Calpain in Breast Cancer: Role in Disease Progression and Treatment Response

Sarah J. Storr Nicola Thompson Xuan Pu Yimin Zhang Stewart G. Martin

Academic Clinical Oncology, Division of Cancer and Stem Cells, School of Medicine, The University of Nottingham, Nottingham University Hospitals NHS Trust, Nottingham City Hospital Campus, Nottingham, UK

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Abstract

The calpains are a family of intracellular cysteine proteases that function in a wide array of cellular activities, including cytoskeletal remodelling, survival and apoptosis. The ubiquitously expressed micro (μ)-calpain and milli (m)-calpain are archetypal family members that require calcium for function and can be inhibited by their endogenous inhibitor calpastatin. This review describes the role of the calpain system in the prognosis of breast cancer and disease progression, in addition to the role of the calpain system in the response to breast cancer treatments, including chemotherapeutic, endocrine and targeted therapies.

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Introduction

The calpain system is a family of intracellular calcium-activated cysteine proteases (EC 3.4.22) that function in a wide range of important cellular activities. The calpain system also contains an endogenous protein inhibitor

named calpastatin (table 1). Calpain was first described over 50 years ago, and much research has amassed in subsequent years. Aberrant activation and expression, as well as gene polymorphisms of various calpain family members are implicated in a number of disease states and pathologies including limb-girdle muscular dystrophy (LGMD2A), neurodegeneration, diabetes and cancer [reviewed in ref. 1–4].

Biology of the Calpain System

There are at least fourteen calpain isoform genes, which have been defined by the presence of a protease domain similar to that found in micro (μ)-calpain. μ -Calpains, along with milli (m)-calpains, are the archetypal calpain family members and have been the most widely studied as they are ubiquitously expressed (table 1) [5–7]. Both μ -calpain and m-calpain were named for the concentration of calcium ions required for in vitro activation [8–10] and are heterodimers of different 80-kDa catalytic subunits [calpain-1 (encoded by *CAPN1*) and calpain-2 (encoded by *CAPN2*), respectively] and a common small (28-kDa) regulatory subunit [calpain-4 (encoded by *CAPNS1*)] [11–13]. The catalytic subunit of

Fig. 1. Schematic structure of μ -calpain. μ -Calpain is composed of an 80-kDa catalytic subunit (encoded by *CAPN1*) and a 28-kDa small regulatory subunit (encoded by *CAPN4*). The large subunit contains DI, the protease domain (DIIa and DIIb), DIII and DIV (with EF hand domains indicated). The small subunit contains DV and DVI (with EF hand domains indicated). In the protease domain of the large subunit, the enzymatic triad residues are indicated.

Table 1. Calpain family members, gene name, protein name, EC number, amino acid length and chromosome location

Gene	Name(s)	EC No.	Amino acids	Chromosome location
<i>CAPN1</i>	Calpain-1, μ -calpain	3.4.22.52	714	11q13
<i>CAPN2</i>	Calpain-2, m-calpain	3.4.22.53	700	1q41–q42
<i>CAPN3</i>	Calpain-3, p94, muscle-specific calpain	3.4.22.54	821	15q15.1–q21.1
<i>CAPN5</i>	Calpain-5		640	11q14
<i>CAPN6</i>	Calpain-6, calpain-like protease X linked		641	Xq23
<i>CAPN7</i>	Calpain-7, PalB homologue		813	3p24
<i>CAPN8</i>	Calpain-8, stomach-specific m-type, new calpain-2		703	1q41
<i>CAPN9</i>	Calpain-9, digestive tract-specific calpain		690	1q42.11–q42.3
<i>CAPN10</i>	Calpain-10		672	2q37.3
<i>CAPN11</i>	Calpain-11		739	6p12
<i>CAPN12</i>	Calpain-12		719	19q13.2
<i>CAPN13</i>	Calpain-13		669	2p22–p21
<i>CAPN14</i>	Calpain-14		684	2p23.1–p21
<i>SOLH</i>	Calpain-15		1,086	16p13.3
<i>CAPNS1</i>	Calpain-4, calpain small subunit 1		269	19q13.12
<i>CAPNS2</i>	Calpain small subunit 2		248	16q12.2
<i>CAST</i>	Calpastatin		791 (isoform 1)	5q15

both μ -calpain and m-calpain is divided into a number of domains, DI, DIIa, DIIb, DIII and DIV, with the regulatory subunit comprised of domains DV and DVI (fig. 1). DI is autolysed when calpain is activated by calcium, DII forms a catalytic cleft upon binding to calcium, DIII contains C2 domains and is involved in structural changes during calcium binding, and DIV and DVI contain EF hands and are involved in calcium binding and aid in dimerisation of the subunits [14–16]. The protease domain (DII) of different calpain isoforms is similar; however, there is divergence of the other domains of calpain family members, which can result in calcium independence or lack of regulatory subunit requirement [reviewed in ref. 7]. The calcium requirement for calpain activation can be reduced by a number of mechanisms, including autolysis, phosphorylation and interaction with phospholipids. Phosphatidylinositol, phosphatidylinositol-4-

monophosphate, and phosphatidylinositol-4,5-bisphosphate can enhance autolysis of m-calpain at the plasma membrane [17–21]. Both μ -calpain and m-calpain can be phosphorylated by protein kinase $C\alpha$ and m-calpain can also be phosphorylated by ERK; both of these events have been shown to alter the invasive phenotype of cancer cells [22, 23].

Calpastatin is the ubiquitously expressed endogenous inhibitor of both μ -calpain and m-calpain and is encoded by a single gene (*CAST*). Studies also suggest that calpastatin may exhibit inhibitory action against calpain-8 and calpain-9, in addition to μ -calpain and m-calpain [24]. Calpastatin is an unstructured protein that can reversibly inhibit up to four calpain heterodimers through four repeating inhibitory domains (I–IV). Inhibition of calpain by calpastatin requires calcium-induced structural changes to have occurred in calpain prior to its binding

[14, 25]. Multiple promoter usage and alternate splicing generates a number of calpastatin isoforms that differ in their N-terminal regions [26–31].

Calpain expression has shown to be altered in cancer in comparison to their normal counterparts. Increased expression of μ -calpain has been observed in schwannomas and meningiomas, with mRNA increased in renal carcinomas [32, 33]. Increased m-calpain has been observed in colorectal cancers and decreased expression of calpastatin in endometrial cancer [34, 35]. The relative level of expression of various calpain system members within a number of different tumours is implicated in disease prognosis and progression in a number of cancers, including breast, ovarian, pancreatic and gastro-oesophageal tumours [36–41].

In summary, the calpains are an influential family of cysteine proteases, and aberrant expression or activity has been described in a number of disease states, including various tumour types. In this review, we will describe the cellular functions of calpain during tumour development and progression, then focus specifically on the links between calpain and breast cancer prognosis and response to anti-cancer therapies.

Cellular Functions of the Calpain System

The calpain degradome is becoming increasingly complex, with new substrates for calpain proteolysis being identified. These substrates appear to differ between cell types and under different cell contexts; importantly, a wide range of cellular actions have been attributed to calpain activation, such as apoptosis, survival and migration amongst others, which will be discussed further in the coming section.

Cellular Survival and Apoptosis

Calpain has been implicated in both cell death and survival, but the roles in these processes seem to be context dependent, including the type of apoptotic stimulus and the cellular localisation of the protein [42]. In addition to survival and apoptosis, calpain also has a role in autophagy, a process whereby autophagosomes fuse with lysosomes to degrade unnecessary or dysfunctional cell components.

In cell survival pathways, calpain has been shown to cleave wild-type p53, regulating protein stability to prevent p53-dependent apoptosis [43–46]. In addition, calpain can activate the transcription factor nuclear factor (NF)- κ B by cleavage of its inhibitor I κ B α . Cleavage of I κ B α by calpain

can happen in response to a number of factors, such as tumour necrosis factor and activation of HER2, a member of the epidermal growth factor receptor family [47, 48]. There are numerous other survival-related pathways that calpain has been shown to be involved in. Increased Myc in cancers has been shown to drive oncogenic transformation, and calpain-mediated cleavage of Myc occurs under stress conditions, such as hypoxia. Cleavage of Myc by calpain converts the active protein to Myc-nick, which is a transcriptionally inactive [49]. Calpain has also been shown to play a role in the regulation of the cell cycle at a number of important steps. In breast cancer, calpain has been shown to cleave cyclin E, a protein required for the transition from G1 to S phase, to a hyperactive low-molecular-weight form [50]; in addition, cyclin E overexpression has been shown to activate m-calpain [51].

In addition to survival, calpain has a clear role in some cellular apoptotic pathways. Calpain-mediated proteolysis of a number of caspases (caspase-7, -9, -10 and -12) has been demonstrated; however, this can result in activation (caspase-7, -10 and -12) or inactivation (caspase-9) of these cysteine proteases [52–54]. Furthermore, calpain is implicated in pro-apoptotic pathways by the cleavage of a number of other apoptosis-associated proteins such as BAX, BCL-2, JNK and JUN, amongst others [54–59]. Autophagy-related gene 5 (Atg5) protein is an ubiquitin ligase required for the formation of autophagosomes. Calpain can cleave Atg5 to switch autophagy to apoptosis via Atg5 translocation to the mitochondria, which results in cytochrome c release [60]. Further review of the action of calpain in these conflicting pathways has been reviewed in depth previously, with much of the evidence described in tumours other than breast cancer [2].

Cellular Migration

The calpain family has an established role in mediating cellular migration through their ability to regulate focal adhesion dynamics. In order to move through the extracellular matrix, cells are dependent on a dynamic process, involving the formation of integrin-mediated focal adhesions on the leading edge of the cell, which connect actin cytoskeleton with the extracellular matrix, and disassembly of these attachments at the rear of the cell [for a review, see 61]. Cleavage of the focal adhesion protein talin at integrin-associated focal adhesion structures by m-calpain was first demonstrated in 1987 [62], which can be regulated by MAP3K1 [63]. Subsequently, calpain has been shown to cleave numerous other substrates to promote cellular motility, such as paxillin and fodrin [64–69]; however, negative regulation of focal adhesion dy-

namics by proteolysis of paxillin has also been observed [70]. The role of calpain in focal adhesion turnover has been reviewed previously [71, 72].

Inhibition of calpain has been shown to stabilise peripheral focal adhesions and subsequently decrease the rate of retraction at the rear of the cell, inhibiting migration [73]. In addition to their role in focal adhesion disassembly, calpains have also been shown to have a role in invadopodia formation. Invadopodia are actin-rich protrusions which form on the leading edge of cells, and are associated with increased invasiveness [for a review, see 74]; they are associated with numerous adhesion receptors and the secretion and activation of proteases such as matrix metalloproteinases (MMP). Calpain-2 has been shown to mediate formation of invadopodia through proteolysis of PTP1B, a protein tyrosine phosphatase that regulates Src and subsequent downstream proteins involved in invadopodia formation [75]. In addition, calpain has also been shown to interact with metalloproteinases, in particular MMP2 and MMP9. Calpain inhibition has been shown to decrease mRNA expression of both MMP2 and MMP9 with associated impaired invasion in vitro [76–78]. Although the mechanism through which calpain modulates MMP expression is currently unknown, one possible mechanism may be through the interaction of calpain with the MAPK signalling pathway, which is known to have a role in regulating MMP9 expression [22, 79], or via NF- κ B activation [80]. Furthermore, calpain can cleave vimentin upstream to membrane type-1 (MT1)-MMP to allow MT1-MMP membrane translocation [81]. In breast cancer, a recent study has shown that the oncoprotein hepatitis B X-interacting protein increased filopodia formation through calpain-4, which resulted in increased cellular migration; the signalling pathway was shown to involve MEKK2 and ERK1/2 [82].

Links between Calpain and Other Cellular Functions

Calpain is linked with numerous other important cellular functions and pathways other than survival, apoptosis and migration. The calpain system appears to play a role in diverse activities, e.g. in hypoxia and DNA repair pathways. Tumour hypoxia is associated with a poor clinical outcome and is linked with angiogenesis and metastasis. In melanoma, hypoxia can induce calpain-mediated cleavage of filamin A (FLNA), a large cytoskeletal actin-binding protein, which results in the up-regulation of hypoxia-inducible factor-1 α (HIF-1 α) target gene expression and potentiates angiogenesis and tumour progression [83]. HIF-1 α has also been shown to increase the

expression and activity of calpains in human umbilical vein endothelial cells [84]. Calpains have also been shown to be involved in hypoxia-induced cell death of the human hepatoblastoma cell line HepG2 [85].

Redox proteins are the intracellular enzymatic redox buffering systems that modulate cellular redox homeostasis. The reduced glutathione (GSH)/oxidised glutathione (GSSG) system, the thioredoxin (Trx)/Trx reductase system and the glutaredoxin system are the three major families of redox proteins. Several studies indicate that the peroxiredoxin (Prx) proteins, which are members of the Trx family, are cleaved by calpain to modulate activity. Calpain-mediated cleavage of Prx III can decrease peroxidase activity in Fanconi anaemia [86]. Inhibition of calpain activity can decrease the abundance of both Prx I and Prx II in Rat2 cells [87], as well as Prx IV in alveolar rhabdomyosarcoma cells [88]. Calpain activity has also been shown to be regulated by the GSH/GSSG system, with activity inhibited by GSH, GSSG and nitric oxide in rat pheochromocytoma PC12 cells [89].

In DNA repair pathways, calpain has been shown to degrade 8-oxoguanine DNA glycosylase 1 (OGG1), an enzyme that repairs 8-oxoguanine lesions in DNA. Calpain-mediated degradation of OGG1 occurs during oxidative stress and during drug-induced apoptosis [90]. In addition, the DNA repair enzyme poly(ADP)ribose polymerase (PARP) has also been shown to be a calpain substrate [91].

The Calpain System and Breast Cancer Prognosis

The role of the calpain system in cancer in general has previously been reviewed [2]; here, we will focus on the importance of the calpain system in breast cancer. A wide range of in vitro and in vivo studies have linked both μ -calpain and m-calpain to various aggressive phenotypes in breast cancer. Knockdown of calpain-2 in mouse mammary carcinoma cells injected into the mammary fat pad of mice results in reduced tumour growth rates and enhanced expression of the tumour suppressor p27^{kip1} [92]. In vitro calpain-2 knockdown in breast cancer cells can impair invadopodia formation [75]. In addition, links between calpain and important prognostic phenotypes of breast cancer have been described; for example, breast cancers that express HER2 are associated with a poor prognosis, and studies have demonstrated that HER2 induces NF- κ B through a phosphatidylinositol 3-kinase/Akt pathway that involves degradation of I κ B α by calpain [48]. Furthermore, links between calpain

Fig. 2. Representative photomicrographs demonstrating calpain-1 (a), calpain-2 (b) and calpastatin (c) expression in breast cancer tissue. $\times 10$. Inset: $\times 20$. Scale bars = 100 μm .

expression and the growth of oestrogen receptor-positive breast cancer cells have been described, and low calpain-9 expression has been linked with adverse survival in breast cancer patients treated with endocrine therapy [93, 94].

Calpain-1, calpain-2, calpain-9 and calpastatin expression has been assessed in large cohorts of tumours from breast cancer patients. Immunohistochemically determined calpain expression in breast cancer has revealed that the majority of tumour cells express calpain-1, calpain-2 and calpastatin, with some heterogeneity observed between adjacent tumour cells. Representative staining patterns for calpain-1, calpain-2 and calpastatin are shown in figure 2. A range of antibodies are available for immunohistochemistry, including those specific for calpain isoforms; however, although calpastatin antibodies are available, there is no commercially available antibody to determine the expression of calpastatin isoforms. One of the difficulties with determining calpain expression in tumour samples is that no inference on calpain activity can be made, which is potentially a much more interesting end point.

In early invasive breast cancer specimens, high expression of calpain-2 has been shown to be associated with the adverse survival of patients with basal-like or triple receptor-negative phenotype tumours [36]. Basal-like and triple receptor-negative tumours are important phenotypic groups of breast cancer that are associated with adverse clinical outcomes in comparison to their luminal and receptor-positive counterparts. This large study investigated 1,371 unselected breast cancer patients and performed further analysis on those patients with triple receptor-negative and basal-like disease, and

validation of results in a separate cohort of 387 oestrogen receptor-negative patients. Interestingly, this study demonstrated that those patients with triple receptor-negative or basal-like disease with low expression of calpain-2 had similar breast cancer-specific survival to their non-basal or receptor-positive disease counterparts. An additional study has investigated calpain-9 expression in 783 unselected breast cancer patients, and although calpain-9 expression was not associated with survival in the total cohort, low calpain-9 expression was associated with adverse survival in patients who received endocrine therapy [94].

As well as phenotypic characteristics of breast tumours, the calpain system has been linked with prognostic factors. The calpain system has been linked with lymphovascular invasion both in patient specimens and in vitro. Lymphovascular invasion is an important initial step of the metastatic cascade, and in breast cancer this occurs primarily via lymphatic vessels as opposed to blood vessels [95]. In tumours, expression of calpastatin is reduced in breast cancer patients with lymphatic vessel invasion, both at the mRNA and the protein level [36, 37]. In vitro, calpain has been shown to aid spheroidogenesis and lymphovascular emboli in models of inflammatory breast cancer via cleavage of E-cadherin [96].

A number of studies have described the association between calpain-1, calpain-2, calpastatin and traditional prognostic variables in various patient cohorts. In unselected breast cancer patients, high calpastatin expression is associated with older patients, low tumour grade, oestrogen- and progesterone-positive tumours; high calpain-1 expression is associated with oestrogen- and

progesterone receptor-positive tumours and low tumour grade [36]. Low expression of calpain-9 is associated with older patients, smaller tumour size and stage, a more favourable Nottingham Prognostic Index value and oestrogen-positive tumours in unselected patients [94].

The Calpain System and Response to Breast Cancer Treatments

Calpains are implicated in breast cancer progression and are considered as a potential anti-cancer target. Standard treatments for breast cancer include surgery, adjuvant systemic therapy (including hormonal therapies, cytotoxic chemotherapy and targeted therapies) and adjuvant radiotherapy. Calpain has been implicated in the response to chemotherapeutic agents in a number of different tumour types, but information in breast cancer is limited. Cisplatin is a platinum-based drug used widely in different types of cancer, and, during cisplatin-induced apoptosis, calpain-1 activation occurs prior to the activation of apoptosis-inducing factor, caspase-3 and caspase-9 and before cytochrome c release in human lung adenocarcinoma cells [66, 97]. Similar results, whereby calpain activation precedes caspase-3 and caspase-7 activation, have been observed in melanoma [98]. Irinotecan is a topoisomerase I inhibitor used in the treatment of colorectal cancer, and in colorectal cancer xenograft models calpain-2 is involved in regulating irinotecan resistance through NF- κ B expression [99]. In addition to calpain-1 and calpain-2, other calpain isoforms have also been implicated in the response to chemotherapeutic agents. Calpain-3 has been implicated in cisplatin-induced apoptosis in melanoma [100]; in cisplatin-treated cervical cancer cells, calpain-6 down-regulates apoptosis and enhances angiogenesis to contribute to cervical cancer progression [101], and in ovarian cancer patients treated with platinum-based chemotherapy calpain-2 expression is significantly associated with progression-free and overall survival [39]. As mentioned above, there is little information linking the calpain system and response to chemotherapeutic agents in breast cancer directly; unpublished data from our laboratory suggest that calpain and calpastatin expression is associated with response to neoadjuvant chemotherapy in non-inflammatory breast cancer. There are also limited studies implicating the calpain system in hormone-sensitive breast cancer. There is evidence to suggest that fulvestrant can activate calpain-1 and enhance cell adhesion [102]. As mentioned previous-

ly, low calpain-9 expression has been linked to adverse survival in patients treated with endocrine therapy containing tamoxifen where expression was determined using immunohistochemistry [94].

The calpain system is more firmly implicated in the response to the targeted agent, trastuzumab, in breast cancer patients. Trastuzumab is a HER2-targeting monoclonal antibody that is used to treat certain HER2-positive breast cancers, and is currently being assessed for the use in other HER2-expressing tumour types. Expression of calpain-1 has been shown to be linked with response to trastuzumab following adjuvant chemotherapy in HER2-positive breast cancer [40]. This study utilised immunohistochemistry on tumours from 93 primary breast cancer patients treated with surgery and adjuvant chemotherapy followed by trastuzumab. The results demonstrated that high expression of calpain-1 is associated with the Nottingham Prognostic Index and lymph node status in these patients in addition to adverse relapse-free survival. Importantly, the association between calpain-1 and relapse-free survival remains significant in multivariate analysis when confounding pathological and treatment variables are included. These findings have been replicated in an expanded cohort and in an independent patient cohort [unpubl. data]. In vitro studies have shown that calpain-4 enhances survival following trastuzumab treatment; however, suppression of calpain-1 activity has also been linked with trastuzumab response in HER2-positive breast cancer cell lines [103, 104]. How the calpain system may regulate the response to trastuzumab is unclear, but evidence indicates that calpain-mediated cleavage of I κ B α is involved in HER2-induced NF- κ B activation and, inversely, that HER2 overexpression blocks calpain activity by up-regulating calpastatin [48, 105].

Conclusions

The calpain system is an important family of enzymes that play a role in a large number of core cellular activities, including cell survival, apoptosis and migration. Altered activity and expression of calpain family members have been implicated in a number of disease states, including cancer. Information has accumulated in breast cancer showing that calpain plays a role in disease progression, prognosis and response to treatment; however, there is a much larger body of research for a role in cancer in general. Current studies in breast cancer suggest that determining the expression and activity of calpain may prove

to have clinical benefit in determining the prognosis of patients and may also prove of use in stratifying patients for personalised medicine. There is still a large amount of research required to fully elucidate the role of calpain in breast cancer and cancer in general.

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