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Cyclin D1 protein overexpression and *CCND1* amplification in breast carcinomas: an immunohistochemical and chromogenic *in situ* hybridisation analysis

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Conflicting results on the prevalence of cyclin D1 ovexpression and its correlation with *CCND1* amplification and outcome of breast cancer patients have been reported. Owing to limited sensitivity and specificity of most antibodies against cyclin D1, evaluation of cyclin D1 immunoexpression is reported to be problematic. The aims of this study were to assess the prevalence of cyclin D1 expression in breast carcinomas using the SP4 rabbit monoclonal antibody; to correlate cyclin D1 expression with amplification, assessed using chromogenic *in situ* hybridisation (CISH); and to analyse the relationship between *CCND1* amplification and overexpression with clinicopathological parameters and outcome in a tissue microarray containing replicate tumour samples from 245 breast cancer patients. Immunohistochemistry for cyclin D1 was performed using the SP4 and the results were scored according to the Allred scoring system. CISH was carried out using the Zymed *CCND1* SpotLight probe. CISH signals were counted in 60 morphologically unequivocal neoplastic cells. Amplification was defined as >5 signals per nucleus in more than 50% of cancer cells, or when large gene copy clusters were seen. Strong cyclin D1 expression and *CCND1* amplification were found in 67.4 and 14.5% of the cases, respectively. A strong correlation between cyclin D1 overexpression and *CCND1* amplification was demonstrated ($P<0.0001$). Cyclin D1 expression showed a positive correlation with hormone receptor expression (both ER and PgR, $P<0.0001$). An inverse correlation was observed between an immunohistochemical panel of 'basal-like' markers and both cyclin D1 overexpression ($P<0.0001$) and *CCND1* amplification ($P<0.0001$). On univariate analysis cyclin D1 expression showed a correlation with longer overall survival (OS). Neither cyclin D1 nor *CCND1* were independent prognostic factors for disease-free survival or OS. The results of this study confirm the association between cyclin D1 overexpression and positivity for hormone receptors and the lack of *CCND1* amplification in basal-like breast carcinomas.
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The *CCND1* gene maps to 11q13 and encodes cyclin D1, the regulatory subunit of a holoenzyme that inactivates the retinoblastoma protein and promotes progression through the G1–S phase of the cell cycle.[1–4] Genetic abnormalities mapping to 11q13 are remarkably frequent in human neoplasms and particularly in breast cancer,[5] where this region is predominantly gained/amplified in grade II breast carcinomas.[6] *CCND1* amplification has been extensively investigated in breast cancer by Southern blotting,[7–10] fluorescent *in situ* hybridisation (FISH)[11–15] and real-time polymerase chain reaction-based methods,[16] with the prevalence ranging from 9 to 24%.[7–10,13,14,16,17] Conflicting data on the associations between *CCND1* amplification and clinicopathological variables and clinical outcome have been reported: while some have found an association between *CCND1* amplification and positivity for oestrogen receptor (ER),[7,12,17] lobular histology[7] and poor outcome,[7,8,16] others have failed to find some of these.[10,12,18]

Cyclin D1 overexpression is reported to be more prevalent than amplification, with the reported frequency ranging from 28 to 83%.[1,2,11,13,14,16,19–23] This variation has been linked with different

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antibodies, techniques and thresholds (cutoff points). Cyclin D1 overexpression, with or without *CCND1* amplification, has received great attention in the literature in the last three years due to results of *in vitro* studies and data from clinical trials implicating cyclin D1 overexpression in resistance to tamoxifen treatment.[13,14,23]

Given the crucial prognostic and predictive information that can be obtained by the analysis of *CCND1* amplification and overexpression, reliable methods for quantification are required. Ideally, these should be applicable to a high throughput methodology such as tissue microarrays (TMAs). In a recent study, it was reported that 44% of TMA cores did not produce interpretable results when FISH probes for *CCND1* were used.[13] Chromogenic *in situ* hybridisation (CISH) is a suitable alternative for FISH and has proven reliable in the assessment of gene copy numbers in TMAs;[24–27] however, this technique has not been employed for the study of *CCND1* amplification.

Immunohistochemical analysis of cyclin D1 expression is fraught with difficulties.[28] Several authorities have reported that immunohistochemical assessment of cyclin D1 in pathology practice is difficult,[28] erratic,[28] 'technically challenging'[29] and 'not routinely used because of the frequent demonstration of equivocal results'.[30] However, recently, a new rabbit monoclonal antibody against cyclin D1 has been developed and provides results that are more consistent than those obtained with other antibodies.[28]

The aims of this study are fourfold: (i) to analyse the expression of cyclin D1 in breast carcinomas using the new rabbit monoclonal antibody anti-cyclin D1; (ii) to correlate cyclin D1 expression with gene amplification as defined by CISH; (iii) to analyse the correlations between *CCND1* amplification and overexpression and the clinicopathological features and patients' outcome in a cohort of 245 patients with invasive breast carcinomas treated with surgery followed by anthracycline-based chemotherapy; and (iv) to define the prevalence of cyclin D1 overexpression and *CCND1* amplification in 'basal-like' breast carcinomas.

Materials and methods

Tissue Microarrays

The TMA contained replicate 0.6 mm cores of 245 invasive breast carcinomas (185 invasive ductal carcinoma, 27 invasive lobular carcinomas, 25 invasive mixed carcinomas and eight invasive breast carcinomas of other special types). All patients were primarily treated with surgery (69 mastectomy and 155 wide local excision), anthracycline-based adjuvant chemotherapy, and hormone therapy for patients with ER-positive tumours. Follow-up was available for 244 patients, ranging from 0.5 to 125 months (median—67 months, mean—67 months). Full details of the characterisation of the TMA and the cohort of patients is described elsewhere.[31] Tumours were graded according to a modified Bloom–Richardson scoring system[32] and size was categorised according to the TNM staging criteria.[33] This study was approved by the Royal Marsden Hospital Ethics Committee.

Immunohistochemistry

Immunohistochemistry for cyclin D1 was performed according to a previously described method,[28] with the SP4 antibody (Neomarkers, Suffolk, UK) at a dilution of 1:50 and developed with the Envision kit (Dako, Glostrup, Denmark). The SP4 antibody is a rabbit monoclonal antibody raised against a synthetic peptide from C-terminus of human cyclin D1, which is deemed to be specific to cyclin D1, identifying a single 36 kDa band on Western blot analysis (http://www.neomarkers.com/AB.CFM?First = AntiBody&Second = 9104). In addition, the SP4 clone is reported to be at least as specific as other monoclonal antibodies against cyclin D1, but is reported to be more sensitive than other clones.[28,34] Antigen retrieval was carried out by pressure cooking the sections for 2 min in EDTA solution, pH 8. Positive control (a translocation confirmed mantle cell lymphoma) and negative controls (omission of the primary antibody and IgG-matched immune serum) were included in each slide run. Cyclin D1 immunohistochemical intensity and distribution were semiquantitatively scored by two of the authors (KS and JSR-F) on a multi-headed microscope using the Allred score method.[35] With this method, the intensity of the immunohistochemical reaction as viewed under the light microscope was recorded as 0, negative (no staining of any nuclei even at high magnification); 1, weak (only visible at high magnification); 2, moderate (readily visible at low magnification); or 3, strong (strikingly positive even at low power magnification). The proportion of tumour nuclei showing positive staining was also recorded as either: 0, none; 1, $<1/100$; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3 and 5, $>2/3$. The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8.[35] Tumours were then categorised into four groups: negative/weak expression (total scores 0–2), intermediate expression (total scores 3–5) and strong expression (total scores 6–8). Only nuclear staining was considered specific. The analysis was performed blinded to the results of the CISH results and patients' outcome.

Ki-67 antigen (MIB1, 1:300, Dako, Glostrup, Denmark) and P53 (DO7, Dako, 1:200, Glostrup, Denmark) immunohistochemical detection was performed under the same conditions.[31] MIB-1 staining was scored low if less than 10% of the nuclei of neoplastic cells were positive, intermediate if 10–30% of the nuclei of neoplastic cells were positive

and high if more than 30% of the nuclei of neoplastic cells were positive. Tumours were scored positive for p53 if >10% of the nuclei of neoplastic cells showed definitive staining. The details of the immunohistochemical methods and scoring systems for ER, PgR, HER2, EGFR, Ck 14, Ck 5/6 and Ck 17 detection are described elsewhere.[31] Based upon the expression of HER2, ER, Ck 5/6 and EGFR, tumours were classified according to the immunohistochemical panel proposed by Nielsen *et al*.[36]

Chromogenic *In Situ* Hybridisation

CISH for *CCND1* was performed as previously described using the ready-to-use digoxigenin-labelled SpotLight cyclin D1 amplification probe (Zymed, South San Francisco, CA). Heat pretreatment of deparaffinised sections consisted of incubation for 15 min at 98°C in CISH pretreatment buffer (SPOT-light tissue pretreatment kit, Zymed) and digested with pepsin for 7 min at room temperature according to the manufacturer's instructions. An appropriate *CCND1* gene-amplified breast tumour control was included in the slide run. CISH experiments were analysed by two of the authors (KS and JSR-F) on a multiheaded microscope. Only unequivocal signals were counted. Signals were evaluated at ×400 and ×630 magnification and 60 morphologically unequivocal neoplastic cells were counted for the presence of the gene probe signals. Amplification was defined as >5 signals per nucleus in more than 50% of cancer cells, or when large gene copy clusters were seen.[25–27] The scoring was evaluated with observers blinded to the results of the immunohistochemical analysis and patients' outcome.

Statistical Analysis

The Statview software package was used for all calculations. Correlations between categorical variables were performed using the χ^2-test and Fisher's exact test. Correlations between continuous and categorical variables were performed with analysis of variance (ANOVA). Disease-free survival (DFS) and overall survival (OS) were expressed as the number of months from diagnosis to the occurrence of an event (local recurrence/metastasis and disease-related death, respectively). Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were tested with the log-rank test. All tests were two-tailed, with a confidence interval of 95%.

Multivariate analysis was performed using the Cox multiple hazards model. A *P*-value of 0.05 in the univariate survival analysis was adopted as the limit for inclusion in the multivariate model, and cases with missing values were excluded from this analysis.

Results

Immunohistochemistry

The correlations between cyclin D1 expression and clinicopathological features and immunohistochemical findings in 245 breast carcinomas are summarised in Table 1. Briefly, 23 cores were either lost/fragmented in the immunohistochemical procedure or did not have invasive tumour. Out of the 224 remaining cores, 13 were scored as 0, 1 as 2, 12 as 3, 16 as 4, 31 as 5, 57 as 6, 59 as 7 and 35 as 8. When classified into negative/low, moderate or strong cyclin D1 expression groups, 26 were classified as low, 47 as intermediate and 151 as strong expressers (Figure 1). Cyclin D1 expression showed a strong direct correlation with expression of ER and PgR (both, $P<0.0001$) and an inverse correlation with the expression of basal markers ($P<0.0001$), including EGFR ($P<0.0001$), Ck 14 ($P=0.0014$), Ck 5/6 ($P<0.0001$) and Ck 17 ($P<0.0001$). Tumours with high levels of cyclin D1 expression less frequently showed p53 immunohistochemical expression ($P<0.0001$) and showed lower proliferation rates when compared to cyclin D1 low and moderate tumours ($P<0.0001$). When tumours were classified according to the immunohistochemical panel proposed by Nielsen *et al*,[36] an inverse correlation between basal-like immunophenotype and cyclin D1 overexpression was found ($P<0.0001$).

Chromogenic *In Situ* Hybridisation

The correlations between *CCND1* amplification and clinicopathological features and immunohistochemical findings in 245 breast carcinomas are summarised in Table 2. Briefly, 39 cores were either lost/fragmented in the CISH procedure, did not have invasive tumour or showed suboptimal *CCND1* signals. Out of the 206 remaining tumours, 30 showed either large clusters of *CCND1* signals (Figure 1) or >5 individual signals/nucleus (Figure 1) in >50% of neoplastic cells. *CCND1* amplification showed an inverse correlation with expression of EGFR ($P=0.0488$) and Ck 5/6 ($P=0.0450$). A trend for an inverse association with HER2 positivity ($P=0.0578$) and expression of Ck 14 ($P=0.0813$) was also observed. When tumours were classified according to the immunohistochemical panel proposed by Nielsen *et al*,[36] an inverse correlation between basal-like immunophenotype and *CCND1* amplification was found ($P<0.0001$). In fact, all basal-like carcinomas did not show *CCND1* amplification.

Correlation between *CCND1* Gene Amplification and Protein Overexpression

Data on *CCND1* amplification and overexpression were available in 197 cases. A good correlation

Table 1 Correlations between Cyclin D1 expression, clinicopathological parameters and immunohistochemical markers in 245 invasive breast carcinomas

Parameter	N	*NA*	*Cyclin D1 weak*	*Cyclin D1 moderate*	*Cyclin D1 strong*	P-*value*
Size	222	23				0.6306*
T1			11	24	83	
T2			12	20	58	
T3			3	2	9	
Grade	220	25				0.0999*
1			3	8	10	
2			4	14	45	
3			19	23	94	
Type	224	21				0.9754*
IDC			19	35	115	
ILC			3	4	18	
Mixed			3	6	14	
Other			1	2	4	
LVI	222	23				0.9092**
−			10	15	53	
+			16	30	98	
LN mets	218	27				0.6519**
−			10	14	55	
+			16	32	91	
ER	224	21				<0.0001**
−			19	12	15	
+			7	35	136	
PgR	224	21				<0.0001**
−			18	17	26	
+			8	30	125	
HER2	224	21				0.0645**
−			26	41	125	
+			0	6	26	
EGFR	224	21				<0.0001**
−			15	41	146	
+			11	6	5	
Ck 14	222	23				0.0014**
−			19	39	143	
+			7	6	8	
Ck 5/6	214	31				<0.0001**
−			16	36	138	
+			8	8	8	
Ck 17	221	24				<0.0001**
−			13	39	143	
+			13	6	7	
Basal markers	222	23				<0.0001**
−			11	33	141	
+			15	12	10	
Nielsen groups	216	29				<0.0001*
Basal			15	8	7	
Luminal			7	32	115	
HER2			0	6	26	
P53	220	25				<0.0001**
−			9	30	116	
+			17	15	33	
MIB-1	219	26				<0.0001*
<10%			5	24	61	
10–30%			6	16	74	
>30%			15	7	11	

Ck: cytokeratin; ER: oestrogen receptor; LN mets: lymph node metastasis; LVI: lymoho-vascular invasion; Nielsen groups: immunophenotypic groups defined based upon the expression of ER, HER2, Ck 5/6 and EGFR; PgR: progesterone receptor.
*χ^2-test; **Fisher's exact test.

between *CCND1* amplification and cyclin D1 overexpression was found (Table 3, $P<0.0001$). In fact, 29/30 tumours with *CCND1* amplification showed strong cyclin D1 expression, whereas 1/30 showed moderate expression.

When data on the replicate cores of each tumour were treated independently, the agreement for cyclin D1 expression considering the categories negative/weak, intermediate and strong expression was good (unweighted kappa score = 0.5787 (0.4678–0.6896)) and for *CCND1* copy numbers (amplification *vs* no amplification) was excellent (unweighted kappa score = 0.9351 (0.8618–1.0084)).

Survival Analysis

In this cohort, size, grade, lymph node metastasis, ER, PgR, Ck 17 and proliferation index assessed by MIB1 were statistically significant prognostic factors for DFS on univariate analysis (Table 4). Cyclin D1 overexpression showed a trend for a longer DFS in tumours with moderate and strong expression when compared to negative/low expression (Figure 2a,

Figure 1 Grade III invasive ductal carcinoma (**a**) lacking cyclin D1 expression (**b**) and *CCND1* gene amplification (**c**). Note the expression of cyclin D1 in inflammatory cells percolating the tumour. Grade I invasive ductal carcinoma (**d**) showing intermediate cyclin D1 expression (**e**) and no *CCND1* amplification (**f**). Grade III invasive ductal carcinoma (**g**) displaying strong cyclin D1 (**h**) expression and lack of *CCND1* gene amplification (**i**). EGFR- and basal keratin-negative grade II invasive ductal carcinoma (**j**) displaying strong cyclin D1 (**k**) expression and *CCND1* gene amplification (**l**) in the form of multiple discrete signals and small clusters in the nuclei of neoplastic cells. EGFR- and basal keratin-negative grade II invasive mixed ductal-lobular carcinoma (**m**) showing strong cyclin D1 (**n**) expression and *CCND1* gene amplification (**o**) in the form of large signal clusters in >50% of the neoplastic cells. In **j** and **m**, note the absence of morphological features of 'basal-like carcinomas' (ie, pushing borders, brisk lymphocytic infiltrate, geographic/central necrosis, spindle and squamous and/or cells) (**a**, **b**, **d**, **e**, **g**, **h**, **j**, **k**, **m** and **n**—original magnification ×200; **c**, **f**, **i**, **l** and **o**—original magnification ×400).

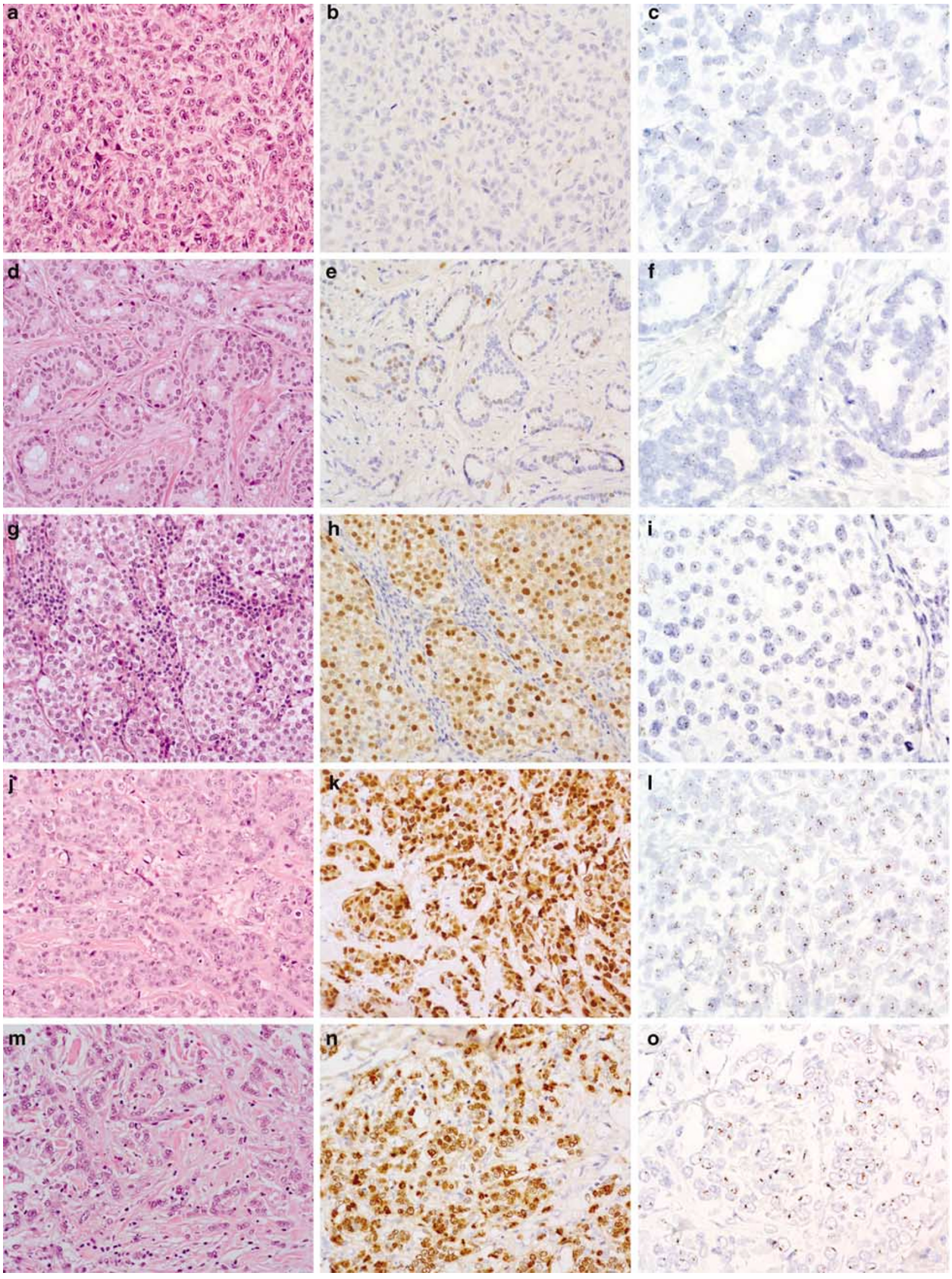
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Table 2 Correlations between *CCND1* amplification, clinicopathological parameters and immunohistochemical markers in 245 invasive breast carcinomas

Parameter	N	*NA*	*CCND1 not amplified*	*CCND1 amplified*	P-*value*
Size	204	41			0.2397*
T1			95	15	
T2			70	11	
T3			9	4	
Grade	203	42			0.1728*
1			19	0	
2			50	10	
3			106	18	
Type	206	39			0.4937*
IDC			134	22	
ILC			18	3	
Mixed			17	5	
Other			7	0	
LVI	205	40			0.2942**
−			61	7	
+			114	23	
LN mets	201	44			0.8378**
−			64	10	
+			108	19	
ER	206	39			0.3054**
−			34	3	
+			142	27	
PgR	206	39			0.6502**
−			44	6	
+			132	24	
HER2	205	40			0.1443**
−			154	23	
+			21	7	
EGFR	206	39			0.0488**
−			155	30	
+			21	0	
Ck 14	205	40			0.0813**
−			157	30	
+			18	0	
Ck 5/6	197	48			0.0450**
−			146	29	
+			22	0	
Ck 17	203	42			0.2819**
−			149	24	
+			28	2	
Basal markers	205	40			0.1201**
−			142	28	
+			33	2	
Nielsen groups	204	41			0.0290*
Basal			27	0	
Luminal			126	23	
HER2			21	7	
P53	199	46			0.6592**
−			119	21	
+			52	7	
MIB-1	197	48			0.1175*
<10%			73	7	
10–30%			71	17	
>30%			26	3	

CCND1: cyclin D1 gene; Ck: cytokeratin; ER: estrogen receptor; LN mets: lymph node metastasis; LVI: lymoho-vascular invasion; Nielsen groups: immunophenotypic groups defined based upon the expression of ER, HER2, Ck 5/6 and EGFR; PgR: progesterone receptor.
*χ^2-test; **Fisher's exact test.

Table 3 Correlation between *CCND1* gene amplification and cyclin D1 immunohistochemical expression

*Cyclin D1 expression**	*CCND1 not amplified*	*CCND1 amplified*
0	11	0
2	0	0
3	12	0
4	12	0
5	24	1
6	52	2
7	41	9
8	16	17

$P<0.0001$; χ^2-test.
*Allred scoring system.

Table 4), whereas *CCND1* amplification showed no correlation with DFS (Figure 2b, Table 4). On multivariate analysis, only size, grade and presence of lymph node metastasis were independent prognostic factors (data not shown).

Univariate survival analysis revealed lymph node metastasis, ER, PgR, Ck 14, Ck 5/6, Ck 17, p53, proliferation index as defined by MIB1, the immunophenotypic groups defined by the immunohistochemical panel described by Nielsen *et al*[36] and cyclin D1 overexpression as prognostic factors for OS (Figure 2c, Table 4). *CCND1* amplification failed to show any correlation with OS (Figure 2d, Table 4). Multivariate analysis revealed only the presence of lymph node metastasis, Ck 17 expression and p53 expression as independent prognostic factors for OS (data not shown).

Discussion

In order to develop immunohistochemical markers that can be utilised as prognostic and predictive factors in breast cancer, it is essential that reagents capable of producing reproducible results are used.

Table 4 Univariate survival analysis of 245 breast cancer patients treated with surgery followed by anthracycline-based adjuvant chemotherapy

Parameter	N	*Events*	*DFS (Mean±s.d.)*	P-*value (Log Rank Test)*	*Events*	*OS (Mean±s.d)*	P-*value (Log Rank Test)*
Size				$P<0.005$			$P>0.1$
T1	127	25	111.7±4.33		20	115.4±4.09	
T2	100	31	99.2±5.41		18	114.9±4.35	
T3	16	9	54.9±7.45		4	76.4±7.47	
Grade				$P<0.005$			$P<0.1$
I	23	1	116.8±3.94		1	117±3.64	
II	69	12	116.4±4.98		8	121±4.93	
III	148	50	95.9±4.52		33	109±4.00	
LN mets				$P<0.0001$			$P<0.0005$
No	83	10	122.2±3.96		5	129±2.60	
Yes	154	54	93.5±4.61		37	105±4.32	
LVI				$P>0.1$			$P>0.1$
No	82	19	109.9±5.18		11	121±4.15	
Yes	161	46	94.9±4.04		31	104±3.73	
ER				$P<0.05$			$P=0.0001$
Negative	48	19	81.2±6.94		17	86.8±6.53	
Positive	191	44	107.9±3.73		24	119.2±3.17	
PgR				$P>0.1$			$P<0.0005$
Negative	64	21	89.3±6.25		20	92.8±5.84	
Positive	175	42	106.9±3.92		21	119.7±3.33	
HER2				$P>0.1$			$P>0.1$
Negative	200	52	104.3±3.75		32	115±3.43	
Positive	36	11	92.9±7.71		9	102±7.28	
EGFR				$P>0.1$			$P<0.1$
Negative	222	57	105±3.55		35	115.5±3.18	
Positive	22	8	86±9.62		7	92.3±8.79	
Ck 14				$P>0.1$			$P<0.05$
Negative	221	57	104.2±3.65		34	116.0±3.13	
Positive	22	8	84.5±10.13		8	86.6±9.47	
Ck 5/6				$P<0.1$			$P<0.01$
Negative	210	53	105.4±3.63		32	116.2±3.19	
Positive	25	10	80.4±9.81		9	86.8±8.95	
Ck 17				$P<0.05$			$P<0.0001$
Negative	213	51	106.5±3.60		28	118.5±3.06	
Positive	28	12	77.2±9.41		12	80.3±8.72	
Basal markers				$P<0.05$			$P<0.001$
Negative	204	49	106.8±3.67		28	117.7±3.19	
Positive	39	16	79.4±7.85		14	87.1±7.13	
Nielsen groups				$P>0.1$			$P<0.005$
Basal	30	12	81.3±8.74		11	87.2±8.01	
Luminal	164	11	92.9±7.71		19	102.2±7.28	
HER2	36	37	107.9±4.13		9	119.1±3.68	
p53				$P<0.05$			$P<0.001$
Negative	158	37	107.8±3.99		18	120±3.52	
Positive	67	23	94.9±6.93		20	103±6.06	
MIB-1				$P<0.05$			$P<0.005$
<10%	96	18	112.2±5.04		11	122.4±3.66	
10–30%	97	29	100.8±5.47		16	111.4±5.48	
>30%	33	15	76.4±8.38		13	88.8±8.44	

Table 4 Continued

Parameter	N	*Events*	*DFS (Mean±s.d.)*	P-*value (Log Rank Test)*	*Events*	*OS (Mean±s.d)*	P-*value (Log Rank Test)*
Cyclin D1				$P<0.1$			$P<0.05$
Negative/weak	26	11	70.8±7.81		26	81.1±7.64	
Moderate	46	12	86.8±6.04		46	106.2±6.29	
Strong	151	36	107.7±4.08		151	118.2±3.51	
CCND1				$P>0.1$			$P>0.1$
Nonamplified	211	56	103.6±3.67		37	114±3.25	
Amplified	30	9	94.8±8.17		5	107±6.81	

CCND1: cyclin D1 gene; Ck: cytokeratin; ER: oestrogen receptor; LN mets: lymph node metastasis; LVI: lymoho-vascular invasion; Nielsen groups: immunophenotypic groups defined based upon the expression of ER, HER2, Ck 5/6 and EGFR; PgR: progesterone receptor; SD: standard deviation.

Conflicting results on the prognostic impact of cyclin D1 overexpression and clinical outcome in breast cancer patients have been reported.[1,11,16–21,37–39] Despite the known problems with commercially available anticyclin D1 antibodies, a considerable amount of data linking cyclin D1 overexpression with lack of response to tamoxifen have been published.[13,14]

After several attempts at optimising mouse monoclonal and rabbit polyclonal antibodies to cyclin D1, we have attained reproducible results with the rabbit monoclonal antibody employing the protocol described by Cheuk *et al*.[28] In contrast to other antibodies, the new rabbit anticyclin D1 monoclonal antibody shows a strong correlation with *CCND1* gene amplification. However, in accordance with previous studies,[1,11,13,14,16,19–21] cyclin D1 overexpression was still more pervasive than gene amplification. In the present study, 67.4% of the cases showed strong cyclin D1 expression, whereas only 11.6% showed very low levels or no expression of this cell cycle regulator. This is not surprising, given that this protein plays a pivotal role in the progression from G1 to S phase[2] and can be upregulated through several different pathways, including the ER, c-myc and fibroblast growth factor receptor pathways[2–4,40] (and references therein). In fact, the cell cycle effects of oestrogen are directly linked with upregulation of cyclin D1 overexpression.[2] Although it is clear that ER-induced cyclin D1 expression is pivotal to induce cell cycle progression in oestrogen stimulated cells,[2] *CCND1* gene regulation by oestrogens appears complex, involving both primary and secondary events that have not yet been fully elucidated.[41] *CCND1* is a transcriptional target of ER[41–43] and this process appears to be directly mediated through coordinate recruitment of specific ER coactivators[2] and activation of IKKα[44] and MAPK pathways.[44] On the other hand, antioestrogens are reported to induce an acute downregulation of cyclin D1.[2,42] Cell line models have demonstrated that when cyclin D1 is inhibited by anticyclin D1 antibodies or by upregulation of $p16^{INK4A}$ oestrogen cannot induce G1–S phase progression.[2,45] Interestingly, cyclin D1 may also regulate ER pathway activity by physically interacting with ER and promoting ER pathway activation in a hormone-independent fashion.

The frequency of *CCND1* amplifications as defined by CISH in the present study (14.6%) is similar to that reported in breast cancer using Southern blot[7–9] or FISH.[1,10,12,13,15,19,39] CISH has several major advantages,[24] which are illustrated in this study; the whole analysis of the 245 replicate cores took less than a week, the neoplastic cells were easily recognisable and only 16% of the cores were not interpretable.

We have confirmed the strong association between cyclin D1 overexpression and positivity for hormone receptors[1,9,10,19,21,39] and have also found a strong inverse correlation with the expression of 'basal-like' markers (ie, EGFR, Ck 14, Ck 5/6 and Ck 17). Although we did not find a statistically significant association between *CCND1* amplification and positivity for hormone receptors, *CCND1* amplification showed an inverse correlation with tumours with basal-like immunophenotype (all cases with basal-like phenotype did not harbour *CCND1* amplification). Therefore, our findings corroborate the results of Vaziri *et al*,[46] who did not find *CCND1* amplifications in *BRCA1* mutation carriers; these tumours frequently harbour a basal-like phenotype[40,41] Taken together, these findings suggest that cyclin D1 is unlikely to play a role in the biology of 'basal-like' carcinomas. In fact, there are several lines of evidence to suggest that cyclin E rather than cyclin D1 would be more biologically significant for this aggressive subgroup of breast carcinomas.[39,47]

In the present study, cyclin D1 overexpression as defined by the SP4 antibody was significantly correlated with OS. Patients whose tumours displayed strong and moderate cyclin D1 expression showed significantly longer OS than those with no or weak expression, which is in agreement with previous studies.[1,19,37] However, others have found an association between cyclin D1 overexpression and poorer clinical outcome.[23,48] This discrepancy may stem from the use of different antibodies, different thresholds for Cyclin D1 positivity and

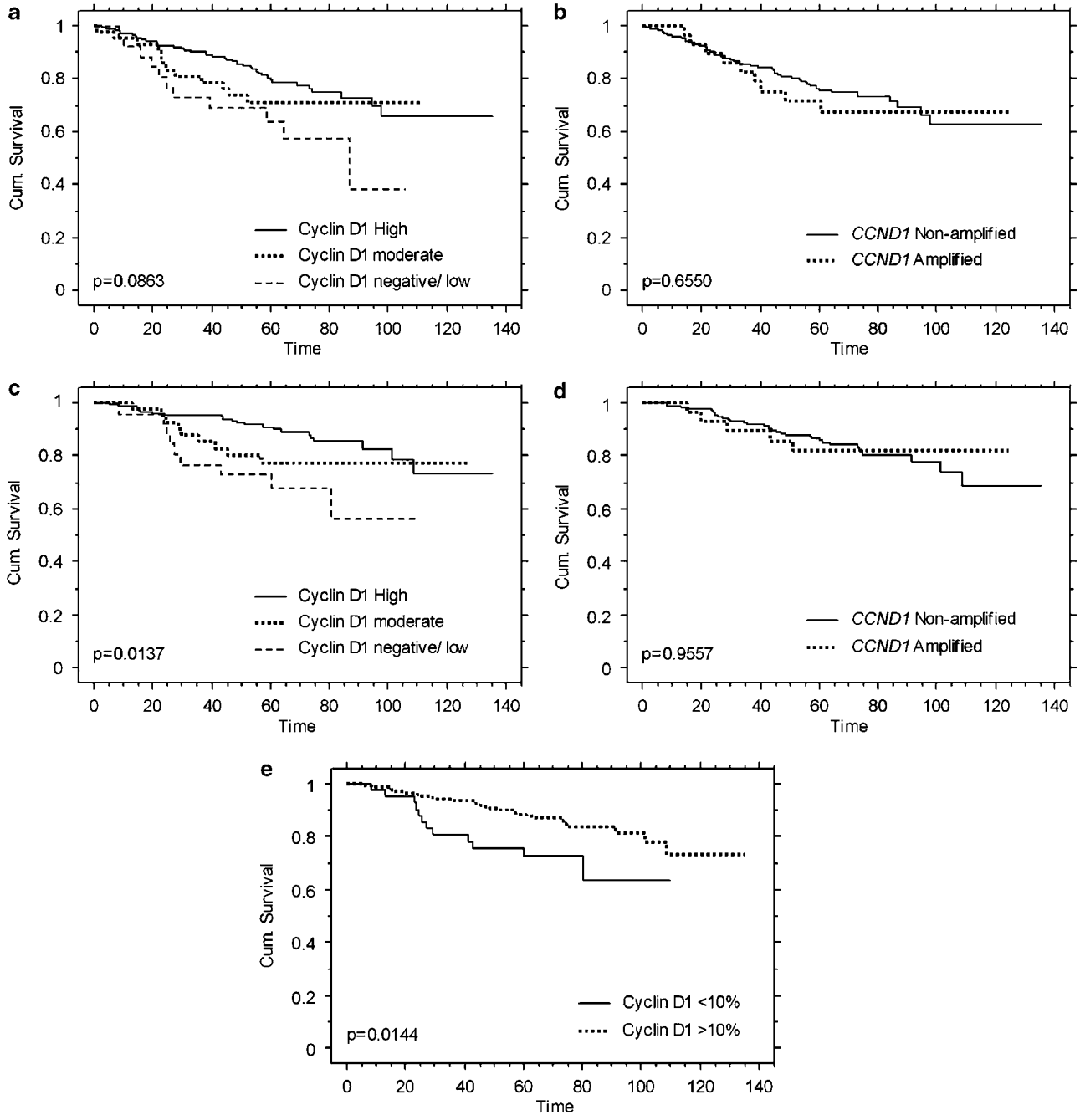

Figure 2 Univariate analysis of the prognostic impact of cyclin D1 overexpression and gene amplification on DFS and OS survival. (**a**)—Cyclin D1 expression—DFS; (**b**)—*CCND1* amplification—DFS; (**c**)—Cyclin D1 expression—OS; (**d**)—*CCND1* amplification—OS; (**g**)—Cyclin D1 expression as defined by the 10% cutoff.

different methods for the analysis of Cyclin D1 expression (Western blotting *vs* imunohistochemistry in the present study and others). Adopting thresholds of ≤ 10 and $>90\%$ as described by Stendahl *et al*,[14] we observed that tumours with Cyclin D1 overexpression still showed a better OS compared to those devoid Cyclin D1 expression (Figures 2e). These differences in the prognostic impact of cyclin D1 may also be explained by the fact that all patients received anthracycline-based adjuvant chemotherapy in the present study. As for the prognostic impact of *CCND1* amplification, our data are in agreement with the results of previous studies showing that *CCND1* amplification is not associated with poor prognosis in a consecutive series of ER-positive and ER-negative breast cancers.[12,17]

Based upon real-time polymerase chain reaction assessment of *CCND1* gene copy numbers and cyclin D1 expression levels performed with nucleic acids obtained from nonmicrodissected breast tissue, Bieche *et al*[16] have called into question the role of

CCND1 as the amplicon driver on 11q13.[16] Moreover, when Jirstrom *et al*[13] compared *CCND1* amplification and overexpression, these authors observed that approx 20% of the cases expressed low levels cyclin D1. One might argue that these results could be interpreted as evidence to suggest that *CCND1* would not be the only amplicon driver of amplifications of 11q.[16] Although up to four distinct amplicon cores have been described on 11q,[49,50] the probes used by Jirstrom *et al*[13] specifically map to *CCND1*. Using an equally specific probe, we observed Cyclin D1 expression, either moderate or strong, in all cases with gene amplification. Given that the methods used by Bieche *et al*[16] cannot differentiate between the expression of cyclin D1 in normal breast tissue and breast cancer cells and that the antibody used by Jirstrom *et al*[13] is reported to have a limited sensitivity (approx 75%),[28,51,52] our results would argue that *CCND1* remains the likeliest candidate of the 11q13 amplicon core mapping to the 69 Mb region.[38]

In conclusion, this study confirms that the cyclin D1 SP4 rabbit monoclonal antibody is capable of producing reliable and reproducible results and that CISH is a remarkably reproducible and easily applied technique for assessing gene amplification. In addition, a strong correlation between cyclin D1 and *CCND1* amplification has been demonstrated in this series of patients. Our data have shown a strong inverse correlation between immunohistochemical 'basal-like' markers and both cyclin D1 overexpression and *CCND1* amplification. Furthermore, all 'basal-like' carcinomas as defined by the immunohistochemical panel proposed by Nielsen *et al*[36] lacked *CCND1* amplification, suggesting that this cell cycle regulator plays a limited role in the biology of 'basal-like' breast cancer.

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