

Calpain-1 expression is associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy

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The calpain family, and their endogenous inhibitor calpastatin, has been implicated in cancer progression, and recent *in vitro* data have indicated a role in trastuzumab resistance. The aims of our study were to examine expression levels of calpastatin, calpain-1 and calpain-2 in breast tumours from patients treated with trastuzumab following adjuvant chemotherapy to determine their potential as biomarkers to predict therapeutic response. The expression of calpastatin, calpain-1 and calpain-2 was determined, using immunohistochemistry (IHC), in tumours from a series of 93 patients with primary breast cancer treated with surgery and adjuvant chemotherapy with or without trastuzumab followed by trastuzumab to complete 1 year of therapy. IHC was performed using tissue microarrays constructed from cores taken from intratumour and peripheral tumour areas. Expression was correlated with clinicopathologic variables and patient outcome. Calpastatin expression was correlated with Nottingham prognostic index ($p = 0.003$) and lymph node status ($p = 0.007$). Trastuzumab resistance was defined as disease relapse during therapy. Calpain-1 expression is associated with relapse-free survival ($p = 0.001$) and remained significant in multivariate analysis accounting for confounding pathological and treatment variables (hazard ratio 4.60, 95% confidence interval 1.05–20.25; $p = 0.043$). Calpain-1 may be a useful biomarker to predict relapse-free survival in breast cancer patients treated with adjuvant trastuzumab and chemotherapy. A larger verification study is warranted.

HER2 belongs to the family of human epidermal growth factor receptors (EGFR, ErbB or HER), which has three other members: HER1 (EGFR), HER3 and HER4, and functions as receptor tyrosine kinases. Activation of the HER1, 3 and 4 occurs *via* a number of ligands promoting dimerisation. HER2 has no direct ligand and acts as a coreceptor by preferential formation of heterodimers with other family members.¹ In breast cancer, a mutation leading to overexpression, or altered receptor structure, can cause the formation of heterodimer and homodimer, which result in overactivation and uncontrolled cellular growth (reviewed in Ref. ²). Amplification of HER2 occurs in 15–30% of breast cancers and results in a more aggressive phenotype.³ Normal HER2 signalling

can act *via* phosphorylation of phosphatidylinositol kinase-3 (PI3K) or through induction of Ras and affects cell survival and cell cycle progression.⁴ Trastuzumab (Herceptin) is a humanised anti-HER2 monoclonal antibody, approved for use in HER2-positive breast cancer that significantly improves disease-free survival after adjuvant chemotherapy.^{5–7} Trastuzumab has been postulated to exert antitumour effects *via* a number of potential mechanisms including antibody-dependent cellular cytotoxicity, inhibition of signal transduction, proteolytic cleavage of HER2, angiogenesis and DNA repair.⁴ Trastuzumab improves the outcome for HER2-positive breast cancers; however, a number of patients can develop resistance to treatment. Certain tests have shown efficacy in predicting response to trastuzumab therapy in patients with metastatic cancer such as the measurement of serum levels of the extracellular domain of HER2,⁸ and there is evidence that low PTEN expression can predict resistance to trastuzumab in patients with metastatic disease treated with trastuzumab and taxane.^{9,10} However, PTEN expression does not reliably predict response in patients treated with trastuzumab containing neoadjuvant chemotherapy.¹¹

The calpains and their endogenous inhibitor calpastatin have a role in a number of disease pathologies, notably carcinogenesis. The calpain family has been implicated in

Key words: calpain, calpastatin, HER2, breast cancer, trastuzumab

DOI: 10.1002/ijc.25832

History: Received 28 Sep 2010; Accepted 19 Nov 2010; Online 7 Dec 2010

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Table 1. Clinicopathologic variables of patient sample cohort

Variables	Number (n = 93)
Age (years)	54.88 ± 9.57
Tumour size (mm)	25.60 ± 18.90
Grade classification	
I	1 (1.1%)
II	22 (23.7%)
III	67 (72.0%)
Unknown	3 (3.2%)
Nottingham prognostic index	5.05 ± 0.96
Lymph node status	
Positive	62 (66.7%)
Negative	28 (30.1%)
Unknown	3 (3.2%)
ER status	
Negative	51 (54.8%)
Positive	42 (45.2%)
PR status	
Low	38 (40.9%)
High	10 (10.8%)
Unknown	45 (48.4%)

Continuous data are shown as mean ± standard deviation. Oestrogen receptor (ER) status was positive with an *H*-score above 10. Age had a minimum value of 30.7 and a maximum value of 74.8 years. Tumour size had a minimum of 2 and a maximum of 90 mm. Nottingham prognostic index (NPI) had a minimum value of 2.28 and a maximum value of 7.28.

various cellular processes including cell differentiation, transcriptional regulation, cytokine processing, migration and apoptosis.^{12,13} The calpains are calcium-dependent cysteine proteases including calpain-1 and -2 (EC3.4.22.52 and EC 3.4.22.53). Activation of calpain-2 can occur *via* the signal-regulated kinase/mitogen-activated protein (ERK/MAPK) pathway, whereas calpain-1 can be activated by physiological calcium levels.¹⁴ Dephosphorylation by protein phosphatase 2A has been shown to reduce activity of calpain-1 and -2 *in vitro*.¹⁵ In cancer, calpain-2 has been implicated in the *in vitro* formation of invadopodia, which are associated with extracellular matrix degradation and increased invasive potential.¹⁶

Calpain has been implicated in HER2 signalling pathways in a number of studies. Calpain-2 can be activated by EGFR through the ERK/MAPK pathway by direct phosphorylation of calpain.^{17,18} HER2 overexpression results in activation of the Akt and ERK pathways. The Akt pathway results in a number of events important in tumorigenesis such as signalling through the mTOR pathway. Importantly, HER2 has been shown to induce NFκB through the AKT pathway, which is maintained by calpain-mediated cleavage of the IκB kinase complex.¹⁹ Recent evidence has indicated a further role for calpain in trastuzumab-resistant breast cancer. Kul-

karni *et al.* investigated the influence of calpain-1 in trastuzumab-treated HER2-positive breast cancer *in vitro*.²⁰ Their data suggested that calpain-1 is activated following trastuzumab treatment and cleaves HER2 to disrupt signalling. In addition, they showed that trastuzumab-sensitive cells have higher calpain-1 activity than resistant cells; however, trastuzumab-resistant cells become dependent on calpain-1 activity for survival.

Interestingly, calpain is implicated in a number of interrelated pathways. For example, integrin engagement can cause focal adhesion kinase (FAK) phosphorylation, which results in ERK activation of calpain-1 to cleave FAK, which results in enhanced cell motility.²¹ FAK, like phosphatidylinositol (3,4,5)-triphosphate (PIP3), can be dephosphorylated by PTEN indicating pathway overlap.

The aims of our study were to (i) analyse the expression pattern of calpastatin, calpain-1 and calpain-2 in HER2-positive breast cancer and (ii) to determine if the expression of these proteins can predict relapse-free survival in HER2-positive breast cancer patients treated with trastuzumab following adjuvant chemotherapy.

Material and Methods

Clinical samples

Our study is reported in accordance with REMARK criteria.²² The expression of calpastatin, calpain-1 and calpain-2 was investigated using a tissue microarray (TMA) of 93 patients with primary breast cancer treated at Nottingham University Hospitals, King's Mill Hospital or Derby Hospitals between June 2004 and December 2009 with a median follow-up time of 30 months. Clinicopathologic variables of the cohort are recorded in Table 1. Patients were all treated with trastuzumab following surgery and chemotherapy. Trastuzumab treatment was given on a 3 weekly regimen for 52 weeks. Chemotherapy consisted of six cycles of 3 weekly fluorouracil (500 mg/m²), epirubicin (100 mg/m²) and cyclophosphamide (500 mg/m²) (FEC) combination (*n* = 53) or FEC-T consisting three cycles of FEC followed by three cycles of trastuzumab with concurrent docetaxel (100 mg/m²) per 3 weeks when patients were in a high-risk prognostic group according to local guidelines (*n* = 40). Patients with oestrogen receptor expression were offered adjuvant hormonal therapy (*n* = 40) following chemotherapy, and the majority of patients received adjuvant radiotherapy (*n* = 78). Treatment resistance was defined as relapse during therapy, and relapse-free survival was defined as the date from diagnosis to relapse with a mean relapse-free survival of 32 months; median survival for the group was not reached. Formalin-fixed paraffin-embedded tissue was used to create the TMA with one core per patient taken from intratumour and peripheral tumour areas. All patients had HER2 expression determined by IHC, and fluorescence *in situ* hybridisation was performed on cases with a score of 2+ by IHC. At the end of the

follow-up period, there had been one death, and 12 patients suffered a recurrence.

TMA construction and immunohistochemistry

Breast cancer TMAs were prepared.²³ 0.6 mm tissue cores from both intratumour and peripheral tumour areas were placed into a single recipient paraffin block. Four-micron sections of the TMA were mounted on poly-L-lysine-coated slides. IHC was performed on the TMA slides, which were initially deparaffinised in histolene followed by rehydration in a series of ethanol baths (100, 90, 70, 50 and 30% in water). Antigen retrieval was performed in 0.01 mol/L sodium citrate buffer (pH 6) in a microwave, 750 W for 10 min followed by 400 W for 10 min. Endogenous peroxidase activity was blocked over 10 min in 0.01% hydrogen peroxide in methanol. Primary antibodies, mouse anti-calpastatin (1:20,000), mouse anti-calpain-1 (1:5,000) and rabbit anti-calpain-2 (1:5,000) (all Chemicon, MA), were diluted in blocking serum and applied to the tissue for 1 hr at room temperature. Staining was achieved using the Vectastain Elite ABC kit (universal), containing blocking serum, biotinylated secondary antibody and ABC reagent (Vector Laboratories, Peterborough, UK). Immunohistochemical reactions were developed with 3,3'-diaminobenzidine as the chromogenic peroxidase substrate (Dako, Glostrup, Denmark). Sections were then counterstained with Gills formula Haematoxylin (Vector Laboratories), dehydrated and fixed in histolene before mounting with DPX. Breast tumour composite sections, which comprised of six Stage 1 breast tumours of Grade 1 to 3, were included as positive and negative controls with each run, with the negative control having primary antibody substituted for PBS.

Assessment of staining was conducted, after scanning of the slides with a Nanozoomer Digital Pathology Scanner (Hamamatsu Photonics), at 20× magnification. The expression of calpastatin, calpain-1 and calpain-2 in tumour cells was manually assessed using an immunohistochemical *H*-score. Staining intensity was assessed as: none (0), weak (1), medium (2) and strong (3) over the percentage area of each staining intensity. *H*-scores were calculated by multiplying the percentage area by the intensity grade (*H*-score range: 0–300). Thirty percent of slides were examined by a second independent assessor blinded to scores and clinicopathologic criteria with good concordance between both scorers (single measure intraclass correlations greater than 0.8). An average *H*-score was generated by taking the mean of intratumour and peripheral tumour area cores and dichotomised using X-tile for analysis.²⁴ Average scores were used for analysis because of the relatively small sample size.

Statistical analysis

The distribution of data was assessed using the Kolmogorov–Smirnov test for goodness of fit. The relationship between categorised protein expression and clinicopathologic factors was analysed using Pearson's Chi-square test of association

(χ^2), where there were more than two variables *e.g.* grade, or Fisher's exact test in a 2 × 2 table. Overall disease-specific survival curves were plotted according to the Kaplan–Meier method, and significance was determined using the log-rank test. Multivariate survival analysis was performed by Cox proportional hazards analysis. Spearman rank order correlations were performed to test the associations between expression of proteins in intratumour and peripheral tumour locations, and between different proteins. All differences were deemed significant at the level of $p < 0.05$. Statistical analysis was performed using SPSS 15.0 software.

Results

Immunohistochemistry

Tissue expression of calpastatin, calpain-1 and calpain-2 was determined in a series of trastuzumab-treated patients. Calpastatin, calpain-1 and calpain-2 demonstrated cytoplasmic staining with some granularity and heterogeneity between adjacent tumour cells, varying from weak to intense staining. Calpastatin had a median *H*-score of 130 and a standard deviation of ± 55 ; calpain-1 had a median value of 155 ± 44 , and calpain-2 core staining had a median value of 165 ± 45 . Representative staining patterns from intracore and peripheral core are displayed in Figure 1.

A marginal biological correlation was observed between the tumour expression of calpastatin and calpain-2 ($r = 0.231$, $p = 0.027$), but not between calpastatin and calpain-1 or calpain-1 and calpain-2. Further relationships were explored by examining the relationship between localisation of expression in tumour cores. Significant correlation was observed between intratumour and peripheral tumour cores for calpastatin ($r = 0.675$, $p < 0.001$) and calpain-1 ($r = 0.391$, $p < 0.001$) but not calpain-2 ($r = 0.028$, $p = 0.808$).

Relationship with clinicopathologic variables

Calpastatin, calpain-1 and calpain-2 *H*-scores were dichotomised using X-Tile software into low and high immunoreactivity and correlated with clinicopathologic criteria. X-tile-generated cut points were as follows: calpastatin had an *H*-score cut point of 150 with 30 cases having a high score; calpain-2 had an *H*-score cut point of 165 with 50 cases having a high score; calpain-1 had an *H*-score of 215 with 10 cases having a high score. A small number of TMA cores were not assessed because of missing cores or insufficient representative tumour. Correlations with clinicopathologic criteria are shown in Table 2 for the three markers. Calpastatin expression correlated with Nottingham prognostic index (NPI) ($p = 0.003$) and lymph node status ($p = 0.007$). No significant correlations were observed with calpain-1 and calpain-2 expression.

The HER2 ratio (HER2/CEN-17) was determined for 78.5% (73/93) of cases using chromogenic *in situ* hybridisation, irrespective of IHC scoring value, and was tested for correlation with calpastatin, calpain-1 and calpain-2 expression using the Spearman rank correlation coefficient. Gene amplification ratios had a mean value of 3.6 ± 0.8 . Calpastatin

expression correlated with the HER2 gene amplification ratio ($r = 0.258$, $p = 0.027$). No correlation was observed between calpain-1 or calpain-2 and HER2 gene amplification. HER2 IHC H -scores were also tested for correlation with calpastatin, calpain-1 and calpain-2 expression using the Spearman rank

correlation coefficient. Calpastatin expression was correlated with the level of HER2 expression ($r = 0.350$, $p = 0.001$), but no correlation was observed with calpain-1 and calpain-2.

Relationship with clinical outcome

Resistance to trastuzumab therapy was determined as patient relapse during therapy. The expression of calpastatin, calpain-1 and calpain-2 was correlated with relapse-free survival using Kaplan–Meier survival curves. The expression of calpain-1 was significantly associated with relapse-free survival ($p = 0.001$), with high expression indicating resistance to treatment (Fig. 2). No correlation was observed for calpastatin or calpain-2 expression and relapse-free survival (Fig. 2). Multivariate Cox regression analysis was performed including potential confounding factors such as tumour size, grade, lymph node status and ER status with individual Kaplan–Meier log-rank statistics of $p = 0.073$, $p = 0.115$, $p = 0.760$ and $p = 0.062$, respectively. In addition, a cohort of other treatment factors was included such as type of surgery (wide local excision or mastectomy), adjuvant hormonal and radiotherapy, anthracycline or anthracycline and taxane chemotherapy and if chemotherapy was given in a neoadjuvant setting with individual Kaplan–Meier log-rank statistics of $p = 0.060$, $p = 0.054$, $p = 0.086$, $p = 0.600$ and $p = 0.554$, respectively. Finally, a cohort including all possible confounding factors, both pathological and treatment bias, was included in multivariate Cox regression analysis. The expression of calpain-1 remained an independent marker for relapse-free survival with inclusion of pathologic confounders in the multivariate model (hazard ratio (HR) 4.265, 95% confidence interval (CI) 1.22–14.95; $p = 0.023$) but also of treatment setting (HR 4.434, 95% CI 1.30–15.13; $p = 0.017$); finally, expression of calpain-1 was an independent marker of relapse-free survival including both pathologic and treatment confounding variables (HR 4.603, 95% CI 1.05–20.25; $p = 0.043$) (Table 3). No other markers were assessed by multivariate Cox regression as they did not correlate with relapse-free survival as single markers using Kaplan–Meier survival curves. No correlations with overall survival were possible as only one event occurred.

Simple tests of association using the Fisher's exact test were performed to assess the relationship between protein

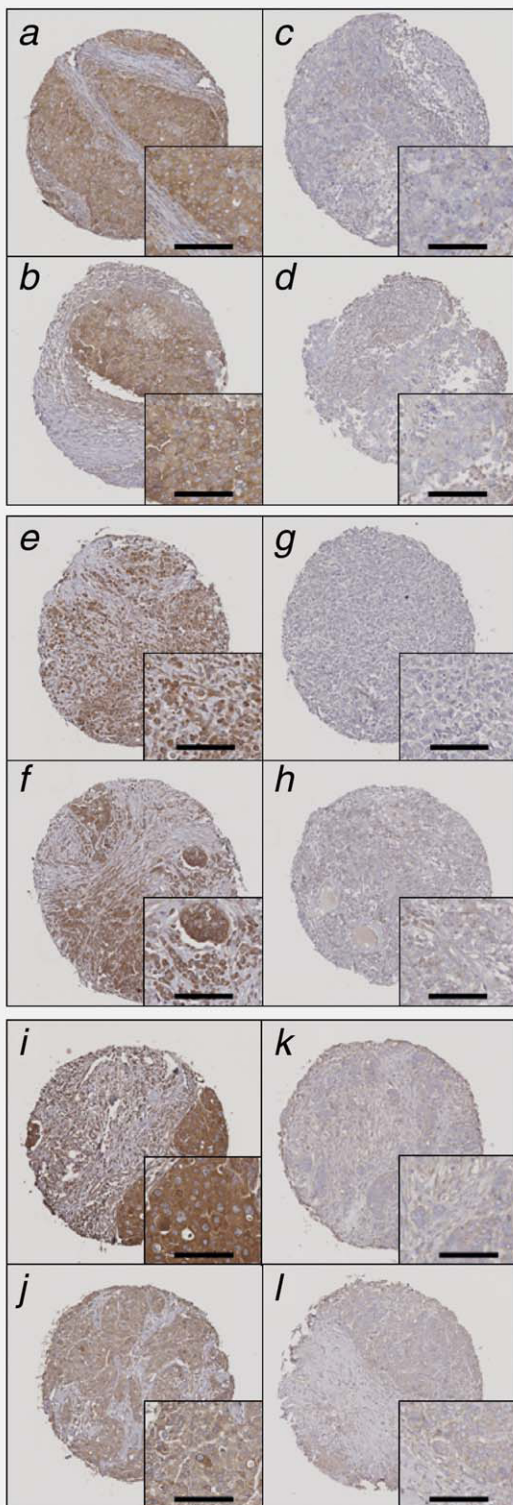


Figure 1. Photomicrographs of representative staining at $\times 10$ magnification with $\times 20$ magnification inset panel with scale bar representing $100 \mu\text{m}$. Intratumour (a) and peripheral (b) tumour high calpastatin staining from the same patient; intratumour (c) and peripheral (d) tumour low calpastatin staining from the same patient; intratumour (e) and peripheral (f) tumour high calpain-1 staining from the same patient; intratumour (g) and peripheral (h) tumour low calpain-1 staining from the same patient; intratumour (i) and peripheral (j) tumour high and moderate calpain-2 staining from the same patient; intratumour (k) and peripheral (l) tumour low calpain-2 staining from the same patient. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 2. Correlation between calpastatin, calpain-1 and calpain-2 protein expression and clinicopathologic variables

Variables	Number <i>n</i> = 93	Statistical <i>p</i> value		
		Calpastatin <i>n</i> = 93	Calpain-1 <i>n</i> = 93	Calpain-2 <i>n</i> = 92
Age (years)				
≤40	6 (6.5%)	0.383	1.000	0.684
>40	87 (93.5%)			
Tumour size (cm)				
≤2	53 (57.0%)	0.178	0.512	0.524
>2	38 (40.9%)			
Unknown	2 (2.2%)			
Grade classification				
I	1 (1.1%)	0.331*	0.484*	0.489*
II	22 (23.7%)			
III	67 (72.0%)			
Unknown	3 (3.2%)			
Nottingham prognostic index				
Good	3 (3.2%)	0.003*	0.711*	0.245*
Moderate	49 (52.7%)			
Poor	25 (26.8%)			
Unknown	16 (17.2%)			
Lymph node status				
Positive	62 (66.7%)	0.007	1.000	0.359
Negative	28 (30.1%)			
Unknown	3 (3.2%)			
ER status				
Negative	51 (54.8%)	0.126	0.503	0.531
Positive	42 (45.2%)			
PR status				
Negative	38 (40.9%)	0.727	1.000	0.724
Positive	10 (10.8%)			
Unknown	45 (48.4%)			
Relapse status				
Relapse free	81 (87.1%)	0.192	0.003	0.766
Relapse	12 (12.9%)			

Fisher's exact (2×2) test was used to determine significance, except where marked by * whereby the Pearson's Chi-square test of association was used.

Bold font indicates $p < 0.05$

expression and relapse. Expression of calpain-1 was correlated with relapse status ($p = 0.003$) with 6.2% (5/81) of patients with low and 41.7% (5/12) of patients with high expression having relapsed disease. None of the other markers was correlated with relapse status (Table 2).

Interestingly, calpain-1 expression in the peripheral tumour core appears to be more informative for relapse-free survival than central tumour expression. By analysing each group using Kaplan–Meier survival curves, calpain-1 in the peritumoural area correlates with relapse-free survival ($p =$

0.014), but not intratumoural staining ($p = 0.247$). It is unclear why a difference between the location of expression would play a role in treatment response.

Discussion

Our study investigated expression levels of calpastatin, calpain-1 and calpain-2 in 93 HER2-positive primary breast cancers treated with trastuzumab following adjuvant chemotherapy. The results show that expression of calpain-1 is able to predict relapse-free survival in patients treated with

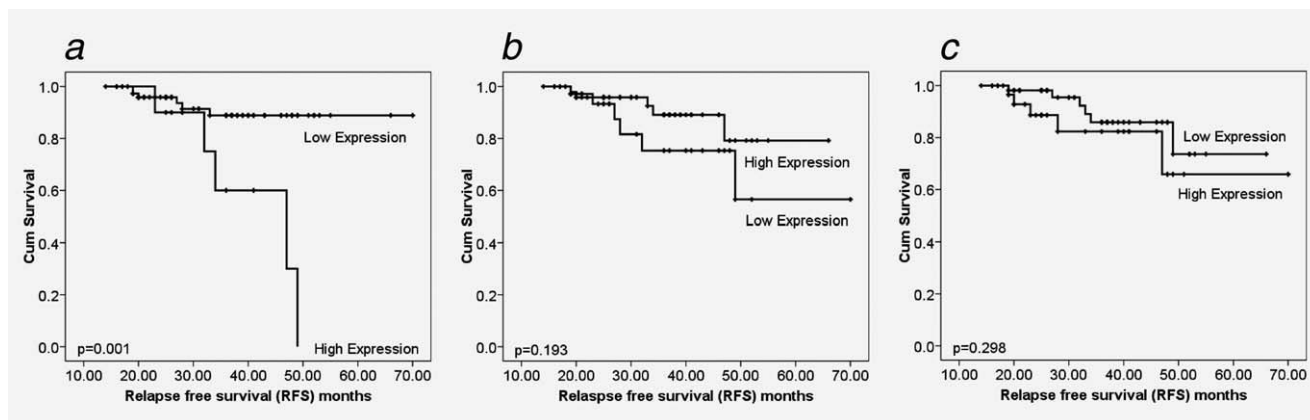


Figure 2. Analysis of relapse-free survival by Kaplan–Meier analysis of expression of calpain-1, calpain-2 and calpastatin determined by IHC. (a) Calpain-1 expression (83 patients low expression, 10 patients high expression); (b) calpain-2 expression (42 patients high expression, 50 patients low expression); (c) calpastatin expression (63 patients high expression, 30 patients low expression). Calpain-1 expression is significantly associated with relapse-free survival ($p = 0.001$), but calpain-2 and calpastatin are not.

Table 3. Multivariate Cox regression analysis showing calpain-1 expression, various pathological and clinical treatment variables and their effects upon relapse-free survival

Multivariate Cox regression analysis	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Calpain-1 expression	1.527	0.756	4.081	1	0.043	4.603	1.046	20.245
Type of surgery	-0.613	0.767	0.639	1	0.424	0.542	0.120	2.435
Adjuvant radiotherapy	-7.514	11.358	0.438	1	0.508	0.001	0.000	2538707
Adjuvant hormonal therapy	1.392	170.427	0.000	1	0.993	4.025	0.000	4.7E + 145
Neoadjuvant/adjuvant chemotherapy	-1.356	1.227	1.221	1	0.269	0.258	0.023	2.856
Chemotherapy regimen	0.829	0.812	1.041	1	0.308	2.291	0.466	11.256
Lymph node status	5.796	11.334	0.262	1	0.609	329.057	0.000	1E + 012
Oestrogen receptor status	-2.933	170.424	0.000	1	0.986	0.053	0.000	6.2E + 143
Tumour grade	10.042	18.386	0.298	1	0.585	22965.297	0.000	1E + 020
Tumour size	3.046	1.182	6.639	1	0.010	21.021	2.073	213.201

The expression of calpain-1 and tumour size are independent predictors of trastuzumab relapse-free survival following adjuvant chemotherapy.

trastuzumab independent of pathological and clinical treatment variables ($p = 0.043$).

The findings presented in our study are interesting in the light of *in vitro* studies highlighting the role of calpain in HER2 signalling.^{19,20,25} The recent study by Kulkarni *et al.* implicated calpain-1 in resistance to trastuzumab treatment.²⁰ Their results suggested that trastuzumab resistance is critically dependent on residual calpain activity through an impaired activation of calpain. It is difficult to draw comparisons between our study and previous *in vitro* work as our work examines calpain and calpastatin protein expression levels before treatment. The level of calpain activity in these specimens is unknown, although calpastatin expression may function to estimate this to some extent. In addition, the activity of calpain can be modulated by a number of factors such as physiological concentrations of calcium-activating

calpain-1 and ERK/MAPK pathway activation of calpain-2.¹⁴ The data presented here show that high protein expression levels of calpain-1 are indicative of treatment outcome, with high expression levels correlating with a worse prognosis. Calpain activity has been implicated in the response to a number of therapeutic agents, such as cisplatin-induced apoptosis in lung adenocarcinoma cells through Bid activation,²⁶ and has also been shown to degrade the DNA repair enzyme OGG1 in response to treatment.²⁷ In addition, calpain-2 has been implicated in androgen receptor proteolysis to the constitutively active low-molecular-weight form in prostate cancer resulting through activation by ERK1 and 2.²⁸

Calpain-1 expression was tested in multivariate analysis against other treatment variables; however, it is possible that calpain-1 expression may be influenced by these other treatment factors as well as trastuzumab alone. Calpain has been

implicated in various tumourigenic processes such as altered adhesion and cellular migration. Previous work in our laboratory has established a link between calpastatin expression and lymphovascular invasion (LVI). This correlation was not made in our study as the data for IHC determination of LVI, as opposed to H&E, were not available.²⁹ In our study, calpastatin expression was significantly associated with NPI and lymph node status, with low levels of expression associated with more aggressive disease. Although calpastatin expression was associated with NPI, it was not associated with relapse-free survival; this was not unexpected as in our study NPI did not correlate with relapse-free survival ($p = 0.132$).

The expression of calpastatin correlates with calpain-2 expression, but not with calpain-1 expression. A correlation was not observed between calpain-1 and calpain-2 expression. It is unclear why a correlation is observed between calpain-2 and calpastatin, but not calpain-1; however, both of these enzymes have different activation requirements. In addition to the proteins investigated in our study, it would be interesting to investigate the activity of calpain. This might be possible using antibodies against calpain-specific degradation products; however, such reagents require further validation in human malignancies such as cancer.^{30,31} It would also be of interest to investigate other proteins that may be indicative of poor trastuzumab treatment response such as PTEN.^{9,11} A difference in expression was noted between calpain-1 intratumour and peripheral tumour cores used to determine the average *H*-score reported, with peripheral tumour staining more closely linked with relapse-free survival ($p = 0.014$ and $p = 0.247$ for peripheral tumour and intratumour, respectively). It is unclear what effect the tumour location of calpain-1 expression would play on response to treatment; however, the scores

between cores taken from peripheral tumour and intratumoural areas had a statistically significant correlation ($p < 0.001$).

The definition of trastuzumab response in patients treated with combination therapy is challenging; however, the results of our preliminary study on a cohort of 93 patients present an exciting indication that calpain-1 expression levels are significantly associated with relapse-free survival in patients treated with trastuzumab following adjuvant chemotherapy. The median follow-up time in our study was 30 months; therefore, more patients in our cohort will be expected to relapse.³² Follow-up studies with larger patient cohorts with longer clinical follow-up are required, incorporating an investigation of calpain-1 expression and the response to other treatment modalities such as adjuvant chemotherapy alone. In particular, it must be noted that calpain appears to impart a more aggressive phenotype in various cancer models, including increased cellular motility.²¹ Although a small sample cohort, we have shown that expression of calpain-1 is significantly associated with relapse-free survival of patients treated with trastuzumab following adjuvant chemotherapy. Interestingly, the opportunity exists that antibodies that estimate the activity of calpain by the detection of specific degradation products, in combination with antibodies detecting calpain expression, may provide a useful tool in determining those patients who will not respond well to trastuzumab treatment following adjuvant chemotherapy. Calpain-1 may be a clinically relevant biomarker to determine HER2-positive patients who will relapse following trastuzumab therapy.

Acknowledgement

The authors gratefully acknowledge Cancer Research UK for funding S.J.S.

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