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# Expression of cyclin Ds in relation to p53 status in human breast carcinomas

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**Abstract** Cyclin D1 has been reported to be overexpressed in many tumours, including breast carcinomas. Cyclin D1 was first identified as a protooncogene (BCL1/PRAD1), and its overexpression was related to tumour proliferation. The product has also recently been identified as important in mediating cell cycle growth arrest via the p53 pathway in murin fibroblast cell lines. Ninety breast carcinomas previously analysed for p53 status were analysed for amplification of cyclin D1, D2 and D3 genes by Southern blot analysis and for protein expression by immunhistochemistry. In 10 samples gene amplification was detected at the cyclin *D1* locus. No gene amplification was detected at the cyclin *D2* and *D3* loci. Immunoreactivity for cyclin D1 was detected in 38 (42.2%) tumour tissue samples. Fifty samples were immunostained for cyclin D2 and D3. Only 2 samples (4%) showed immunoreactivty for cyclin D2, and 9 samples (18%) for cyclin D3. Cyclin D1 protein overexpression was significantly more often found in tumours with wild type p53 and in tumours with higher grades of differentiation expressing ER. No association was seen between gene amplification of the cyclin D1 gene and p53 status. We conclude there is a relationship between wild type p53 and cyclin D1 protein overexpression in clinical material, indicating that cyclin D1 may be another downstream effector of p53.

**Key words** Breast cancer · Cyclin D1 · p53 · Immunohistochemistry

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## Introduction

When DNA is damaged the level of the p53 protein is elevated [21]. This accumulation of the p53 protein results in either cell cycle arrest and DNA repair, or apoptosis [23, 28]. What pathway DNA-damaged cells undergo depends on the extracellular signal, the threshold in different cell types for apoptotic inducers, and expression of other cellular and/or viral proteins [15]. p53-Induced cell cycle arrest is due to the transcriptional activity of p53 in regulating cell cycle checkpoint-related genes, such as *mdm-2*, *GADD 45* and *p21 WAF1/CIP1* [11, 21, 36]. Accumulation of the wild-type p53 protein primarily arrests cells in $G_1$–S [28]. Cell cycle transition from $G_1$ to S phase requires sequential events involving the formation, activation and subsequent inactivation of a series of cyclin/CDK complexes. In addition, regulation of the synthesis and activity of other cyclin/CDK complexes also play a major part in cell cycle control [19, 30].

The D-type cyclins (D1, D2, D3) are important in regulating the $G_1$ checkpoint [19]. The human cyclin D1 gene (*CCND1*) is located on chromosome band 11q13, the cyclin D2 gene (*CCND2*) on 12p13 and the cyclin D3 gene (*CCND3*) on 6p21 [20]. Cyclin D1, D2 and D3 promote progression through the $G_1$ phase of the cell cycle by regulating the activity of the cyclin-dependent protein kinases Cdk4 and Cdk6. In their activated form these kinases are capable of phosphorylating the retinoblastoma protein pRB [25, 30], a critical target of $G_1$ CDKs that is also thought to be of importance for the stability of D-cyclin kinases [4, 34].

The three D-type (D1, D2 and D3) cyclins are closely related but may have tissue-specific roles. Cyclin D1 seems to have a role in development of the mammary gland in mice [13]. It is not clear whether this mechanism operates in human tissue.

D cyclins are differently expressed in different cell types; some cells express all three, but many express only one or two [2, 35]. Normal human mammary epithelial cells express all three genes [5]. Human breast cancer cell lines fail to express cyclin D2 or express it at levels con-

siderably lower than those in normal cells [24]. The functional consequences of the differential expression of cyclin D2 in normal and neoplastic breast epithelial cells are still unclear. One possibility may be that these genes are involved in differentiation as well as proliferation [29].

The role of cyclin D1 in breast tumorigenesis is uncertain, but it is thought to be of importance in progression of the disease [5, 17]. Approximately one in six breast carcinomas show gene amplification of q13 sequences of the long arm of chromosome 11, where cyclin D1 is located [22]. However, stable overexpression of exogenous cyclin D1 cDNA in the human mammary epithelial cell line HBL-100 has been found to inhibit growth [18]. Thus, it appears that increased levels of cyclin D1 can be found in two opposing aspects of cell cycle control; growth promotion and growth arrest.

Previously, Spitovsky et al. [33] found evidence for the involvement of wild type p53 protein in the regulation of cyclin D1 gene expression. In fibroblasts, cyclin D1 mRNA and protein expression were dramatically reduced when p53 function was inhibited. With a p53 protein level back to normal, the level of cyclin D1 mRNA and protein was elevated. Chen et al. and Del Sal et al. studying murine cell lines also found evidence that wild type p53 protein may play an important part in the regulation of cyclin D1 expression at $G_1$–S [8, 10].

Barbareschi et al. [3] have recently demonstrated divergent results between cyclin D1 protein expression and cyclin D1 gene amplification, indicating that cyclin D1 protein may be upregulated by other mechanisms than gene amplification. However, they did not find any association between p53 protein overexpression and cyclin D1 protein overexpression.

To investigate whether a relationship exists between cyclin D1,2 and 3 gene amplification and/or protein expression and the p53 status in breast carcinomas, we analysed a series of tumours from 90 patients with breast carcinoma and with previously examined p53 status.

Expression of pRb has been associated with cyclin D1/Cdk stabilisation in experimental studies [4]. The expression of this protein was also investigated to clarify whether any relationship exists between expression of pRb and cyclin D1 in breast carcinoma.

## Materials and methods

Material for this study was obtained from 90 primary breast carcinomas selected from 212 previously described cases [6]. The series included 38 tissue samples from patients with abnormal p53, and 52 tissue samples from patients without detectable p53 alteration. The mean age at diagnosis for this group of patients was 63.7 years (range 28–88 years). In 33 patients no lymph node metastases were present at the time of surgery, while 47 did already have lymph node metastases at the time of surgery. No lymph node dissection was performed in 10 of patients. Ten of the primary tumours were classified as invasive lobular carcinomas, 72 as invasive ductal carcinomas, and 8 as other types. Four of the tumours were classified as grade 1, 56 as grade 2, and 26 as grade 3. No grading was performed on 4 of the tumours. Grading of the tumours was based on recommendations made by Elston and Ellis 1991 [12]. All samples included in this study were judged to contain more than 20% tumour tissue after histological evaluation. The mean observation time for this group of patients was 2.3 years (range 2–5 years). Thirty-five patients had recurrences or distant metastases (to liver, lungs, skeleton, CNS). Twelve patients died of disseminated breast cancer and 2 of other causes.

Isolation of genomic DNA was performed by phenol/chloroform extraction and ethanol precipitation by standard methods. Aliquots (7 µg) of DNA were digested with *Hin*dIII, separated on 0.8% agarose gels and transferred by alkaline blotting onto Hybond N+ membranes (Amersham) according to the manufacturer's manual. After UV cross-linking for 5 min, the blots were prehybridised for 30 min and subsequently hybridised with DNA probes labelled with $^{32}P$ by the random primer technique [14]. The hybridisation was carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM sodium EDTA overnight at 65°C. The membranes were subsequently washed three times for 20 min in 40 mM sodium phosphate (pH 7.2) and 1% SDS. For multiple hybridisations, the bound probe was removed by incubating the filters for 20 min at room temperature in 100 mM sodium hydroxide and 1 mM sodium EDTA. Membranes were exposed to Fuji Medical X-ray films, RX. Several exposures were performed for each probe (24 h to 6 days). Signals were scored with the naked eye by three of the investigators. A signal at least 3 times more intense than signals from samples with a normal copy number was scored as an amplification. To adjust for unequal DNA loading, the blots were rehybridised to a control probe encoding apolipoprotein B located on chromosome 2.

cDNA probes for cyclin D, CCND1,CCND2 and CCND3 were kindly provided by Dr. D. Beach. The probe for APOB (clone pB27), used as control for loading, was kindly provided by Dr. J. Breslow.

Tumour samples were examined for cyclin D1, D2 D3 and pRb protein expression by immunohistochemistry (IHC). Sections 4–6 mm thick from formalin-fixed, paraffin-embedded material were made on coated slides. After antigen retrieval by microwave oven the staining was performed in an Optimax plus, Automated Cell Stainer; Model 1.5 (BioGenix, USA) following the operating manual. The monoclonal antibodies used for cyclins were supplied by Nota Bene Scientific, Denmark (cyclin D1 Ab-3, clone DCS-6, dilution 1:40, cyclin D2 and D3 concentrated solution). The monoclonal antibody for pRb (C-15) was supplied from Santa Cruz, Calif. (dilution 1:700). All series included positive and negative controls. For each sample at least 100, and usually more than 1000, cells were analysed. The amount of immunopositive cells was estimated semiquantitiatively: grade + corresponded to 5–10%, grade ++ to 10–50% and grade +++ to more than 50% positive cells.

The results of ER and p21 (WAF1/CIP1) immunostaining in this material have been described previously [7]. Samples were immunostained for ER protein and scored as either positive or negative (cut-off value less than 5% of the cells positives). Of these 90 samples 29 did not show any immunoreactivity, while 61 of the samples were positive. Strong immunoreactivity of p21 (++/+++) was detected in 18 samples, presence of a few cells immunoreactive for p21 (+) in 35 samples, and no immunoreactivity in 37 samples.

The results of the p53 analysis in this series have also been previously reported [6]. Gene mutations were detected using CDGE analyses followed by sequencing, and altered protein expression was detected by immunohistochemistry using antibody PAB 1801 (Oncogene Science) [6]. Thirty-one tumours showed protein accumulation and 25 tumours had a mutation. In total, 38 of the tumours showed p53 protein alteration detected as an accumulation and/or as a mutation.

Statistical analysis was performed by a Chi-square test with Yates correction and Fisher's exact test when appropriate (total number<50).

## Results

Gene amplification of the cyclin *D1* locus was detected in 10 of the 90 samples (11.1%) analysed (Fig. 1A). No amplification was detected in any of the tumour samples when they were hybridised with the cyclin D2 and cyclin D3 probes.

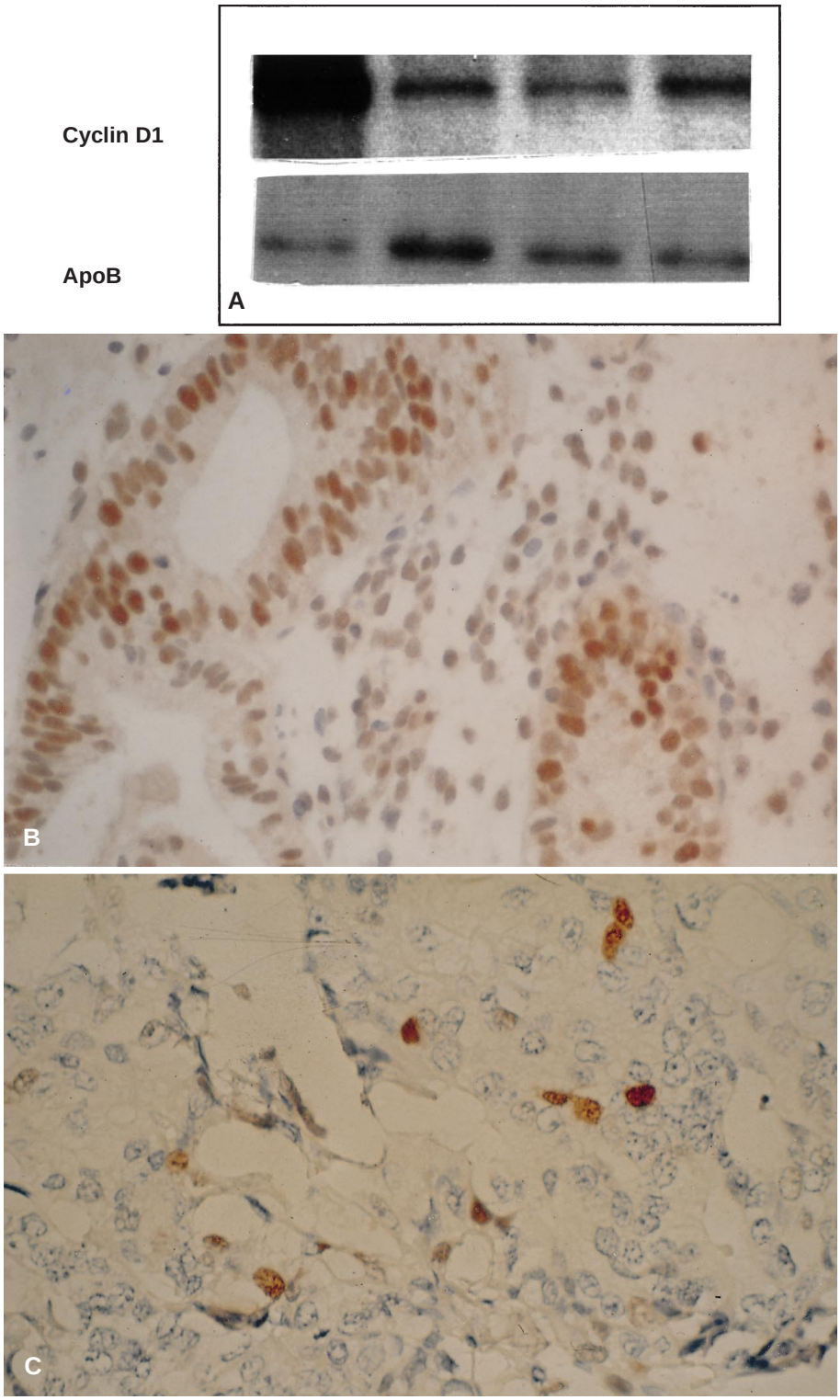


**Fig. 1** **A** Southern blot analysis of DNA from four different tumours probed with cyclin D1 and ApoB. Amplification of cyclin D1 is seen in one tumour (*first lane*). **B** Immunohistochemical analysis of the same tumour with gene amplification as in A, showing strong immunoreactivity for cyclin D1 protein. **C** Expression pattern of cyclin D1 protein seen in a tumour with wild type p53 protein and no gene amplification

Expression of the cyclin D1 protein was found in 38 (42.2%) of the tumour samples. Only cells with nuclear staining were scored as positive. In all these samples immunoreactivity was seen in at least 15%–20% of the cells. Three samples showed immunoreactivity in more than 50% of the cells (Fig. 1B, C). No immunoreactivity was seen in the stromal tissue, and normal breast epithelium was rarely immunoreactive for cyclin D1 protein. Only 2 (5.3%) samples with positive immunoreactivty also showed amplification at the gene level, giving no association between gene amplification and expression of cyclin D1. However, of the three samples with the highest expression of cyclin *D1*, 2 were also amplified at the gene level. No association was seen between gene amplification at the cyclin D1 locus and p53 protein status.

**Table 1** Relationship between p53, cyclin D1 and pRb protein expression in human breast carcinomas

| | | Cyclin D1 protein expression | | |
|---|---|---|---|---|
| | | 0 | + | |
| *Tp53* gene mutation | Negative | 32 (49.2%) | 33 (50.7%) | |
| | Positive | 20 (80.0%) | 5 (20.0%) | *P*=0.016 |
| p53 protein accumulation | no accumulation | 24 (42.9%) | 32 (57.1%) | |
| | accumulation | 25 (80.6%) | 6 (19.4%) | *P*=0.001 |
| p53 protein alterations[a] | p53 normal | 22 (42.3%) | 30 (57.7%) | |
| | p53 abnormal | 30 (78.9%) | 8 (21.1%) | *P*=0.001 |
| pRb protein expression | 0/+ | 21 (70.0%) | 9 (30.0%) | |
| | ++/+++ | 31(51.7%) | 29 (48.3%) | *P*=0.152 |

[a] Scored as protein accumulation and/or gene mutations

**Table 2** Relationship between cyclin D1 protein expression and tumour grade in human breast carcinomas

| | | Cyclin D1 protein expression | | |
|---|---|---|---|---|
| | | 0 | + | |
| Tumour grade | 2 | 28 (50.0%) | 28 (50.0%) | |
| | 3 | 20 (76.9%) | 6 (23.1%) | *P*=0.039 |

**Table 3** Relationship between cyclin D1 protein expression and ER immunoreactivity in human breast carcinomas

| | | Cyclin D1 protein expression | | |
|---|---|---|---|---|
| | | 0 | + | |
| ER protein | 0 | 25 (86.2%) | 4 (13.8%) | |
| | + | 27 (44.3%) | 34 (55.7%) | *P*<0.001 |

Comparison of cyclin D1 protein expression and p53 status, both at the gene and at the protein level, revealed a highly significant association (Table 1). In samples with no detectable p53 alterations, 30 (57.7%) showed cyclin D1 protein expression. In samples with altered p53 protein only 8 (21.1%) showed immunoreactivity to cyclin D1 in tumour cells. All 3 tumour samples with immunoreactivity in more than 50% of the cells, 2 of which also showed gene amplification, were found in the group with altered p53 protein. No association was seen between p21(WAF1/CIP1) protein and cyclin D1 protein expression.

Immunoreactivity for the pRb was detected in 88 (97.8%) of the samples. Only nuclear staining was scored as positive. Normal breast tissue, when present, showed strong immunoreactivity. Samples were scored as positive when at least 5% of the tumour cells were immunoreactive. No association was seen between expression of pRb and any of the cyclin Ds. However, when pRb protein expression was subdivided into high protein expression (immunoreactivity in more than 40% of the tumour cells) and low protein expression, a trend was seen for an association between high pRb protein expression and presence of cyclin D1 immunoreactivity (*P* corrected 0.152, *P* uncorrected 0.097). In the group of tumours with high pRb expression 29 of 60 (48.3%) showed cyclin D1 immunoreactivity, as against 9 of 30 (30.0%) in the group with low expression of pRb (Table 1). No association was seen between pRb protein expression and amplification of cyclin D1 at the gene level.

Expression of cyclin D1 was compared with tumour grade, and a significant association was found between expression of cyclin D1 and tumours of higher differentiation grade (grade 2 tumours; Table 2). In samples without detectable cyclin D1 protein immunoreactivty, 28 out 48 (58.3%) were grade 2 tumours. In samples with positive cyclin D1 immunoreactivty, 28 out of 34 (82.4%) were grade 2 tumours. All 3 tumour samples with strong cyclin D1 immunoreactivity were found among grade 3 tumours. There was no association between cyclin D1 protein expression and tumours of different histological type (invasive ductal carcinomas, invasive lobular carcinomas).

Only 50 samples were analysed for cyclin D2 and D3 protein expression. Cyclin D2 protein expression was detected in 2 samples in under 20% of the tumour cells. When analysing the protein expression of cyclin D3, immunoreactivity was detected in 9 samples (18%). No association was seen between p53 protein status and cyclin D3 protein expression. No association was seen between cyclin D1 and D3 protein expression. Cyclin D3 did not correlate to tumour grade, metastasis or overall survival.

Comparison of cyclin D1 gene amplification and ER immunoreactivity disclosed no significant association. However, when cyclin D1 protein expression was compared with ER-immunoreactivity, a significant association was seen (Table 3). No association was observed between cyclin D3 and ER immunoreactivity.

## Discussion

We have found evidence of a relationship between cyclin D1 protein expression, p53 status and ER status in human breast carcinomas. Cyclin D1 is an early-delayed growth factor-induced gene and is required for $G_1$ transition in both human and murine cells [2, 29]. It has been reported that overexpression of cyclin D1 promotes cell progres-

sion and differentiation, which are generally observed as shortened $G_1$–S transition [30, 31]. However, in some cases, increased levels of cyclin D1 have been reported to be characteristic of cell cycle arrest [18, 27]. The biological effect of cyclin D1 overexpression seems to depend on how, when and on what level this protein is induced.

Expression of cyclin D1 was found in two different modes in the present study. Most of the positive samples showed expression of cyclin D1 in 15–20% of the tumour cells. This expression was highly associated with p53 status without detectable alterations. Gene amplification was seen in only 11.1% of cases. This is relatively low compared with previous studies. However, previous studies have shown amplification of chromosome 11q13 in 10–30% of cases [3, 9, 16, 32, 37]. This discrepancy may be caused by differing composition of patient groups or different detection levels of the gene in amplifications.

There was no association between expression of cyclin D1 protein and amplification at the gene level. In 3 cases, however, a high level of cyclin D1 protein was detected in more than 80% of the tumour cells. These 3 tumours were found in the group with altered p53 protein and strong amplification at the gene level. Our data support the hypothesis that there are two different mechanisms causing elevated cyclin D1 protein expression: induction through the wild type p53 protein after $G_1$ cell cycle arrest and gene amplification at the cyclin *D1* locus. Whether the loss of wild type p53 protein function has a role is not clear, since there are only 3 samples in this group. It is worth noting that all these 3 samples were found in the group with altered p53 protein.

We observed a trend towards an association between high pRb protein expression and expression of cyclin D1 protein. No association was seen between gene amplification of cyclin D1 and pRb protein expression. However, Barbareschi et al. [3] found a significant association between high-grade pRb protein expression and expression of cyclin D1 protein in a study of 64 breast carcinoma patients. This difference may be a result of differing composition of the patient groups in these two studies.

We also found significant associations between cyclin D1 protein expression and ER immunoreactivty and between ER immunoreactivity and wild type p53 protein. 17β-Oestradiol may induce cyclin D1 gene transcription in the MCF-7 cell line [1]. Whether the elevation of cyclin D1 protein expression seen in the present study is induced by wild type p53 or oestrogen receptor protein is still not known.

Cyclin D1 seems to activate transcription on oestrogen receptor-regulated genes. This activation is independent of complex formation to the CDK partner, and the activation of oestrogen receptors is not inhibited by anti-oestrogens [26, 38].This mechanism may play an important part in the treatment of breast cancer patients by anti-oestrogen therapy. If transcription of oestrogen-responsive genes is enhanced by cyclin D1, and not by oestrogen, patients may not respond to anti-oestrogen treatment. Thus, evaluation of cyclin D1 expression may be of clinical relevance.

Del Sal et al. [10] and Chen et al. [8] found evidence of involvement of *p21 WAF1/CIP1* in cyclin D1 expression. In the present study no association was seen between *p21 WAF1/CIP1* immunoreactivity and cyclin D1 protein expression. This may be because p21 is modulated by other proteins. In a previous study we have demonstrated that *p21 WAF1/CIP1* is suppressed by *bcl-2* overexpression [7].

Cyclin D3 expression was not associated with p53 protein or any other parameters, and its role in breast cancer is unclear. Only 2 samples showed cyclin D2-immunoreactivity. This finding is in accordance with results from studies performed on breast cancer cell lines, where low or absent expression of cyclin D2 has been found.

In summary, the present study demonstrates a relationship between wild type p53 and cyclin D1 protein expression in human breast carcinomas. Cyclin D1 may be another effector protein of p53, and moderate expression of cyclin D1 may be a favourable prognostic factor associated with high differentiation grade, positive ER status and wild type status of p53 protein.

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