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β-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with *CTNNB1* mutation

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Aberrant β-catenin expression as determined by assessment of its subcellular localization constitutes a surrogate marker of Wnt signalling pathway activation and has been reported in a subset of breast cancers. The association of β-catenin/Wnt pathway activation with clinical outcome and the mechanisms leading to its activation in breast cancers still remain a matter of controversy. The aims of this study were to address the distribution of β-catenin expression in invasive breast cancers, the correlations between β-catenin expression and clinicopathological features and survival of breast cancer patients, and to determine whether aberrant β-catenin expression is driven by *CTNNB1* (β-catenin encoding gene) activating mutations. Immunohistochemistry was performed on a tissue microarray containing 245 invasive breast carcinomas from uniformly treated patients, using two anti-β-catenin monoclonal antibodies. Selected samples were subjected to *CTNNB1* exon 3 mutation analysis by direct gene sequencing. A good correlation between the two β-catenin antibodies was observed (Spearman's $r > 0.62$, $P < 0.001$). Respectively, 31 and 11% of the cases displayed lack/reduction of β-catenin membranous expression and nuclear accumulation. Complete lack of β-catenin expression was significantly associated with invasive lobular carcinoma histological type. Subgroup analysis of non-lobular cancers or non-lobular grade 3 carcinomas revealed that lack/reduction of β-catenin membranous expression and/or nuclear accumulation were significantly associated with oestrogen receptor negativity, absence of *HER2* gene amplification and overexpression, lack/reduction of E-cadherin expression and tumours of triple-negative and basal-like phenotype. Univariate survival analysis revealed a significant association between β-catenin nuclear expression and shorter metastasis-free and overall survival in the whole cohort; however, β-catenin nuclear expression was not an independent predictor of outcome in multivariate analysis. No *CTNNB1* mutations were identified in the 28 selected breast carcinomas analysed. In conclusion, β-catenin/Wnt pathway activation is preferentially found in triple-negative/basal-like breast carcinomas, is associated with poor clinical outcome and is unlikely to be driven by *CTNNB1* mutations in breast cancer.
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β-Catenin is a multifunctional protein located to the intracellular side of the cytoplasmic membrane coded by the *CTNNB1* gene, which maps to chromosome 3p22.1. It has a critical role in cell-to-cell adhesion by linking cadherins to the actin cytoskeleton and has a central role in transcriptional regulation in the Wnt signalling pathway.[1] Indeed, upon Wnt activation, β-catenin is translocated from the membrane to the cytoplasm and nucleus, where it interacts with transcriptional activators to modulate a number of target genes associated with increased growth, invasion and cellular transformation, such as *c-MYC*[2] or *cyclin D1*.[3,4]

There are numerous lines of evidence to implicate the importance of β-catenin deregulation and

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CTNNB1 activating mutations in carcinogenesis.[1,3–9] Several studies have addressed the issue of β-catenin/Wnt pathway dysfunction in breast cancer.[3,10–17] Our previous study on spindle lesions of the breast demonstrated that aberrant β-catenin expression is often observed in metaplastic carcinomas of the breast, indicating that Wnt canonical pathway activation is found in at least a subset of breast cancers.[18] Expression of β-catenin in breast cancer and its association with outcome have been a matter of controversy. Although some have reported that aberrant β-catenin expression is found in breast cancers of poor outcome[3,13,15] and of the basal-like phenotype,[19] others have failed to demonstrate a correlation between β-catenin aberrant expression and outcome.[11,14,16,17,20] This is not surprising, given the difficulties in assessing β-catenin/Wnt pathway activation in breast cancer, because of the complexity of this molecular pathway, the challenging interpretation of β-catenin subcellular localization, specificity of different antibodies and other technical issues.[12,20]

The mechanisms underpinning β-catenin/Wnt pathway activation in breast cancer remain poorly understood. *CTNNB1* activating mutations and *APC* inactivating mutations are some of the possible mechanisms leading to β-catenin nuclear accumulation. The prevalence of these molecular abnormalities is highly debated in breast cancer.[21–24] Although some authors have described *CTNNB1* mutations in up to 26% of metaplastic carcinomas of the breast,[25] we[18] and others[7] have failed to confirm the presence of *CTNNB1* mutations in this histological type of breast tumours. Notwithstanding the fact that most recent studies agree in that β-catenin/Wnt pathway is activated in at least a subset of breast cancers,[15,18,19] the molecular alterations underlying aberrant β-catenin expression are yet to be elucidated.

The aims of this study were threefold: (1) to address the distribution of β-catenin expression as defined by immunohistochemistry using two antibodies in a cohort of 245 invasive breast carcinomas uniformly treated with anthracycline-based chemotherapy, (2) to correlate β-catenin expression with clinicopathological characteristics and outcome of primary breast cancer patients and (3) to investigate whether activation of β-catenin/Wnt pathway in breast cancer is driven by *CTNNB1* gene mutation.

Materials and methods

Case Selection and Tissue Microarray

A cohort of 245 patients with invasive breast cancer was included in a tissue microarray containing three 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 followed by anthracycline-based chemotherapy. Adjuvant endocrine therapy was prescribed for patients with oestrogen receptor-positive tumours (tamoxifen alone in 96% 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,[26] and size was categorized according to the TNM staging.[27] The study was approved by the Royal Marsden Hospital Ethics Committee.

Immunohistochemistry

Immunohistochemistry was performed on 3 μm-thick tissue microarray sections. For β-catenin immunohistochemistry, two commercially available monoclonal antibodies raised against the C-terminal domain of β-catenin were used, clone 14/β-catenin (BD Transduction Laboratories, San Jose, CA, USA) and 17C2 (Novocastra/Leica, Newcastle Upon Tyne, UK), which were used in 1:6000 and 1:100 dilutions, respectively, as previously described[18] and summarized in Supplementary Table 1. Immunohistochemical analysis with the 14/β-catenin clone was performed with the observers blinded to the results of the analysis of 17C2 clone. Results of β-catenin immunohistochemistry obtained with each antibody were analysed independently by two of the authors (ML-T and FCG) using the Allred scoring system for cytoplasmic and nuclear reactivity. This scoring system combines the staining intensity and the percentage of stained cells (intensity score 0–3 + % score 0–5).[28] An Allred score of >2 was considered as positive. β-catenin membranous staining was scored according to a previously used system for E-cadherin.[29] Briefly, the proportion of stained cells with complete membranous staining was recorded in four categories: 0, 0–10%; 1, 10–<25%; 2, 25–<50%; 3, 50–75%; and 4, >75%. Expression of β-catenin was considered normal when scores were ≥ 3, reduced when equal to 2, and negative when scores were <2.

β-Catenin expression was correlated with the expression of oestrogen receptor, progesterone receptor, HER2, epidermal growth factor receptor (EGFR), cytokeratin (CK) 14, CK5/6 and CK17, Ki-67, p53, topoisomerase II α (TOP2A), caveolin-1 (CAV1), caveolin-2 (CAV2), FOXA1, E-cadherin, CD44, Bcl2, nestin and cyclin D1, and with amplification of *CCND1*, *HER2*, *TOP2A* and *MYC*. Details of the methods and results of the above proteins are described elsewhere[30,31] and summarized in Supplementary Table 1. The prevalence of *CCND1*, *HER2*, *TOP2A* and *MYC* gene amplification was assessed by chromogenic *in situ* hybridization with SpotLight CISH probes (Invitrogen, Paisley, UK) and results not in relation to β-catenin expression were reported elsewhere.[32,33] Based upon the expression of HER2, oestrogen receptor, CK5/6 and EGFR,

tumours were classified into basal, HER2 and luminal according to the immunohistochemical panel proposed by Nielsen *et al*.[34]

Microdissection and DNA Extraction

Cases with β-catenin nuclear expression and/or of triple-negative phenotype (ie, oestrogen receptor-, progesterone receptor- and HER2-negative) from the tissue microarray were selected for *CTNNB1* sequencing analysis based on an Allred score for β-catenin nuclear staining = 2 ($n = 9$) or > 2 ($n = 19$). All cases were microdissected to ensure > 75% of purity of neoplastic cells. Microdissection of formalin-fixed paraffin-embedded samples was performed with a sterile needle under a stereomicroscope (Olympus SZ61, Tokyo, Japan) from ten consecutive 8 μm thick sections stained with nuclear fast red as previously described.[35] DNA was extracted using the DNeasy Kit (Qiagen, Crawley, UK) according to the manufacturer's recommendations. DNA concentration was measured with the PicoGreen- assay as per the manufacturer's instructions (Invitrogen).[35] Out of the 30 carcinomas selected for the *CTNNB1* mutation analysis, microdissection yielded sufficient DNA of optimal quality in 28 samples.

CTNNB1 Mutation Analysis

Sequencing of known mutation hotspots of *CTNNB1* on exon 3[5,9,21,23,25] was performed in 19 invasive carcinomas of the breast displaying β-catenin nuclear expression, and 9 cases with a β-catenin nuclear Allred score of 2 (ie, considered as negative) and of triple-negative phenotype. As previously described,[18] positive controls (ie, DNA samples of the HCT116 colon cancer cell line[36] and of one formalin-fixed paraffin-embedded breast fibromatosis, which harbored an exon 3 *CTNNB1* mutation) were included in each experiment. The primers used for *CTNNB1* sequencing were previously described.[25] A total of 50 ng tumour DNA was amplified and sequencing reactions were carried out using the DNA Sequencing Kit BigDye Terminator v 1.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Warrington, UK) as previously described.[36] Sequences of the forward and reverse strands were analysed with Mutation Surveyor software (Softgenetics, State College, PA, USA). All reactions were carried out in duplicate from the original DNA sample.

Statistical Analysis

Data analysis of β-catenin expression was performed with the results obtained with each antibody. The SPSS statistical software package was used for all statistical analysis. Spearman's correlations coefficient and unweighted κ scores were assessed to determine the concordance between results obtained with the two anti-β-catenin antibodies used in this study. For each parameter, correlations between categorical variables were performed using the χ^2 test and Fisher's exact test. Metastasis-free survival was expressed as the number of months from diagnosis to the occurrence of distant relapse. Disease-free survival 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

β-Catenin Expression in Invasive Breast Cancer

The results of the immunohistochemical analysis of β-catenin expression are summarized in Table 1. Owing to lost/fragmented cores or cores without invasive tumour on tissue microarray sections, β-catenin expression data with both antibody clones were available in 221 out of 245 tumours. As previously described,[18] a good correlation between the two commercially available antibodies raised against β-catenin, clones 14/β-catenin and 17C2, was found. We observed statistically significant correlations between both antibodies for the semi-quantitative assessment of β-catenin membranous (Spearman's $r = 0.863$, $P < 0.0001$), cytoplasmic (Spearman's $r = 0.620$, $P < 0.0001$), and nuclear expression (Spearman's $r = 0.676$, $P < 0.0001$) (Table 1). Analysis of agreement between the two antibodies revealed a substantial to nearly perfect agreement, with κ scores of 0.801, 0.893 and 0.672 for membranous (negative *vs* reduced *vs* normal), cytoplasmic (positive *vs* negative) and nuclear (positive *vs* negative) reactivity, respectively.

In the 221 cases with available data for 14/β-catenin clone, β-catenin membranous expression was normal in 152 (69%) cases, reduced in 19 (9%) cases and negative in 50 (22%) cases. β-catenin cytoplasmic and nuclear expression (Allred score > 2) was observed in 195 (88%) and 25 (11%) tumours, respectively (Table 1). A weak cytoplasmic β-catenin expression was found in the neoplastic cells (ie, Allred score > 2) in most of the tumours, rendering the objective interpretation of β-catenin expression in this subcellular compartment challenging. Therefore, although cytosolic accumulation of β-catenin is considered to be an aberrant form of β-catenin expression and, potentially, indicative of Wnt pathway activation,[19,25] in this study, aberrant expression was defined as lack/reduction of β-catenin membrane expression and/or β-catenin nuclear expression ($n = 83$, 37%). Of the total of

69 cases displaying lack/reduction of β-catenin membrane expression, 11 also showed nuclear accumulation.

Table 1 Comparison of β-catenin immunostainings in 221 invasive carcinomas of the breast using two commercially available antibodies (clones 14/β-catenin and 17C2)

	Clone 14/β-catenin n = *221*	*Clone 17C2* n = *221*
β-*Catenin membranous expression*		
Reduction score		
0	35 (15.8%)	33 (15%)
1	15 (6.8%)	15 (6.8%)
2	19 (8.6%)	24 (10.8%)
3	42 (19%)	39 (17.6%)
4	110 (49.8%)	110 (49.8%)
Spearman's *r*, two tailed	0.863 ($P<0.0001$)	
β-*Catenin cytoplasmic expression*		
Allred score		
0	24 (10.9%)	23 (10.4%)
2	2 (0.9%)	4 (1.8%)
3	6 (2.7%)	11 (5%)
4	19 (8.6%)	24 (10.9%)
5	53 (24%)	100 (45.2%)
6	105 (47.5%)	53 (24%)
7	11 (5%)	5 (2.3%)
8	1 (0.4%)	1 (0.4%)
Spearman's *r*, two tailed	0.620 ($P<0.0001$)	
β-*Catenin nuclear expression*		
Allred score		
0	174 (78.7%)	198 (89.6%)
2	22 (10%)	8 (3.6%)
3	14 (6.4%)	6 (2.7%)
4	5 (2.3%)	3 (1.4%)
5	1 (0.4%)	3 (1.4%)
6	1 (0.4%)	2 (0.9%)
7	3 (1.4%)	0
8	1 (0.4%)	1 (0.4%)
Spearman's *r*, two tailed	0.676 ($P<0.0001$)	

β-Catenin Pattern of Expression Differs According to the Histological Types of Breast Cancer

β-Catenin expression was significantly correlated with histological type (Table 2 and Figure 1). The majority of invasive ductal carcinomas, in particular those of low histological grade, displayed a normal pattern of β-catenin expression, that is, normal membranous expression (81%) without nuclear localization (88%; Figures 2a and b); whereas, in a way akin to E-cadherin expression in lobular carcinomas of the breast,[29] a strong correlation between lack of β-catenin membranous expression and lobular histological type was found (82%, $P<0.001$, Figures 1a, c, 2c and d). Likewise, the vast majority of lobular carcinomas did not display cytoplasmic and nuclear expression of β-catenin (Table 2, Figure 2d and Supplementary Table 2). Furthermore, reduced or negative membranous expression (40%) and positive nuclear expression (30%) were more prevalent in carcinomas of other histological types (Figure 1), such as in a metaplastic carcinoma (Figures 2e and f). It is noteworthy that this is a pattern frequently observed in this subtype of breast cancer.[18]

β-Catenin Aberrant Expression Correlates with Triple-Negative and Basal-Like Phenotypes

β-Catenin expression in the distinct subcellular compartments was correlated with clinicopathological parameters in the whole cohort. The results with 14/β-catenin clone in relation to membranous and nuclear β-catenin expression are summarized in Table 3. Aberrant β-catenin expression (ie, lack/reduction of membranous expression and/or nuclear expression) was significantly correlated with histo-

Table 2 β-Catenin membranous and nuclear expression in 222 invasive breast carcinomas according to histological type

14/β-catenin clone

		Membranous expression				*Nuclear expression*		
Type	N = *222*	*Negative*	*Reduced*	*Positive*	P-*value*	*Negative*	*Positive*	P-*value*
IDC		16	15	129		140	20	
ILC		28	1	5	<0.001	32	2	0.091
Mixed		4	2	12		18	0	
Other		3	1	6		7	3	

17C2 clone

		Membranous expression				*Nuclear expression*		
Type	N = *222*	*Negative*	*Reduced*	*Positive*	P-*value*	*Negative*	*Positive*	P-*value*
IDC		17	17	127		149	12	
ILC		25	4	5	<0.001	34	0	<0.001
Mixed		3	3	12		18	0	
Other		3	0	6		6	3	

IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma.

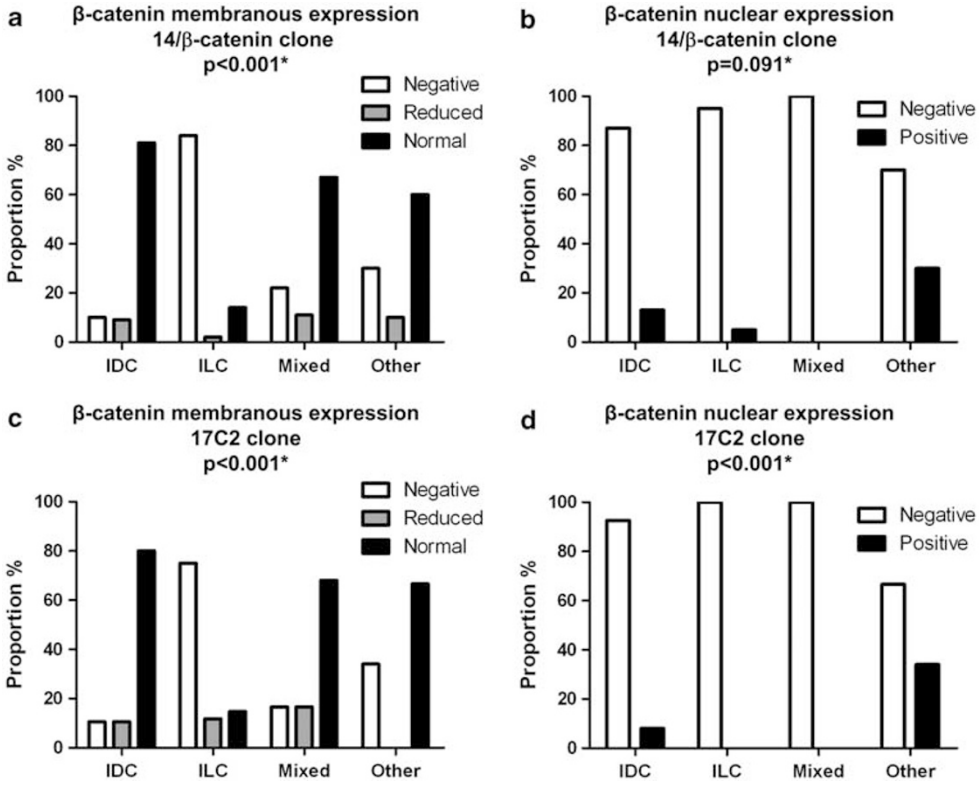

Figure 1 β-Catenin expression in invasive breast carcinomas according to histological type. β-Catenin membranous and nuclear expression using 14/β-catenin clone (**a**, **b**) and 17C2 clone (**c**, **d**) according to distinct histological type in the whole cohort ($n=222$). *χ^2 test.

logical grade, presence of lympho-vascular invasion and lymph node metastasis, triple negativity (ie, oestrogen receptor-, progesterone receptor- and HER2-negative), basal-like phenotype and basal-like features, as described below ($P<0.05$, Table 3). As expected, given that the great majority of lobular carcinomas displayed complete lack of β-catenin expression, a significant correlation between E-cadherin and β-catenin membranous expression was found ($P<0.001$, Table 3). However, β-catenin nuclear expression was also associated with lack/reduction of E-cadherin in the whole cohort ($P=0.027$, Table 3), indicating that the association between lack/reduction of both E-cadherin and aberrant β-catenin expression may be due not only to the lobular histology. Analysis of β-catenin expression with the clone 17C2 revealed similar associations (Table 4). Owing to the subjective nature of the analysis of β-catenin cytoplasmic expression, it is not surprising that no associations between β-catenin cytoplasmic expression and clinicopathological parameters were observed (Supplementary Table 2).

To avoid the confounding factor of the distinctive pattern of β-catenin expression in lobular carcinomas, β-catenin expression in the distinct subcellular compartments was investigated after the exclusion of all lobular carcinomas ($n=186$ cases). Associations similar to those observed in the analysis of β-catenin expression in the whole cohort were found (Tables 5 and 6, and Supplementary Table 2). Using 14/β-catenin clone, a significant inverse correlation between aberrant (reduced/negative membranous and/or positive nuclear expression) β-catenin expression and oestrogen receptor, oestrogen receptor pathway-associated parameters, such as progesterone receptor, FOXA1, cyclin D1 and Bcl2, and *HER2* gene amplification and overexpression was found ($P<0.05$, Table 5). Furthermore, a positive correlation was observed between aberrant β-catenin expression and expression of EGFR, basal CKs (CK5/6, CK14, CK17) and other markers typically found in basal-like breast carcinomas,[34,37–40] such as p53 positivity, high proliferation indices as defined by MIB-1 expression, expression of CAV1, CAV2 and nestin ($P<0.05$, Table 5). Not surprisingly, aberrant β-catenin expression was significantly associated with triple-negative and basal-like phenotypes (Table 5 and Figures 3a, b and 4). It is noteworthy that despite the exclusion of lobular carcinomas, the association between reduction/lack of E-cadherin expression and both lack/reduction of β-catenin membranous staining and nuclear β-catenin accumulation was still significant (Table 5). Similar observations were obtained when tumours were analysed with the 17C2 clone (Table 6 and Figures 3c and d).

As the vast majority of basal-like and triple-negative tumours are of histological grade 3, one

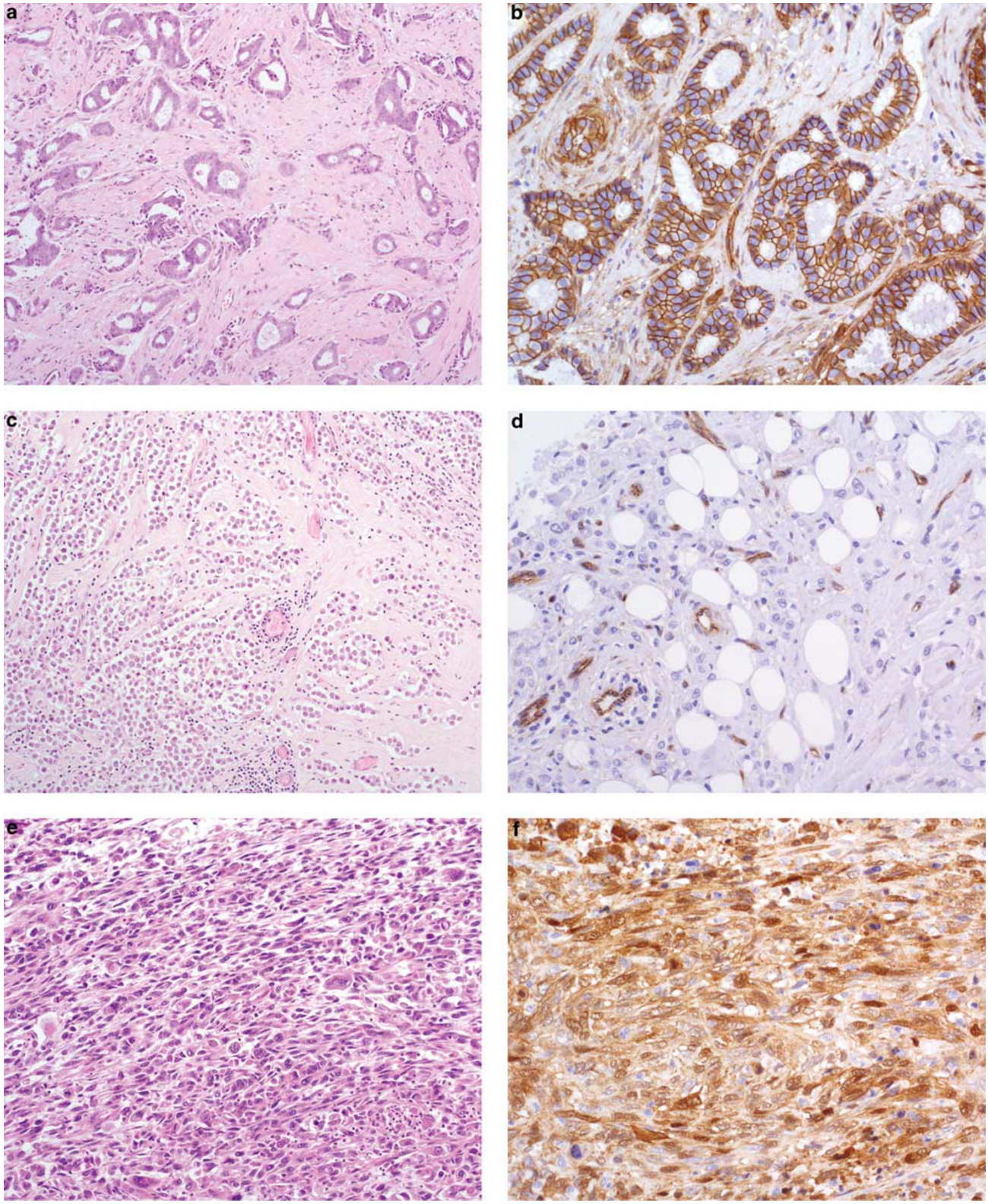

Figure 2 β-Catenin expression in invasive breast carcinomas according to histological type. The vast majority (>80%) of invasive ductal carcinomas (**a**, **b**) displayed normal β-catenin membranous expression and lacked β-catenin nuclear expression, whereas invasive lobular carcinomas lacked any β-catenin expression (**c**, **d**). Lack/reduction of β-catenin membranous expression and positive nuclear expression were more prevalent in carcinomas of other histological subtype, such as metaplastic carcinomas (**e**, **f**).

Table 3 β-Catenin membranous and nuclear expression in 222 invasive breast carcinomas (14/β-catenin clone)

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Size—TNM	221				0.4679			0.108
T1		24	7	83		106	8	
T2		21	11	60		77	15	
T3		5	1	9		13	2	
Grade	217				0.1706			**0.029**
1		3	1	19		23	0	
2		19	3	40		58	4	
3		27	15	90		111	21	
LVI	220				**0.0428**			0.374
Negative		23	7	41		61	10	
Positive		27	12	110		134	15	
LN mets	215				0.9061			**0.044**
Negative		18	6	48		59	13	
Positive		32	13	98		131	12	
ER	222				**0.0432**			**<0.001**
Negative		11	7	22		25	15	
Positive		40	12	130		172	10	
PR	222				0.091			**<0.001**
Negative		19	7	35		46	15	
Positive		32	12	117		151	10	
HER2	222				**0.0424**			**0.03**
Negative		47	13	130		165	25	
Positive		4	6	22		32	0	
HER2 CISH	211				0.0442			**0.029**
Not amp		47	13	120		156	24	
Amp		4	6	21		31	0	
EGFR	222				0.513			**0.003**
Negative		46	16	140		184	18	
Positive		5	3	12		13	7	
CK14	220				0.0671			**<0.001**
Negative		45	15	142		187	15	
Positive		5	4	9		8	10	
CK5/6	213				0.8458			**<0.001**
Negative		43	15	133		178	13	
Positive		6	2	14		11	11	
CK17	219				0.0601			**<0.001**
Negative		43	14	137		178	16	
Positive		7	5	13		16	9	
Basal CKs	220				0.214			**<0.001**
Negative		42	14	133		176	13	
Positive		8	5	18		19	12	
Basal CKs or EGFR	220				0.0921			**<0.001**
Negative		40	13	131		174	10	
Positive		10	6	20		21	15	
P53	208				**0.0206**			**0.007**
Negative		35	8	106		138	11	
Positive		15	10	34		46	13	
MIB-1					0.3661			**<0.001**
<10%		20	4	60		78	6	
10–30%		23	9	61		85	8	
>30%		7	5	18		19	11	

Table 3 Continued

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Molecular subtype[a]	216				**0.0042**			**<0.001**
Basal		8	5	14		14	13	
HER2		4	6	23		33	0	
Luminal		37	6	113		146	10	
Triple-negative	218				**0.0072**			**<0.001**
No		41	13	135		178	11	
Yes		10	6	13		15	14	
E-cadherin	201				**<0.001**			**0.027**
Negative		33	6	19		49	9	
Reduced		2	2	10		12	2	
Normal		12	10	107		118	11	
TOP2A	196				0.3746			0.373
Low		17	8	65		82	8	
High		29	9	68		92	14	
TOP2A CISH	211				0.0142			0.234
Not amp		47	14	132		169	24	
Amp		3	5	10		18	0	
Cyclin D1	208				0.0646			**0.002**
Low		11	3	10		17	7	
Intermediate		9	3	30		35	7	
High		29	13	100		133	9	
CCND1 CISH	221				0.4839			0.542
Not amp		43	15	133		168	23	
Amp		8	4	18		28	2	
MYC CISH	184				0.1075			0.418
Not amp		40	12	115		149	18	
Amp		1	3	13		14	3	
Caveolin 1	222				0.2008			**<0.001**
Negative		43	17	141		185	16	
Positive		8	2	11		12	9	
Caveolin 2	196				0.3686			**<0.001**
Negative		43	16	125		169	15	
Positive		1	2	9		5	7	
Nestin	166				0.0751			**<0.001**
Negative		31	10	106		136	11	
Positive		5	4	10		10	9	
FOXA1	175				**0.0303**			**<0.001**
Negative		13	7	23		31	12	
Positive		31	7	94		125	7	
Bcl2	172				0.0891			**0.027**
Negative		15	9	37		51	10	
Positive		24	6	80		104	6	

amp: amplified; CISH: chromogenic *in situ* hybridization; ER: oestrogen receptor; IDC: invasive ductal carcinoma; LN mets: lymph node metastasis; LVI: lympho-vascular invasion; PR: progesterone receptor.

[a]Molecular subtypes as defined by Nielsen immunohistochemical surrogate panel.[34]

Significant *P*-values are shown in bold.

could argue that the above correlations would be a mere reflection of the associations between high histological grade and aberrant β-catenin expression. However, subgroup analysis after the exclusion of grade 1 and 2 cancers and lobular carcinomas still revealed significant correlations between β-catenin aberrant expression and triple-negative and basal-like phenotypes, as well as with markers characteristically expressed by basal-like breast cancers, regardless of the antibody used

Table 4 β-Catenin membranous and nuclear expression in 222 invasive breast carcinomas (17C2 clone)

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Size—TNM	221				0.300			0.917
T1		22	9	83		107	7	
T2		20	14	58		85	7	
T3		5	1	9		14	1	
Grade	217				0.426			0.203
1		2	2	19		23	0	
2		17	6	40		60	3	
3		27	15	89		119	12	
LVI	220				**0.012**			0.570
Negative		23	9	39		65	6	
Positive		24	15	110		140	9	
LN mets	215				0.514			**0.009**
Negative		14	10	48		62	10	
Positive		33	13	97		138	5	
ER	222				0.074			**<0.001**
Negative		14	4	22		30	10	
Positive		34	20	128		177	5	
PR	222				**0.017**			**0.001**
Negative		21	5	35		51	10	
Positive		27	19	115		156	5	
HER2	222				0.485			0.138
Negative		43	19	129		176	15	
Positive		5	5	21		31	0	
HER2 CISH	211				0.236			0.228
Not amp		43	18	120		167	14	
Amp		5	6	19		30	0	
EGFR	222				0.719			**0.034**
Negative		43	21	138		191	11	
Positive		5	3	12		16	4	
CK14	220				0.244			**<0.001**
Negative		41	21	140		193	9	
Positive		6	3	9		12	6	
CK5/6	214				0.894			**0.002**
Negative		40	21	131		183	9	
Positive		5	3	14		16	6	
CK17	219				0.159			0.076
Negative		40	19	135		183	11	
Positive		7	5	13		21	4	
Basal CKs	220				0.421			**0.002**
Negative		39	19	131		181	8	
Positive		8	5	18		24	7	
Basal CKs or EGFR	220				0.224			**<0.001**
Negative		36	19	129		178	6	
Positive		11	5	20		27	9	
P53	208				0.218			**0.027**
Negative		30	15	104		143	6	
Positive		18	7	34		51	8	
MIB-1	207				0.788			**0.001**
<10%		16	10	59		81	4	
10–30%		22	10	60		88	4	
>30%		9	3	18		23	7	

Table 4 Continued

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Molecular subtype[a]	216				0.096			**<0.001**
Basal		10	3	14		19	8	
HER2		5	6	21		32	0	
Luminal		30	14	113		152	5	
Triple-negative	218				**0.006**			**<0.001**
No		35	21	133		183	6	
Yes		13	3	13		20	9	
E-cadherin	201				**<0.001**			0.710
Negative		33	7	18		53	5	
Reduced		3	2	9		13	1	
Normal		9	13	107		122	7	
TOP2A	196				0.365			0.266
Low		16	10	65		87	4	
High		27	12	66		95	10	
TOP2A CISH	211				0.201			0.610
Not amp		44	19	131		180	14	
Amp		4	4	9		17	0	
Cyclin D1	208				**0.044**			0.057
Low		11	3	10		20	4	
Intermediate		9	3	30		38	4	
High		27	17	98		136	6	
CCND1 CISH	221				0.185			1.000
Not amp		42	18	132		179	13	
Amp		6	6	17		27	2	
MYC CISH	184				0.725			**0.021**
Not amp		36	18	113		158	9	
Amp		3	1	13		13	4	
Caveolin 1	222				**0.040**			**<0.001**
Negative		39	23	139		193	8	
Positive		9	1	11		14	7	
Caveolin 2	196				0.484			**0.001**
Negative		41	20	123		175	9	
Positive		3	0	9		7	5	
Nestin	167				0.187			**0.005**
Negative		28	16	104		141	7	
Positive		7	2	10		14	5	
FOXA1	175				**0.046**			**0.001**
Negative		16	4	23		34	9	
Positive		25	13	94		128	4	
Bcl2	171				0.098			0.170
Negative		19	6	36		55	6	
Positive		19	10	81		106	4	

amp: amplified; CISH: chromogenic *in situ* hybridization; ER: oestrogen receptor; IDC: invasive ductal carcinoma; LN mets: lymph node metastasis; LVI: lympho-vascular invasion; PR: progesterone receptor.

[a]Molecular subtypes as defined by Nielsen immunohistochemical surrogate panel.[34]

Significant *P*-values are shown in bold.

($P<0.05$, Tables 7 and 8). Taken together, these results provide strong circumstantial evidence to suggest that Wnt pathway is preferentially activated in triple-negative/basal-like breast cancers.

Given the association between aberrant β-catenin expression and clinicopathological features associated with poor outcome, it is not surprising that univariate survival analysis revealed that β-catenin nuclear expression as defined by 14/β-catenin clone was significantly associated with decreased metastasis-free survival ($P=0.0216$, Figure 5) and overall survival ($P=0.0237$, Figure 5). Furthermore, a trend

Table 5 β-Catenin membranous and nuclear expression in 186 non-lobular invasive breast carcinomas (14/β-catenin clone)

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous normal*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Size—TNM	186				0.534			0.067
T1		9	7	83		92	7	
T2		11	9	56		62	14	
T3		2	1	8		10	1	
Grade	183				0.398			**0.043**
1		2	1	19		22	0	
2		3	3	39		42	3	
3		17	13	86		97	19	
Type	186				0.342			0.148
IDC		16	15	129		140	20	
Mixed		4	2	12		18	0	
Other		2	0	6		6	2	
LVI	185				0.433			0.322
Negative		8	7	41		47	9	
Positive		14	10	105		116	13	
LN mets	181				0.304			**0.017**
Negative		11	6	47		51	13	
Positive		11	11	95		108	9	
ER	186				**0.013**			**<0.001**
Negative		8	6	22		21	15	
Positive		14	11	125		143	7	
PR	186				**0.018**			**<0.001**
Negative		11	6	33		35	15	
Positive		11	11	114		129	7	
HER2	186				**0.029**			**0.028**
Negative		21	11	125		135	22	
Positive		1	6	22		29	0	
HER2 CISH	176				**0.031**			**0.028**
Not amp		21	11	116		127	21	
Amp		1	6	21		28	0	
EGFR	186				0.200			**0.002**
Negative		18	14	135		152	15	
Positive		4	3	12		12	7	
CK14	185				**0.019**			**<0.001**
Negative		18	13	137		156	12	
Positive		4	4	9		7	10	
CK5/6	179				0.214			**<0.001**
Negative		17	13	128		148	10	
Positive		5	2	14		10	11	
CK17	184				**0.007**			**<0.001**
Negative		16	12	132		147	13	
Positive		6	5	13		15	9	
Basal CKs	185				**0.021**			**<0.001**
Negative		15	12	128		145	10	
Positive		7	5	18		18	12	
Basal CKs or EGFR	185				**0.006**			**<0.001**
Negative		14	11	126		144	7	
Positive		8	6	20		19	15	
P53	172				**0.005**			**0.005**
Negative		10	8	103		112	9	
Positive		11	8	32		39	12	
MIB-1	171				0.221			**<0.001**
<10%		5	4	57		61	5	
10–30%		10	9	59		71	7	
>30%		6	3	18		17	10	

Table 5 Continued

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous normal*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Molecular subtype[a]	182				**<0.001**			**<0.001**
Basal		7	5	14		13	13	
HER2		1	6	23		30	0	
Luminal		13	5	108		119	7	
Triple-negative	182				**0.001**			**<0.001**
No		14	12	130		148	8	
Yes		8	5	13		12	14	
E-cadherin	166				**0.016**			**0.047**
Negative		8	4	16		21	7	
Reduced		1	2	10		12	1	
Normal		10	10	105		114	11	
TOP2A	163				0.566			0.473
Low		7	6	60		66	7	
High		13	9	68		77	13	
TOP2A CISH	175				**0.009**			0.223
Not amp		20	12	127		138	21	
Amp		1	5	10		16	0	
Cyclin D1	173				**0.001**			**<0.001**
Low		8	2	9		12	7	
Intermediate		3	3	29		28	7	
High		10	12	97		112	7	
CCND1 CISH	186				0.757			0.320
Not amp		19	14	130		142	21	
Amp		3	3	17		22	1	
MYC CISH	156				0.710			1,000
Not amp		17	12	111		123	17	
Amp		1	2	13		14	2	
Caveolin 1	186				**0.016**			**<0.001**
Negative		16	15	136		154	13	
Positive		6	2	11		10	9	
Caveolin 2	164				0.679			**<0.001**
Negative		18	14	120		139	13	
Positive		1	2	9		5	7	
Nestin	141				**0.026**			**<0.001**
Negative		12	9	102		114	9	
Positive		5	3	10		9	9	
FOXA1	144				**0.001**			**<0.001**
Negative		10	6	22		27	11	
Positive		8	7	91		100	6	
Bcl2	142				**0.048**			0.087
Negative		9	8	36		44	9	
Positive		7	6	76		83	6	

amp: amplified; CISH: chromogenic *in situ* hybridization; ER: oestrogen receptor; IDC: invasive ductal carcinoma; LN mets: lymph node metastasis; LVI: lympho-vascular invasion; PR: progesterone receptor.

[a]Molecular subtypes as defined by Nielsen immunohistochemical surrogate panel.[34]

Significant *P*-values are shown in bold.

for an association between β-catenin nuclear expression and decreased disease-free survival was observed ($P = 0.0873$, Figure 5). We did not observe any significant correlation between β-catenin membranous or cytoplasmic expression and survival (data not shown). Multivariate survival analysis demonstrated that β-catenin nuclear expression was not associated with the outcome of breast cancer patients when other clinicopathological parameters were included in the model (ie, tumour size, lymph node

Table 6 β-Catenin membranous and nuclear expression in 187 non-lobular invasive breast carcinomas (17C2 clone)

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Size—TNM	187				0.314			0.954
T1		10	7	83		93	7	
T2		10	12	54		70	6	
T3		2	1	8		10	1	
Grade	184				0.353			0.291
1		1	2	19		22	0	
2		3	4	39		43	3	
3		18	13	85		105	11	
Type	187				0.473			0.082
IDC		17	17	127		149	12	
Mixed		3	3	12		18	0	
Other		2	0	6		6	2	
LVI	186				0.191			0.363
Negative		10	7	39		50	6	
Positive		12	13	105		122	8	
LN mets	182				0.450			**0.007**
Negative		10	8	47		55	10	
Positive		12	11	94		113	4	
ER	187				**0.001**			**<0.001**
Negative		11	3	22		26	10	
Positive		11	17	123		147	4	
PR	187				**0.001**			**<0.001**
Negative		13	4	33		40	10	
Positive		9	16	112		133	4	
HER2	187				0.461			0.132
Negative		19	15	124		144	14	
Positive		3	5	21		29	0	
HER2 CISH	177				0.182			0.227
Not amp		19	14	116		136	13	
Amp		3	6	19		28	0	
EGFR	187				0.269			**0.040**
Negative		18	17	133		158	10	
Positive		4	3	12		15	4	
CK14	186				**0.028**			**<0.001**
Negative		17	17	135		161	8	
Positive		5	3	9		11	6	
CK5/6	180				0.379			**0.002**
Negative		16	17	126		151	8	
Positive		4	3	14		15	6	
CK17	185				**0.015**			0.089
Negative		16	15	130		151	10	
Positive		6	5	13		20	4	
Basal CKs	186				**0.037**			**0.002**
Negative		15	15	126		149	7	
Positive		7	5	18		23	7	
Basal CKs or EGFR	186				**0.007**			**<0.001**
Negative		13	15	124		147	5	
Positive		9	5	20		25	9	
P53	173				**0.004**			**0.059**
Negative		9	12	101		116	6	
Positive		13	6	32		44	7	
MIB-1	172				**0.026**			**0.014**
<10%		2	9	56		63	4	
10–30%		12	8	58		74	4	
>30%		7	2	18		21	6	

Table 6 Continued

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Molecular subtype[a]	183				**<0.001**			**<0.001**
Basal		9	3	14		18	8	
HER2		3	6	21		30	0	
Luminal		8	11	108		123	4	
Triple-negative	183				**<0.001**			**<0.001**
No		11	18	128		152	5	
Yes		11	2	13		17	9	
E-cadherin	167				**0.004**			0.269
Negative		9	4	15		24	4	
Reduced		2	2	9		12	1	
Normal		9	12	105		119	7	
TOP2A	164				0.490			0.146
Low		7	7	60		71	3	
High		13	11	66		80	10	
TOP2A CISH	176				0.091			0.611
Not amp		19	15	126		147	13	
Amp		3	4	9		16	0	
Cyclin D1	174				**0.002**			**0.021**
Low		8	2	9		15	4	
Intermediate		3	3	29		31	4	
High		11	14	95		115	5	
CCND1 CISH	187				0.181			1.000
Not amp		20	15	129		151	13	
Amp		2	5	16		22	1	
MYC CISH	157				0.860			0.108
Not amp		17	15	109		132	9	
Amp		2	1	13		13	3	
Caveolin 1	187				**0.002**			**<0.001**
Negative		15	19	134		161	7	
Positive		7	1	11		12	7	
Caveolin 2	165				0.238			**0.001**
Negative		18	17	118		145	8	
Positive		3	0	9		7	5	
Nestin	142				**0.001**			**0.005**
Negative		10	14	100		118	6	
Positive		7	1	10		13	5	
FOXA1	145				**<0.001**			**0.003**
Negative		12	4	22		30	8	
Positive		6	10	91		103	4	
Bcl2	143				**0.003**			0.292
Negative		12	6	35		48	5	
Positive		4	9	77		86	4	

amp: amplified; CISH: chromogenic *in situ* hybridization; ER: oestrogen receptor; IDC: invasive ductal carcinoma; LN mets: lymph node metastasis; LVI: lympho-vascular invasion; PR: progesterone receptor.

[a]Molecular subtypes as defined by Nielsen immunohistochemical surrogate panel.[34]

Significant *P*-values are shown in bold.

metastasis, oestrogen receptor, progesterone receptor and HER2 status, proliferation index as assessed by MIB-1 immunostaining, and basal-like markers; data not shown). No significant correlation between β-catenin expression and outcome could be observed when using 17C2 clone (data not shown).

Absence of *CTNNB1* Mutation in Breast Carcinomas Displaying Aberrant β-Catenin Expression

Given that aberrant β-catenin nuclear expression was found in 11% of the breast cancers studied, we investigated the presence of *CTNNB1* mutations in

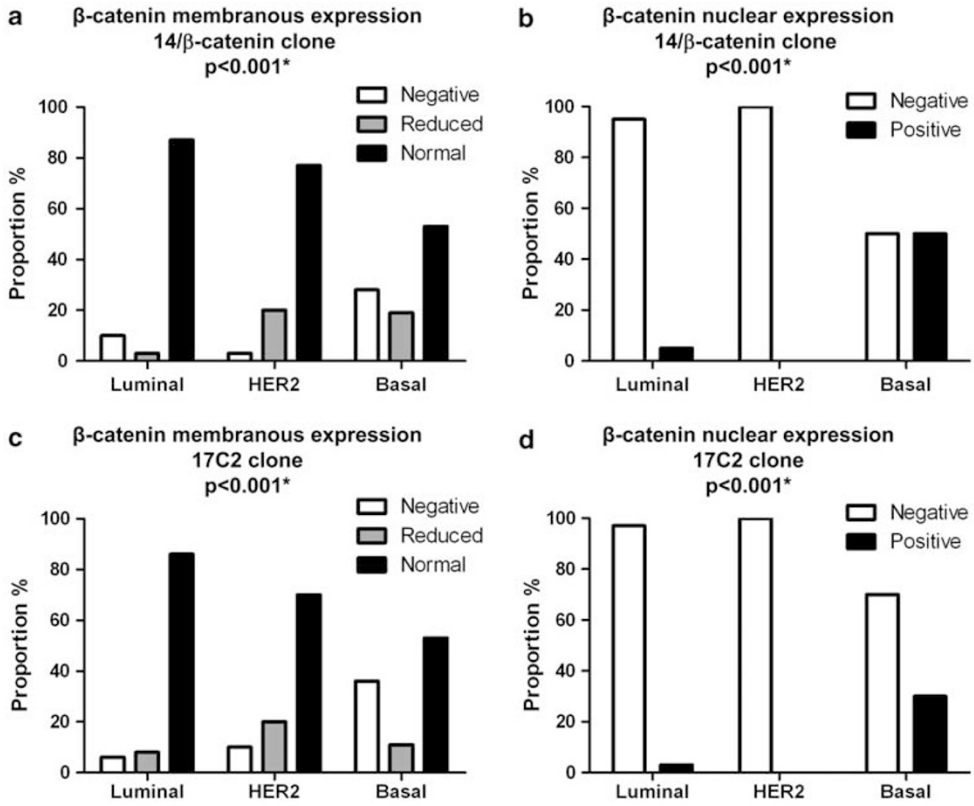

Figure 3 β-Catenin expression in invasive breast carcinomas according to the molecular subtypes. β-Catenin membranous and nuclear expression using 14/β-catenin (**a**, **b**) or 17C2 (**c**, **d**) clones in the cohort after exclusion of lobular carcinomas ($n = 182/183$, respectively) according to the molecular subtypes of breast cancer as defined by the immunohistochemical surrogate described by Nielsen *et al*.[34] *χ^2 test.

28 cases, 19 with β-catenin nuclear accumulation and nine without, all of triple-negative phenotype. No exon 3 *CTNNB1* gene mutations were found. Positive controls, included in each experiment, showed the expected presence of the previously reported *CTNNB1* mutation on exon 3 (deletion of codon 45) in the HCT116 colorectal cancer cell line.[36] In addition, one formalin-fixed paraffin-embedded sample of breast fibromatosis previously described[18] and displaying a *CTNNB1* mutation on exon 3 (25195T > TC:45S > S/P) was also used as a positive control, confirming the accuracy of our sequencing technique. Taken together with our previous observations derived from the *CTNNB1* sequencing of 21 metaplastic breast carcinomas,[18] these results suggest that β-catenin/Wnt pathway activation in breast cancer is not commonly driven by *CTNNB1* mutations.

Discussion

In this study we report that β-catenin expression in a large series of invasive breast carcinomas is aberrant in invasive lobular carcinomas and in a subgroup of triple-negative and basal-like breast cancers, as defined by a validated immunohistochemical surrogate panel.[34] Our results expand on those of Khramtsov *et al*,[19] as we have not only confirmed the association between β-catenin nuclear expression in basal-like cancers, but also demonstrated its association with proliferation markers, p53, CAV1, CAV2 and nestin.[34,37–39] We have also observed a statistically significant inverse correlation between β-catenin nuclear expression and lymph node metastasis at presentation, in the whole cohort ($P = 0.044$) and in the non-lobular ($P = 0.017$) and non-lobular grade 3 ($P = 0.009$) subgroups. This should not come as a surprise, given the reported lower frequency of lymph node metastasis in triple-negative[30] and basal-like[41] cancers. Finally, our results further support our recent observations that metaplastic breast carcinomas,[7,18] tumours that consistently display a basal-like phenotype,[42–44] often display β-catenin aberrant expression but lack *CTNNB1* gene mutations.

The prevalence of β-catenin/Wnt pathway activation and its association with outcome in breast cancer are contentious issues. Several studies have failed to demonstrate any association between β-catenin aberrant expression and outcome of breast cancer patients.[11,12,14,16,20,45] Our data derived from the analysis of a cohort of 245 breast cancer patients uniformly treated with anthracycline-based chemotherapy and those from recent studies[3,13,15,19] provide strong circumstantial evidence that aberrant

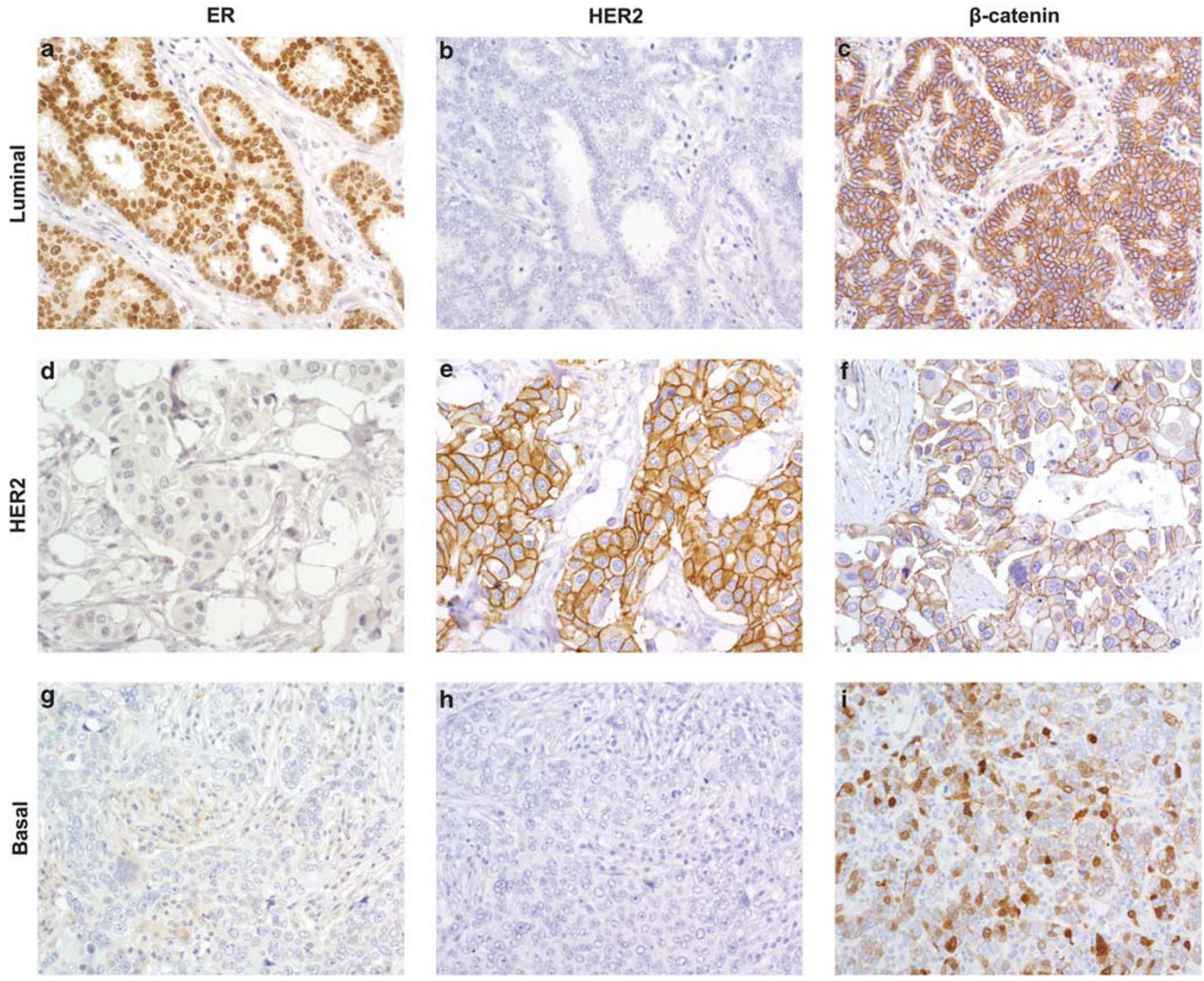

Figure 4 β-Catenin expression in invasive breast carcinomas according to the molecular subtypes. One representative case of each subtype is shown: a luminal (ie, oestrogen receptor-positive (**a**) and HER2-negative (**b**)) carcinoma displaying β-catenin membranous expression without nuclear expression (**c**); a HER2 (ie, oestrogen receptor-negative (**d**) and HER2-positive (**e**)) carcinoma displaying β-catenin membranous expression without nuclear expression (**f**); and a basal-like (ie, oestrogen receptor-negative (**g**) and HER2-negative (**h**)) carcinoma showing strong β-catenin nuclear accumulation and lack of β-catenin membranous expression (**i**).

β-catenin expression is associated with a subset of patients of adverse outcome. It should be noted, however, that although β-catenin nuclear expression is significantly correlated with shorter metastasis-free and overall survival, it is not an independent predictor of outcome.

Although current evidence supports the contention that the β-catenin/Wnt pathway is activated in a subgroup of breast cancers, the mechanisms leading to β-catenin nuclear accumulation in breast cancer remain elusive. In this study we investigated the hypothesis that β-catenin nuclear expression would be driven by *CTNNB1* activating gene mutations as previously suggested by Hayes *et al*.[25] No *CTNNB1* mutations on exon 3 in 28 invasive breast carcinomas displaying β-catenin nuclear expression and/or triple-negative phenotype were found. This observation, which is in agreement with our previous *CTNNB1* mutation analysis in metaplastic breast carcinoma and studies from other groups,[7,18] supports the hypothesis that β-catenin nuclear accumulation in breast cancer is not driven by mutations in *CTNNB1* exon 3. Alternatively, β-catenin nuclear expression may stem from mutations affecting other exons of the *CTNNB1* gene or other genes in the Wnt pathway (eg, the *APC* gene). It has been demonstrated that in colorectal cancers, copy number losses of *APC* gene may cooperate with inactivating *APC* mutations for complete APC protein loss of function and promote nuclear β-catenin translocation in tumour cells.[46,47] Importantly, there is evidence to suggest that in breast cancer, APC mRNA expression levels are determined by *APC* gene copy numbers.[47] We, therefore, performed a hypothesis generating re-analysis of microarray-based comparative genomic hybridization and gene

Table 7 β-Catenin membranous and nuclear expression in 116 grade 3 non-lobular invasive breast carcinomas (14/β-catenin clone)

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous normal*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Size—TNM	116				0.504			0.071
T1		7	5	50		56	6	
T2		9	7	30		34	12	
T3		1	1	6		7	1	
Grade	116				NP			NP
3		17	13	86		97	19	
Type	116				0.211			0.265
IDC		14	12	82		91	17	
Mixed		1	1	1		3	0	
Other		2	0	3		3	2	
LVI	116				0.382			0.284
Negative		6	6	24		28	8	
Positive		11	7	62		69	11	
LN mets	113				0.804			**0.009**
Negative		8	5	32		32	13	
Positive		9	8	51		62	6	
ER	116				0.073			**<0.001**
Negative		8	6	21		20	15	
Positive		9	7	65		77	4	
PR	116				**0.039**			**0.000**
Negative		11	6	28		30	15	
Positive		6	7	58		67	4	
HER2	116				0.195			**0.012**
Negative		16	9	66		72	19	
Positive		1	4	20		25	0	
HER2 CISH	111				0.209			**0.021**
Not amp		16	9	64		71	18	
Amp		1	4	17		22	0	
EGFR	116				0.489			**0.015**
Negative		13	10	74		85	12	
Positive		4	3	12		12	7	
CK14	116				0.083			**<0.001**
Negative		13	9	77		90	9	
Positive		4	4	9		7	10	
CK5/6	112				0.392			**<0.001**
Negative		12	9	71		85	7	
Positive		5	2	13		9	11	
CK17	116				**0.026**			**0.003**
Negative		11	8	74		83	10	
Positive		6	5	12		14	9	
Basal CKs	116				0.087			**<0.001**
Negative		10	8	69		80	7	
Positive		7	5	17		17	12	
Basal CKs or EGFR	116				**0.037**			**<0.001**
Negative		9	7	67		79	4	
Positive		8	6	19		18	15	
P53	109				**0.031**			**0.008**
Negative		8	4	56		62	6	
Positive		8	8	25		29	12	
MIB-1	108				0.722			**0.009**
<10%		1	2	11		12	2	
10–30%		9	7	51		60	7	
>30%		6	3	18		17	10	

Table 7 Continued

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous normal*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Molecular subtype[a]	112				**0.023**			**<0.001**
Basal		7	5	14		13	13	
HER2		1	4	19		24	0	
Luminal		8	3	51		58	4	
Triple-negative	114				**0.007**			**<0.001**
No		9	8	71		83	5	
Yes		8	5	13		12	14	
E-cadherin	105				**0.027**			**0.015**
Negative		7	2	10		12	7	
Reduced		1	2	6		8	1	
Normal		7	8	62		69	8	
TOP2A	104				0.152			0.789
Low		4	3	35		36	6	
High		12	9	41		51	11	
TOP2A CISH	108				0.362			0.120
Not amp		15	10	70		77	18	
Amp		1	3	9		13	0	
Cyclin D1	110				**0.008**			**<0.001**
Low		7	2	7		9	7	
Intermediate		3	2	15		14	6	
High		6	9	59		69	5	
CCND1 CISH	116				0.638			0.126
Not amp		16	11	74		82	19	
Amp		1	2	12		15	0	
MYC CISH	99				0.635			1.000
Not amp		13	8	66		73	14	
Amp		1	2	9		10	2	
Caveolin 1	116				**0.048**			**<0.001**
Negative		11	11	76		88	10	
Positive		6	2	10		9	9	
Caveolin 2	105				0.672			**<0.001**
Negative		15	10	69		84	10	
Positive		1	2	8		4	7	
Nestin	89				**0.091**			**<0.001**
Negative		8	6	57		65	6	
Positive		5	3	10		9	9	
FOXA1	90				**0.016**			**<0.001**
Negative		8	5	15		17	11	
Positive		6	5	51		58	4	
Bcl2	88				0.424			0.245
Negative		7	7	28		33	9	
Positive		6	4	36		41	5	

amp: amplified; CISH: chromogenic *in situ* hybridization; ER: oestrogen receptor; IDC: invasive ductal carcinoma; LN mets: lymph node metastasis; LVI: lympho-vascular invasion; PR: progesterone receptor.

[a]Molecular subtypes as defined by Nielsen immunohistochemical surrogate panel.[34]

Significant *P*-values are shown in bold.

expression data from a previously published cohort of 95 grade 3 invasive breast cancers,[48,49] which revealed a significant correlation between heterozygous deletions of *APC* locus and the basal-like phenotype (*APC* loss in the basal-like subtype $n = 10/25$, *vs* 2/25 in the HER2 subtype and 4/45 in the luminal subtype, $P = 0.003$, 2×3 Fisher's exact test), and that in the basal-like tumours with *APC* loss, APC mRNA expression levels were significantly lower (Figures 6 and 7). Taken together, our results warrant further studies to determine whether *APC* deletions, mutations and gene promoter methy-

Table 8 β-Catenin membranous and nuclear expression in 116 grade 3 non-lobular invasive breast carcinomas (17C2 clone)

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Size—TNM	116				0.446			0.847
T1		9	4	49		57	5	
T2		8	8	30		41	5	
T3		1	1	6		7	1	
Type	116				0.056			0.052
IDC		14	13	81		99	9	
Mixed		2	0	1		3	0	
Other		2	0	3		3	2	
LVI	116				0.290			0.313
Negative		8	5	23		31	5	
Positive		10	8	62		74	6	
LN mets	113				0.772			**0.006**
Negative		8	6	31		36	9	
Positive		10	7	51		66	2	
ER	116				**0.008**			**<0.001**
Negative		11	3	21		25	10	
Positive		7	10	64		80	1	
PR	116				**0.007**			**<0.001**
Negative		13	4	28		35	10	
Positive		5	9	57		70	1	
HER2	116				0.503			0.118
Negative		16	10	65		80	11	
Positive		2	3	20		25	0	
HER2 CISH	111				0.398			0.206
Not amp		16	9	64		79	10	
Amp		2	4	16		22	0	
EGFR	116				0.551			0.080
Negative		14	10	73		90	7	
Positive		4	3	12		15	4	
CK14	116				0.114			**0.001**
Negative		13	10	76		94	5	
Positive		5	3	9		11	6	
CK5/6	112				0.586			**0.004**
Negative		12	10	70		87	5	
Positive		4	3	13		14	6	
CK17	116				**0.036**			0.225
Negative		12	8	73		86	7	
Positive		6	5	12		19	4	
Basal CKs	116				0.120			**0.005**
Negative		11	8	68		83	4	
Positive		7	5	17		22	7	
Basal CKs or EGFR	116				**0.043**			**<0.001**
Negative		9	8	66		81	2	
Positive		9	5	19		24	9	
P53	109				**0.020**			**0.039**
Negative		6	7	55		65	3	
Positive		12	4	25		34	7	
MIB-1	108				0.203			0.057
<10%		0	3	11		13	1	
10–30%		10	7	50		63	4	
>30%		7	2	18		21	6	

Table 8 Continued

Parameter	N	*Membranous negative*	*Membranous reduced*	*Membranous positive*	P-*value*	*Nuclear negative*	*Nuclear positive*	P-*value*
Molecular subtype[a]	112				**0.014**			**<0.001**
Basal		9	3	14		18	8	
HER2		2	4	18		24	0	
Luminal		5	6	51		61	1	
Triple-negative	114				**<0.001**			**<0.001**
No		7	11	70		86	2	
Yes		11	2	13		17	9	
E-cadherin	105				**0.005**			0.083
Negative		8	2	9		15	4	
Reduced		2	2	5		8	1	
Normal		7	7	63		73	4	
TOP2A	104				0.184			0.197
Low		6	2	34		40	2	
High		12	9	41		54	8	
TOP2A CISH	108				0.341			0.606
Not amp		16	9	70		85	10	
Amp		2	3	8		13	0	
Cyclin D1	110				**0.003**			**0.018**
Low		8	1	7		12	4	
Intermediate		3	2	15		17	3	
High		7	9	58		71	3	
CCND1 CISH	116				0.593			0.354
Not amp		17	11	73		90	11	
Amp		1	2	12		15	0	
MYC CISH	99				0.969			0.299
Not amp		13	9	65		80	7	
Amp		2	1	9		10	2	
Caveolin 1	116				**0.011**			**<0.001**
Negative		11	12	75		94	4	
Positive		7	1	10		11	7	
Caveolin 2	105				0.364			**0.001**
Negative		15	11	68		89	5	
Positive		3	0	8		6	5	
Nestin	89				**0.010**			**0.008**
Negative		7	8	56		68	3	
Positive		7	1	10		13	5	
FOXA1	90				**0.001**			**0.001**
Negative		11	2	15		20	8	
Positive		4	7	51		60	2	
Bcl2	88				**0.030**			0.251
Negative		11	3	28		37	5	
Positive		3	7	36		44	2	

amp: amplified; CISH: chromogenic *in situ* hybridization; ER: oestrogen receptor; IDC: invasive ductal carcinoma; LN mets: lymph node metastasis; LVI: lympho-vascular invasion; PR: progesterone receptor.

[a]Molecular subtypes as defined by Nielsen immunohistochemical surrogate panel.[34]

Significant *P*-values are shown in bold.

lation may be the driver of β-catenin/Wnt pathway activation in a subgroup of basal-like and triple-negative breast cancers.

A significant correlation between β-catenin aberrant expression and reduction/lack of E-cadherin was found in the non-lobular and non-lobular grade 3 subgroups ($P<0.05$, Tables 5–8). We and others[29,50] have previously shown that reduction of E-cadherin expression, one of the surrogate markers of epithelial-to-mesenchymal transition,[50] is associated with non-lobular breast carcinomas of basal-like and triple-negative phenotypes.[29] Hence, it is plausible

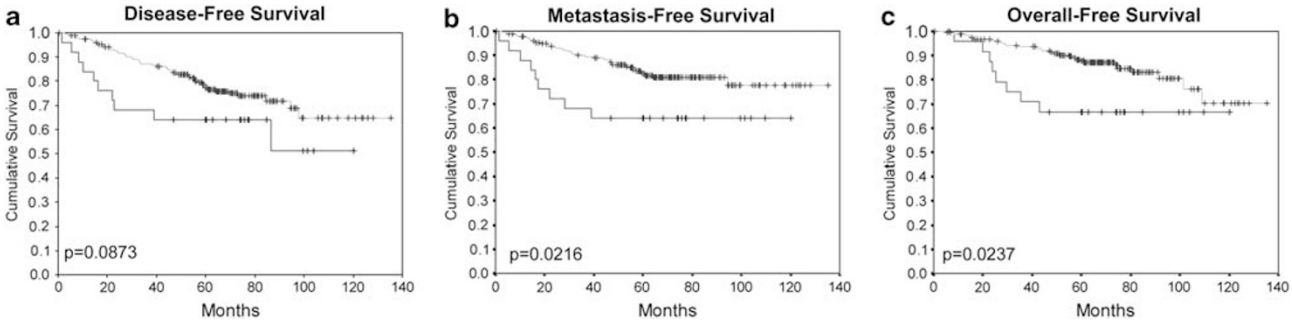

Figure 5 β-Catenin nuclear expression is associated with poor survival. Kaplan–Meier curves for disease-free survival (**a**), metastasis-free survival (**b**) and overall survival (**c**) for 221 patients uniformly treated with anthracycline-based chemotherapy. Invasive breast carcinomas with β-catenin nuclear expression as defined by the 14/β-catenin clone (dark grey) are significantly associated with shorter metastasis-free survival and overall survival ($P<0.05$). A similar but non-significant trend was also observed with disease-free survival.

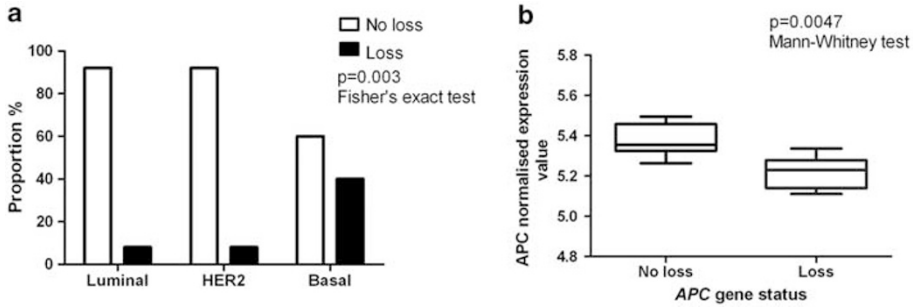

Figure 6 *APC* (5q22.2) copy number and expression in the molecular subtypes of breast cancer. (**a**) Frequency of *APC* (5q22.2) loss, as defined by microarray-based comparative genomic hybridization performed with the 32K bacterial artificial chromosome tiling path array,[48] in a previously published cohort of 95 grade 3 invasive breast cancers[48,49] according to the molecular subtypes of breast cancer as defined by the immunohistochemical surrogate described by Nielsen *et al*.[34] (**b**) Correlations between APC mRNA expression levels, as defined by the WG6 Illumina expression arrays (ArrayExpress http://www.ebi.ac.uk/microarray-as/ae/; accession number E-TABM-543)[49] and *APC* loss (as defined by microarray-based comparative genomic hybridization performed with the 32K bacterial artificial chromosome tiling path array[48]) in basal-like breast cancers ($n=13$).

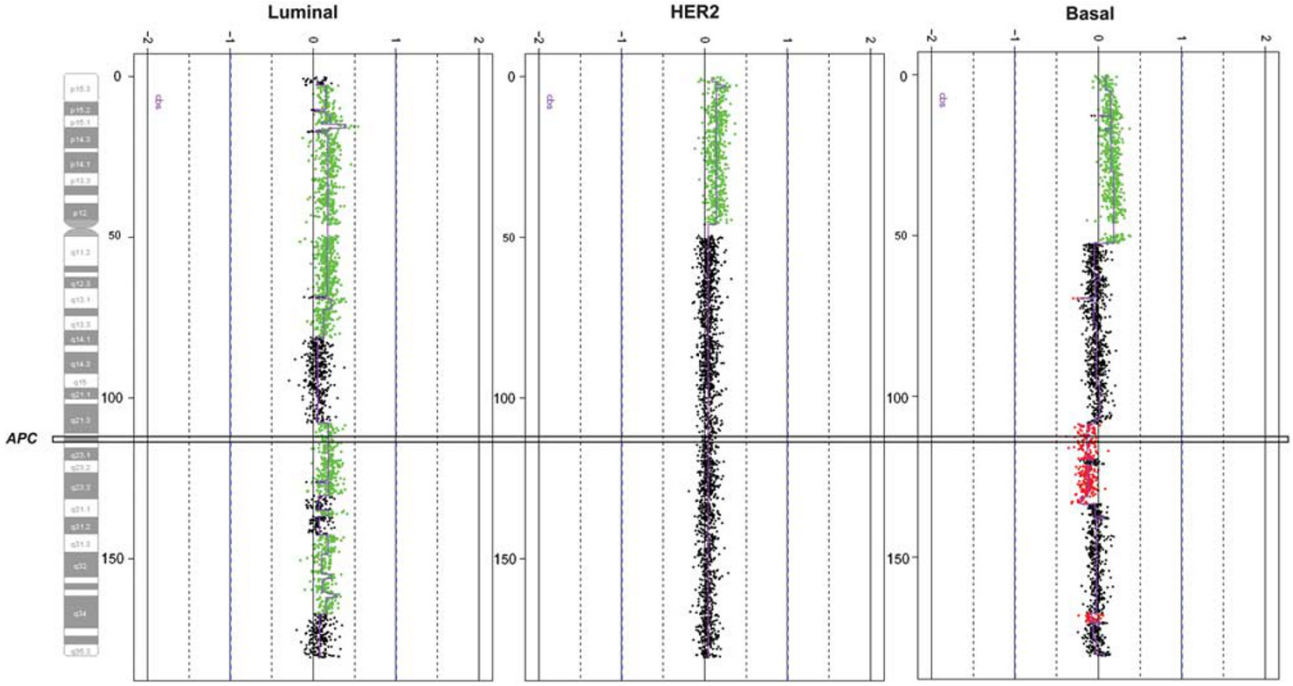

Figure 7 *APC* (5q22.2) copy number in the molecular subtypes of breast cancer. Representative chromosome 5 plots of luminal, HER2 and basal-like cancers obtained from re-analysis of the data from Natrajan *et al*.[48] Log_2 ratios are plotted on the *x* axis against each clone according to genomic location on the *y* axis. BACs categorized as displaying genomic gains or amplification are plotted in green and those categorized as genomic losses in red, as defined by previously validated cutoffs for circular binary segmentation-smoothed log_2 ratios.

that the activation of the β-catenin/Wnt pathway in these tumours may be the consequence of a global epithelial-to-mesenchymal transition programme, which is characteristic of at least a subset of basal-like tumours, including metaplastic breast cancers.[50,51]

In conclusion, β-catenin loss of membrane expression is a characteristic feature of invasive lobular carcinomas. β-catenin/Wnt canonical pathway activation, as defined by nuclear expression, is found in a subgroup of invasive breast cancers of triple-negative and basal-like phenotypes and is unlikely to be driven by *CTNNB1* activating gene mutations. Studies exploring alternative mechanisms of β-catenin/Wnt pathway activation are warranted.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Hatsell S, Rowlands T, Hiremath M, *et al.* Beta-catenin and Tcfs in mammary development and cancer. J Mammary Gland Biol Neoplasia 2003;8:145–158.
2 He TC, Sparks AB, Rago C, *et al.* Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–1512.
3 Lin SY, Xia W, Wang JC, *et al.* Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc Natl Acad Sci USA 2000;97:4262–4266.
4 Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–426.
5 Cui J, Zhou X, Liu Y, *et al.* Wnt signaling in hepatocellular carcinoma: analysis of mutation and expression of beta-catenin, T-cell factor-4 and glycogen synthase kinase 3-beta genes. J Gastroenterol Hepatol 2003;18:280–287.
6 Deng J, Miller SA, Wang HY, *et al.* beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. Cancer Cell 2002;2:323–334.
7 Hennessy BT, Gonzalez-Angulo AM, Stemke-Hale K, *et al.* Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. Cancer Res 2009;69:4116–4124.
8 Nagasawa Y, Miyoshi Y, Iwao K, *et al.* Transformation and morphological changes of murine L cells by transfection with a mutated form of beta-catenin. Cancer Res 1999;59:3539–3542.
9 Sparks AB, Morin PJ, Vogelstein B, *et al.* Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 1998;58:1130–1134.
10 Bankfalvi A, Terpe HJ, Breukelmann D, *et al.* Immunophenotypic and prognostic analysis of E-cadherin and beta-catenin expression during breast carcinogenesis and tumour progression: a comparative study with CD44. Histopathology 1999;34:25–34.
11 Bukholm IK, Nesland JM, Karesen R, *et al.* E-cadherin and alpha-, beta-, and gamma-catenin protein expression in relation to metastasis in human breast carcinoma. J Pathol 1998;185:262–266.
12 Chung GG, Zerkowski MP, Ocal IT, *et al.* beta-Catenin and p53 analyses of a breast carcinoma tissue microarray. Cancer 2004;100:2084–2092.
13 Dolled-Filhart M, McCabe A, Giltnane J, *et al.* Quantitative in situ analysis of beta-catenin expression in breast cancer shows decreased expression is associated with poor outcome. Cancer Res 2006;66:5487–5494.
14 Gillett CE, Miles DW, Ryder K, *et al.* Retention of the expression of E-cadherin and catenins is associated with shorter survival in grade III ductal carcinoma of the breast. J Pathol 2001;193:433–441.
15 Lopez-Knowles E, Zardawi SJ, McNeil CM, *et al.* Cytoplasmic localization of beta-catenin is a marker of poor outcome in breast cancer patients. Cancer Epidemiol Biomarkers Prev 2010;19:301–309.
16 Pedersen KB, Nesland JM, Fodstad O, *et al.* Expression of S100A4, E-cadherin, alpha- and beta-catenin in breast cancer biopsies. Br J Cancer 2002;87:1281–1286.
17 Wong SC, Lo SF, Lee KC, *et al.* Expression of frizzled-related protein and Wnt-signalling molecules in invasive human breast tumours. J Pathol 2002;196:145–153.
18 Lacroix-Triki M, Geyer FC, Lambros MB, *et al.* beta-catenin/Wnt signalling pathway in fibromatosis, metaplastic carcinomas and phyllodes tumours of the breast. Mod Pathol 2010; doi:10.1038/modpathol.2010.141 (e-pub ahead of print).
19 Khramtsov AI, Khramtsova GF, Tretiakova M, *et al.* Wnt/beta-catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome. Am J Pathol 2010;176:2911–2920.
20 Dillon DA, D'Aquila T, Reynolds AB, *et al.* The expression of p120ctn protein in breast cancer is independent of alpha- and beta-catenin and E-cadherin. Am J Pathol 1998;152:75–82.
21 Abraham SC, Reynolds C, Lee JH, *et al.* Fibromatosis of the breast and mutations involving the APC/beta-catenin pathway. Hum Pathol 2002;33:39–46.
22 Candidus S, Bischoff P, Becker KF, *et al.* No evidence for mutations in the alpha- and beta-catenin genes in human gastric and breast carcinomas. Cancer Res 1996;56:49–52.
23 Lazar AJ, Tuvin D, Hajibashi S, *et al.* Specific mutations in the beta-catenin gene (CTNNB1) correlate with local recurrence in sporadic desmoid tumors. Am J Pathol 2008;173:1518–1527.
24 Miyaki M, Konishi M, Kikuchi-Yanoshita R, *et al.* Coexistence of somatic and germ-line mutations of APC gene in desmoid tumors from patients with familial adenomatous polyposis. Cancer Res 1993;53:5079–5082.
25 Hayes MJ, Thomas D, Emmons A, *et al.* Genetic changes of Wnt pathway genes are common events in metaplastic carcinomas of the breast. Clin Cancer Res 2008;14:4038–4044.

26 Elston CW, Ellis IO. Pathological prognostic factors in breast cancer I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–410.
27 Singletary SE, Connolly JL. Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual. CA Cancer J Clin 2006;56:37–47, quiz 50-1.
28 Harvey JM, Clark GM, Osborne CK, *et al.* Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999;17:1474–1481.
29 Mahler-Araujo B, Savage K, Parry S, *et al.* Reduction of E-cadherin expression is associated with non-lobular breast carcinomas of basal-like and triple negative phenotype. J Clin Pathol 2008;61:615–620.
30 Tan DS, Marchio C, Jones RL, *et al.* Triple negative breast cancer: molecular profiling and prognostic impact in adjuvant anthracycline-treated patients. Breast Cancer Res Treat 2008;111:27–44.
31 Lambros MB, Natrajan R, Geyer FC, *et al.* PPM1D gene amplification and overexpression in breast cancer: a qRT-PCR and chromogenic in situ hybridization study. Mod Pathol 2010;23:1334.
32 Arriola E, Marchio C, Tan DS, *et al.* Genomic analysis of the HER2/TOP2A amplicon in breast cancer and breast cancer cell lines. Lab Invest 2008;88:491–503.
33 Reis-Filho JS, Savage K, Lambros MB, *et al.* Cyclin D1 protein overexpression and CCND1 amplification in breast carcinomas: an immunohistochemical and chromogenic in situ hybridisation analysis. Mod Pathol 2006;19:999–1009.
34 Nielsen TO, Hsu FD, Jensen K, *et al.* Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 2004;10:5367–5374.
35 Marchio C, Iravani M, Natrajan R, *et al.* Genomic and immunophenotypical characterization of pure micropapillary carcinomas of the breast. J Pathol 2008;215:398–410.
36 Sekine S, Shibata T, Sakamoto M, *et al.* Target disruption of the mutant beta-catenin gene in colon cancer cell line HCT116: preservation of its malignant phenotype. Oncogene 2002;21:5906–5911.
37 Parry S, Savage K, Marchio C, *et al.* Nestin is expressed in basal-like and triple negative breast cancers. J Clin Pathol 2008;61:1045–1050.
38 Savage K, Lambros MB, Robertson D, *et al.* Caveolin 1 is overexpressed and amplified in a subset of basal-like and metaplastic breast carcinomas: a morphologic, ultrastructural, immunohistochemical, and in situ hybridization analysis. Clin Cancer Res 2007;13:90–101.
39 Savage K, Leung S, Todd SK, *et al.* Distribution and significance of caveolin 2 expression in normal breast and invasive breast cancer: an immunofluorescence and immunohistochemical analysis. Breast Cancer Res Treat 2008;110:245–256.
40 Subhawong AP, Subhawong T, Nassar H, *et al.* Most basal-like breast carcinomas demonstrate the same Rb-/p16+ immunophenotype as the HPV-related poorly differentiated squamous cell carcinomas which they resemble morphologically. Am J Surg Pathol 2009;33:163–175.
41 Rakha EA, El-Sayed ME, Reis-Filho J, *et al.* Patho-biological aspects of basal-like breast cancer. Breast Cancer Res Treat 2009;113:411–422.
42 Reis-Filho JS, Milanezi F, Steele D, *et al.* Metaplastic breast carcinomas are basal-like tumours. Histopathology 2006;49:10–21.
43 Weigelt B, Horlings HM, Kreike B, *et al.* Refinement of breast cancer classification by molecular characterization of histological special types. J Pathol 2008;216:141–150.
44 Weigelt B, Kreike B, Reis-Filho JS. Metaplastic breast carcinomas are basal-like breast cancers: a genomic profiling analysis. Breast Cancer Res Treat 2009;117:273–280.
45 Bukholm IK, Nesland JM, Borresen-Dale AL. Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [see comments]. J Pathol 2000;190:15–19.
46 Fodde R, Tomlinson I. Nuclear beta-catenin expression and Wnt signalling: in defence of the dogma. J Pathol 2010;221:239–241.
47 Segditsas S, Rowan AJ, Howarth K, *et al.* APC and the three-hit hypothesis. Oncogene 2009;28:146–155.
48 Natrajan R, Lambros MB, Rodriguez-Pinilla SM, *et al.* Tiling path genomic profiling of grade 3 invasive ductal breast cancers. Clin Cancer Res 2009;15:2711–2722.
49 Natrajan R, Weigelt B, Mackay A, *et al.* An integrative genomic and transcriptomic analysis reveals molecular pathways and networks regulated by copy number aberrations in basal-like, HER2 and luminal cancers. Breast Cancer Res Treat 2010;121:575–589.
50 Sarrio D, Rodriguez-Pinilla SM, Hardisson D, *et al.* Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 2008;68:989–997.
51 Lien HC, Hsiao YH, Lin YS, *et al.* Molecular signatures of metaplastic carcinoma of the breast by large-scale transcriptional profiling: identification of genes potentially related to epithelial-mesenchymal transition. Oncogene 2007;26:7859–7871.

Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)