

12. Jung KW, Park S, Kong HJ et al. Cancer statistics in Korea: incidence, mortality and survival in 2006–2007. *J Korean Med Sci* 2010; 25: 1113.
13. Yu B, Douglas N, Ferin MJ et al. Changes in markers of ovarian reserve and endocrine function in young women with breast cancer undergoing adjuvant chemotherapy. *Cancer* 2010; 116: 2099–2105.
14. Kil WJ, Ahn SD, Shin SS et al. Treatment-induced menstrual changes in very young (< 35 years old) breast cancer patients. *Breast Cancer Res Treat* 2006; 96: 245–250.
15. Swain SM, Jeong JH, Geyer CE, Jr et al. Longer therapy, iatrogenic amenorrhea, and survival in early breast cancer. *N Engl J Med* 2010; 362: 2053–2065.
16. Swain SM, Jeong JH, Wolmark N. Amenorrhea from breast cancer therapy—not a matter of dose. *N Engl J Med* 2010; 363: 2268–2270.
17. Karlsson P, Sun Z, Braun D et al. Long-term results of International Breast Cancer Study Group Trial VIII: adjuvant chemotherapy plus goserelin compared with either therapy alone for premenopausal patients with node-negative breast cancer. *Ann Oncol* 2011; 22: 2216–2226.
18. Jonat W, Kaufmann M, Sauerbrei W et al. Goserelin versus cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy in premenopausal patients with node-positive breast cancer: the Zoladex Early Breast Cancer Research Association Study. *J Clin Oncol* 2002; 20: 4628.
19. Colleoni M, Cole BF, Viale G et al. Classical cyclophosphamide, methotrexate, and fluorouracil chemotherapy is more effective in triple-negative, node-negative breast cancer: results from two randomized trials of adjuvant chemoendocrine therapy for node-negative breast cancer. *J Clin Oncol* 2010; 28: 2966.

*Annals of Oncology* 23: 2289–2296, 2012  
doi:10.1093/annonc/mds176  
Published online 27 June 2012

## Calpain system protein expression in basal-like and triple-negative invasive breast cancer

S. J. Storr<sup>1</sup>, K. W. Lee<sup>1</sup>, C. M. Woolston<sup>1</sup>, S. Safuan<sup>1</sup>, A. R. Green<sup>2</sup>, R. D. Macmillan<sup>3</sup>, A. Benhasouna<sup>2</sup>, T. Parr<sup>4</sup>, I. O. Ellis<sup>2</sup> & S. G. Martin<sup>1\*</sup>

<sup>1</sup>Academic Oncology; <sup>2</sup>Histopathology, School of Molecular Medical Sciences, University of Nottingham; <sup>3</sup>The Breast Institute, Nottingham University Hospitals NHS Trust, Nottingham; <sup>4</sup>Division of Nutritional Sciences, School of Biosciences, University of Nottingham, Loughborough, Leicestershire, UK

Received 26 January 2012; revised 28 March 2012 & 25 April 2012; accepted 30 April 2012

**Background:** Basal-like and triple-negative breast tumours encompass an important clinical subgroup and biomarkers that can prognostically stratify these patients are required.

**Materials and methods:** We investigated two breast cancer tissue microarrays for the expression of calpain-1, calpain-2 and calpastatin using immunohistochemistry. The first microarray was comprised of invasive tumours from 1371 unselected patients, and the verification microarray was comprised of invasive tumours from 387 oestrogen receptor (ER)-negative patients.

**Results:** The calpain system contains a number of proteases and an endogenous inhibitor, calpastatin. Calpain activity is implicated in important cellular processes including cytoskeletal remodelling, apoptosis and survival. Our results show that the expression of calpastatin and calpain-1 are significantly associated with various clinicopathological criteria including tumour grade and ER expression. High expression of calpain-2 in basal-like or triple-negative disease was associated with adverse breast cancer-specific survival ( $P = 0.003$  and  $< 0.001$ , respectively) and was verified in an independent cohort of patients. Interestingly, those patients with basal-like or triple-negative disease with a low level of calpain-2 expression had similar breast cancer-specific survival to non-basal- or receptor- (oestrogen, progesterone or human epidermal growth factor receptor 2 (HER2)) positive disease.

**Conclusions:** Expression of the large catalytic subunit of m-calpain (calpain-2) is significantly associated with clinical outcome of patients with triple-negative and basal-like disease.

**Key words:** basal, breast cancer, calpain, calpastatin, triple negative

### introduction

Breast cancer is a heterogeneous disease that displays a range of phenotypes with different clinical characteristics

including altered clinical outcome, varying prognostic characteristics and differential response to treatment. The triple-negative and basal-like subgroups of breast cancer are of considerable clinical interest as they exhibit an aggressive phenotype and a higher metastatic potential. Triple-negative breast cancer lacks expression of both oestrogen receptor (ER) and progesterone receptor (PgR) and do not overexpress human epidermal growth factor receptor 2

\*Correspondence to: Dr S. G. Martin, Academic Oncology, School of Molecular Medical Sciences, University of Nottingham, Nottingham University Hospitals NHS Trust, City Hospital Campus, Nottingham NG5 1PB, UK. Tel: +44-0115-823-1846; Fax: +44-0115-823-1849; E-mail: stewart.martin@nottingham.ac.uk

(HER2); this subgroup accounts for 10–24% of invasive breast cancers [1]. Although basal differentiation of breast cancer was shown in 1971 [2], the molecular classification of distinct classes of breast cancer, including basal-like, was shown by microarray analysis in 2000 by Perou and colleagues; the results of which have been verified in subsequent studies [3–5]. There is a substantial overlap between the two groups; however, not all basal-like breast cancers are triple-negative [6]. There are currently no specific therapies for either triple-negative or basal-like breast cancer. A number of biomarkers have emerged, which show an association with clinical outcome in basal-like or triple-negative disease, including solute carrier organic anion transporter family member 1B1 (OATP2) and fatty acid-binding protein 7 (FABP7) [7], and forkhead box protein C1 (FOXC1) [8] in basal-like cancers. In triple-negative disease, a number of biomarkers are associated with clinical outcome including p53 [9] and DNA repair proteins—ERCC4 (XPF), MAP kinase-activated protein-2 (pMK2), DNA mismatch repair protein MLH1 (MLH1), fanconi anaemia complementation group D2 (FANCD2) [10] and high-molecular weight cytokeratin (34betaE12) [11]. There is a need to identify additional prognostic biomarkers to aid stratification of breast cancer patients diagnosed with triple-negative and basal-like disease, and that could potentially identify novel treatment regimens.

The calpains are a family of neutral cysteine proteases that require calcium for their catalytic activity. The ubiquitously expressed micro ( $\mu$ ) and milli (m) calpains are named for the concentration of calcium required for their activation, and are part of a family of at least 15 members [12]. The cellular activity of calpain is, in part, regulated by their endogenous inhibitor calpastatin. The calpains function in the controlled proteolysis of a large number of specific substrates involved in various cellular processes such as migration, cell signalling and apoptosis. The activity and expression of calpains are altered in a number of tumour types [13]; and in breast cancer, high calpain-1 expression has been associated with relapse-free survival of HER2-positive patients treated with trastuzumab following adjuvant chemotherapy [14]. In addition, low expression of calpastatin has been associated with lymphatic vessel invasion in breast cancer [15]. Calpain expression in basal-like and triple-negative tumours has not been previously described; however, a relationship exists between calpain-2 and cyclin-E, which has been associated with basal-like phenotype [16, 17]. Furthermore, recent analysis has established a role for an extracellular-signal-related kinase (ERK) 1/2 subunit preferentially activated in basal breast cancer, in which calpain-2 is implicated [18]. Due to the *in vitro* importance attributed to calpain activity and limited but positive clinical studies previously performed in breast cancer, the aim of the current study was to investigate the expression of calpain and calpastatin in a large cohort of breast cancers with long-term follow-up. This allowed assessment of protein expression in subgroups such as triple-negative and basal-like breast cancer.

## materials and methods

### study patients

This retrospective study was conducted using two independent cohorts of patients; an initial biomarker discovery cohort consisting of 1371 patients and a verification cohort of 387 ER-negative patients. The discovery cohort comprises a well-characterised consecutive series of early-stage invasive breast cancer patients treated at Nottingham University Hospitals, between 1987 and 1998 with long-term follow-up. The median age of the cohort was 55 years (ranging from 18 to 72) and 62% (850 of 1371) of patients had stage I disease. Information on clinical history and outcome is prospectively maintained and patients were assessed in a standardised manner for clinical history and tumour characteristics (Table 1). Data on a wide range of biomarkers are available; ER, PgR, HER2 status and p53 expression were available for this cohort and have been described previously [19]. ER and PgR were positive if staining was above 1%, and HER2 was positive if staining by immunohistochemistry (IHC) was 3+, with 2+ confirmed by fluorescence *in situ* hybridisation. A pragmatic definition of basal phenotype, whereby expression of cytokeratin (CK)-5/6 and/or CK-14 above 10% was defined as basal phenotype, irrespective of ER, PgR or HER2 receptor status, was used for this study [20]. Breast cancer-specific survival was defined as the time interval (in months) between the start of primary surgery and death resultant from breast cancer. Similarly, relapse-free survival was defined as the time interval (in months) between the start of primary treatment and date of disease relapse.

Patients were managed under a uniform protocol, where all underwent mastectomy or wide local excision followed by radiotherapy. Patients received systemic adjuvant treatment on the basis of Nottingham prognostic index (NPI), ER and menopausal status. Patients with an NPI score <3.4 did not receive adjuvant treatment and patients with an NPI score of 3.4 were candidates for CMF chemotherapy (cyclophosphamide, methotrexate and 5-fluorouracil) if they were ER negative or premenopausal; and hormonal therapy if they were ER positive.

The verification cohort used in the current study comprised of patients with ER-negative tumours, who were independent from those investigated in the discovery cohort. Patients in this cohort were treated at Nottingham University Hospitals between 1998 and 2006. The median age of the cohort was 53 years (ranging from 24 to 71) and 63% (245 of 387) of patients had stage I disease. As before, clinical history and outcome are prospectively maintained and patients were assessed in a standardised manner for clinical history and tumour characteristics (Table 1).

This study is reported in accordance with REMARK criteria [21] and was approved by Nottingham Research Ethics Committee 2 under the title 'development of a molecular genetic classification of breast cancer'.

### Tissue Microarray and IHC

Expression of calpastatin, calpain-1 and calpain-2 was investigated using two tissue microarrays (TMAs) which were prepared using 0.6-mm cores as described previously [22]. Freshly cut 4- $\mu$ m sections of the TMAs were stained using a streptavidin–biotin method using the primary antibodies; mouse anti-calpastatin (1 : 20 000), mouse anti-calpain-1 (1 : 5000) and rabbit anti-calpain-2 (1 : 5000) (all Chemicon, MA, USA, clones PI-11, P-6 and rabbit polyclonal AB1625, respectively with specificity confirmed by western blotting) as previously described [14]. Assessment of staining was conducted at  $\times 20$  magnification using an immunohistochemical *H*-score after scanning of the slides with a Nanozoomer Digital Pathology Scanner (Hamamatsu Photonics). Expression of Calpastatin, calpain-1 and calpain-2 was assessed semi-quantitatively 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).

**Table 1.** Clinicopathological variables of the primary cohort of 1371 patients and the verification cohort of 387 patients assessed using immunohistochemistry for markers, calpain-1, calpain-2 and calpastatin

Variable	Primary cohort N (%)	Validation cohort N (%)
Age (years)		
<40	95 (7)	56 (14)
≥40	1275 (93)	331 (86)
ND	1	0
Size (cm)		
<2	686 (50)	184 (50)
≥2	683 (50)	187 (50)
ND	2	16
Stage		
I	850 (62)	245 (64)
II	394 (29)	94 (24)
III	125 (9)	45 (12)
ND	2	3
Grade		
I	242 (18)	1 (0)
II	456 (33)	40 (10)
III	673 (49)	346 (89)
ND	0	0
Node status		
Negative	727 (61)	243 (64)
Positive	458 (39)	138 (36)
ND	186	6
NPI		
Good (<3.4)	393 (29)	23 (6)
Intermediate (3.4–5.4)	743 (55)	271 (70)
Poor (>5.4)	225 (17)	91 (24)
ND	10	2
ER		
Negative	347 (26)	387 (100)
Positive	983 (74)	0 (0)
ND	41	0
PgR		
Negative	548 (42)	313 (98)
Positive	744 (58)	7 (2)
ND	79	67
HER2		
Negative	1159 (87)	276 (83)
Positive	175 (13)	58 (17)
ND	37	53
Classification		
Non-triple negative	1088 (79)	58 (19)
Triple negative	236 (18)	253 (81)
ND	47	76
Basal phenotype		
Non-basal	1009 (79)	165 (53)
Basal	270 (21)	149 (47)
ND	92	73
LVI		
Negative	748 (67)	ND
Positive	361 (33)	
ND	262	

Continued

**Table 1.** Continued

Variable	Primary cohort N (%)	Validation cohort N (%)
IT-LVI		
Negative	969 (87)	ND
Positive	139 (13)	
ND	263	
PT-LVI		
Negative	812 (73)	ND
Positive	297 (27)	
ND	262	

ND, not determined; NPI, Nottingham prognostic index; ER, oestrogen receptor; PgR, progesterone receptor; LVI, lymphatic vessel invasion; IT-LVI, intra-tumoural LVI; PT-LVI, peri-tumoural LVI.

Greater than 30% of cores were double assessed for each protein in each TMA. Single measure intraclass correlation coefficients between scores for the discovery TMA were 0.891, 0.886 and 0.860 for calpastatin, calpain-1 and calpain-2, respectively showing excellent concordance between scorers.

### statistical analyses

The relationship between categorised protein expression and clinicopathological variables was assessed using the Pearson  $\chi^2$  test of association. Survival curves were plotted according to the Kaplan–Meier method and significance determined using the log-rank test. Multivariate survival analysis was performed by the Cox proportional hazards regression model. All differences were deemed statistically significant at the level of  $P < 0.05$ . Statistical analysis was performed using SPSS 17.0 software. Stratification cut points were determined using X-Tile software and were determined before statistical analyses [23]. X-tile is a programme designed for assessing the relationships between clinical outcome and biomarker expression capable of cut-point selection.

## results

### staining location and frequency—discovery cohort

As previously observed calpastatin, calpain-1 and calpain-2 demonstrated cytoplasmic staining with some granularity and heterogeneity between adjacent tumour cells, varying from weak to intense staining. Nuclear staining was observed extremely infrequently for calpastatin (10 cases), calpain-1 (3 cases) and calpain-2 (7 cases), with no overlap on stained cases between proteins. Calpastatin had a median  $H$ -score of 100 and ranged between 0 and 280; calpain-1 had a median value of 100 and ranged between 0 and 300 and calpain-2 staining had a median value 70 and ranged between 0 and 240. Photomicrographs showing representative staining patterns are shown in Figure 1. Of the tumours analysed 57% (776 of 1371) were invasive ductal carcinomas of no special type, 16% (226 of 1371) were tubular mixed, 7% (98 of 1371) were classic lobular, all other subcategories did not have an individual frequency above 5% of all observations.

Calpastatin, calpain-1 and calpain-2  $H$ -scores were dichotomised using X-Tile software into low and high immunoreactivity and correlated with clinicopathological

criteria. X-tile generated cut points were as follows: calpastatin had a H-score cut point of 35 with 13% (177 of 1266) of cases having a low score; calpain-1 had a H-score cut point of 140 with 75% (1024 of 1315) of cases having a low score; calpain-2 had a H-score cut point of 60 with 25% (342 of 1286) of cases having a low score. A small number of TMA cores were not assessed due to missing cores or insufficient representative tumour.

### relationship with clinicopathological variables—discovery cohort

The expression of calpain-1, calpain-2 and calpastatin was assessed for association with a number of clinicopathological variables (Table 2). High calpastatin expression was significantly associated with older patients ( $\chi^2 = 9.7$ ; 1 *df*;  $P = 0.002$ ), low grade ( $\chi^2 = 12.6$ ; 2 *df*;  $P = 0.002$ ), low NPI score ( $\chi^2 = 13.3$ ; 2 *df*;  $P = 0.001$ ), ER- and PgR-positive tumours ( $\chi^2 = 11.9$ ; 1 *df*;  $P = 0.001$  and  $\chi^2 = 13.1$ ; 1 *df*;  $P < 0.001$ , respectively), non-basal phenotype ( $\chi^2 = 8.7$ ; 1 *df*;  $P = 0.003$ ), tumours <2 cm ( $\chi^2 = 5.2$ ; 1 *df*;  $P = 0.022$ ) and the absence of intra-tumoural lymphovascular invasion ( $\chi^2 = 6.1$ ; 1 *df*;  $P = 0.014$ ).

High calpain-1 expression was significantly associated with ER- and PgR-positive tumours ( $\chi^2 = 10.2$ ; 1 *df*;  $P = 0.001$  and  $\chi^2 = 7.9$ ; 1 *df*;  $P = 0.005$ , respectively) and low grade tumours ( $\chi^2 = 8.6$ ; 2 *df*;  $P = 0.014$ ). Calpain-2 expression was significantly associated with the presence of peri-tumoural lymphovascular invasion ( $\chi^2 = 5.2$ ; 1 *df*;  $P = 0.022$ ).

### relationship with clinical outcome—discovery cohort

The expression of calpastatin and calpain-1 were not significantly associated with breast cancer-specific survival or relapse-free interval in the total patient cohort. High calpain-2 expression was associated with worse relapse-free survival ( $P = 0.043$ ); however, in multivariate Cox regression calpain-2 expression was not an independent indicator of relapse-free survival (HR = 1.178; 95% CI = 0.914–1.520;  $P = 0.206$ ). In the multivariate Cox regression, the potential confounding factors of patient age, tumour size, stage and grade, node status, NPI, ER status, PgR status, HER2, basal-like and lymphovascular invasion were included [with individual Kaplan–Meier statistics of  $P < 0.001$  for all variables except age ( $P = 0.001$ ), ER status ( $P = 0.007$ ), PgR status ( $P = 0.001$ ) and basal-like ( $P = 0.009$ )].

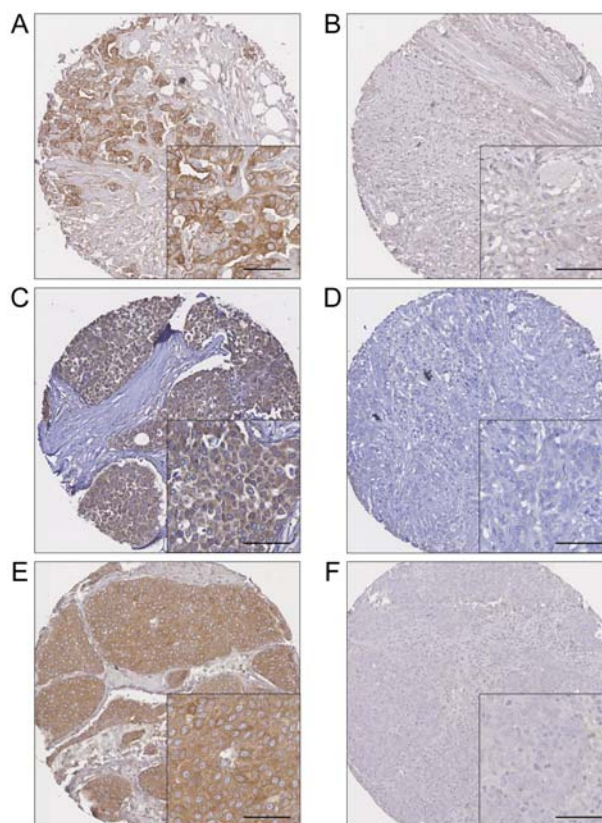
The expression of calpastatin and calpain-1 was not associated with breast cancer specific- or relapse-free survival of patients with triple-negative or basal-like tumours. However, Kaplan–Meier analyses demonstrated that patients with triple-negative or basal-like tumours had a significantly worse prognosis if they had high expression of calpain-2 ( $P = 0.003$  and  $<0.001$ , respectively; Figure 2). Furthermore, Kaplan–Meier analyses were performed on only triple-negative and basal-like subgroups, where calpain-2 expression remained significant ( $P = 0.043$  and  $0.025$ , respectively; Figure 3). If the alternative ‘core basal’ definition is used to define the basal phenotype (triple-negative cases with any expression of CK5/6 and/or

epidermal growth factor receptor (EGFR) [24]), calpain-2 expression remains statistically associated with survival for patients with core basal tumours ( $P = 0.050$ ).

### Verification cohort

To verify the finding that calpain-2 expression is significantly important in triple-negative and basal-like disease and could prognostically stratify patients, an independent patient cohort was investigated.

Clinicopathological criteria of this cohort are shown in Table 1. Of the 387 ER-negative patients, 65% (253 of 387) had triple-negative tumours and 39% (149 of 387) had basal-like tumours. A similar staining pattern was observed in comparison to the discovery TMA. The cohort had a median H-score of 135 and ranged between 10 and 280. A new X-tile cut point was determined for the cohort due to the complete ER-negative bias of this cohort (H-score of 145). Kaplan–Meier analysis was performed on the patients with triple-negative and basal-like disease within the cohort, and high calpain-2 expression was significantly associated with breast cancer-specific survival in both subgroups ( $P = 0.033$  and  $0.020$ , respectively; Figure 4).



**Figure 1.** Representative photomicrographs of high and low levels of expression. (A) High calpain-1 and (B) low calpain-1 expression. (C) High calpain-2 and (D) low calpain-2 expression. (E) High calpastatin and (F) low calpastatin expression. Photomicrographs are at  $\times 10$  magnification with  $\times 20$  magnification inset box where scale bar shows 50  $\mu\text{m}$ .

## discussion

This study investigated the expression levels of calpastatin, calpain-1 and calpain-2 in breast cancer using

immunohistochemistry. Calpastatin expression is significantly associated with patient age, tumour size and grade, NPI, ER and PgR status, triple-negative and basal-like disease and intra-tumoural lymphatic vessel invasion. Calpain-1 expression was

**Table 2.** Association between calpastatin, calpain-1 and calpain-2 protein expression and clinicopathological variables

Variable	Calpastatin (n = 1266)			Calpain-1 (n = 1315)			Calpain-2 (n = 1286)		
	Low	High	P-value	Low	High	P-value	Low	High	P-value
Age (years)									
<40	22	66	<b>0.002</b>	78	14	0.097	25	65	0.796
≥40	154	1023		945	277		317	878	
Size (cm)									
<2	74	555	<b>0.022</b>	505	148	0.616	172	473	0.929
≥2	103	532		518	142		169	470	
Stage									
I	106	671	0.624	637	170	0.387	216	574	0.754
II	51	318		288	94		96	277	
III	20	98		97	27		30	91	
Grade									
I	23	197	<b>0.002</b>	175	57	<b>0.014</b>	58	172	0.412
II	45	378		328	114		107	323	
III	109	514		521	120		177	449	
Node status									
Negative	94	571	0.847	522	165	0.500	195	477	0.203
Positive	59	371		331	115		111	324	
NPI									
Good (<3.4)	30	329	<b>0.001</b>	294	84	0.646	91	279	0.501
Intermediate (3.4–5.4)	111	572		548	160		193	500	
Poor (>5.4)	35	179		176	43		55	158	
ER									
Negative	64	265	<b>0.001</b>	275	52	<b>0.001</b>	83	242	0.667
Positive	106	794		718	232		247	676	
PgR									
Negative	90	408	<b>&lt;0.001</b>	422	98	<b>0.005</b>	142	368	0.383
Positive	75	623		537	185		180	523	
HER2									
Negative	149	923	0.726	853	253	0.280	299	783	0.058
Positive	21	142		139	33		35	134	
Classification									
Non-triple negative	117	881	<b>&lt;0.001</b>	802	250	<b>0.016</b>	266	760	0.315
Triple negative	52	172		185	36		64	155	
Basal phenotype									
Non-basal	112	813	<b>0.003</b>	745	219	0.552	257	689	0.487
Basal	49	205		207	55		64	191	
LVI									
Negative	85	603	0.408	548	168	0.627	194	507	0.464
Positive	48	290		271	77		86	251	
IT-LVI									
Negative	106	786	<b>0.014</b>	711	216	0.609	241	666	0.410
Positive	26	107		107	29		39	91	
PT-LVI									
Negative	96	654	0.798	602	177	0.696	220	542	<b>0.022</b>
Positive	37	239		217	68		60	216	

The *P*-values are resultant from the Pearson  $\chi^2$  test of association. Significant *P*-values are indicated by bold. The cohort was comprised of 1371 patients (total clinicopathological variables shown in Table 1); however, scores were not available for every patient for each marker (calpastatin: 1266 of 1371; calpain-1: 1315 of 1371; calpain-2: 1286 of 1371). The number of observations for each marker is shown for each clinicopathological variable; the table does not include the number of observations where clinicopathological data were not available.

NPI, Nottingham prognostic index; ER, oestrogen receptor; PgR, progesterone receptor; LVI, lymphatic vessel invasion; IT-LVI, intra-tumoural LVI; PT-LVI, peri-tumoural LVI.

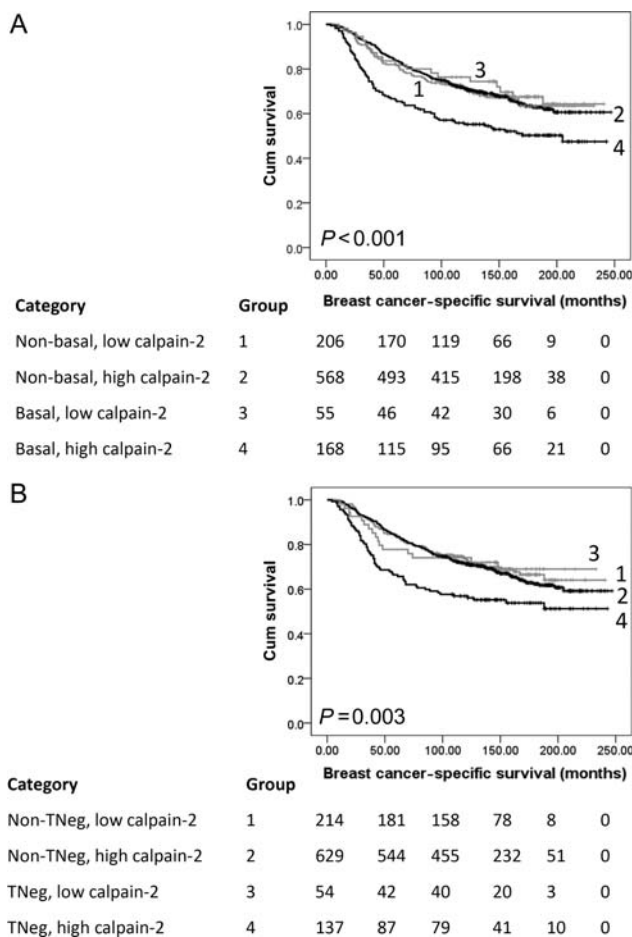
associated with grade, ER and PgR status and triple-negative disease, and calpain-2 expression was associated with peritumoural lymphatic vessel invasion.

The calpain system has been implicated in various aspects of cancer progression, including migration and cell survival [13]. It functions as a cysteine protease that is responsible for the controlled proteolysis of a number of important cellular substrates. Both  $\mu$ -calpain (calpain-1 is the large catalytic subunit) and m-calpain (calpain-2 is the large catalytic subunit) require calcium for their activity and are named for the calcium concentration required for their activity *in vitro* [12]. There are several mechanisms that enhance calpain activation through reducing the concentration of calcium required, such as interaction with membrane phospholipids and autolysis of the protein itself. Furthermore, m-calpain can be phosphorylated by ERK and protein kinase C $\alpha$  (PKC $\alpha$ ), which has been shown to alter cell adhesion and migration [25, 26].

This study used a large consecutive series of invasive breast carcinoma patients to demonstrate an association between breast cancer-specific overall survival and calpain-2 expression in both pragmatically defined basal-like and triple-negative phenotype subgroups. Furthermore, the importance of calpain-2 expression was verified in a separate, defined cohort of invasive breast cancer patients. This provides strong evidence that determining calpain-2 expression is of prognostic significance. Interestingly, determining calpain-2 expression could stratify patients with basal-like and triple-negative disease into two groups, one with a poor prognosis and one that behaved like patients with non-basal-like or receptor-positive disease. The pragmatic immunohistochemical definition of basal-like breast cancers used in this study was expression of the basal cytokeratins, CK5/6 and/or CK14. There are a number of immunohistochemical markers that have been used for determination of basal-like phenotype, varying from those that include EGFR and p53 [6]. There is no current information that would explain why calpain-2 expression is of such importance in basal-like and triple-negative breast cancer; however, overexpression of m-calpain in these patients could be expected to alter both cellular migration and invasion, which would have an important impact on patient outcome in terms of breast cancer-specific survival.

The current study examined the expression levels of calpain-1, calpain-2 and calpastatin, and cannot predict the subsequent activity levels of the protease. It would be of interest to determine calpain activity, which might be possible using antibodies against the products of calpain proteolysis; however, such reagents would require validation to determine their suitability for use in malignant tissue [27, 28]. In addition, the small regulatory subunit of both  $\mu$ -calpain and m-calpain, calpain-4 (CAPNS1) was not investigated as part of this study. In future works, it may be of interest to determine the expression level of calpain-4.

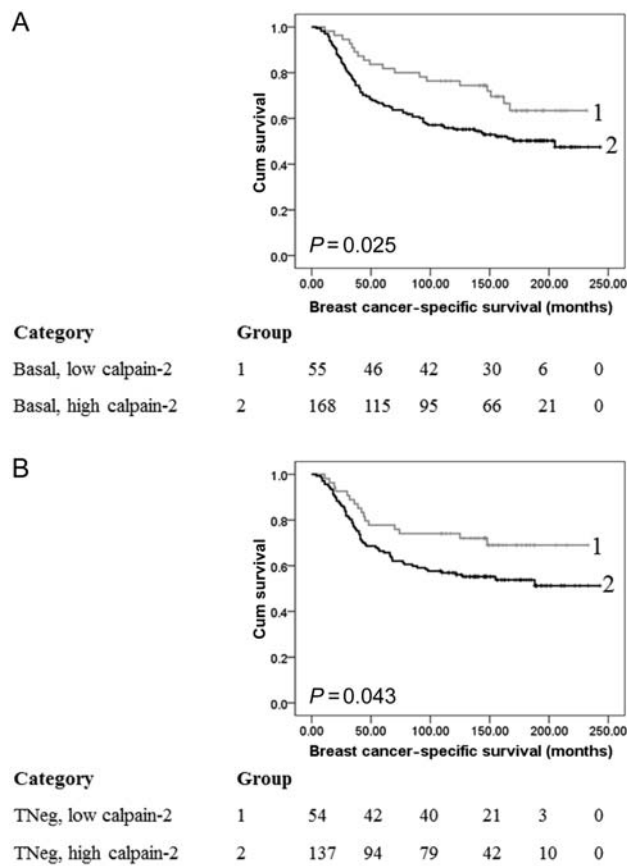
The expression of calpain-1, calpain-2 and calpastatin has been previously reported in HER2-positive breast cancer patients treated with trastuzumab following adjuvant chemotherapy to demonstrate that calpain-1 expression is associated with relapse-free survival [14]. Calpain-1 and calpain-2 expression were not associated with any



**Figure 2.** Kaplan–Meier analysis of breast cancer-specific survival showing the impact of calpain-2 expression in the primary cohort of 1371 patients with significance determined using the log-rank test. The numbers shown below the Kaplan–Meier survival curves are the number of patients at risk at the specified month. (A) Subgroup analysis of basal-like disease where, 1: non-basal-like disease with low calpain-2 expression ( $n = 206$ ); 2: non-basal-like disease with high calpain-2 expression ( $n = 568$ ); 3: basal-like disease with low calpain-2 expression ( $n = 55$ ); 4: basal-like disease with high calpain-2 expression ( $n = 168$ ). (B) Subgroup analysis of triple-negative disease where, 1: non-triple-negative disease with low calpain-2 expression ( $n = 214$ ); 2: non-triple-negative disease with high calpain-2 expression ( $n = 629$ ); 3: triple-negative disease with low calpain-2 expression ( $n = 54$ ); 4: triple-negative disease with high calpain-2 expression ( $n = 137$ ).

clinicopathological variables and calpastatin was significantly associated with NPI and lymph node status; these observations differ to the current data, but may be due to the defined HER2-positive cohort reported previously. We have previously reported an association between low calpastatin expression and lymphatic vessel invasion [15], which was also observed in the current study; however, this association is limited to intra-tumoural lymphatic vessel invasion.

Expression of the large catalytic subunit of m-calpain (calpain-2) is significantly associated with clinical outcome of patients with triple-negative and pragmatically defined basal-like disease in terms of breast cancer-specific survival. As part of future research, multi-centre, non-selected breast cancers



**Figure 3.** Kaplan–Meier analysis of breast cancer-specific survival showing the impact of calpain-2 expression in the basal-like (A) and triple-negative (B) subgroups of the primary cohort of patients with significance determined using the log-rank test. The numbers shown below the Kaplan–Meier survival curves are the number of patients at risk at the specified month. (A) Basal-like disease where, 1: low calpain-2 expression ( $n = 55$ ); 2: high calpain-2 expression ( $n = 168$ ). (B) Triple-negative disease where, 1: low calpain-2 expression ( $n = 54$ ); 2: high calpain-2 expression ( $n = 137$ ).

must be investigated to test if determining calpain-2 expression would be of clinical benefit and produce recommendations for standardisation of staining and assessment. Inhibition of the proteolytic functions of calpain may provide a therapeutic opportunity for certain subgroups of patients.

## acknowledgements

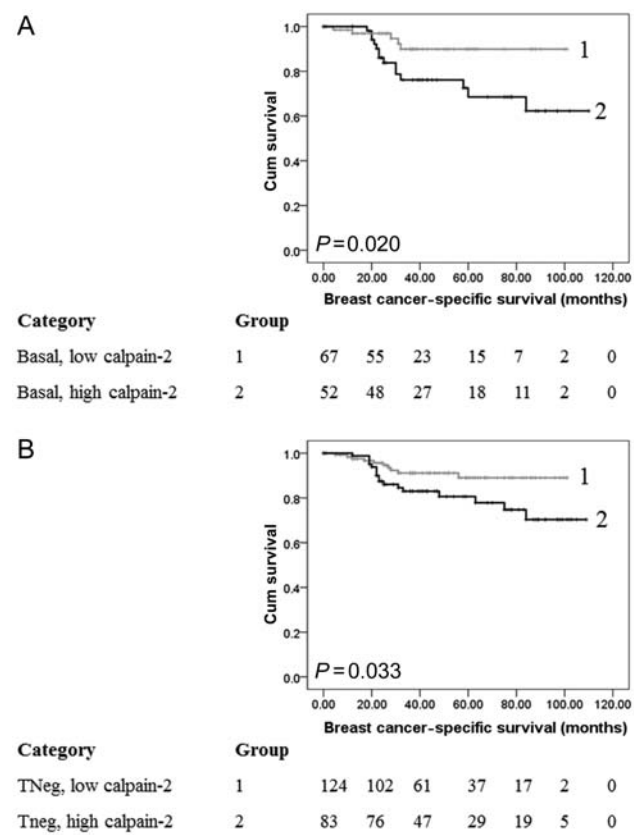
The authors acknowledge Christopher Nolan for his excellent technical assistance.

## funding

The authors acknowledge the Breast Cancer Campaign for funding this research (Ref: 2011MayPR35).

## disclosure

The authors have declared no conflicts of interest.



**Figure 4.** Kaplan–Meier analysis of breast cancer-specific survival showing the impact of calpain-2 expression in the basal-like (A) and triple-negative (B) subgroups of the verification cohort of patients with significance determined using the log-rank test. The numbers shown below the Kaplan–Meier survival curves are the number of patients at risk at the specified month. (A) Basal-like disease where, 1: low calpain-2 expression ( $n = 67$ ); 2: high calpain-2 expression ( $n = 52$ ). (B) Triple-negative disease where, 1: low calpain-2 expression ( $n = 124$ ); 2: high calpain-2 expression ( $n = 83$ ).

## references

- Carey L, Winer E, Viale G et al. Triple-negative breast cancer: disease entity or title of convenience? *Nat Rev Clin Oncol* 2010; 7: 683–692.
- Murad TM. A proposed histochemical and electron microscopic classification of human breast cancer according to cell of origin. *Cancer* 1971; 27: 288–299.
- Sorlie T, Perou CM, Tibshirani R et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; 98: 10869–10874.
- Sorlie T, Tibshirani R, Parker J et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* 2003; 100: 8418–8423.
- Perou CM, Sorlie T, Eisen MB et al. Molecular portraits of human breast tumours. *Nature* 2000; 406: 747–752.
- Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol* 2008; 26: 2568–2581.
- Zhang H, Rakha EA, Ball GR et al. The proteins FABP7 and OATP2 are associated with the basal phenotype and patient outcome in human breast cancer. *Breast Cancer Res Treat* 2010; 121: 41–51.
- Ray PS, Wang J, Qu Y et al. FOXC1 is a potential prognostic biomarker with functional significance in basal-like breast cancer. *Cancer Res* 2010; 70: 3870–3876.

9. Biganzoli E, Coradini D, Ambrogio F et al. p53 status identifies two subgroups of triple-negative breast cancers with distinct biological features. *Jpn J Clin Oncol* 2011; 41: 172–179.
10. Alexander BM, Spratt K, Farrow DA et al. DNA repair protein biomarkers associated with time to recurrence in triple-negative breast cancer. *Clin Cancer Res* 2010; 16: 5796–5804.
11. Mehta R, Jain RK, Sneige N et al. Expression of high-molecular-weight cytokeratin (34betaE12) is an independent predictor of disease-free survival in patients with triple-negative tumours of the breast. *J Clin Pathol* 2010; 63: 744–747.
12. Goll DE, Thompson VF, Li H et al. The calpain system. *Physiol Rev* 2003; 83: 731–801.
13. Storr SJ, Carragher NO, Frame MC et al. The calpain system and cancer. *Nat Rev Cancer* 2011; 11: 364–374.
14. Storr SJ, Woolston CM, Barros FF et al. Calpain-1 expression is associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy. *Int J Cancer* 2010; 129: 1773–1780.
15. Storr SJ, Mohammed RA, Woolston CM et al. Calpastatin is associated with lymphovascular invasion in breast cancer. *Breast* 2011; 20: 413–418.
16. Libertini SJ, Robinson BS, Dhillon NK et al. Cyclin E both regulates and is regulated by calpain 2, a protease associated with metastatic breast cancer phenotype. *Cancer Res* 2005; 65: 10700–10708.
17. Agarwal R, Gonzalez-Angulo AM, Myhre S et al. Integrative analysis of cyclin protein levels identifies cyclin b1 as a classifier and predictor of outcomes in breast cancer. *Clin Cancer Res* 2009; 15: 3654–3662.
18. Heiser LM, Sadanandam A, Kuo WL et al. Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc Natl Acad Sci USA* 2012; 109: 2724–2729.
19. Abdel-Fatah TM, Powe DG, Agboola J et al. The biological, clinical and prognostic implications of p53 transcriptional pathways in breast cancers. *J Pathol* 2010; 220: 419–434.
20. Rakha EA, El-Rehim DA, Paish C et al. Basal phenotype identifies a poor prognostic subgroup of breast cancer of clinical importance. *Eur J Cancer* 2006; 42: 3149–3156.
21. McShane LM, Altman DG, Sauerbrei W et al. REporting recommendations for tumor MARKer prognostic studies (REMARK). *Nat Clin Pract Urol* 2005; 2: 416–422.
22. Abd El-Rehim DM, Ball G, Pinder SE et al. High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int J Cancer* 2005; 116: 340–350.
23. Camp RL, Dolled-Filhart M, Rimm DL. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. *Clin Cancer Res* 2004; 10: 7252–7259.
24. Cheang MC, Voduc D, Bajdik C et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res* 2008; 14: 1368–1376.
25. Glading A, Uberall F, Keyse SM et al. Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. *J Biol Chem* 2001; 276: 23341–23348.
26. Xu L, Deng X. Protein kinase Ciota promotes nicotine-induced migration and invasion of cancer cells via phosphorylation of micro- and m-calpains. *J Biol Chem* 2006; 281: 4457–4466.
27. Neumar RW, Meng FH, Mills AM et al. Calpain activity in the rat brain after transient forebrain ischemia. *Exp Neurol* 2001; 170: 27–35.
28. Goncalves I, Nitulescu M, Saido TC et al. Activation of calpain-1 in human carotid artery atherosclerotic lesions. *BMC Cardiovasc Disord* 2009; 9: 26.

*Annals of Oncology* 23: 2296–2300, 2012  
doi:10.1093/annonc/mdr630  
Published online 21 February 2012

## A randomised trial of primary tamoxifen versus mastectomy plus adjuvant tamoxifen in fit elderly women with invasive breast carcinoma of high oestrogen receptor content: long-term results at 20 years of follow-up

S. J. Johnston<sup>1,†</sup>, F. S. Kenny<sup>1,†</sup>, B. M. Syed<sup>1</sup>, J. F. R. Robertson<sup>1</sup>, S. E. Pinder<sup>2</sup>, L. Winterbottom<sup>3</sup>, I. O. Ellis<sup>2</sup>, R. W. Blamey<sup>1</sup> & K. L. Cheung<sup>1\*</sup>

Divisions of <sup>1</sup>Breast Surgery; <sup>2</sup>Pathology, University of Nottingham, Nottingham; <sup>3</sup>Nottingham Breast Institute, Nottingham University Hospitals, Nottingham, UK

Received 15 November 2011; accepted 20 December 2011

**Background:** Long-term analysis of a randomised trial in Nottingham comparing tamoxifen versus surgery as initial treatment demonstrated that in oestrogen receptor (ER)-unselected cases, surgery achieved better local control, with

\*Correspondence to: Mr K. L. Cheung, Division of Breast Surgery, School of Graduate Entry Medicine and Health, University of Nottingham, Royal Derby Hospital, Uttoxeter Road, Derby DE22 3DT, UK. Tel: +44-0-1332-724881; Fax: +44-0-1332-724880; E-mail: kl.cheung@nottingham.ac.uk

<sup>†</sup>Joint first authors.