ashry D, Miller D. L., Kharbanda S., Lippman M. E., and Kern F. G. Constitutive Raf-1 kinase activity in breast cancer cells induces both estrogen-independent growth and apoptosis. Oncogene 1997;15:423-35.

46. Mercer K, Giblett S., Oakden A., Brown J., Marais R., and Pritchard C. A-Raf and Raf-1 work together to influence transient ERK phosphorylation and Gl/S cell cycle progression. Oncogene 2005;24:5207-17.

47. Leng Q and Mixson A. J. Small interfering RNA targeting Raf-1 inhibits tumor growth in vitro and in vivo. Cancer Gene Ther 2005;12:682-90.

48. Osborne CK, Shou Jiang, Massarweh Suleiman, and Schiff Rachel. Crosstalk between Estrogen Receptor and Growth Factor Receptor Pathways as a Cause for Endocrine Therapy Resistance in Breast Cancer. Clin Cancer Res 2005;11:865s-870.

49. Kurokawa H, Lenferink A. E., Simpson J. F.et al. Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. Cancer Res 2000;60:5887-94.

50. Mouridsen HT, Keshaviah A., Mauriac L.et al. BIG 1-98: A randomized double-blind phase III study comparing letrozole and tamoxifen given in sequence vs. alone as adjuvant endocrine therapy for postmenopausal women with receptor-positive breast cancer. Journal of Clinical Oncology 2006;24:10S.

51. Howell A, Cuzick J., Baum M.et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60-2.

		STB Patients		BR9601 Patients	
		Number/total	**%**	**Number/Total**	**%**
Grade	**1**	99/391	**25.32**	20/305	**6.55**
	2	193/391	**49.36**	100/305	**32.79**
	3	99/391	**25.32**	185/305	**60.66**
	unknown	11		13	
Nodal status	**0**	193/369	**52.3**	43/313	**13.74**
	1-3	107/369	**29.0**	176/313	**56.23**
	4+	69/369	**18.7**	94/313	**30.03**
	unknown	33		5	
Size	**T1 (<20mm)**	154/380	**40.53**	94/308	**30.52**
	T2 (20-50mm)	204/380	**53.68**	203/308	**65.91**
	T3 (>50mm)	22/380	**5.79**	11/308	**3.57**
	unknown	22			
NPI	**<3.5**	128/344	**37.21**	65/311	**20.9**
	3.5-4.5	106/344	**30.81**	148/311	**47.59**
	4.5+	110/344	**31.98**	98/311	**12.86**
	Missing	58		7	
Age	**<50 years**	73/401	**18.2**	90/318	**28.3**
	>50 years	328/401	**81.8**	228/318	**71.7**
	Missing	1			
ER Status	**+ve**	402/402	**100.0**	174/278	**62.59**
	-ve			104/278	**37.41**
	Unknown			40	
Treatment	**Epi & CMF**	n/a		155/318	**48.74**
	CMF Alone	n/a		163/318	**51.26**

Table 1: Patient clinical and pathological variables
Grade = Bloom and Richardson grade. Nodal status = number of positive nodes.
NPI = Nottingham Prognostic Index = grade+nodal status+0.02*size in mm.

Protein	Cytoplasmic Expression Median Histoscore (IQ Range)	Nuclear Expression Median Histoscore (IQ Range)
H-Ras	125 (90-157)	40 (20-70)
K-Ras	53 (27-85)	23 (8-50)
N-Ras	147 (113-180)	100 (80-117)
Raf-1	123 (83-153)	108 (91-123)
pRaf(ser259)	71 (30-120)	0 (0-3)
pRaf(ser338)	157 (120-185)	137 (113-160)
MAPK	110 (68-147)	75 (50-100)
pMAPK	60 (25-95)	68 (40-95)

Table 2: Protein Expression in Breast Tumours
Table 2 shows the median histoscore and interquartile range (IQ) for protein expression in the cytoplasm and nuclei of breast tumour cells.

		pRaf(ser338)	
		Cytoplasmic	Nuclear
H-Ras	Cyto	**R^2 = 0.130 p=0.015**	ns
	Nuc	**R^2= -0.163 p=0.002**	ns
K-Ras	Cyto	**R^2 = 0.226 p<0.0005**	ns
	Nuc	ns	**R^2 = 0.143 p = 0.007**
N-Ras	Cyto	**R^2 = 0.274 p<0.0005**	ns
	Nuc	**R^2 =-0.217 p<0.0005**	ns
pMAPK	Cyto	**R^2 = 0.240 p<0.0005**	**R^2 = 0.246 p<0.0005**
	Nuc	**R^2 = 0.217 p<0.0005**	**R^2 = 0.334 p<0.0005**

Table 3: Correlations between Ras and Raf

Spearman Rank Tests were performed to analyse the relationship between overexpression of the three Ras isoforms and phosphorylation of Raf at serine 259 and serine 338 in the cytoplasm and nuclei. Only cytoplasmic pRaf(ser259) was analysed because only very low levels of nuclear pRaf(ser259) were detected. R^2 = correlation coefficient. p values < 0.05 were deemed statistically significant. ns = non-significant.

Factor	Hazard Ratio	p-value
Nodal Status (0, 1-3, 4+)	2.01 (1.41 – 2.87)	p=0.0002
Tumour Grade (1, 2, 3)	1.47 (0.99 – 2.20)	p=0.0552
Tumour Size (T1 <20mm, T2 20-50mm, T3 >50mm)	2.23 (1.31 – 3.81)	p=0.0027
Nuclear pRaf(ser338) (IHC score < 160, IHC score >160)	1.94 (1.09 – 3.45)	p=0.0307

Table 4: Factors that independently influence disease-free survival time of patients treated only with tamoxifen

Cox-Regression multivariate analysis revealed that nuclear pRaf(Ser338) expression was independent of known prognostic markers; nodal status, tumour size and grade, in influencing disease-free survival time in patients treated only with tamoxifen. p-values < 0.05 were deemed statistically significant. Hazard ratio = relative risk associated with development of tamoxifen resistance (95% CI).

Figure Legends

Figure 1: Immunohistochemical Staining of Breast Tumour Tissue

Immunohistochemistry pictures of breast tumour tissue stained with (a),H-Ras (b)K-Ras, (c) N-Ras, (d)Raf-1, (e) pRaf(ser259), (f) pRaf(ser338), (g)p44/42MAPK and (h) phospho-p44/42MAPK antibodies. For all antibodies the proteins are detected in both the cytoplasm and the nuclei of tumour cells.

Figure 2: pRaf(ser338) Disease Free & Overall Survival Curves in 402 STB patients

Kaplein Meier survival curves showing disease-free survival (DFS) in patients whose tumours express cytoplasmic and nuclear pRaf(ser338). (a) Survival curve showing a significant reduction in DFS in patients whose tumours express high levels of cytoplasmic pRaf(ser338) (p=0.0022). (b) Survival curve showing a significant reduction in DFS in patients whose tumours express high levels of nuclear pRaf(ser338) (p=0.0064). (c) Survival curve showing a significant reduction in overall survival time in STB patients treated with tamoxifen and chemotherapy, whose tumours express high levels of cytoplasmic pRaf(ser338) (p=0.0229). (d) Survival curve showing a significant reduction in overall survival time in STB patients treated with tamoxifen and chemotherapy, whose tumours express high levels of nuclear pRaf(ser338) (p=0.0006). High levels were defined as scores ≥ upper quartile value. p values represent log rank testing of the differences in survival. HR=Hazard Ratio (95% CI)

Figure 3: Tamoxifen only treated patients disease free survival curves

Kaplein Meier survival curves showing disease-free survival in patients treated only with tamoxifen whose tumours express pRaf(ser338) and pMAPK. (a) Survival curve showing a significant reduction in disease-free survival time in patients whose tumours express high levels of cytoplasmic pRaf(ser338) (p=0.0023). (b) Survival curve showing a significant reduction in disease survival time in patients whose tumours express high levels of nuclear pRaf(ser338) (p=0.0020). (c) Survival curve showing a significant reduction in disease-free survival time in patients whose tumours express high levels of cytoplasmic pMAPK (p=0.0104). High levels were defined as scores ≥ upper quartile value. p values represent log rank testing of the differences in survival. HR=Hazard Ratio (95% CI).

Figure 4: Overall survival curves for 303 tamoxifen only treated STB patients

Kaplein Meier survival curves showing overall survival (OS) in 303 STB patients treated only with tamoxifen. (a) Survival curve showing a significant reduction in OS time in STB patients whose tumours express high levels of cytoplasmic pRaf(ser338) (p=0.0154). (b) Survival curve showing a significant reduction in OS time in STB patients, whose tumours express high levels of nuclear pRaf(ser338) (p=0.0008). (c) Survival curve showing a significant reduction in OS time in STB patients whose tumours express high levels of cytoplasmic MAPK (p=0.0331). (d) Survival curve showing a significant reduction in OS time in STB patients whose tumours express high levels of nuclear MAPK (p=0.0395) (e) Survival curve showing a significant reduction in OS time in STB patients, whose tumours express high levels of nuclear pMAPK (p=0.0336).High levels were defined as scores ≥ upper quartile value, p values represent log rank testing of the differences in survival. HR=Hazard Ratio (95% CI)