J Cancer Res Clin Oncol (1999) 125:505–512

ORIGINAL PAPER

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Cyclin D1 overexpression in invasive breast cancers: correlation with cyclin-dependent kinase 4 and oestrogen receptor overexpression, and lack of correlation with mitotic activity

Received: 18 November 1998 / Accepted: 22 February 1999

Abstract ***Background***: Cyclin D1 (CCND1) and its catalytic partner cyclin-dependent kinase 4 (cdk4) are known to play important roles in the G1/S check point of the cell cycle, and CCND1 overexpression has been reported to correlate with progression and prognosis of breast cancers. Oestrogen receptor (ER) levels determine the proliferative response to oestrogen by regulating binding. It has been postulated that CCND1 and cdk4 exert effects on mammary carcinogenesis in co-operation with ER. ***Patients and methods***: CCND1 and cdk4 overexpression in 117 breast cancer cases with long-term follow-up were investigated by means of immunohistochemistry and differential polymerase chain reaction (PCR), using formalin-fixed and paraffin-embedded samples, and compared with ER status and mitotic indices. Additional Western blotting and reverse transcription (RT)/PCR/Southern blot hybridization were performed for 4 breast cancer cell lines and 15 fresh-frozen breast cancer samples to confirm CCND1 and cdk4 data. ***Results***: Immunohistochemically 27 cases were CCND1-positive (23.0%), and *CCND1* amplification was evident in 21 (21/86; 24.4%). The two methods in combination demonstrated 37 cases (31.6%) to be positive for CCND1 overexpression. Western blotting revealed 60% of samples of fresh tissue to overexpress CCND1, corresponding well with the results of RT-PCR. There was thus a strong discrepancy between results for paraffin block and fresh samples, probably because of the short life of CCND1. In the case of cdk4, the respective percentages for positive cases were 54.7% and 73%. CCND1 and cdk4 overexpression ($P < 0.0001$), and CCND1 and ER positivity ($P = 0.0128$) correlated. In addition, samples overexpressing CCND1, cdk4 and ER tended to have slightly lower proliferative activity than samples where these were absent. However, no association with clinicopathological parameters was evident. cdk4 overexpression had no linkage with ER status or clinicopathological status. Neither CCND1 nor cdk4 expression affected prognosis. ***Conclusion***: CCND1 overexpression does not correlate with cancer progression or prognosis or with mitotic activity. The results may suggest that an excess accumulation of CCND1 in breast cancer cells tends to suppress entry into the S phase of the cell cycle.

Key words Breast cancer · Cyclin D1 · Cyclin-dependent kinase 4 · Oestrogen receptor · Proliferation

Abbreviations *CCND1* cyclin D1 · *cdk4* cyclin-dependent kinase 4 · *ER* oestrogen receptor · *PR* progesterone receptor · *RT-PCR* reverse transcription/polymerase chain reaction

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Introduction

The cyclin D1 (CCND1) gene, cloned in 1991, was detected as a defective gene mapping to chromosome 11q13 and was thought to be the cause of parathyroid adenoma (Motokura et al. 1991; Xiong et al. 1991). Two further types of D cyclins, named cyclins D2 and D3, were discovered soon after (Sherr 1993). Transcription of D cyclin mRNA and protein synthesis is

highest in middle–late G1 and lowest during S phase (Baldin et al. 1993). They map to different chromosomes but have an identical cyclin box, a region of conserved sequence homology and the so-called PEST sequence near their carboxy termini. The open reading frames encode proteins of around 34 kDa, which are between 57% and 62% identical in pairwise comparisons (Motokura et al. 1991; Peters 1994). CCND1 is a labile protein and its half-life is short (Sewing et al. 1993).

Overexpression and rearrangement of the *CCND1* gene have been previously reported to be associated with tumour progression and/or poor prognosis in many different tumour types, such as carcinomas of the breast (Gillet et al. 1994), oesophagus (Gramlich et al. 1994), pancreas (Gansauge et al. 1997) and head and neck (Michalides 1997) and mantle cell lymphomas (Nakamura et al. 1997). Gene rearrangements result in the transcriptional activation of *CCND1* (Motokura et al. 1991) and the commonest genetic abnormality affecting *CCND1* is DNA amplification (Peters 1994). Deregulation of other cyclins has also been found in tumours, but CCND1 appears to be of singular importance in the multistep process of oncogenesis (Keyomarsi and Pardee 1993).

Chromosome 12q13 encompasses the gene for cyclin-dependent kinase 4 (cdk4), one of the catalytic partners for CCND1. DNA amplification of cdk4 is also observed, for example, in human sarcomas and gliomas, presumably leading to increased activity of cyclin-D-dependent kinase complexes (Khatib et al. 1993). It has been reported that association with cdk4 is required for CCND1 to function in G1 progression (Tam et al. 1994).

The oestrogen receptor (ER) is a nuclear regulatory protein that functions as a hormone-activated transcription factor. Its activation is considered to be a consequence of ligand-induced conformation changes in the structure (Kumar and Chambon 1988). Extracellular oestrogen freely enters across the cell membrane and binds to ER, leading to ER dimerization and translocation to the nucleus. The oestrogen-ER complex binds with high affinity to a well-defined 13-bp palindromic sequence, the oestrogen response element (ERE) (Beato et al. 1995). Enhancer-like ERE sequences are located near or within oestrogen-responsive genes (Beato 1991). After ERE binding, the liganded ER activates transcription, resulting in stimulation of proliferation (Dubik and Shiu 1988).

In the present study, the possible relationship between ER, CCND1/cdk4 and proliferative activity in breast tumours was examined.

Materials and methods

Cases and histology

A group of 117 cases of surgically resected invasive ductal carcinoma of the breast were selected from the patients' charts of Kitasato University Hospital from 1989 to 1991. All cases underwent total mastectomy and removement of regional lymph nodes, and were followed until August, 1998. Adjuvant chemotherapy and/or irradiation were performed in all cases. The majority of cases were examined for their ER and progesterone receptor (PR) status by biochemical methods. ER and PR contents were assessed with the dextran-coated charcoal assay on tumour cytosols, receptor levels above 10 fmol/mg cytosolic protein being taken as positive.

The patients were all female, and their mean age was 57.2 years. Histopathological diagnoses were made by the classification of Bloom and Richardson, described elsewhere (Bloom and Richardson 1957), with grades I (36 cases), II (53) and III (28).

Mitotic indices were calculated after counting 2000 nuclei in randomly selected fields for each case under a light microscope (using ×40 objective and ×10 ocular lenses).

For additional experiments to confirm the results of the present study, Western blotting, reverse transcription/polymerase chain reaction (RT-PCR) and differential PCR were performed for 4 breast cancer cell lines (HCF7, HBC7, HBC9 and HBC4p) and 15 snap-frozen (−80°C) fresh samples of breast cancer tissue obtained surgically at Kitasato University Hospital from 1996 to 1997.

Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded tissue, 4 μm thick, were employed for the study with a combination of the ordinary labelled streptavidin-biotin-peroxidase (LSAB kit, Dako, Denmark) method and heating in a microwave oven. Antibodies used were DCS-6 mouse monoclonal antibodies (×10 dilution; YLEM, Roma, Italy) for CCND1 protein and cdk4 (c-22) rabbit polyclonal antibody (×1000 dilution; Santa Cruz Biotechnology, USA) for cdk4 protein. Counterstaining was achieved with 0.3% methyl green solution.

Cases were defined as positive for CCND1 immunostaining when over 5% of the cells were stained in each section in accordance with the criteria described elsewhere (Gillet et al. 1996). The same criteria were employed for cdk4. Breast cancer cases positive for CCND1 and cdk4, proven by Western blotting, were included in each run as positive controls. Samples of lymph nodes with lymphadenitis, negative for CCND1 and cdk4 on Western blotting, were used as negative controls.

Western blotting

Proteins were extracted routinely and samples (40 μg protein) were electrophoresed on 8% sodium dodecyl sulfate/polyacrylamide gels at 30 mA for 3 h, and transferred onto 0.45-mm polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.), in a semi-dry system (Biocraft, Tokyo, Japan) at 200 mA for 30 min. Membranes were blocked with 15% skimmed milk in phosphate-buffered saline and then incubated with DCS-6 and c-22 antibodies (×20 and ×2000 dilutions) at 4°C overnight, followed by exposure to horseradish-peroxidase-conjugated rabbit anti-(mouse IgG) and swine anti-(rabbit IgG) (Dako). Specific binding of antibody was determined by enhanced chemiluminescence (Amersham, Arlington Heights, III) of X-ray films (Fuji RX-U, Japan).

RT-PCR Southern blot hybridization

mRNA was isolated by the phenol and guanidinium thiocyanate method. A 1-mg sample of mRNA was dissolved in 20 μl reaction buffer containing reverse transcriptase (RAV-2, Takara, Ohtu, Japan), random primers (Takara) and ribonuclease inhibitor (Takara). After incubation of 37°C for 1 h, cDNA was obtained. Total cDNA was amplified in a final volume of 100 μl reaction mixture containing 2 U Taq polymerase (Takara), using 1 μM primers for the 434-bp *CCND1* fragment (5′-CTGGAGCCCGTGAAAAAGAGC-3′ and 5′-CTGGAGAGGAAGCGTGTGAGG-3′), the 396-bp cdk4 fragment (5′-ATCAGCACAGTTCGTGAGGTGGC-3′ and 5′-AGCTCGGTACCAGAGTGTAACAAC-3′) and the 412-bp β-actin fragment (5′-TGATGATATCGCCGCGCTCGTCGT-3′ and 5′-CACAGCCTGGATAGCAACGTACAT-3′) under the following cycling conditions: 94°C for 1 min, 55°C for 1 min

and 72°C for 2 min for 25 cycles. The PCR products were electrophoresed through 2% agarose gels containing ethidium bromide and photographed under UV light. After the gels had been denatured, neutralized and transferred by Southern blotting onto nylon membranes (Hybon N+, Amersham, UK), hybridization was performed, using 10 pmol/ml digoxygenin-labelled oligonucleotide probes for the respective samples (CCND1: 5′-GCTGTGCATCTACACCGACGGCTCCATCCG-3′, cdk4: 5′-GCTGATGGACGTCTGTGCCACATCCCGAAC-3′ and β-actin: 5′-CATCGAGCACGGCATCGTCACCAACTGGGA-3′) with DIG Easy Hyb (Boehringer Mannheim, GmbH, Germany) at 60°C for 16 h. After the membrane had been washed with 2× standard saline citrate (SSC)/0.1% SDS and 0.1× SSC/0.1% SDS at 60°C, luminescence was detected with X-ray films (Fuji RX-U, Japan) employing a Dig luminescent detection kit for nucleic acids (Boehringer Mannheim).

Differential PCR

Routine DNA extraction with phenol/chloroform was performed for cell lines, fresh-frozen and paraffin-embedded tissue. For the latter, the microdissection method for inoculating only cancer cells from 5-μm-thick sections on uncoated slides under a microscope was carried out after routine removal of paraffin and staining with haematoxylin.

Two sets of primers were employed for differential PCR: for the 151-bp CCND1 gene (*CCND1*), 11q13 (5′-ACCAGCTCCTGTGCTGCGAA-3′ and 5′-CAGGACCTCCTTCTGCACAC-3′), and for the 112-bp dopamine D2 receptor gene (*DR*), 11q22–23 (5′-CCACTGAATCTGTCCTGGTATG-3′ and 5′-GCGTGGCATAGTAGTTGTAGTGG-3′).

A 25-μl reaction mixture containing 0.5 μg DNA, 0.5 U Taq polymerase and 0.5 μM each primer was amplified with PCR under the following conditions: 2 min at 95°C, followed by 30 cycles for fresh tissue DNA and 35 cycles for microdissected DNA with 1 min each at 95°C, 55°C and 72°C. Following amplification, the samples were electrophoresed on 8% polyacrylamide gels and stained with ethidium bromide. UV-illuminated gels were photographed and data were transformed to a computer (Power Macintosh 7600/120) by scanner (Epson GT-9500). Densities of bands for *CCND1* and *DR* were calculated with NIH Image software to obtain *CCND1*/*DR* ratios. The average *CCND1*/*DR* ratio for 20 samples of normal breast epithelium microdissected from paraffin-embedded material was 0.43 ± 0.05 (SE). The deviation of the *CCND1*/*DR* ratio for normal breast epithelium from the expected value of 1.0 was due to the greater efficiency of the *DR* primers. Since this bias in the *CCND1*/*DR* ratio would be expected to occur consistently in all specimens, it was of no significance in interpreting the results of differential PCR assays. The criteria for amplification to values greater than the *CCND1*/*DR* ratio of normal epithelium were in line with those defined in the previous report (Gramlich et al. 1994).

Statistical evaluation

Statistical analysis was performed using the Mann-Whitney U-test and the χ^2-test for demonstration of the relation between CCND1, cdk4 and ER positivity and other data. Survival time was defined as the time from the operation to death from cancer. Differences between the survival curves generated by the Kaplan-Meier method were statistically analysed by the generalised Wilcoxon test.

Results

Cell lines and fresh cancer tissue

The upper portion of Fig. 1 shows the results of Western blotting with DCS-6 for breast cancer cell lines (lane 1, HCF7; lane 2, HBC7; lane 3, HBC9; lane 4, HBC4p).

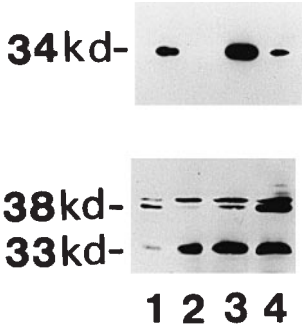

Fig. 1 The *upper portion* demonstrates Western blotting of DCS-6. Clear single bands around 34 kDa (*kD*) are apparent in lanes *1*, *3* and *4*. Blotting of c-22 is shown *below*: a doublet at around 38 kDa and a single band at 33 kDa. The single band is cdk4, but the doublet bands are of unknown origin

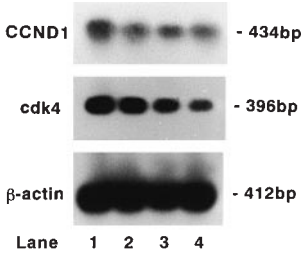

Fig. 2 Results of reverse transcription/polymerase chain reaction (RT-PCR)/Southern blot hybridization for breast cancer cell lines. In the *upper portion* clear bands around 434 bp in length, identified with the sequence of the cyclin D1 gene (*CCND1*), are visible in all lanes. In the *middle portion* single bands 396 bp in length, identified with the *cdk4* sequence, are found in all lanes. The *lower portion* indicates successful RT-PCR/Southern blot hybridization, in this case using the internal standard of β-actin fragment of 412 bp

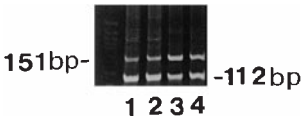

Fig. 3 Results of differential PCR. *CCND1*/dopamine D2 receptor gene (*DR*) ratios are 0.40, 0.43, 1.10 and 0.98 for lanes *1–4* respectively, *CCND1* amplification is demonstrated by HBC9 and HBC4p

Similar bands around 34 kDa were visible in three of the four cell lines. In the lower portion, all cell lines demonstrated a doublet at around 38 kDa and a single band at 33 kDa, the latter corresponding to cdk4: the former was an unknown antigen. The results of RT-PCR/Southern blot hybridization for the breast cancer cell lines are shown in Fig. 2. The upper portion demonstrates positive results of CCND1 mRNA overexpression in all cases and the middle portion shows positive results of cdk4 mRNA overexpression. The quality of cDNA obtained from breast cancer cell lines was proven to be reliable by the results of β-actin in the lower portion. The results of differential PCR are demonstrated in Fig. 3. The *CCND1*/*DR* ratio was 0.40 in lane 1, 0.43 in lane 2, 1.10 in lane 3 and 0.98 in lane 4. Thus, *CCND1* amplification was noted in HBC9 and HBC4p.

Out of 15 fresh material samples, 9 (60%) demonstrated clear single or doublet bands on Western blotting, as shown in Fig. 4. Western blotting for cdk4 revealed 87% positivity. RT-PCR/Southern blot hybridization of the *CCND1* and *cdk4* fragments showed overexpression in 60% and 87% of samples respectively. There was no discrepancy between Western blotting and RT-PCR/Southern blot hybridization results, unlike the results in breast cancer cell lines. Differential PCR detected *CCND1* amplification in 27% of samples (case 3, *CCND1/DR* ratio = 1.06; case 5, 1.00; case 10, 0.94; case 12, 0.93). Immunohistochemistry showed CCND1 positivity in 47% and cdk4 in 87%.

The data, summarized in Table 1, indicate:

1. CCND1 is overexpressed in around 60% of cancers, Western blotting findings being mostly supported by the mRNA overexpression.
2. CCND1 overexpression does not require *CCND1* amplification, although the two may be associated.
3. CCND1 immunohistochemistry of formalin-fixed material is less sensitive than Western blotting, possibly because of the short half-life of CCND1.
4. cdk4 is overexpressed in the majority of breast cancers and is not always linked to CCND1 overexpression.

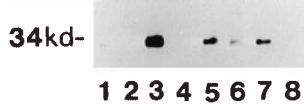

Fig. 4 Results of Western blotting for CCND1 of frozen samples of breast cancer. Clear single bands of 34 kDa are visible in lanes *3*, *5*, *6* and *7*

Clinical cases

Immunohistochemistry for CCND1 demonstrated 27 of 117 breast cancer cases (23%) to show positive nuclear staining (Fig. 5). Cytoplasmic staining was observed in 6 cases (5.1%), these being treated as negative. cdk4 was positive in 64 (54.7%) and most of the cdk4-positive cases displayed staining mainly in the nucleus with only weak cytoplasmic binding (Fig. 6). *CCND1* amplification on the basis of differential PCR was noted in 21 out of 86 informative cases (24.4%). Thirty-one cases were not included because there was no PCR amplification or inappropriate results on analysis by the NIH Image program. Cases with CCND1-positive staining and/or *CCND1* amplification were treated as $CCND1^+$. Table 2 shows the relation between CCND1 and cdk4 positivity and clinicopathological data. Statistically significant differences were noted between $CCND1^+$ and $cdk4^+$ and between $CCND1^+$ and ER status.

The data suggest that:

1. ER overexpression correlates with CCND1 overexpression.
2. ER overexpression is independent of cdk4.
3. CCND1 overexpression is not related to malignant histological grading or cancer progression.
4. CCND1 overexpression correlates with cdk4 overexpression in general.

Figure 7a–c illustrates Kaplan-Meier survival curves comparing $CCND1^+$ with $CCND1^-$, $cdk4^+$ with $cdk4^-$, and ER^+ with ER^-. No significant differences were apparent in any of the comparisons. Thus, CCND1 and cdk4 overexpression does not affect prognosis. Also, cases with *CCND1* amplification do not show a worse prognosis. The ER^+ group appeared to have a better

Table 1 Results for cyclin D1 (*CCND1*) and cyclin-dependent kinase 4 (*cdk4*) overexpression demonstrated by Western blotting, reverse transcription/polymerase chain reaction (RT-PCR)/Southern blot hybridization, differential PCR and immunohistochemistry in cell lines and fresh samples of breast cancer. *Western blot.* Western blotting, *RT-PCR Southern* RT-PCR/Southern blot hybridization, *Gene amp.* gene amplification proved by differential PCR, *ND* not done

Cell line or case no.	Western blot.		RT-PCR/Southern		Gene amp. *CCND1*	Immunohistochemistry	
	CCND1	cdk4	CCND1	cdk4		CCND1	cdk4
HCF7	+	+	+	+	–	ND	ND
HBC7	–	+	+	+	–	ND	ND
HBC9	+	+	+	+	+	ND	ND
HBC4p	+	+	+	+	+	ND	ND
Case							
1	–	+	–	+	–	–	+
2	–	+	–	+	–	–	+
3	+	+	+	+	+	+	+
4	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+
6	+	+	+	+	–	–	+
7	+	+	+	+	–	–	+
8	–	+	–	+	–	–	+
9	+	+	+	+	–	+	+
10	+	+	+	+	+	+	+
11	–	+	–	+	–	+	+
12	+	+	+	+	+	+	+
13	–	–	–	–	–	–	–
14	+	+	+	+	–	–	+
15	+	+	+	+	–	–	+

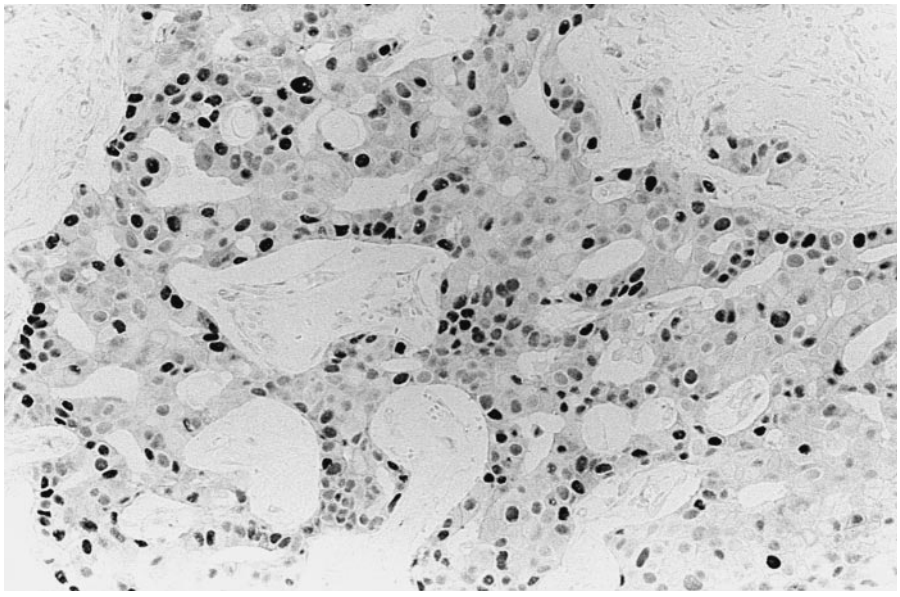

Fig. 5 Immunohistochemistry of CCND1. Strong or weak positive staining, restricted to nuclei, is found in cancer cells. ×200

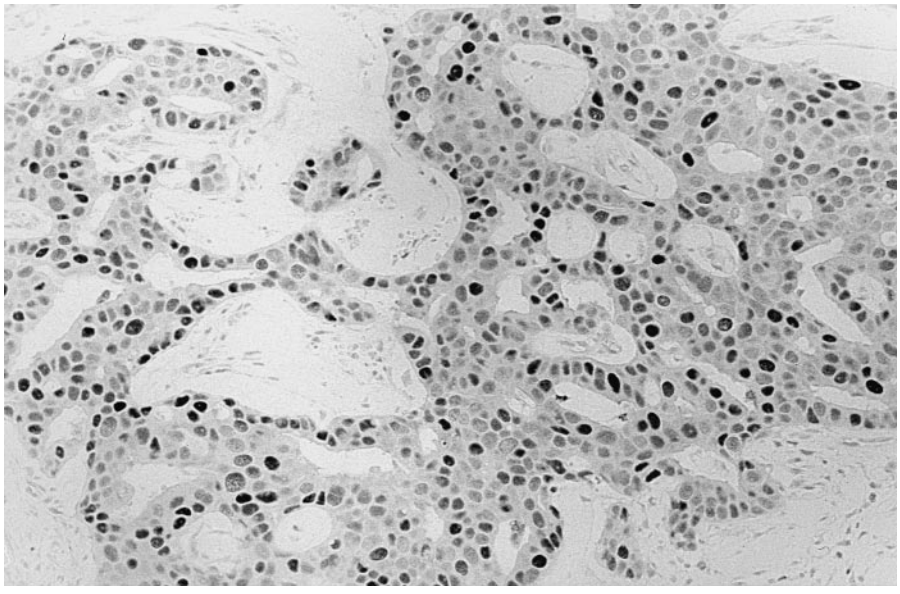

Fig. 6 Immunohistochemical staining for cdk4 in the same case as illustrated in Fig. 5. Relatively homogeneous positive binding, restricted to nuclei, is apparent. ×200

prognosis in the short term, but similar survival curves emerged in the longer term for ER^+ and ER^- groups.

Data relating $CCND1^+/CCND1^-$, $cdk4^+/cdk4^-$ and ER^+/ER^- to mitotic index are shown in Table 3. A slightly lower mitotic index was recognized in $CCND1^+$, $cdk4^+$ and ER^+ cases.

Discussion

The fact that CCND1 is a labile nuclear protein, with a half-life of about 40 min (Sewing et al. 1993), may explain the discrepancy between results for formalin-fixed and fresh tissue in the present series. Previous immunohistochemical evaluation of CCND1 gave varying positivity between 20% and 81% for breast cancers, possibly because of the variety of antibodies and evaluation criteria (Gillet et al. 1994, 1996; van Diest et al. 1997) . It is possible that previous reports showing much higher positivity of CCND1 may include overestimation and/or false positive results. Our differential PCR finding of 24% positive for gene amplification is in line with the previous observations of about 15% (Peters et al. 1995). Since *CCND1* amplification may correlate with increased mRNA and CCND1 overexpression, although this does not always occur (Jares et al. 1997), cases of *CCND1* amplification were combined with immunohistochemically CCND1-positive cases for clinical comparisons. Even when amplification cases were added, 32% positivity for CCND1 is still lower than the results of Western blotting and even immunostaining of fresh samples. This may represent

Table 2 Comparisons of CCND1 and cdk4 overexpression with clinicopathological data for invasive breast cancers. *LN* lymph node involvement *ER* oestrogen receptor, *PR* progesterone receptor. The total numbers in each category are shown in parentheses

Clinicopathological status	Total (117)	CCND1$^+$ (37)	cdk4 (64)
Histology grade			
I	36	10*	17
II	53	19*	32
III	28	8*	15
ER$^+$	77	29**	44
ER$^-$	26	3**	10
PR$^+$	46	18	26
PR$^-$	56	14	28
LN$^+$	65	24	34
LN$^-$	52	13	30

*$P < 0.0001$
**$P = 0.0128$

the limit for investigating CCND1 in long-term follow-up cases using formalin-fixed and paraffin-embedded samples.

The results of Western blotting and RT-PCR/Southern blot hybridization for *CCND1* and *cdk4* showed no discrepancy except for *CCND1* in 1 breast cancer cell line. It seems likely that mRNA overexpression directly links to protein overexpression for both CCND1 and cdk4. The one exception, in cell line HBC7, was difficult to explain since no further intensive investigation was performed.

We could not use paired normal breast tissue to examine overexpression of protein and mRNA of *CCND1* and *cdk4* since it was difficult to obtain paired normal breast samples. However, the comparison between cancerous and normal tissues in the same case should be used to investigate the overexpression of CCND1 and cdk4 protein and mRNA. In our experience, they are seldom overexpressed in normal gastric epithelium even when paired cancerous samples show overexpression (data not shown). In contrast, normal breast epithelium seems to show different results, overexpressing CCND1 in 11.7% of cases compared to 48.3% for invasive carcinoma according to Western blotting and immunohistochemistry (Alle et al. 1998). Benign breast lesions also overexpressed *CCND1* mRNA in 18% of cases compared to 83% for invasive carcinoma, on the basis of in situ hybridization (Weinstat-Saslow et al. 1995). There has been no report of overexpression of cdk4 and *cdk4* mRNA in normal breast epithelium.

Contradictory reports have been published on the correlation between *CCND1* amplification and CCND1 overexpression and clinicopathological parameters. An association with a poor prognosis of breast cancers was found in one study (Adnane et al. 1989), but this was not observed in other recent studies (Jares et al. 1997; Worsley et al. 1996) and the present series. Our results are similar to those of Michalides et al. (1996), who investigated 248 breast cancer cases with long-term follow-up, showing 34% CCND1 positivity. In addition most

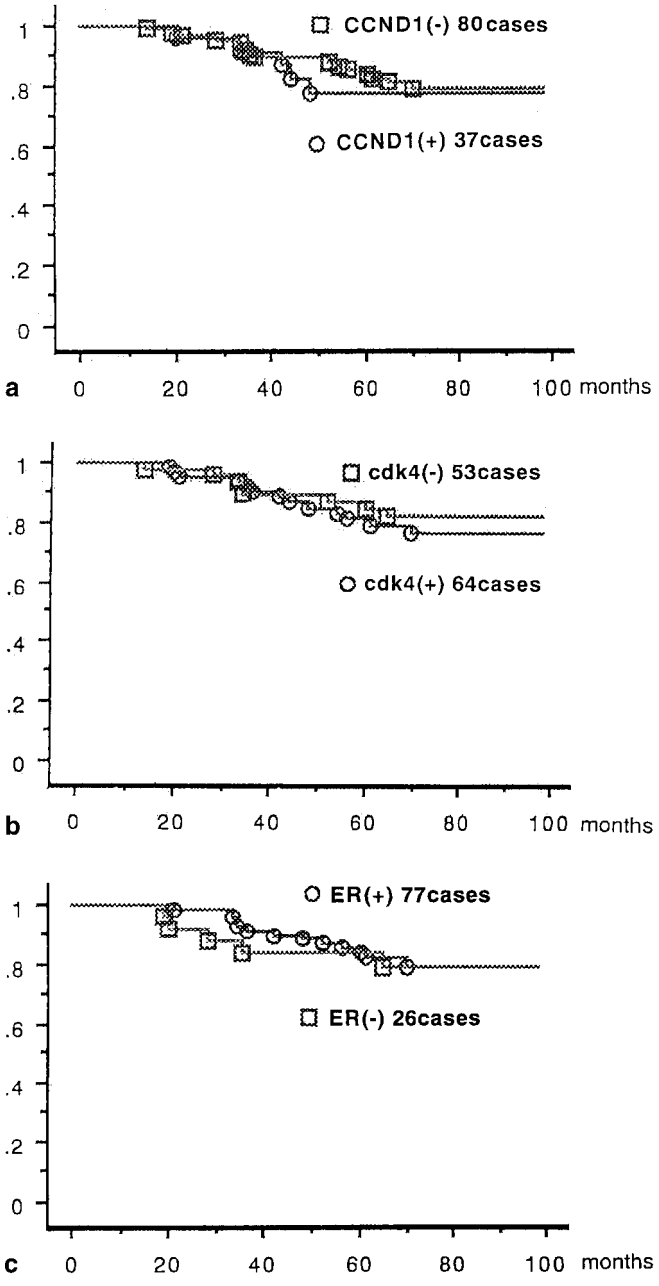

Fig. 7a–c Kaplan-Meier survival curves comparing (**a**) CCND1$^+$ with CCND1$^-$, (**b**) cdk4$^+$ with cdk4$^-$ and (**c**) oestrogen receptor positive (ER$^+$) with ER$^-$

Table 3 Comparisons of mitotic index with CCND1, cdk4 and ER status. The values are shown + standard error. The total numbers in each category are shown in parentheses

Marker	Mitotic index
CCND1$^+$ (37)	0.299 + 0.047
CCND1$^-$ (80)	0.316 + 0.031
cdk4$^+$ (63)	0.309 + 0.035
cdk4$^-$ (54)	0.313 + 0.039
ER$^+$ (76)	0.296 + 0.032
ER$^-$ (26)	0.357 + 0.064
Total (117)	0.311 + 0.026

of the results of other recent studies (Jares et al. 1997; van Diest 1997) are similar to ours.

ER expression is usually observed in carcinomas with a better prognosis and this forms the basis for the therapeutic efficacy of anti-oestrogen drugs for ER-positive breast cancer cases. In the present study, this tendency was recognized, albeit without statistical significance, and a close positive correlation was found between ER and CCND1 expression, in line with recent studies (Jares et al. 1997; van Diest 1997). ER is linked to proliferative activity in collaboration with CCND1 in normal breast epithelial cells. ER are thought to be inactive within the cell before binding to the hormone. The attachment of the hormone results in a conformational change in its cognate protein, which leads to intranuclear translocation and allows association with a specific DNA sequence in the regulatory region of target genes. As a result of ER-ERE binding, transcription of *cis*-linked target genes will be stimulated. Zwijsen et al. (1997) showed that ER transactivation by CCND1 is associated with a direct physical interaction between the two. CCND1 enhances binding of both liganded and unliganded ER to ERE sequences, and the activation is entirely independent of cdk4 binding. In breast cancer cells, it seems likely that an association between ER and CCND1 is maintained, and ER has no linkage to cdk4. It is also reported that ER overexpression relates to differentiation and CCND1 overexpression correlates with a better histological grade in breast cancer (van Diest 1997). There was a tendency for cases showing ER expression to be well differentiated in our series, but this was not significant.

Cases with CCND1 overexpression showed a tendency for lower proliferative activity than negative cases. This is consistent with other reports where flow cytometry (Jares et al. 1997) or bromodeoxyuridine labelling (Zukerberg et al. 1995) was used in breast cancer series. Transfection of cells with vectors expressing CCND1 from constitutive or inducible promoters has been demonstrated to accelerate the G1/S transition (van Diest 1997). However, some authors testified to the toxicity of CCND1 in transfection assays (Quelle et al. 1993), indicating that a toxic effect when CCND1 is in excess may explain this finding (Gillet et al. 1994). In addition, cancer cells that overexpress CCND1 tend to retain wild-type retinoblastoma protein, as one of the observations in the retinoblastoma pathway in cancer cells is that inactivation of any one component of this pathway decreases the probability of identifiable damage to other components (Sherr 1996). Alternatively, CCND1 expression may only be important in the first step of mammary carcinogenesis, being clonally inherited and observed in clinically evident tumours. In fact, the observation of CCND1 expression in pre-invasive lesions has led to the suggestion that this alteration is an early event in breast carcinogenesis (Bartkova et al. 1995). This hypothesis is supported by studies with transgenic mice, in which targeted expression of CCND1 in mammary epithelial cells leads to ductal hyperproliferation and eventual tumour formation (Wang et al. 1994). However, it is most likely that other factors cooperate with this gene in the pathogenesis of the neoplasms.

In conclusion, the present study indicated that CCND1 overexpression does not correlate with cancer progression or prognosis or with mitotic activity in invasive breast cancers. It may also be suggested that an excess accumulation of CCND1 in breast cancer cells instead tends to suppress entry into the S phase of the cell cycle.

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