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Abdulkader Albasri, Mohammed Aleskandarany, Ahmed Benhasouna, Desmond G. Powe, Ian O. Ellis, et al.. CTEN (C-terminal tensin-like), a novel oncogene overexpressed in invasive breast carcinoma of poor prognosis. *Breast Cancer Research and Treatment*, 2010, 126 (1), pp.47-54. 10.1007/s10549-010-0890-3 . hal-00561316

HAL Id: hal-00561316

<https://hal.science/hal-00561316v1>

Submitted on 1 Feb 2011

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CTEN (C-terminal tensin-like), a novel oncogene overexpressed in invasive breast carcinoma of poor prognosis

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Running title: CTEN expression in breast cancer

Keywords: CTEN, Immunohistochemistry, tissue microarray, Poor prognosis, Breast cancer

ABSTRACT

Background: C-terminal tensin-like (*CTEN*) gene is a member of the *TENSIN* gene family, involved in cell migration and localised at focal adhesion sites.

Material and Methods: This study was designed to explore the prevalence and clinical significance of CTEN expression in a large and well-characterised series (n=1,409) invasive breast cancer (BC) cases with long term follow-up (median 11 years), using immunohistochemistry and tissue microarray.

Results: Moderate to strong cytoplasmic immunoreactivity for CTEN was observed in 90% of the studied cases. CTEN expression was significantly associated with poor prognostic variables including larger tumour size ($P=0.044$), higher histological grade ($P=0.019$), axillary nodal involvement ($P=0.035$) and poor Nottingham Prognostic Index ($P=0.016$). Significant associations were observed between increased CTEN expression and up-regulation of phosphorylated–Akt (P-Akt), PIK3 and N-cadherin proteins ($P<0.001$). Kaplan-Meier survival analysis demonstrated that patients with high CTEN expression had significantly shorter Breast Cancer Specific Survival ($P=0.004$) and Metastasis-Free Survival ($P=0.041$) than those with low CTEN expression. Multivariate analysis showed that CTEN was not an independent prognostic marker in BC.

Conclusion: Our results demonstrated the oncogenetic role of increased CTEN expression and its association with poor prognostic parameters. These data could help in prognostic assessment in BC patients.

INTRODUCTION

Breast cancer (BC) is the second most common cause of cancer related death in women, after lung cancer, in the Western World [1]. Worldwide, more than one million new cases of BC are diagnosed annually. In the UK, BC is now the most common cancer and almost 46,000 new cases are diagnosed every year [2].

The *TENSIN* comprises three proteins (*TNS1*, *TNS2* and *TNS3*), which are localised to cytoplasmic tail of integrin focal adhesion molecule, and are involved in various biological processes such as cell adhesion, migration, proliferation, differentiation, apoptosis and invasion [3]. They interact with several structural and signalling molecules such as vinculin, paxillin, Src, Focal Adhesion Kinase (FAK), phosphatidylinositol-3-kinase (PI3-K) and Crk-associated substrate p130^{CAS} as well as actin [4]. The COOH-terminus Tensin-like molecule (*CTEN*) gene is the fourth member of the *TENSIN* family, maps to chromosome 17q12-q21 and encodes a 715 amino acid protein [5]. It shares an extensive homology with other members at its C-terminal. However, in comparison to others, *CTEN* is a shorter polypeptide lacking the N-terminal actin-binding domain [3]. *CTEN* has a very restricted pattern of expression and was originally observed to be highly expressed in normal prostate and placenta [5].

CTEN expression has been detected in various human tumours, but its role in cancer remains uncertain, because it behaves differently depending on the cancer tissue type. For example, we and others have reported, that *CTEN* acts as an oncogene in colorectal cancer (CRC) and correlates with changed cell morphology and increased cell motility. This is complemented by decreased E-cadherin protein levels, resistance to staurosporine-induced apoptosis and increased colony forming properties [6, 7]. In addition, *CTEN* has been reported to have an oncogenic activity in thymomas, lung and gastric cancers, and in general, its over-expression has been associated with poor prognosis [8-10]. In BC, *CTEN* has been reported to stimulate mammary tumour migration through disruption of the link between integrins and cortical

actin fibres at focal adhesion sites and its expression was highly linked to epidermal growth factor (EGFR) activation [11]. Contrasting this, Lo and colleague reported down-regulation of *CTEN* in prostate cancer compared to normal prostate, and therefore CTEN might act as a tumour suppressor gene in this instance [5].

In the current study we evaluated CTEN protein expression in a large series of breast cancers using immunohistochemistry on a tissue microarrays (TMAs), in an attempt to assess if CTEN has prognostic significance on the outcome of patients with invasive breast cancer.

MATERIALS AND METHODS

Patient selection and characterisation

TMAAs were prepared from a series of 1,409 cases of primary operable invasive breast carcinoma from patients under the age of 70 years, diagnosed between 1987 and 1998 and entered into the Nottingham Tenovus Primary Breast Carcinoma Series. Patients' clinical and pathological data including patients' age, histological tumour type, primary tumour size, lymph node status, histological grade, Nottingham Prognostic Index (NPI), vascular invasion (VI) and development of local, region and distant recurrence were available and maintained on a prospective basis. Survival data including Breast Cancer Specific Survival (BCSS) and Disease-Free Interval (DFI) were available. The BCSS is defined as the time (in months) from the date of the primary surgery to the time of death from breast cancer. DFI is defined as the duration (in months) from the date of the primary surgery to the appearance of first local regional, recurrences or distant metastasis. Data on other biomarkers of known clinical and biological relevance to breast cancer including estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), BRCA1, p53, Phosphatidylinositol 3- kinase (PI3-K), phospho-Akt473 (P-Akt), the cell-cycle-associated molecule Ki-67, N-cadherin, E-cadherin, P-cadherin, epidermal growth factor receptor (EGFR), HER1, HER2, basal and luminal cytokeratins (CKs) (CK5/6, CK14, CK18, CK19) were also available as previously described [12-17].

The median age of the patients was 55 years (range 18–70 years) with a median overall survival of 133 months (range 4–247 months) and the median time of event-free survival of 115 months (range 2–247 months). Distant recurrence occurred in 453 cases (31%), 395 (21%) patients died from breast cancer, while 770 (44%) patients were alive at the end of follow-up.

This research was approved by Nottingham Research Ethics Committee 2 under the title of "Development of a molecular genetic classification of breast cancer". The Reporting Recommendations for Tumour

Marker Prognostic Studies (REMARK) criteria, recommended by McShane et al [18], throughout this study.

Immunohistochemistry

Four μm tissue TMA sections were immunohistochemically stained using the standard streptavidin–biotin complex method as previously described [18]. Water bath-induced method for retrieval of antigen was performed in EDTA buffer (Sigma), at pH 8.0. Primary mouse anti-CTEN (Abcam, ab57940) antibody [6], diluted 1:75 (optimum working dilution) in normal swine serum (NSS), was applied to each slide, and incubated for 45 minutes at room temperature. Strept ABC complex (Dako) diluted in 1:100 in NSS was applied and incubated for 55 minutes. After washing with TBS, slides were incubated using freshly prepared Diaminobenzidine (DAB; Sigma) solution for 10 minutes at room temperature. Slides were counterstained with haematoxylin (Dako). Negative controls were performed by omitting the primary antibody and substitution with a diluent. Whole tissue sections of HER2 positive cases were used as positive controls.

Scoring of immunohistochemical staining:

Histochemical score (H-score) was used to assess the intensity and percentage of stained cells [19]. Only staining of the invasive malignant cells within the tissue cores was considered. Staining intensity was scored from 0, 1, 2, or 3, (0; no staining, 1; weak, 2; moderate, 3; strong). The percentage of positive cells was subjectively estimated and scored from 0 to 100%. Percentage staining was multiplied by corresponding intensity with products added representing the H-score ranging from 0-300. Immunostaining was evaluated by two Histopathologists (MI, AA) without prior knowledge of the clinicopathologic data or the patient outcome.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc. USA). Determination of the optimal CTEN cut-off was performed using X-tile bioinformatics software, version 3.6.1, 2003–2005, (Yale University, USA) [20]. Association between the CTEN immunoreactivity expression and different clinicopathological parameters was evaluated using Chi-square test. Survival analyses were performed using the Kaplan–Meier plot with a log rank test to assess significance. Multivariate Cox hazard analysis was used to test the statistical independence and significance of the variables. A *P*-value of <0.05 was considered significant.

RESULTS

The staining pattern for CTEN in breast tumour cells was homogenous cytoplasmic staining (Figure 1). In contrast, no tumour cell membrane or stromal expression of CTEN was detected in any specimen. Normal breast epithelium, entrapped in some cores, did not show any CTEN staining (Figure 1). The X-tile bio-informatics software was used to define optimal cut-off points of CTEN H-score values (<85, negative/low; ≥85, moderate/strong expression). This program randomly divides the total patients' cohort into two separate equal training and validation sets ranked by patients' follow-up time. The optimal cut-points were determined by locating the brightest pixel on the X-tile plot diagram of the training set. Statistical significance was tested by validating the obtained cut-point to the validation set. Using this cut-off point, 90% of studied tumours showed positive expression for CTEN.

Correlation between CTEN expression and clinicopathological parameters

Table 1 summarises the associations between CTEN expression and clinicopathological parameters. CTEN expression was significantly associated with larger tumour size ($P=0.044$), higher tumour grade ($P=0.019$), axillary lymph node metastasis ($P=0.035$), occurrence of local and regional recurrence ($P=0.039$, $P=0.042$ respectively) and higher Nottingham prognostic index (NPI, $P=0.016$). A significant association ($P=0.012$) was found between CTEN expression and the histologic tumour type with more frequent expression of CTEN in invasive ductal carcinoma of no special type and invasive lobular carcinoma (92.9% and 92.6%, respectively). Furthermore, there was a significant association between increased CTEN expression and P-Akt, PIK3CA and N-cadherin ($P<0.001$). However, CTEN expression was not significantly associated with patients' age, menopausal status, vascular invasion, p53, HER2, EGFR, ER, MIB1, BRCA1 expression or any of Nielson's group subtypes [21] (Table 2).

Correlation between CTEN expression and patients' outcome

Kaplan-Meier curve demonstrated that patients with high CTEN expression had significantly poorer prognosis than those with low CTEN expression. Moreover, CTEN expression was associated with an increased likelihood to develop distant metastasis ($P=0.041$), but this was not significantly associated with any particular metastatic site. Univariate survival analyses showed that patients with tumours positive for CTEN had shorter BCSS ($P=0.004$, Figure 2 a). Positive expression of CTEN also showed association with shorter distant metastasis-free interval ($P=0.041$, Figure 2 b). However, multivariate Cox proportional hazard analysis including tumour size, histological grade, lymph node stage, adjuvant hormonal, chemotherapy and CTEN expression showed that CTEN expression was not an independent predictor of BCSS and distant metastasis-free interval ($P=0.213$, $P=0.874$ respectively, Table 3).

DISCUSSION

In this study, the expression of CTEN protein was evaluated using immunohistochemistry in a large well-characterised cohort of BC cases, to evaluate its biological and prognostic relevance. To the best of our knowledge, this is the largest study to date pertaining to CTEN protein expression in BC. We found that CTEN expression was significantly associated with poor prognostic clinicopathologic parameters, including larger tumour size, higher tumour grade, axillary lymph node metastasis and poor NPI. Moreover, our data showed that CTEN was more frequently over-expressed in invasive duct carcinoma NST and invasive lobular carcinoma, histologic types known to be associated with poor prognosis [22].

Recently, we have reported that CTEN acts as an oncogene in CRC and correlates with changed cell morphology and increased cell motility through repression of E-cadherin protein [6]. In support of this hypothesis, we have found a significant association, in the current study, between CTEN expression and lobular carcinoma that is known to be associated with loss of E-cadherin expression [23]. Although, CTEN expression did not show a significant association with E-cadherin protein expression in this series, there was a significant association with N-cadherin and a trend of positive association with P-cadherin expression; markers which have been reported to over-express frequently in high-grade invasive breast carcinomas, conferring an increased motility of breast cancer cells and being associated with tumour aggressiveness [24, 25]. A growing body of evidence has been shown that decreased expression of E-cadherin does not necessarily lead to induced cell motility in breast cancer cells. In addition, forced expression of E-cadherin in invasive, N-cadherin-expressing MDA-MB-435 cells does not reduce their motility or invasive capacity. However, forced expression of N-cadherin in non-invasive, E-cadherin-positive BT-20 cells would confer the capacity to invade on these cells, even though they continued to express high levels of E-cadherin. Taken together, these data demonstrate that BC invasiveness might be correlated with N-cadherin expression rather than lack of E-cadherin expression as reported in CRC [26].

Although CTEN expression showed a lack of association with other markers of aggressive tumour phenotype including EGFR and p53, it showed significant associations with PIK3CA and P-Akt which have been reported as oncogenic biomarkers associated with poor prognosis in BC [15, 27]. The close localisation of CTEN and PI3K at the cytoplasmic tail of integrin raises the possibility that CTEN-mediated effect may be related to PIK3. Recently, we found that CTEN forced expression and knock-down in CRC cell lines resulted in up-regulation and down-regulation of both focal adhesion kinase (FAK) and activated phosphorylated-FAK (P-FAK), respectively (unpublished data), indicating that CTEN may be considered as a key target in FAK-PI3K-Akt signalling pathway.

Our findings are in agreement with previous studies showing association between CTEN and both high tumour grade and the ability to metastasise to axillary lymph nodes [11]. Moreover, we showed that patients with CTEN over-expressing tumours had shorter BCSS and distant metastasis-free interval, supporting the oncogenic role of *CTEN* in BC. However, our data showed that CTEN expression was not significantly associated with increased expression of HER2 or EGFR and loss of ER, as shown previously [11]. This discrepancy may be due to the variations in the number of cases involved in both studies. They relatively investigated a small cohort of primary breast tumours compared with the present study.

In summary, we have shown that CTEN expression is a prognostic biomarker in BC, and its elevated expression is positively associated with markers of poor prognosis. The strong significant association between CTEN expression and both PIK3CA and P-Akt raised the possibility that *CTEN* may be involved in FAK-PI3K-Akt signalling pathway. Taken together, this information indicates that CTEN protein expression in breast cancer could have a role in prognostic assessment of breast cancer patients.

Legends:

Figure 1. CTEN protein expression in TMA cores: (A x100 and B x200) normal breast showing negative CTEN expression. (C x100 and D x200) low grade invasive ductal carcinoma with moderate CTEN expression. (E x100 and F x200) high grade invasive ductal carcinoma showing strong CTEN Expression.

Table 1. Correlations between CTEN expression and clinico-pathological parameters.

Table 2. Correlations between CTEN expression and other biomarkers.

Table 3. Cox-proportional hazards analysis for predictors of breast cancer specific survival and distant metastasis-free survival: effects of tumour grade, size, lymph node stage, chemotherapy, endocrine therapy and CTEN expression in whole series.

Figure 2. Kaplan–Meier plots for CTEN expression in invasive breast cancer showed that patients with tumours positive for CTEN had shorter BCSS (a), ($P=0.004$) and shorter distant metastasis-free interval (b), ($P=0.041$).

Acknowledgements

ARG was funded by the Breast Cancer Campaign.

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Table 1

Parameter	Low CTEN No.(%)	High CTEN No. (%)	χ^2	P value
Age				
<50	47 (9.4%)	451 (90.6%)	2.047	0.152
>50	66 (7.3%)	842 (92.7%)		
Menopausal status				
Premenopausal	52 (9.9%)	475 (90.1%)	3.820	0.064
Postmenopausal	61 (6.9%)	818 (93.1%)		
Tumour size				
<1.5 cm	80 (9.2%)	791 (90.8%)	4.254	0.044
≥1.5 cm	29 (5.1%)	501 (94.9%)		
Lymph Node stage				
1 (Negative)	82 (9.8%)	787 (90.2%)	5.842	0.035
2/3 (Definite)	21 (4.6%)	602 (96.4%)		
Grade				
1	30 (12.2%)	215 (87.8%)	7.555	0.019
2	38 (7.9%)	445 (92.1%)		
3	45 (6.7%)	629 (93.3%)		
NPI				

Good (<3.4)	45 (10.1%)	402 (89.9%)	7.578	0.016
Moderate (3.41–4.4)	59 (8.1%)	671 (91.9%)		
Poor (4.41–5)	9 (4%)	218 (96%)		
Vascular invasion				
Negative	81 (10%)	722 (90%)	1.185	0.286
Definite	42 (9.2%)	411 (90.8%)		
Local recurrence				
Negative	49 (12.1%)	381 (87.9%)	5.211	0.039
Positive	75 (9.1%)	822 (90.9%)		
Regional recurrence				
Negative	45 (10.1%)	442 (90.8%)	4.778	0.042
Positive	35 (6.7%)	769 (93.3%)		
Distant metastases				
Negative	80 (9.4%)	791 (90.6%)	4.780	0.041
Positive	36 (5.2%)	499 (94.8%)		
Histological type				
Ductal/NST	55 (7.1%)	735 (92.9%)	7.634	0.012
Lobular	10 (7.4%)	148 (92.6%)		
Tubular mixed	21 (11.3%)	229 (88.7%)		
Medullary	5 (13.2%)	33 (86.8%)		
Other special types ^a	14 (22.4%)	56 (77.6%)		

NPI = Nottingham prognostic index

^aIncludes Mucoïd and invasive cribriform carcinoma

Table 2

Parameter	Negative CTEN No.(%)	Positive CTEN No. (%)	χ^2	P value
ER				
Negative	24 (7.0%)	319 (93.0%)	0.650	0.477
Positive	68 (8.4%)	741 (91.6%)		
PR				
Negative	32 (5.7%)	525 (94.3%)	6.119	0.016
Positive	70 (9.5%)	668 (90.5%)		
AR				
Negative	27 (8.0%)	315 (91.0%)	0.669	0.457
Positive	58 (7.4%)	731 (93.6%)		
HER2				
Negative	99 (8.4%)	947 (91.6%)	1.623	0.654
Positive	11 (5.9%)	174 (94.1%)		
EGFR				
Negative	70 (7.6%)	855 (92.4%)	0.022	0.890
Positive	18 (7.9%)	211 (92.1%)		
P53				

Negative	74 (7.7%)	889 (92.3%)	0.092	0.814
Positive	25 (7.2%)	323 (92.8%)		
E-cadherin				
Negative	35 (6.9%)	471 (93.1%)	0.124	0.345
Positive	69 (8.5%)	739 (91.5%)		
P-cadherin				
Negative	47 (9.2%)	465 (90.8%)	5.147	0.058
Positive	37 (6.3%)	548 (93.7%)		
N-cadherin				
Negative	72 (11.1%)	578 (88.9%)	26.91	<0.001
Positive	11 (2.5%)	424 (97.5%)		
PIK3				
Negative	46 (14.2 %)	278 (85.8%)	37.22	<0.001
Positive	41 (4.3 %)	911 (95.7 %)		
Akt				
Negative	55 (10.2%)	551 (87.0%)	27.23	<0.001
Positive	14 (2.5%)	427 (97.5%)		
MIB1				
Low proliferative	38 (9.8%)	348 (90.2%)	2.567	0.131
High proliferative	51 (7.1%)	668 (92.9%)		
Nielsen groups				
Luminal	74 (8.4%)	804 (91.6%)	2.053	0.562
HER2	12 (6.2%)	183 (93.8%)		
Basal-like	3 (4.8%)	60 (95.2%)		

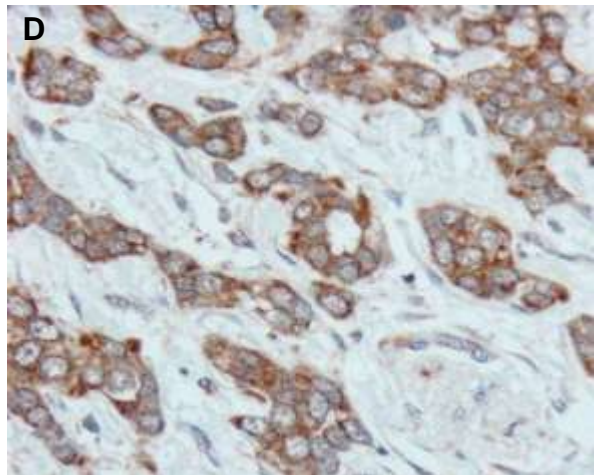
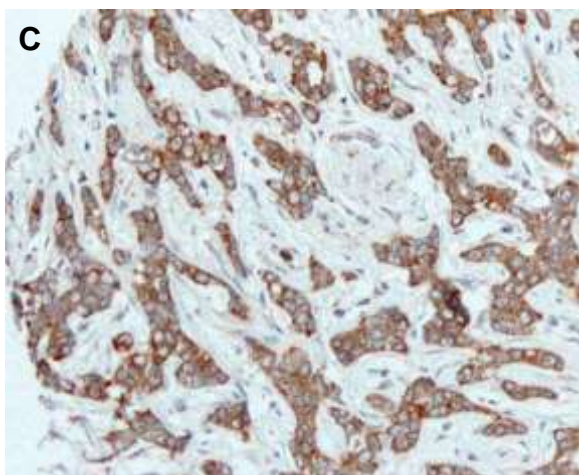
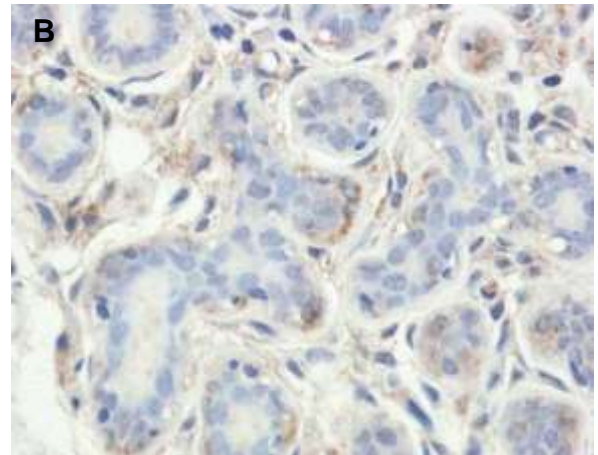
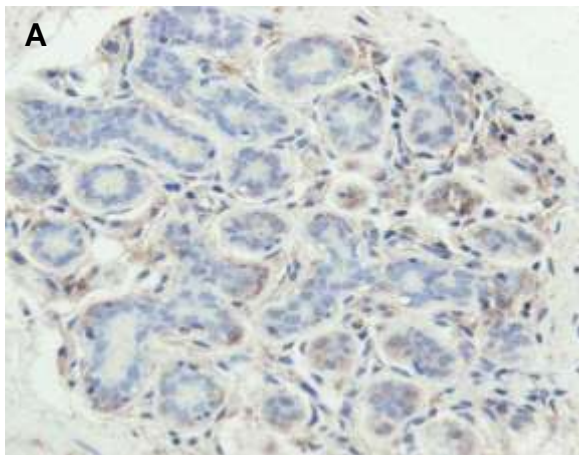
Table 3

Parameter	Hazard ratio	95% CI	P value
BCSS			
Tumour size	1.328	1.181–1.493	<0.001
Nodal stage	1.874	1.600–2.196	<0.001
Tumour grade	2.106	1.745–2.542	<0.001
Chemotherapy	0.679	0.511–0.901	0.007
Endocrine therapy	0.924	0.845–1.011	0.084
CTEN expression	1.379	0.832–2.287	0.213
DM-free interval			
Tumour size	1.408	1.254–1.581	<0.001

Nodal stage	1.899	1.633–2.208	<0.001
Tumour grade	1.682	1.425–1.987	<0.001
Chemotherapy	0.673	0.513–0.883	0.004
Endocrine therapy	0.931	0.856–1.012	0.093
CTEN expression	1.034	0.681–1.571	0.874

DM=distant metastasis

Figure 1



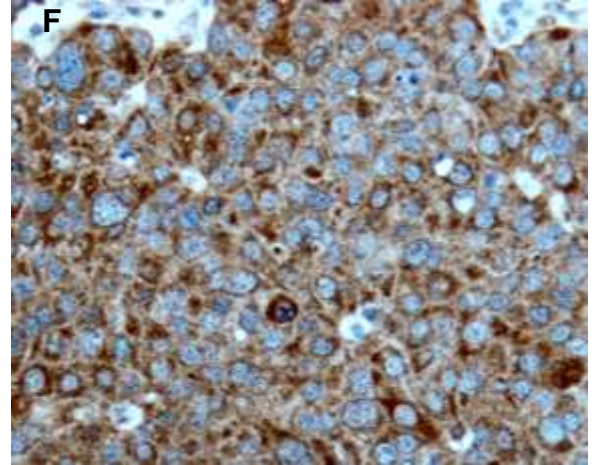
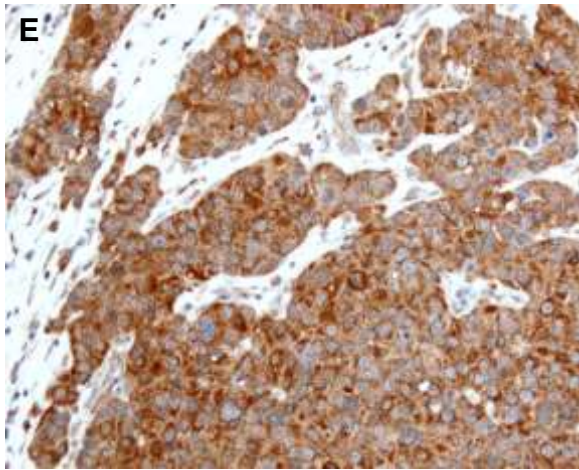
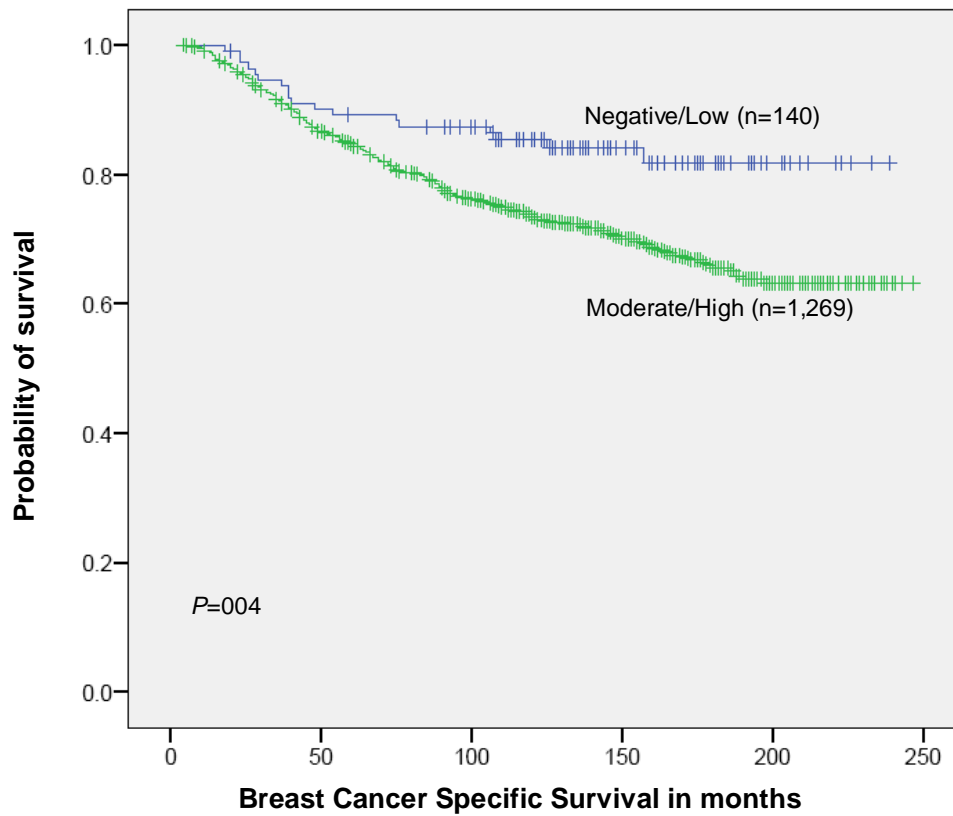


Figure 2

a



b

