Phosphorylated cSrc in the nucleus is associated with improved patient outcome in ER positive breast cancer.

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Elevated c-Src protein expression has been demonstrated in breast cancer and *in vitro* evidence suggests a role in endocrine resistance. To investigate whether c-Src is involved in endocrine resistance, we examined the expression of both total and activated c-Src in human breast cancer specimens from a cohort of oestrogen receptor (ER) positive tamoxifen treated breast cancer patients.

Tissue microarray technology was employed to analyse 262 tumour specimens taken prior to tamoxifen treatment. Immunohistochemistry using total c-Src and activated c-Src antibodies was performed. Kaplan-Meier survival curves were constructed and log rank test were performed.

High level of nuclear activated Src was significantly associated with improved overall survival ($p=0.047$) and lower recurrence rates on tamoxifen ($p=0.02$). Improved patient outcome was only seen with activated Src in the nucleus. Nuclear activated Src expression was significantly associated with node negative disease and a lower NPI ($p<0.05$). On subgroup analysis, only ER positive/ Progesterone receptor (PgR) positive tumours were associated with improved survival ($p=0.004$).

This demonstrates that c-Src activity is increased in breast cancer and that activated Src within the nucleus of ER positive tumours predicts an improved outcome. In ER/PgR positive disease activated Src Kinase does not appear to be involved in endocrine resistance. Further work is required in ER negative breast cancer as this may represent a cohort in which it is associated with poor outcome.

Key words. ER positive breast cancer, c-Src activation, nuclear, tamoxifen

Introduction

Adjuvant hormone therapy results in substantial improvements in disease free and overall survival for woman with early hormone receptor positive breast cancer (Early Breast Cancer Trialists' Collaborative Group, 1998 and 2005). Despite these benefits, a substantial proportion of patients will develop de novo or acquired resistance to hormone therapy and this presents a significant clinical problem.

The precise molecular mechanisms involved in breast cancer cell resistance to endocrine therapy is poorly understood but there is strong evidence suggesting it involves crosstalk between the ER, growth factor receptors and other downstream cellular signalling pathways (Osborne & Schiff, 2004) resulting in ligand-independent activation of the ER and tumour cell growth. Indeed, we previously demonstrated that HER 1-3 expression is significantly associated with early relapse in an ER+, tamoxifen treated cohort (Tovey *et al,* 2005). Evidence is now emerging that endocrine resistance not only results in oestrogen independent growth but is also associated with altered cell-cell and cell-matrix adhesive interactions, promoting a more invasive phenotype (Hiscox *et al*, 2006).

c-Src non receptor tyrosine kinase is over expressed and activated in a large number of human malignancies and the relationship between activation and cancer progression appears significant (Irby & Yeatman, 2000). The precise mechanism of its action has not been fully elucidated, but c-Src is known to interact with a diverse array of molecules, including growth factor receptors and cell-cell adhesion receptors, integrins and steroid receptors including the ER (Ishizawar & Parsons, 2004 and Shupnik, 2004) promoting tumour cell proliferation, survival, differentiation, migration and invasion (Yeatman, 2004 and Frame, 2004). Recent in vitro studies

have demonstrated the over expression and over activity of Src during the acquisition of tamoxifen resistance in ER positive cell lines (Planas-Silva *et al,* 2006 and Hiscox *et al*, 2005). Src inhibition was seen to significantly reduce the invasive behaviour of these cells. In addition, inhibition of c-Src has been shown to reduce the incidence of breast cancer metastases and increases survival in mice. Progress in knowledge of c-Src in tumour genesis and has resulted in Src Kinase inhibition being investigated as a therapeutic target for anti invasive therapies in breast cancer (Finn *et al,* 2007 and Rucci *et al,* 2006).

This study, using a large cohort of ER positive (+) tamoxifen treated patients, was undertaken to examine if c-Src expression is involved in *de novo* resistance to tamoxifen treatment. We examine the role of c-Src expression in human ER positive breast cancers, to determine if *in vivo* c-Src expression, activation or cellular location is associated with response to tamoxifen therapy and patient survival.

Material & Methods

Patients and tissues

The local ethics committee granted ethical approval for this study to utilise a database that details the outcome of ER positive patients diagnosed with primary operable breast cancer between 1980 and 1999 treated with adjuvant tamoxifen. Within this cohort all patients received adjuvant tamoxifen (mean time 4.8 years), 26 % of patients received adjuvant chemotherapy and 18% received adjuvant radiotherapy. Formalin fixed paraffin embedded tissue, taken at time of surgical resection, was used for tissue microarray (TMA) construction, as described previously (Tovey *et al*, 2005).

Immunohistochemistry.

Immunohistochemistry was performed on 10 normal breast sections and 10 prostate cancer samples, in addition to the 262 ER positive breast cancer specimens. Full activation of c-Src requires phosphorylation at tyrosine (Tyr) 419 in addition to the absence of phosphorylation at tyrosine 519. A phosphospecific antibody (Cell Signalling, Technology) raised in rabbit to phosphorylated Y416, SrcpY^{416} which corresponds to human Tyr 419 was used, as described in the literature (Planas-Silva *et al,* 2006). In addition an antibody recognising Total Src (36D10, Cell Signalling Technology) was used. Prior to performing immunohistochemistry, antibody specificity was confirmed by western blotting (figure 1). As expected, activated c-Src, SrcpY^{416}, was detected as a single 60kDa band and decreased in response to the Src kinase inhibitor dasatinib. Titration of the optimal antibody dilution was undertaken in breast tumour specimens prior to the procedure.

Tissue sections were dewaxed and rehydrated through graded alcohols and then subject to heat induced antigen retrieval by pressure steaming in preheated 10mM citrate buffer for 5 mins. Immunostaining was then performed; sections were first treated with hydrogen peroxide and then blocked using horse serum, followed by incubation in primary antibody (1: 50 dilution, SrcpY^{416} overnight) (1: 200 for Src36D10, 1 hour). DakoCytomation EnVision was applied for 30 mins and sections incubated with DAB (1:50 dilution). Finally, sections were counterstained, dehydrated and mounted. Positive and negative (isotype matched antibody) control slides were incorporated in each run.

Tissue staining intensity was scored blind by 2 independent observers using a weighted histoscore method (Kirkegaard *et al*, 2006) also known as the Hscore system (McCarty *et al*,1986). Histoscores were calculated from the sum of (1 x % cells staining weakly positive) + (2 x % cell staining moderately positive) + (3 x % cells staining strongly positive) with a maximum of 300. Each cellular location was separately assessed with a weighted histoscore assigned to any membrane, cytoplasm and nucleus staining. The histoscores for each core were then averaged. Where one core was missing the remaining core(s) scores were used. To determine high and low expression the median value for all scores was used. The inter-class correlation coefficient (ICCC) for each protein was calculated to confirm consistency between observers.

Western blot analysis.

MCF-7 cells treated with varying concentrations of dasatinib were lysed in RIPA buffer (50 mM Tris pH7.6, 150 mM sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate and 1:100 Calbiochem protease inhibitor cocktail set 1) and centrifuged at 12 000 rpm for 10 min, the supernatant removed and protein concentration determined using BCA/$CuSO_4$ assay. 40 µg of protein per well was resolved by 4-12% gradient Bis-Tris gel electrophoresis (Invitrogen, UK); proteins were transferred to nitrocellulose membranes (Millipore, UK), which were blocked for 1 hour in 5% BSA and probed with primary antibodies: anti-phospho $SrcY^{419}$ (1:10000) and anti-total Src (1:10000 Cell Signaling Technologies, UK) at 4^0C overnight. Membranes were then incubated with secondary antibodies (anti-rabbit 1:5000 or anti-mouse 1:5000, Cell Signalling

Technologies) and visualized with ECL kit (Amersham, UK). Where necessary, the membranes were stripped by incubating with Re-Blot Plus stripping buffer (Chemicon, UK) before re-probing with other antibodies including anti-αTubulin (1:8000 Santa Cruz, USA) to confirm equal protein loading.

Statistical analysis.

The statistical software package SPSS version 11.5 was used for all analysis. Interclass correlation coefficient was employed to confirm consistency between observers. Protein expression data were not normally distributed and are shown as median and inter quartile ranges. Pearson Chi Square test was employed to assess association between staining intensity and known clinical parameters and survival analyses were conducted using Kaplan-Meier method, curves were compared with the log-rank test. Hazard ratios (HR) were calculated using Cox Regression analysis.

Results

Clinical & pathological characteristics.

Clinical and pathological characteristics for all patients (n=262), including age, grade, nodal status, histology, size and Nottingham Prognostic Index are detailed in Table 1. The mean duration of tamoxifen therapy was 4.82 years. 55 patients (21%) had breast cancer specific deaths, 77 patients (29.4%) had breast cancer relapse, 60 of these patients while receiving tamoxifen therapy.

Localisation of Total Src and activated c-Src in normal breast

Ten normal breast sections were stained for total Src and activated c-Src. Low expression oftotal Src was observed in the cytoplasm of 60% and nucleus of 40%, however no activated c-Src expression was observed at any location

Localisation of activated c-Src expression in ER positive breast cancer tissue

A total of 262 ER positive tumour samples were analysed for activated c-Src expression. 57.3% (150/262) of tumours expressed activated Src in the cytoplasm; median histoscore 20 (interquartile range 0-61.5). 58.4% (153/262) of tumours expressed activated c-Src in the nucleus; median histoscore 10 (interquartile range 0-45). High levels (greater than the median value) of activated c-Src expression in the cytoplasm or nucleus was therefore detectable in over 50% (n=153) of all ER positive breast tumours analysed. 2.7% (7/260, 2 samples missing) of tumours expressed activated Src in the membrane, median histoscore 0. Due to the low rate of membrane expression observed it was not deemed appropriate to apply these results to further statistical tests. In order to confirm that the antibody was able to detect membrane staining 10 prostate tumours were also stained for activated c-Src. Activated c-Src was much more commonly located to the cell membrane of prostate cells compared to the ER positive breast carcinomas. Figure 2 illustrates the staining patterns observed in the ER positive breast cancer specimens compared to prostate cancer specimens.

Activated c-Src and patient outcome

High expression level (above the median value) of activated c-Src within the nucleus of tumour cells was significantly associated with improved overall survival (p=0.047) and decreased recurrence in tamoxifen treated patients (p=0.02), figure 3a-b. On cox regression analysis this was not demonstrated to be independent for survival or recurrence. The location of activated c-Src around the nucleus was also significant, tumours with uniform staining had improved outcome in comparison to patients with only perinuclear staining (figure 3c, p=0.0153). Activated c-Src within the cell cytoplasm was not significantly associated with patient outcome.

Activated c-Src and prognostic indices

Activated c-Src within the nucleus was associated with node negativity and low NPI (Pearson-Chi Square, p=0.03 and p=0.046 respectively). Activated c-Src within the cytoplasm of cells was not associated with nodal status, NPI, tumour grade or size. No significant correlation was found with Ki67 (proliferative index). In contrast when the cohort was subdivided by Progesterone receptor (PgR) status (histoscore >10), activate c-Src nuclear expression remained highly significant in the ER and PgR + subgroup (n=165, p=0.004). However in the ER+/ PgR negative subgroup significance was lost (n=93, p=0.56). PgR status was not available for 4 tumours from our cohort of 262 patients. The cohort was not stratified for HER2 status as only 4 tumours were found to be positive using the Herceptest.

Total Src expression in ER positive breast cancer

Of the 262 patients only 231 tumour samples were scored for total Src expression. 95.8% (220/231) of tumours expressed total Src in the cytoplasm, median histoscore 97 (interquartile range 40-150). 70.6% (153/230) of tumours expressed total Src in the cell membrane, median histoscore 26 (interquartile range 0-95). No total Src was seen within the cell nucleus. Total Src expression (at any location) was not significantly associated with any clinical parameters or patient outcome.

Discussion

Although cell line studies strongly support the role of c-Src in endocrine resistant breast cancer progression, translational studies investigating human breast tumour

expression, activation and correlation with clinical parameters are surprisingly limited. Using a large cohort of ER positive breast cancer patients treated with adjuvant tamoxifen we have shown that high levels of activated c-Src are present in over 50% of tumour specimens and we also demonstrate that nuclear c-src activation is significantly associated with improved overall and disease free survival. Subgroup analysis demonstrates that this benefit is only seen in ER+/PgR+ patients and not within ER+/PgR negative group.

As c-Src is a non receptor tyrosine kinase that is localized to the intracellular membranes and cytoplasm of the cell (Biscardi *et al*, 2000) it was surprisingly that in the current study we rarely observed activated c-Src in the cell membrane. However antibody specificity was confirmed by western blotting. A single 60kDa band suggesting that phosphorylated Src kinase was detected. In addition, phosphorylation of c-Src (but not total c-Src) was observed to fall following treatment with increasing concentrations of the Src kinase inhibitor dasatinib confirming that the antibody detected phosphorylated Src only (figure 1). Although these experiments confirmed that the antibody used in the study was specific for phosphorylated Src kinase, it did not answer the question about the location of phosphorylated Src observed in this cohort. We therefore stained prostate tumours to assess the localisation of activated Src in a different tumour type. In prostate cancer the majority of staining observed for phosphorylated Src was located to the membrane and nuclear expression was rarely observed. These results suggest that the lack of membrane staining and high level of nuclear staining observed in the current study was associated with our ER positive breast cancer cohort, and was not a characteristic of the antibody used. However the Y^{416} sequence is highly conserved amongst the src kinases so this does not exclude detection of other src family kinases along with c-Src using this antibody. Our

detection of nuclear c-src expression and activation is in line with recent literature as c-Src has been reported both within the nucleus and nucleolus (David-Pfeuty *et al,* 1993 & 1995) of other solid tumours. Previous immunohistochemical work demonstrated that in non malignant breast cells c-Src is distributed within the cytoplasm, whereas in malignant breast cells the majority of c-Src appears concentrated around the nucleus (Verbeek *et al,*1996).

In this present study we found that high levels of activated c-Src was present in over 50% tumour specimens analysed and nuclear activated c-Src was significantly associated with improved overall survival and decreased recurrence. Ito *et al,* 2001 also found that elevated activated cSrc was inversely correlated with biological aggressiveness in 73 breast cancer specimens and suggested that c-Src may have an important role in malignant transformation of breast cells rather than malignant progression. Madan *et al*, 2006 subsequently demonstrated that c-Src activation did not correlate with the development of invasive tumour properties but correlated with malignant transformation. In ductal carcinoma *in situ*, activated c-Src was found to correlate with high tumour grade, high proliferation and HER 2 positivity, suggesting that high cSrc activity may identify a subset of DCIS at risk of disease progression to invasive carcinoma (Wilson *et al,* 2006).

The body of evidence does, however, still support a role for c-Src in malignant progression. Compared with adjacent normal tissues, elevated Src expression and/or activity has been reported in a wide range of tumour types, including breast cancer (Verbeek *et al,* 1996) and in many of these tissues, an increase in Src activity correlates with disease stage or malignant potential (Aligayer *et al* 2002 and Weiner *et al,* 2003). Tumour cell lines possessing elevated Src activity are often highly

metastatic, displaying an increased capacity for migration and invasion in vitro (Mao *et al* 1997, Jackson *et al* 2000, Slack *et al 2001*).

Recent in vitro breast cell line work, demonstrate over expression and over activity of Src during the acquisition of tamoxifen resistance in ER+ cell line (Planas-Silva *et al*, 2006 and Hiscox *et al*, 2005). Src inhibition was seen to significantly reduce the invasive behaviour of cells. Hiscox *et al* found elevated Src kinase activity in endocrine resistance models was independent of Src gene or protein level. Tamoxifen resistance may be either *de novo* (present before tamoxifen treatment) or "acquired". In this present study all analysis was performed on tumour samples taken prior to tamoxifen treatment and whilst we do not find that active c-Src correlates with *de novo* endocrine resistance it is interesting that within our cohort the survival benefit was only in ER+/PgR+ patients and not in the ER+/PgR negative group. PgR expression is a marker of a functional ER and a number of laboratory studies have demonstrated the importance of molecular characteristics such as PgR and HER2 in predicting tumour response to endocrine therapy. We have previously reported that ER+/PgR negative tumours are more likely to relapse on tamoxifen (Tovey *et al*, 2005) and a number of other laboratory studies report a reduction in PgR expression in ER+ cells is consistent with acquired tamoxifen resistance (Scott *et al*, 2007). It is therefore possible that in tumours with a functioning ER (ER+/PgR+) "active" cSrc is in the nucleus and not able to perform its role in promoting tumour progression. Tumours acquiring tamoxifen resistance over time have an adaptive change in growth factor signalling (such as a reduction in PgR expression, increased EGFR expression), therefore Src kinase being downstream of such signalling networks may not become fully active until later during the development of tamoxifen resistance. High levels of activated cSrc expression in the cell cytoplasm have been reported in recurrent breast

carcinoma samples (Planas-Silva *et al*, 2006) although expression was not compared with the primary tumour sample. Comparison of primary breast tumour cSrc expression with expression in recurrent or metastatic tumours following endocrine resistance would be a preferable model. Within our laboratory we have examined this in prostate cancer specimens. In hormone sensitive prostate cancer active cSrc was associated with improved survival but after development of hormone therapy resistance, active cSrc was associated with reduced time to death (unpublished data). It is also likely that our patient cohort represents a good prognostic group and that the aggressive phenotype associated with Src kinase is limited to poor prognostic cancers. Indeed Finn *et al,* 2007 recently reported a highly significant relationship between breast cancer cell line sub type based on gene expression of cytokeratins and sensitivity to src kinase inhibition, suggesting that the "triple negative" breast cancers were most likely to benefit from Src inhibition. ER negative tumours correlate with poor tumour differentiation, high proliferation rate and other unfavourable characteristics, and are in general considered a more aggressive breast carcinoma. An inverse correlation between Src and ER levels has been reported, ER negative primary breast cancer and cell lines showed increased Src levels and/ or activity compared to ER + cancers (Chu *et al,* 2007).

In conclusion, we found elevated levels of activated cSrc within the cell nucleus of ER+ breast cancer was associated with improved patient outcome in a large cohort of Tamoxifen treated ER positive patients. Although we are unable to substantiate the *in vitro* studies suggesting a role for c-Src in tamoxifen resistance we feel that further clarification defining the role of cSrc in the different subtypes of breast cancer, particularly in ER negative breast cancer and recurrent tumours, is warranted as this

likely represents the group in which targeted Src Kinase inhibition may be beneficial to patient outcome.

References

Aligayer H, Boyd DD, Heiss MM, Abdalla EK, Curley SA & Gallick GE (2002) Activation of Src Kinase in primary colorectal carcinoma: an indicator of poor clinical prognosis. *Cancer* **94**: 344-351

Biscardi JS, Ishizawar RC, Silva CM & Parsons SJ (2000) Tyrosine kinase signalling in breast cancer, epidermal growth factor receptor and cSrc interactions in breast cancer. *Breast Cancer Res* **2**: 203-210

Chu I, Arnaout A, Loiseau S, Sun J, Seth J, McMahon C, Chun K, Hennessy B, Mills GB, Nawaz Z & Slingerland JM (2007) Src promotes estrogen-dependant estrogen receptor alpha proteolysis in human breast cancer. *The Journal of Clinical Investigation* **117** (8):2206-2215

David-Pfeuty T, Bagrodia S & Shalloway D (1993) Differential localization patterns of myristoylated and nonmyristoylated c-Src proteins in interphase and mitotic c-Src overexpresser cells. *Journal of Cell Science* **105**: 613-628

David-Pfeuty T & Nouvian-Dooghe Y (1995) Highly specific antibody to Rous sarcoma virus src gene product recognizes nuclear and nucleolar antigens in human cells. *Journal of Virology* **69**: 1699-1713

Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: An overview of the randomised trials. *Lancet* **351**:1451-1467

Early Breast Cancer Trialists' Collaborative Group (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15 year survival: an overview of the randomised trials. *Lancet* **365**:1687-1717

Finn RS, Dering J, Ginther C, Wilson CA, Glaspy P, Tchekmedyian N & Slamon DJ (2007) Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/ "triple negative" breast cancer cell lines growing in vitro. *Breast Cancer Research and Treatment* **105**(3): 319-326

Frame MC (2004) Newest findings on the oldest oncogene; how activated src does it. *Journal of Cell Science* **117**(7): 989-998

Hiscox S, Morgan L, Green TP, Barrow D, Gee J & Nicholson RI (2005) Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Research and Treatment* **97**: 263-274

Hiscox S, Morgan L, Green T & Nicholson RI (2006) Src as a therapeutic target in anti-hormone/anti-growth factor-resistant breast cancer. *Endocrine-Related Cancer* **13**: S53-S59

Irby RB & Yeatman T (2000) Role of Src expression and activation in human cancer. *Oncogene* **19**: 5636-5642

Ishizawar R & Parsons SJ (2004) cSrc and cooperating partners in human cancer. *Cancer Cell* **6**: 209-214

Ito Y, Kawakatsu H, Takeda T, Tani N, Kawaguchi N, Noguchi S, Sakai T & MatsuuraN (2002) Activation of cSrc is inversely correlated with biological aggressiveness of breast carcinoma. *Breast Cancer Research and Treatment* **76**: 261-267

Jackson JG, Yoneda T, Clark GM &Yee D (2000) Elevated levels of p66Shc are found in breast cancer cell lines and primary tumours with metastatic potential. *Clinical Cancer Research* **6**: 1135-1139

Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, Tam L, Munro AF, Dunne B, Bartlett JM (2006) Observer variation in immunohistochemical analysis of protein expression, time for a change? *Histopathology* **48**: 787-794

Madan R, Smolkin MB, Cocker R, Fayyad R & Oktay MH (2006) Focal adhesion proteins as markers of malignant and prognostic indicators in breast carcinoma. *Human Pathology* **37**: 9-15

Mao W, Irby R, Coppola D, Fu L, Wloch M, Turner J, Yu H, Garcia R, Jove R & Yeatman TJ (1997) Actication of cSrc by receptor tyrosine kinases in human colon cancer cells with high metastatic potential. Oncogene 15: 3083-3090

McCarty KS, Jr., Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, Konrath J, Soper JT, Budwit DA, Creasman WT (1986) Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res* **46**: 4244s-4248s

Osborne, CK & Schiff, R (2004) Crosstalk between estrogen receptor and growth factor receptor pathways as a mechanism of endocrine therapy resistance. In: Advances in Endocrine Therapy of Breast Cancer, Ingle & Dowsett. Informa Health Care p215-228

Planas-Silva MD, Bruggeman RD, Grenko RT & Stanley Smith J (2006) Role of cSrc and focal adhesion kinase in progression and metastasis of estrogen receptor-positive breast cancer. *Biochemical and Biophysical Research Communications* **341**: 73-81

Rucci N, Rechia I, Angelucci A, Alamanou M, Del Fattore A, Fortunati D, Susa M, Fabbro D, Bologna M & Teti A (2006) Inhibition of protein kinase cSrc reduces the

incidence of breast cancer metastases and increases survival in mice: implications for therapy. *The Journal of Pharmacology and Experimental Therapeutics* **318**(1): 161-172

Shupnik MA (2004) Crosstalk between steroid receptors and the c-Src receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* **23**: 7979-7989

Slack JK, Adams RB, Rovin JD, Bissonette EA, Stocker CE & Parsons JT (2001) Alterations in the focal adhesion/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells. *Oncogene* **20**: 1152-1163

Tovey S, Dunne B, Witton CJ, Forsyth A, Cooke TG & Bartlett JM (2005) Can molecular markers predict when to implement treatment with aromatase inhibitors in invasive breast cancer? *Clinical Cancer Research* **11**: 4835-4842

Verbeek BS, Vroom TM, Adriaansen-Slot SS, Ottenhoff-Kalff AE, Geertzema JGN, Hennipman A & Ruksen G (1996) cSrc protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis. *Journal of Pathology* **180**: 383-388

Weiner JR, Windham TC, Estrella VC, Parikh NU, Thall PF, Deavers MT, Bast RC, Mills GB & Gallick GE (2003) Activated SRC protein tyrosine kinases is overexpressed in late stage human ovarian cancers. *Gynecologic Oncology* **88**: 73-79

Wilson GR, Cramer A, Welman A, Knox F, Swindell R, Kawakatsu H, Clarke RB, Dive C & Bundred NJ (2006) Activated cSrc in ductal carcinoma in situ correlates with high tumour grade, high proliferation and HER2 positivity. *British Journal of Cancer*: 1-5

Yeatman TJ (2004) A renaissance for Src. *Cancer* **4**: 470-480

Table 1. Patient clinical and pathological variables

	Number	Valid%
Grade		
1	60	23.3%
2	124	48.2%
3	73	28.4%
unknown	5	
Nodal Status		
0	128	53.3%
1-3	72	30%
4+	40	16.7%
unknown	22	
Histological type	218	83.2%
ductal	20	7.6%
lobular	24	9.2%
Other (incl. unknown)		
Size (mm)	89	36.3%
T1(<20)	149	59.1%
T2 (20-50)	14	5.6%
T3 (>50)	10	
unknown		
NPI	66	35.5%
<3.5	94	50.5%
3.5-5.5	26	14%
>5.5	76	
unknown		
Age (years)	42	16%
</=50	220	84%
>50		
Chemotherapy	68/262	26%
yes	194/262	74%
no		
Progesterone Receptor (PgR) status		
PgR +	165	
PgR-	93	
PgR unknown	4	

Note: Grade= Bloom and Richardson grade. Nodal status= number of positive nodes, Histological type: ductal, invasive ductal carcinoma; lobular, invasive lobular carcinoma; other includes mucinous, mucoid etc.
Abbreviation: NPI, Nottingham Prognostic Index (grade+ nodal status+ 0.02x size in mm)

Figure Legends

Figure 1 shows western blot experiment. Phosphospecific antibody recognising activated c-Src (SrcpY^{416}) is demonstrated as a single 60kDa band (lane 1-control, C). In addition phosphorylation of c-Src was observed to fall following

treatment with increasing concentrations of the Src kinase inhibitor dasatinib (lanes2-6) and total Src were not affected by this. Tubulin was used as a control.

Figure 2 demonstrates the different localisation of activated c-Src, SrcpY^{416} in prostate and breast tumour samples. In breast tumours activated c-Src was most commonly present in the cell cytoplasm and cell nucleus (2a & 2b). In the prostate cancer (2c & 2d) the majority of staining observed for phosphorylated c-Src was located to the membrane and nuclear expression was rarely observed.

Figure 3. Kaplan Meier survival curves, 3a shows overall survival difference between ER+ patients with high (above the median value) and low expression of activated nuclear c-Src. p=0.047; 3b one minus survival curve demonstrating disease recurrence in ER+ patients with high and low expression of activated nuclear c-Src. p=0.02; 3c demonstrates overall survival differences between activated c-Src depending on pattern of nuclear staining. Uniform nuclear staining was significantly associated with improved survival compared to no nuclear staining or only perinuclear. p=0.0153.

Figure 1

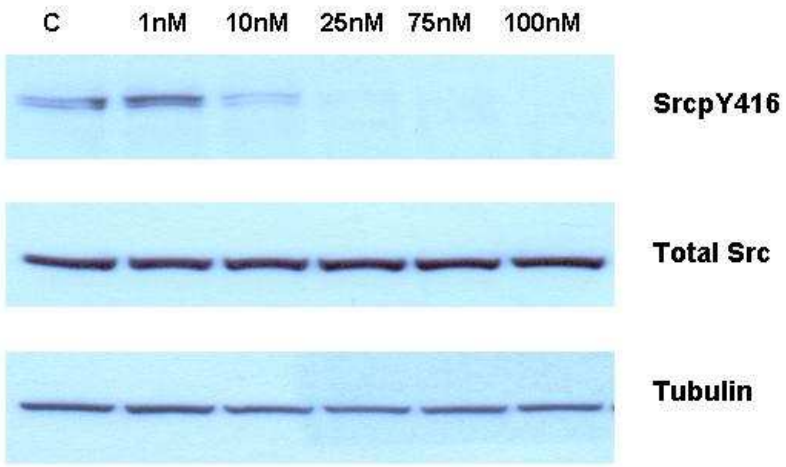
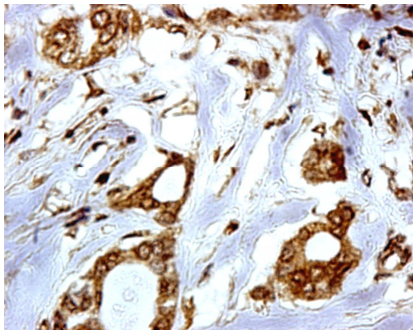
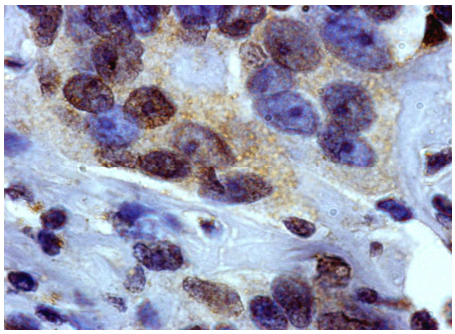

C
1nM
10nM
25nM
75nM
100nM
SrcpY416
Total Src
Tubulin

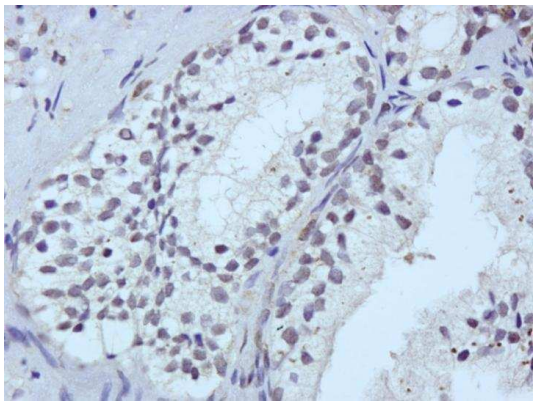
Figure 2

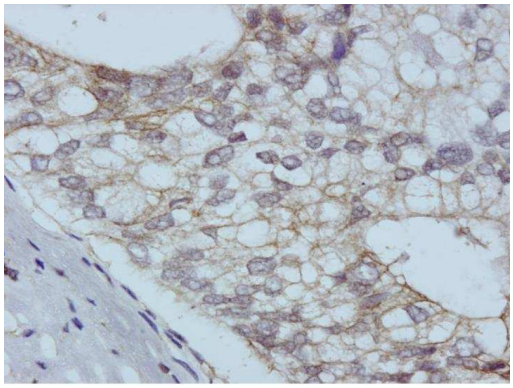
Breast

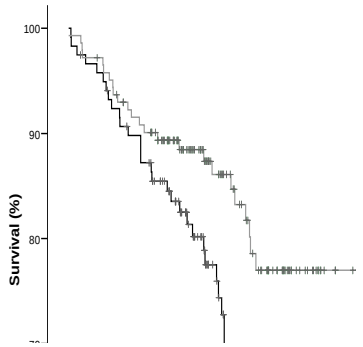




Prostate







Overall survival (Years)