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Cortactin gene amplification and expression in breast cancer: a chromogenic *in situ* hybridisation and immunohistochemical study

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ABSTRACT

Amplification of 11q13 is found in approximately 15% of breast cancers. Cyclin D1 (*CCND1*) has been reported to be the 'driver' of this amplicon, however multiple genes map to the smallest region of amplification of 11q13. Out of these genes, cortactin (*CTTN*) has been shown to be consistently overexpressed at the mRNA level in tumours harbouring 11q13 amplification. The aims of this study were to define whether *CTTN* is consistently co-amplified with the main core of the 11q13 amplicon, whether it is consistently overexpressed when amplified and to determine correlations between *CTTN* amplification and overexpression with clinicopathological features of breast cancers and survival of breast cancer patients. *CTTN* and *CCND1* chromogenic *in situ* hybridisation (CISH) probes and a validated monoclonal antibody against *CTTN* were applied to a tissue microarray of a cohort of breast cancers from patients treated with anthracycline-based chemotherapy. *CTTN* and *CCND1* amplification was found in 12.3% and 12.4% of cases, respectively. All cases harbouring *CTTN* amplification also displayed *CCND1* amplification. High expression of *CTTN* was found in 10.8% of cases and was associated with *CTTN* amplification, expression of 'basal' markers and topoisomerasell α . Exploratory subgroup analysis of tumours devoid of 11q13 amplification revealed that high expression of *CTTN* in the absence of *CTTN* gene amplification was associated with lymph node negative disease, lack of hormone receptors and FOXA1, expression of 'basal' markers, high Ki-67 indices, p53 nuclear expression, and basal-like and triple negative phenotypes. *CTTN* expression and *CTTN* gene amplification were not associated with disease-free, metastasis-free and overall survival. In conclusion, *CTTN* is consistently co-amplified with *CCND1* and expressed at higher levels in breast cancers harbouring 11q13 amplification, suggesting that *CTTN* may also constitute one of the drivers of this amplicon. *CTTN* expression is not associated with the outcome of breast cancer patients treated with anthracycline-based chemotherapy.

Key words: breast cancer, chromogenic *in situ* hybridisation, immunohistochemistry, 11q13 amplification, *CCND1*, *CTTN*

INTRODUCTION

The cortactin gene (*CTTN*, formerly known as *EMS1*) maps to 11q13 [1-3] and produces a cytoplasmic protein that is a key regulator of actin polymerisation through binding and activating the actin regulated protein complex (ARP2/3) [1]. *CTTN* has been shown to play a critical role in various actin-mediated processes such as cell migration, adhesion and receptor mediated endocytosis [2, 4].

11q13 amplification is found in up to 15% of breast cancers [5-8] and has been suggested to be associated with oestrogen receptor (ER)-positive disease [9-11], lobular histological type [11, 12], and poor prognosis [11, 13-16]. The 11q13 amplicon is complex and encompasses multiple cores [6, 8, 14, 16-23]. Out of the genes mapping to this amplicon, cyclin D1 (*CCND1*) has been suggested as the main 'driver' (i.e. the gene whose expression would confer advantage to cancer cells harbouring its amplification) [9, 15, 16, 18, 19, 23-25]. Contrary to the initial concept that each amplicon would have one 'driver', recent studies have demonstrated that within each amplicon, there may be more than one gene whose expression and activity is selectively required for the survival of cancer cells harbouring its amplification. For instance, on the 17q12 amplicon, *HER2* has been shown to be the main 'driver'; however, expression of *GRB7* and *STARD3* has also been shown to be required for the survival of cancer cells with 17q12 amplification [26].

We [6, 27-30] and others [20, 31] have demonstrated that in tumours with 11q13 amplification, *CTTN* maps to the smallest region of amplification of the 11q13 amplicon and is consistently overexpressed at the mRNA level when amplified. This observation would suggest that *CTTN* may constitute a potential amplicon driver. *CTTN* has been shown to be overexpressed in many types of human cancers, including head and neck and esophageal squamous cell carcinomas [21, 32, 33], hepatocellular cancer [34], and breast cancer [8, 19]. In agreement with these observations, *in vitro* and *in vivo* studies of breast cancer [35-37], hepatocellular carcinoma [34], and head and neck squamous cell carcinoma (HNSCC) [33,

38, 39] have suggested that *CTTN* plays a role in tumour invasion and metastasis. In breast cancer, *CTTN* overexpression is often reported to be underpinned by chromosomal amplification of the 11q13.3 region [8, 19], however, overexpression has also been reported in tumours without amplification [8, 19].

The aims of this study were i) to define whether *CTTN* is consistently co-amplified with the main core of the 11q13 amplicon, ii) to determine the correlations between *CTTN* amplification and overexpression with clinicopathological features of breast cancers and survival of breast cancer patients and iii) to test whether tumours with 11q13 amplification consistently overexpress *CTTN* at the protein level.

MATERIAL AND METHODS

Tissue microarrays

A cohort of 245 patients with invasive breast cancer (185 invasive ductal carcinomas, 27 invasive lobular carcinomas, 25 invasive mixed carcinomas and 8 invasive breast carcinomas of other special types) treated with therapeutic surgery followed by adjuvant anthracycline-based chemotherapy were included in a tissue microarray (TMA) containing two replicate 0.6 mm cores. All patients were diagnosed and managed at the Royal Marsden Hospital, London, UK, between 1994 and 2000. All patients were primarily treated with surgery (69 mastectomy and 156 wide local excision) followed by anthracycline-based chemotherapy. Adjuvant endocrine therapy was prescribed for patients with ER-positive tumours (tamoxifen alone in 96.4% of the patients for the available follow-up period). Complete follow-up was available for 244 patients, ranging from 0.5 to 125 months (median = 67 months, mean = 67 months). Tumours were graded according to a modified Bloom-Richardson scoring system [40] and size was categorised according to the TNM staging [41]. The study was approved by the Royal Marsden Hospital Ethics Committee.

Chromogenic In Situ Hybridisation

We developed an in-house generated *CTTN* probe composed of three bacterial artificial chromosomes (BACs, RP11-240K12, RP11-517E18 and RP11-347I13), which map to 69,867Kb – 70,330Kb on chromosome 11 and encompass the genes *CTTN*, *ANO1*, *FADD*, *PPFA1* and part of *SHANK2*. BACs were digoxigenin labelled as previously described [42]. In addition, chromogenic *in situ* hybridisation (CISH) with probes for *CCND1*, *MYC*, *HER2* and *TOP2A* was performed as previously described [9, 15, 42]. The *CCND1* probe maps to the smallest region of amplification on 11q13 as previously defined by high resolution microarray-based comparative genomic hybridisation analysis [6, 27-30]. Hybridisation using the digoxigenin-labelled in-house probe for *CTTN* and the ready-to-use digoxigenin-labelled SpotLight amplification probes for *CCND1*, *MYC*, *HER2* and *TOP2A* amplification (Invitrogen, Carlsbad, California) were hybridised to sections of the TMAs as previously described [42]. CISH experiments were analysed by three of the authors (MAL-G, FCG and JSR-F) on a multiheaded microscope with observers blinded to *CTTN* expression levels and clinicopathological features. Amplification was defined as >5 signals per nucleus or large gene copy clusters in more than 50% of cancer cells [9, 15, 42]. [The results of the correlations between *CCND1* gene amplification and clinicopathological features and patients' survival in this cohort of breast cancers are reported elsewhere \[9\].](#)

Immunohistochemistry

Sections of the TMA were cut at 4 µm and mounted on polylysine-coated slides and immunohistochemistry was performed with antibodies raised [against *CTTN*](#) (clone 30, dilution 1:1000, BD Transduction Laboratories, cat no. 610049). Slides were blocked in 1.5% H₂O₂ in methanol for 10 minutes and detection achieved using the Vector avidin-biotin complex (ABC) system (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's recommendations. The antibody was applied for 60 minutes following heat-induced antigen retrieval for 18 minutes in citrate buffer pH 6.0 in a microwave oven. Development was carried out using the chromogen 3,3'-Diaminobenzidine (Dako, Glostrup, Denmark). Positive controls included formalin fixed pellets of ovarian and breast cancer cell

lines, whose CTTN mRNA and protein expression levels were determined by analysis of unfixed cells with Illumina WG6 v2 expression arrays [43, 44] or in-house cDNA arrays, and western blotting using the same antibody employing a previously validated method [43], respectively. Negative controls included omission of the primary antibody and substitution of the primary antibody by IgG-matched control.

CTTN immunohistochemical intensity and distribution were semiquantitatively scored by three of the authors (MAL-G, FCG and JSR-F) on a multiheaded microscope using the 'quick score' system [45] (intensity 0-3 x distribution 0-6) with observers blinded to 11q13 status and clinicopathological features. Membranous and cytoplasmic expression was considered specific. Tumours were then categorised in three groups according to their CTTN expression levels as low <5, intermediate 5-12 and >12 as high. The details of the immunohistochemical methods and scoring systems for ER, progesterone receptor (PR), HER2, epidermal growth factor receptor (EGFR), cytokeratin (Ck) 14, Ck 5/6 and Ck 17, Ki-67, p53, topoisomerase II alpha, caveolin-1 (CAV1) and caveolin-2 (CAV2), FOXA1, E-cadherin, CD44, Bcl2, nestin and cyclin D1 detection are described elsewhere [46-51] and summarised in Supplementary Table 1. Based upon the expression of HER2, ER, Ck 5/6 and EGFR, tumours were classified into basal, HER2 and luminal according to the immunohistochemical panel proposed by Nielsen et al. [52].

Statistical Analysis

The SPSS statistical software package was used for all statistical analysis. Correlations between categorical variables were performed using the chi-square test and Fisher's exact test. Metastasis-free survival (MFS) was expressed as the number of months from diagnosis to the occurrence of distant relapse. Disease-free survival (DFS) was expressed as the number of months from diagnosis to the occurrence of distant, local relapse or death (disease-related death). Overall survival was expressed as the number of months from diagnosis to the occurrence of breast-cancer related death. Cumulative survival probabilities

were calculated using the Kaplan–Meier method. Differences between survival rates were tested with the log-rank test. A p value of 0.05 was considered as statistically significant.

RESULTS

CTTN is co-amplified with CCND1

To test whether the *CTTN* gene maps to the smallest region of amplification on 11q13, we subjected a series of breast cancers to CISH with probes for *CTTN* and *CCND1*, which maps to the smallest region of amplification on chromosome 11. CISH for *CTTN* and *CCND1* was interpretable in 146 and 242 cases, respectively. Eighteen (12.3%) and 30 (12.4%) cases harboured amplification of *CTTN* and *CCND1*, respectively (Table 1). All cases with *CTTN* amplification displayed *CCND1* amplification and all cases harbouring *CCND1* amplification and interpretable results for *CTTN* were also co-amplified. Taken together, these CISH results validate the results of previous microarray-based comparative genomic hybridisation analysis which suggested that *CTTN* is part of the smallest region of amplification [6, 19, 27] of the 11q13 amplicon and is consistently co-amplified with *CCND1*.

CTTN amplification

Amplifications of the *CTTN* gene were found in 18 out of 146 cases (12.3%). Table 2 summarises the correlations between *CTTN* amplification and clinicopathological features and immunohistochemical findings in breast carcinomas. In brief, *CTTN* amplification showed a statistically significant association with cyclin D1 expression (Table 2).

CTTN expression

The correlations between *CTTN* expression and clinicopathological features and immunohistochemical findings in breast carcinomas are summarised in Table 3. Briefly, out of the 245 cases included in the TMAs, cores from 59 cases were either lost/ fragmented in the immunohistochemical procedure or did not have invasive tumour. Out of the 186 remaining cores, 25 (13.4%) cases displayed low (quick score <5) expression of *CTTN*, 141

(75.8%) cases had intermediate expression (quick score 5-12) and 20 (10.8%) cases exhibited high expression (quick score >12) of CTTN (Figure 1).

Expression of CTTN was significantly associated with expression of 'basal' markers, including Ck14, Ck17 and Caveolin 1 expression, topoisomerase II α expression but not *TOPO2A* gene amplification, and normal levels of E-cadherin expression (Table 3).

As described for the associations between *CTTN* amplification and mRNA expression [6], CTTN protein expression was strongly associated with *CTTN* amplification (Mann-Whitney U test, $p=0.00002$, Table 1).

Expression of CTTN in breast cancers lacking 11q13 amplification

To investigate the associations between CTTN expression levels and clinicopathological features in the absence of 11q13 amplification, we removed cases harbouring 11q13 amplification ($n=30$; i.e. all cases harbouring amplification of *CTTN* and/ or *CCND1*) and cases where 11q13 status could not be defined by at least one of the probes ($n=3$). In this series of 212 breast cancers, data on CTTN expression was available for 156 cases, of which 24 cases were classified as CTTN low, 126 as intermediate and 6 as high (Table 4).

Expression of CTTN in this subgroup of cancers was significantly associated with lymph node negative disease, lack of markers of luminal differentiation (i.e. ER, PR and FOXA1), expression of 'basal' markers (EGFR, Ck5/6, Ck14 and Ck17, Caveolin 1, Caveolin 2, and nestin), high Ki-67 labelling indices and p53 nuclear expression. All cases with high CTTN expression lacked *HER2* gene amplification. Not surprisingly, CTTN expression in the absence of 11q13 amplification was significantly more prevalent in breast cancers of basal-like and triple negative phenotype (Table 4).

Survival analysis

Univariate survival analysis failed to show any association between MFS, DFS and OS for CTTN expression, *CTTN* amplification or CTTN expression among cases without 11q13-amplification.

DISCUSSION

Amplification of *CCND1* is reported to be found in up to 15% of invasive breast cancers. Here, we have confirmed that amplification of *CTTN* is found in 12.3% of cases, whereas amplification of *CCND1* was found in 12.4% of cases. Importantly, in all cases harbouring *CTTN* amplification *CCND1* was also co-amplified. Likewise, in all cases where both *CCND1* and *CTTN* were interpretable, *CCND1* was always found to be co-amplified with *CTTN*. These results corroborate previous observations derived from microarray-based comparative genomic hybridisation studies [6, 27] that suggested that *CTTN* is part of the smallest region of amplification of the *CCND1* amplicon on 11q13.

High expression of CTTN has been previously reported to be present in 27.1% of breast cancers at the protein level [19] and in 18.5% at the mRNA level [8]. Here, high expression, as defined by immunohistochemical analysis, was observed in 10.8% of cases. Within cases harbouring *CTTN* amplification, CTTN is consistently expressed at higher levels, supporting the evidence of previous studies on breast cancer [8, 19, 20, 27, 31] and other tumour types [21, 32, 33] that *CTTN* may be one of the 11q13 amplicon drivers. In fact, out of our 17 cases where both *CTTN* amplification and expression could be analysed, only 1 case had low CTTN expression. When 11q13 amplification, as defined by *CTTN* or *CCND1* amplification, was considered, out of the 27 cases where both 11q13 amplification and CTTN levels could be analysed, 48% of cases with 11q13 amplification displayed high protein levels of CTTN. This is in agreement with Lundgren et al. [19], who reported that 44% of cases with 11q13 amplification display CTTN overexpression.

Previous studies have suggested that CTTN mRNA levels would be of higher levels in ER-positive breast cancers [8]; however, this observation may have stemmed from the higher prevalence of 11q13 amplification in ER-positive breast cancers and the correlation between 11q13 amplification and CTTN mRNA overexpression [5, 6, 27, 31, 53, 54]. At the protein level, Lundgren et al. did not find an association between ER status and CTTN expression [19], which is in accordance with our observations. However, low expression of CTTN was predictive of response to tamoxifen treatment, suggesting that CTTN would be expressed at higher levels in ER-negative than in ER-positive cancers [19]. Here we demonstrate that when cases harbouring 11q13 amplification were excluded, CTTN was indeed expressed at higher levels in ER-negative breast cancers of triple negative and basal-like phenotype.

We have also observed an association between high expression of CTTN and EGFR overexpression, which was previously reported in HNSCC [55] but not in breast cancers. This observation may be explained by results from studies in HNSCC cell lines [56, 57] which indicate that CTTN could participate in receptor-mediated endocytosis of EGFR. Thus, CTTN overexpression attenuated ligand-induced downregulation of EGFR and led to sustained activation of EGFR signalling. Conversely, reduction of CTTN expression in an 11q13-amplified HNSCC cell line by RNA interference accelerated EGFR degradation [57].

Recent studies have demonstrated that CTTN constitutes an important signalling pathway that is functionally linked to E-cadherin adhesion [58]. CTTN, as a target of E-cadherin-activated Src-signalling, was necessary to preserve the integrity of cadherin contacts and the perijunctional actin cytoskeleton [58]. Consistent with this observation, here we demonstrate that cases with higher levels of CTTN expression displayed normal levels of E-cadherin expression significantly more frequently.

Here we describe a significantly higher prevalence of overexpression of CTTN expression in a subgroup of basal-like and triple negative cancers when compared to other breast cancers

lacking *CTTN* and/ or 11q13 amplification. *CTTN* has been shown to play pivotal roles in the migration and invasion of cancer cells [39]. Consistent with these observations, basal-like and triple negative breast cancer cell lines have been shown to have a more invasive phenotype than non-triple negative breast cancer cell lines [59]. This is not surprising, given that *CTTN* is phosphorylated by SRC, a tyrosine kinase whose *in vitro* inhibition is lethal in a subgroup of basal-like and triple negative breast cancers [60, 61] and shown to be involved in breast cancer cell migration and invasion [62]. Further functional studies to investigate whether *CTTN* overexpression in a subgroup of basal-like and triple negative breast cancers is driven by a global transcriptomic programme that promotes a more invasive phenotype or if it is a consequence of SRC activation are warranted.

Despite the numerous lines of evidence to demonstrate that *CTTN* may be one of the drivers of the 11q13 amplicon and that *CTTN* has oncogenic properties, previous studies have failed to find an association between *CTTN* expression and outcome in breast cancer [8, 19]. In our analysis of a homogeneous cohort of breast cancer patients treated with anthracycline-based chemotherapy ± endocrine therapy, which would be representative of up to 70% of all breast cancer patients, no associations between *CTTN* expression or *CTTN* gene amplification and outcome were found. Our findings should be interpreted with caution, however, as this is a retrospective, single institution study. Furthermore, the prevalence of cases displaying *CTTN* overexpression and *CTTN* gene amplification is relatively low (<15%); therefore, it is plausible that in larger cohorts with longer follow-up, associations with survival may be identified. Further studies testing the prognostic impact of *CTTN* gene amplification in larger cohorts of breast cancer patients are warranted.

In conclusion, high expression of *CTTN* is present in a small proportion (~12%) of invasive breast cancers and is strongly associated with *CTTN* gene amplification and co-amplification with *CCND1*. We also demonstrate that all cases harbouring *CTTN* amplification also displayed *CCND1* amplification and that >90% of cases with *CTTN* amplification display

moderate-to-high levels of CTTN expression, providing another line of evidence to suggest that *CTTN* may be one of the drivers of the 11q13 amplicon. CTTN expression and *CTTN* gene amplification failed to show an association with outcome in a cohort of breast cancers homogeneously treated with anthracycline-based chemotherapy. In cases devoid of 11q13 (i.e. *CTTN* and/ or *CCND1*) amplification, CTTN was expressed at higher levels in ER-negative cancers of triple negative and basal-like phenotype. These observations illustrate the importance of reducing the complexity of breast cancers *a priori* in studies aiming to determine whether a given gene is consistently overexpressed when amplified, as the molecular subtype of the tumours may influence the baseline expression levels of a gene. Finally, our data warrant further studies [to define whether *CTTN* is one of the 'drivers' of the 11q13 amplicon in breast cancer](#) and to determine whether basal-like cancer cells or luminal cancer cells with 11q13 amplification would require expression of CTTN for their survival and migration.

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Table 1: Correlation between *CTTN* gene amplification and *CCND1* gene amplification and cortactin protein expression.

		<i>CTTN</i>		
		Not amplified	Amplified	P value
<i>CCND1</i>				<0.00001*
	Not amplified	128	0	
	Amplified	0	18	
CTTN expression				0.00002**
	0	5	0	
	2	1	0	
	3	1	0	
	4	9	1	
	5	21	0	
	6	22	2	
	8	14	0	
	9	1	1	
	10	18	1	
	12	20	3	
	15	3	4	
	18	1	5	

*: Fisher's exact test; **: Mann-Whitney U test.

Table 2: Correlations between *CTTN* gene amplification and clinicopathological features, immunohistochemical markers and amplification of key oncogenes.

Parameter	n	<i>CTTN</i> not amplified	<i>CTTN</i> amplified	p value
Size (TNM)	146			0.22863*
pT1		68 (90.7)	7 (9.3)	
pT2		52 (86.7)	8 (13.3)	
pT3		8 (72.7)	3 (27.3)	
Grade	143			0.34766*
1		13 (100)	0 (0)	
2		32 (88.9)	4 (11.1)	
3		81 (86.2)	13 (13.8)	
Histological type	144			0.62810*
IDC		96 (88.9)	12 (11.1)	
ILC		17 (85)	3 (15)	
Mixed		10 (100)	0 (0)	
Other		5 (83.3)	1 (16.7)	
LVI	145			0.11431**
Absent		47 (94)	3 (6)	
Present		80 (84.2)	15 (15.8)	
LN metastasis	141			0.42355**
Absent		43 (91.5)	4 (8.5)	
Present		81 (86.2)	13 (13.8)	
ER	146			1.00000**
Negative		27 (90)	3 (10)	
Positive		101 (87.1)	15 (12.9)	
PR	146			0.39949**
Negative		37 (92.5)	3 (7.5)	
Positive		91 (85.8)	15 (14.2)	
HER2	146			0.08670**
Negative		109 (90.1)	12 (9.9)	
Positive		19 (76)	6 (24)	
EGFR	146			0.36215**
Negative		116 (86.6)	18 (13.4)	
Positive		12 (100)	0 (0)	
Ck 14	145			0.59605**
Negative		119 (86.9)	18 (13.1)	
Positive		8 (100)	0 (0)	
Ck 5/6	139			0.36069**
Negative		110 (86.6)	17 (13.4)	
Positive		12 (100)	0 (0)	
Ck 17	143			1.00000**
Negative		111 (87.4)	16 (12.6)	
Positive		14 (87.5)	2 (12.5)	
Basal keratins	145			1.00000**
Negative		110 (87.3)	16 (12.7)	
Positive		17 (89.5)	2 (10.5)	
Any basal marker	145			0.73806**
Negative		105 (86.8)	16 (13.2)	
Positive		22 (91.7)	2 (8.3)	

p53		138			0.79130**
	Negative		81 (86.2)	13 (13.8)	
	Positive		39 (88.6)	5 (11.4)	
Ki-67		140			0.22714*
	Low		52 (92.9)	4 (7.1)	
	Intermediate		51 (82.3)	11 (17.7)	
	High		19 (86.4)	3 (13.6)	
HER2 – CISH		138			0.08831**
	Not amplified		102 (89.5)	12 (10.5)	
	Amplified		18 (75)	6 (25)	
TOPO2A - CISH		139			0.06695**
	Not amplified		112 (88.9)	14 (11.1)	
	Amplified		9 (69.2)	4 (30.8)	
TOPO2A - IHC		136			1.00000**
	Low		59 (86.8)	9 (13.2)	
	High		59 (86.8)	9 (13.2)	
Molecular subtypes***		140			0.07025*
	Basal-like		17 (100)	0 (0)	
	HER2		19 (76)	6 (24)	
	Luminal		86 (87.8)	12 (12.2)	
Triple negative		144			0.13037**
	No		107 (85.6)	18 (14.4)	
	Yes		19 (100)	0 (0)	
MYC – CISH		127			0.11304**
	Not amplified		99 (87.6)	14 (12.4)	
	Amplified		10 (71.4)	4 (28.6)	
Caveolin 1		146			1.00000**
	Negative		116 (87.2)	17 (12.8)	
	Positive		12 (92.3)	1 (7.7)	
Caveolin 2		136			0.60659**
	Negative		109 (85.8)	18 (14.2)	
	Positive		9 (100)	0 (0)	
FOX1A		121			0.56167**
	Negative		23 (82.1)	5 (17.9)	
	Positive		80 (86)	13 (14)	
E-Cadherin		137			0.78599*
	Absent		34 (89.5)	4 (10.5)	
	Reduced		9 (90)	1 (10)	
	Normal		76 (85.4)	13 (14.6)	
Nestin		132			0.21629**
	Negative		102 (86.4)	16 (13.6)	
	Positive		14 (100)	0 (0)	
Bcl2		123			0.28558**
	Negative		41 (91.1)	4 (8.9)	
	Positive		65 (83.3)	13 (16.7)	
CD44		129			0.49573*
	Low		24 (88.9)	3 (11.1)	
	Intermediate		21 (80.8)	5 (19.2)	
	High		68 (89.5)	8 (10.5)	
Cyclin D1		138			0.03955*

Low	17 (100)	0 (0)
Intermediate	25 (96.2)	1 (3.8)
High	78 (82.1)	17 (17.9)

*: Chi-squared test; **: Fisher's exact test; *** Breast cancer molecular subtypes as defined by Nielsen et al. [52] criteria.

CISH: chromogenic *in situ* hybridisation; Ck: cytokeratin; EGFR: epidermal growth factor receptor; ER: oestrogen receptor; IHC: immunohistochemistry; LN: lymph node; LVI: lympho-vascular invasion.

Table 3: Correlations between cortactin expression and clinicopathological features, immunohistochemical markers and amplification of key oncogenes.

Parameter	n	CTTN Low	CTTN Intermediate	CTTN High	p value*
Size (TNM)	185				0.08760
pT1		11 (11.3)	76 (78.4)	10 (10.3)	
pT2		12 (15.8)	58 (76.3)	6 (7.9)	
pT3		2 (16.7)	6 (50)	4 (33.3)	
Grade	181				0.56311
1		2 (10.5)	17 (89.5)	0 (0)	
2		7 (13.5)	39 (75)	6 (11.5)	
3		16 (14.5)	81 (73.6)	13 (11.8)	
Histological type	183				0.40273
IDC		14 (10.6)	105 (79.5)	13 (9.8)	
ILC		7 (24.1)	17 (58.6)	5 (17.2)	
Mixed		2 (14.3)	10 (71.4)	2 (14.3)	
Other		1 (12.5)	7 (87.5)	0 (0)	
LVI	184				0.34389
Absent		9 (15.3)	41 (69.5)	9 (15.3)	
Present		16 (12.8)	98 (78.4)	11 (8.8)	
LN metastasis	179				0.20491
Absent		12 (19)	43 (68.3)	8 (12.7)	
Present		12 (10.3)	92 (79.3)	12 (10.3)	
ER	185				0.30084
Negative		2 (6.1)	26 (78.8)	5 (15.2)	
Positive		23 (15.1)	114 (75)	15 (9.9)	
PR	185				0.10020
Negative		3 (6.1)	38 (77.6)	8 (16.3)	
Positive		22 (16.2)	102 (75)	12 (8.8)	
HER2	185				0.73544
Negative		22 (13.9)	118 (74.7)	18 (11.4)	
Positive		3 (11.1)	22 (81.5)	2 (7.4)	
EGFR	185				0.44540
Negative		24 (14.3)	127 (75.6)	17 (10.1)	
Positive		1 (5.9)	13 (76.5)	3 (17.6)	
Ck 14	184				0.00426
Negative		25 (14.7)	130 (76.5)	15 (8.8)	
Positive		0 (0)	9 (64.3)	5 (35.7)	
Ck 5/6	177				0.26880
Negative		23 (14.3)	121 (75.2)	17 (10.6)	
Positive		0 (0)	14 (87.5)	2 (12.5)	
Ck 17	182				0.03541
Negative		24 (14.9)	122 (75.8)	15 (9.3)	
Positive		0 (0)	16 (76.2)	5 (23.8)	
Basal keratins	184				0.01412
Negative		25 (15.7)	120 (75.5)	14 (8.8)	
Positive		0 (0)	19 (76)	6 (24)	
Any basal marker	184				0.05904
Negative		24 (15.5)	117 (75.5)	14 (9)	
Positive		1 (3.4)	22 (75.9)	6 (20.7)	
p53	174				0.50510
Negative		19 (15.4)	92 (74.8)	12 (9.8)	

Ki-67	Positive	174	5 (9.8)	39 (76.5)	7 (13.7)	0.13136
	Low		12 (16.4)	58 (79.5)	3 (4.1)	
	Intermediate		11 (14.1)	56 (71.8)	11 (14.1)	
	High		1 (4.3)	18 (78.3)	4 (17.4)	
HER2 – CISH		177				0.83162
TOPO2A - CISH	Not amplified	179	21 (14)	112 (74.7)	17 (11.3)	0.69125
	Amplified		4 (14.8)	21 (77.8)	2 (7.4)	
TOPO2A - IHC	Not amplified	166	21 (13)	121 (75.2)	19 (11.8)	0.01437
	Amplified		3 (16.7)	14 (77.8)	1 (5.6)	
Molecular subtypes**	Low	181	17 (21)	57 (70.4)	7 (8.6)	0.59085
	High		5 (5.9)	69 (81.2)	11 (12.9)	
Triple negative	Basal-like	183	1 (4.3)	18 (78.3)	4 (17.4)	0.44713
	HER2		4 (14.8)	21 (77.8)	2 (7.4)	
	Luminal		19 (14.5)	98 (74.8)	14 (10.7)	
CCND1 - CISH	No	183	23 (14.3)	122 (75.8)	16 (9.9)	<0.00001
	Yes		2 (9.1)	16 (72.7)	4 (18.2)	
MYC – CISH	Not amplified	158	24 (15.4)	126 (80.8)	6 (3.8)	0.45331
	Amplified		1 (3.7)	13 (48.1)	13 (48.1)	
Caveolin 1	Not amplified	185	20 (14.3)	106 (75.7)	14 (10)	0.03959
	Amplified		1 (5.6)	14 (77.8)	3 (16.7)	
Caveolin 2	Negative	168	24 (14.4)	128 (76.6)	15 (9)	0.02434
	Positive		1 (5.6)	12 (66.7)	5 (27.8)	
FOX1A	Negative	149	23 (14.7)	118 (75.6)	15 (9.6)	0.22441
	Positive		0 (0)	8 (66.7)	4 (33.3)	
E-Cadherin	Negative	171	6 (16.7)	23 (63.9)	7 (19.4)	0.04565
	Positive		15 (13.3)	87 (77)	11 (9.7)	
Nestin	Absent	160	13 (25)	34 (65.4)	5 (9.6)	0.48634
	Reduced		2 (20)	8 (80)	0 (0)	
	Normal		9 (8.3)	86 (78.9)	14 (12.8)	
Bcl2	Negative	151	20 (14.2)	106 (75.2)	15 (10.6)	0.78453
	Positive		1 (5.3)	15 (78.9)	3 (15.8)	
CD44	Negative	161	8 (15.4)	39 (75)	5 (9.6)	0.82229
	Positive		13 (13.1)	73 (73.7)	13 (13.1)	
Cyclin D1	Low	174	6 (18.2)	23 (69.7)	4 (12.1)	0.71721
	Intermediate		4 (13.3)	24 (80)	2 (6.7)	
	High		13 (13.3)	72 (73.5)	13 (13.3)	
	Low		2 (10.5)	14 (73.7)	3 (15.8)	
	Intermediate	4 (11.1)	30 (83.3)	2 (5.6)		
	High	17 (14.3)	88 (73.9)	14 (11.8)		

*: Chi-squared test; **: Breast cancer molecular subtypes as defined by Nielsen et al. [52] criteria.

CISH: chromogenic *in situ* hybridisation; Ck: cytokeratin; EGFR: epidermal growth factor receptor; ER: oestrogen receptor; IHC: immunohistochemistry; LN: lymph node; LVI: lympho-vascular invasion.

Table 4: Correlations between cortactin expression and clinicopathological features, immunohistochemical markers and amplification of key oncogenes in cases lacking *CTTN* and/ or *CCND1* gene amplification.

Parameter	n	CTTN Low	CTTN Intermediate	CTTN High	p value*
Size (TNM)	156				0.66290
pT1		10 (12)	70 (84.3)	3 (3.6)	
pT2		12 (18.2)	51 (77.3)	3 (4.5)	
pT3		2 (28.6)	5 (71.4)	0 (0)	
Grade	154				0.32648
1		2 (10.5)	17 (89.5)	0 (0)	
2		7 (16.7)	35 (83.3)	0 (0)	
3		15 (16.1)	72 (77.4)	6 (6.5)	
Histological type	156				0.31385
IDC		14 (12.2)	96 (83.5)	5 (4.3)	
ILC		7 (31.8)	15 (68.2)	0 (0)	
Mixed		2 (16.7)	9 (75)	1 (8.3)	
Other		1 (14.3)	6 (85.7)	0 (0)	
LVI	155				0.59721
Absent		9 (17.3)	40 (76.9)	3 (5.8)	
Present		15 (14.6)	85 (82.5)	3 (2.9)	
LN metastasis	151				0.01427
Absent		11 (20.4)	38 (70.4)	5 (9.3)	
Present		12 (12.4)	84 (86.6)	1 (1)	
ER	156				0.00517
Negative		2 (6.7)	24 (80)	4 (13.3)	
Positive		22 (17.5)	102 (81)	2 (1.6)	
PR	156				0.00220
Negative		3 (7.1)	34 (81)	5 (11.9)	
Positive		21 (18.4)	92 (80.7)	1 (0.9)	
HER2	156				0.41515
Negative		22 (16.3)	107 (79.3)	6 (4.4)	
Positive		2 (9.5)	19 (90.5)	0 (0)	
EGFR	156				0.00496
Negative		23 (16.5)	113 (81.3)	3 (2.2)	
Positive		1 (5.9)	13 (76.5)	3 (17.6)	
Ck 14	155				0.00000
Negative		24 (17)	116 (82.3)	1 (0.7)	
Positive		0 (0)	9 (64.3)	5 (35.7)	
Ck 5/6	149				0.02572
Negative		23 (17.3)	107 (80.5)	3 (2.3)	
Positive		0 (0)	14 (87.5)	2 (12.5)	
Ck 17	153				0.00006
Negative		23 (17.2)	109 (81.3)	2 (1.5)	
Positive		0 (0)	15 (78.9)	4 (21.1)	
Basal keratins	155				0.00000
Negative		24 (18.2)	107 (81.1)	1 (0.8)	
Positive		0 (0)	18 (78.3)	5 (21.7)	
Any basal marker	155				0.00003
Negative		23 (18)	104 (81.3)	1 (0.8)	

	Positive		1 (3.7)	21 (77.8)	5 (18.5)	
		148				0.03620
p53	Negative		18 (17.5)	84 (81.6)	1 (1)	
	Positive		5 (11.1)	36 (80)	4 (8.9)	
Ki-67		148				0.01751
	Low		12 (18.2)	54 (81.8)	0 (0)	
	Intermediate		10 (16.1)	50 (80.6)	2 (3.2)	
	High		1 (5)	16 (80)	3 (15)	
HER2 – CISH		150				0.57085
	Not amplified		21 (16.3)	102 (79.1)	6 (4.7)	
	Amplified		3 (14.3)	18 (85.7)	0 (0)	
TOPO2A - CISH		151				0.71590
	Not amplified		21 (15.3)	110 (80.3)	6 (4.4)	
	Amplified		2 (14.3)	12 (85.7)	0 (0)	
TOPO2A - IHC		141				0.00064
	Low		17 (25.4)	50 (74.6)	0 (0)	
	High		4 (5.4)	65 (87.8)	5 (6.8)	
Molecular subtypes**		152				0.00502
	Basal-like		1 (4.3)	18 (78.3)	4 (17.4)	
	HER2		3 (14.3)	18 (85.7)	0 (0)	
	Luminal		19 (17.6)	87 (80.6)	2 (1.9)	
Triple negative		154				0.00077
	No		22 (16.7)	108 (81.8)	2 (1.5)	
	Yes		2 (9.1)	16 (72.7)	4 (18.2)	
MYC – CISH		134				0.56866
	Not amplified		19 (15.7)	98 (81)	4 (3.3)	
	Amplified		1 (7.7)	11 (84.6)	1 (7.7)	
Caveolin 1		156				0.00003
	Negative		23 (16.5)	114 (82)	2 (1.4)	
	Positive		1 (5.9)	12 (70.6)	4 (23.5)	
Caveolin 2		143				0.00000
	Negative		22 (16.8)	108 (82.4)	1 (0.8)	
	Positive		0 (0)	8 (66.7)	4 (33.3)	
FOX1A		124				0.00776
	Negative		6 (20)	20 (66.7)	4 (13.3)	
	Positive		14 (14.9)	79 (84)	1 (1.1)	
E-Cadherin		145				0.04277
	Negative		13 (28.9)	31 (68.9)	1 (2.2)	
	Reduced		2 (22.2)	7 (77.8)	0 (0)	
	Normal		8 (8.8)	79 (86.8)	4 (4.4)	
Nestin		133				0.00642
	Negative		20 (17.5)	92 (80.7)	2 (1.8)	
	Positive		1 (5.3)	15 (78.9)	3 (15.8)	
Bcl2		127				0.51193
	Negative		8 (17)	36 (76.6)	3 (6.4)	
	Positive		12 (15)	66 (82.5)	2 (2.5)	
CD44		135				0.69801
	Low		6 (21.4)	21 (75)	1 (3.6)	
	Intermediate		4 (16)	21 (84)	0 (0)	
	High		13 (15.9)	64 (78)	5 (6.1)	

Cyclin D1		147			0.20043
	Low	2 (11.1)	14 (77.8)	2 (11.1)	
	Intermediate	4 (11.4)	29 (82.9)	2 (5.7)	
	High	16 (17)	77 (81.9)	1 (1.1)	

*: Chi-squared test; **: Breast cancer molecular subtypes as defined by Nielsen et al. [52] criteria.

CISH: chromogenic *in situ* hybridisation; Ck: cytokeratin; EGFR: epidermal growth factor receptor; ER: oestrogen receptor; IHC: immunohistochemistry; LN: lymph node; LVI: lympho-vascular invasion.

Figure Legend

Figure 1: Cortactin (CTTN) expression, as defined by immunohistochemistry, and *CTTN* gene copy number status, as defined by chromogenic *in situ* hybridisation.

Representative micrographs of an invasive ductal carcinoma carcinoma (a) lacking CTTN expression (b) and lacking *CTTN* gene amplification (c); an invasive ductal carcinoma (d) displaying intermediate levels of cytoplasmic and membranous CTTN expression (e) and lacking *CTTN* gene amplification (f); and an invasive ductal carcinoma (g) displaying high levels of cytoplasmic and membranous CTTN expression (h) and *CTTN* gene amplification (i) in the form of gene clusters.

Supplementary Materials

Supplementary Table 1: Summary of antibodies, clones, dilutions and antigen retrieval methods.

Figure 1