J Clin Pathol:Mol Pathol 1998;**51**:30–34

Correlation of cyclin D1 and Rb gene expression with apoptosis in invasive breast cancer

J S de Jong, P J van Diest, R J A M Michalides, P van der Valk, C J L M Meijer, J P A Baak

Abstract

***Background*—In vitro studies have shown that amplification and overexpression of the cyclin D1 gene can accelerate the progress of cells through the G_1 phase. Therefore, cyclin D1 may have an apoptosis inhibiting effect. The retinoblastoma (Rb) gene was shown recently to be an important regulator of apoptosis.**

***Aims*—To evaluate whether expression of the cyclin D1 and Rb genes correlated with apoptotic counts in a group of 97 invasive breast cancers.**

***Methods*—Expression of the cyclin D1 and Rb genes was detected by standard immunohistochemistry using paraffin wax embedded sections. Apoptotic cells were counted according to a strict protocol, in 10 fields of vision systematically spread over the most poorly differentiated area of the tumour, at a magnification of ×630. Apoptotic cells counts were expressed as apoptotic cells/mm^2.**

***Results*—Cyclin D1 overexpression was found in 49% of cases. Loss of Rb expression was found in 44% of cases, and occurred particularly in poorly differentiated tumours. Cyclin D1 and Rb expression showed a positive correlation ($p = 0.003$). Apoptotic counts varied from 1 to 62/mm^2. There were no significant correlations between cyclin D1 overexpression and apoptotic counts in the total group or in the retinoblastoma protein (pRb) positive tumours. Loss of Rb expression also showed no correlation with apoptotic counts.**

***Conclusions*—Cyclin D1 is frequently overexpressed in pRb positive tumours, but no evidence has been found for an anti-apoptotic effect of cyclin D1 overexpression or Rb expression in invasive breast cancer.**

(*J Clin Pathol:Mol Pathol* 1998;**51**:30–34)

Keywords: breast cancer; cyclin D1; retinoblastoma susceptibility gene; immunohistochemistry; apoptosis; histological type; histological grade

Department of Pathology, Free University Hospital, Amsterdam, Netherlands
J S de Jong
P J van Diest
J P A Baak
P van der Valk
C J L M Meijer

Department of Tumour Biology, The Netherlands Cancer Institute, Amsterdam, Netherlands
R J A M Michalides

Correspondence to:
Dr P J van Diest,
Department of Pathology,
Free University Hospital, PO Box 7057, 1007 MB,
Amsterdam, Netherlands.
email: pj.vandiest@azvu.nl

Accepted for publication
11 November 1997

Apoptosis, a form of programmed cell death with a typical morphology, is an important biological process that is thought to play an important role in the aetiology of many cancers. In breast cancer, there are also indications that the numbers of apoptotic cells are of prognostic value.[1] During recent years, much has been learned about the different proteins that play a role in the regulation of apoptosis. Such proteins include p53,[2–4] bax ,[3 5–8] Bak,[9–11] and bcl-x_s,[12] which seem to promote apoptosis, together with and bcl-2[13–15] and bcl-x_L,[12–16] which appear to inhibit apoptosis. These proteins act largely during the G_0 and G_1 phases of the cell cycle. However, little is known about the role of cyclin D1, another important G_1 regulator,[17–23] in the process of apoptosis. Amplification of cyclin D1 is found frequently in invasive breast carcinomas.[23 24] Overexpression of the cyclin D1 gene is found in up to half of invasive breast cancer cases,[25–29] but lacks prognostic value,[20 25] or relation to improved survival.[26] Cyclin D1 overexpression might result from amplification,[19 20 23 24] chromosomal translocation (as has been found in parathyroid adenomas[30] and centrocytic lymphomas[31]), or increased hormone sensitivity.[20 25] Selective induction of cyclin D1 is sufficient for cell line cells arrested in the early G_1 phase to complete the cell cycle.[21] In order to complete the G_1 phase, apoptotic mechanisms must be suppressed. Therefore, cyclin D1 expression might counteract apoptosis. In contrast, many neurons in the developing nervous system undergo apoptosis under the regulation of cyclin D1,[32 33] thereby pointing to a possible stimulatory role for cyclin D1 in apoptosis. It is not clear which of these two effects of cyclin D1 on apoptosis prevails in invasive breast cancer.

The retinoblastoma gene product (pRb) inhibits apoptosis induced by various stimuli such as ionising radiation,[34] tumour growth factor (TGF) β1,[35] interferon (IFN) γ,[36] and wild-type p53 overexpression.[37] The effect of pRb on apoptosis has not yet been studied in invasive breast cancer.

The aim of this study was to explore the possible role of cyclin D1 and pRb in apoptosis in invasive breast cancer by correlating immunohistochemical cyclin D1 and pRb staining patterns with counts of apoptotic cells.

Methods

PATIENTS

Patients were selected from a previously described group of 189 cases with invasive breast cancer, diagnosed between 1971 and 1981 in the Free University Hospital or the Netherlands Cancer Institute, Amsterdam, Netherlands.[25] In 92 cases, no tumour material remained in the original blocks, leaving only 97 cases.

SPECIMEN PREPARATION

Fresh surgical specimens were cut into slices at approximately 0.5 cm and the material was fixed in 4% neutral buffered formalin. Representative tumour samples were taken, with care that the periphery of the tumour was sampled, and embedded in paraffin wax.

Table 1 Correlations between expression patterns of cyclin D1 and pRb in 97 cases of invasive breast cancer

	pRb		
Cyclin D1	Positive	Negative	p value*
Positive	34	14	0.003
Negative	20	29	

*χ^2 test.

Table 2 Correlation between Rb and cyclin D1 expression and apoptosis counts in invasive breast cancer

	pRb		cyclin D1	
Apoptotic cells	Positive	Negative	Positive	Negative
≤ 6/mm²	28	23	28	23
> 6/mm²	26	20	20	26
	p = 0.87		p = 0.26	

Sections (4 µm thick) were cut and mounted on poly-L-lysine coated slides for cyclin D1 and pRb immunohistochemistry. Also, routine staining was performed with haematoxylin and eosin for the counting of apoptotic cells, and for both histological typing (according to the WHO system) and grading.[38]

IMMUNOHISTOCHEMISTRY

For immunohistochemical staining of the cyclin D1 and Rb proteins, a previously well characterised affinity purified rabbit polyclonal antibody against cyclin D1 (B19S)[19] and a monoclonal antibody against pRb, (1F8, Novocastra, Newcastle upon Tyne, UK) were used. The slides were dewaxed and endogenous peroxidase activity was blocked by incubation for 30 minutes in 3% hydrogen peroxide in methanol. Antigen retrieval was performed by heating the sections in a 0.01 M citrate buffer (pH 6.0) at 100°C for 15 minutes. The slides were preincubated for one hour with normal goat serum (1/20 dilution) for cyclin D1 and normal rabbit serum (1/50 dilution) for pRb, to diminish nonspecific binding of the secondary antibody. Slides were then incubated overnight at 4°C with the primary antibodies against cyclin D1 (1/80 dilution) and pRb (1/100 dilution). Thereafter, slides were incubated for 30 minutes at room temperature with biotinylated goat antirabbit antibody (1/500 dilution) for cyclin D1 and biotinylated rabbit antimouse antibody (1/500 dilution) for pRb. Subsequently, slides were incubated with avidin–biotin–peroxidase complex (Dako Duet Kit; Dako, Glostrup, Denmark) (1/200 dilution) for one hour at room temperature. 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used as chromogen. Between steps, the slides were rinsed three times for 10 minutes in phosphate buffered saline (PBS). After counterstaining with haematoxylin, slides were dehydrated and mounted. Negative controls were obtained by omission of the primary antibodies from the incubation.

As a positive control for cyclin D1 expression, a head and neck squamous cell carcinoma with known cyclin D1 amplification and overexpression[19] was used. In all tumours there was a non-uniform nuclear cyclin D1 expression pattern resulting from the oscillating expression pattern of cyclin D1 protein with a peak in G_1, also seen in tumours with overexpression of cyclin D1.[18,19,39] The percentages of positive nuclei were estimated by two observers. In accordance with previous studies,[19,20,25] cases were regarded as negative when < 5% nuclei showed staining, and as positive when ≥ 5% nuclei stained. The cytoplasmic staining that was observed in some cases was ignored.

The retinoblastoma protein was localised in the nucleus. pRb was present in normal tissue adjacent to the tumour tissue, thereby providing an internal positive control. Rb gene expression was regarded as negative when staining was heterogeneous or staining for Rb was not seen.

COUNTING OF APOPTOTIC CELLS

As shown in previous studies,[1,40,41] apoptotic cells can be recognised easily in haematoxylin and eosin stained tissue sections. Apoptotic cells show retracted and strongly eosinophilic cytoplasm. The nuclear DNA condenses at the nuclear membrane, later forming clumps, and often falling apart into round and homogeneously dark nuclear fragments. Apoptosis involves individual cells and does not provoke an inflammatory reaction.

Apoptotic cells were counted using a standard light microscope at a ×630 magnification (×63 objective, field diameter 275 µm) in the most poorly differentiated area of the tumour (0.5 × 0.5 cm in size) according to a strict protocol that has been described previously.[40] In short, the total numbers of apoptotic cells were counted in 10 fields of vision, systematically spread over the selected area. This procedure was shown to provide good intraobserver and interobserver reproducibility.[40] All apoptotic counts were expressed as apoptotic cells/mm².

STATISTICS

For correlation between cyclin D1 overexpression and loss of Rb expression (grouped as positive versus negative) on the one hand, and the numbers of apoptotic cells (using the median as the cut off point) and histological type and grade on the other hand, confusion matrices were composed and tested for significance with the χ^2 test. The percentage of cyclin D1 positive cells was also compared with the numbers of apoptotic cells by linear regression analysis, registering the correlation coefficient (R) and the p value. These tests were performed with the Biomedical Package (BMDP; Statistical Solutions, Cork, Ireland). p values below 0.05 were regarded as significant.

Results

Overexpression of the cyclin D1 gene was found in 48 of 97 cases (49%). Loss of Rb expression was found in 43 of 97 cases (44%). As shown in table 1, the expression patterns of the cyclin D1 and Rb genes showed a positive correlation (p = 0.003). Of the pRb positive tumours, 63% (34 of 54) showed positive staining for cyclin D1, while 67% (29 of 43) of the pRb negative tumours were also negative

Table 3 Expression of Rb and cyclin D1 in 97 cases of invasive breast cancer in relation to histological tumour type

	pRb		Cyclin D1	
Tumour type	Positive	Negative	Positive	Negative
Tubular	6	3	7	2
Mucinous	1	0	1	0
Invasive cribriform	2	0	0	2
Lobular	7	1	6	2
Ductal	35	37	34	38
Medullary	3	2	0	5
	p = 0.014*		p = 0.039*	

The cyclin D1 data have been adapted from van Diest *et al* 1997.[25]
*χ^2 test.

Table 4 Expression of Rb and cyclin D1 in 97 cases of invasive breast cancer in relation to histological grade

	pRb		Cyclin D1	
Grade	Positive	Negative	Positive	Negative
I	27	13	29	11
II	18	14	14	18
III	9	16	5	20
	p = 0.015*		p = 0.001*	

*χ^2 test.

for cyclin D1. The average number of apoptotic cells/mm^2 was nine (median (SD), 6 (8.2); range 1–44). Correlations between apoptotic counts and cyclin D1 overexpression are shown in table 2. Of the 51 patients with a low apoptotic count ($\leq$ 6 mm^2), 28 showed cyclin D1 overexpression and 23 did not. Of the 46 patients with a high apoptotic count (> 6 mm^2), 20 showed cyclin D1 overexpression and 26 did not. This did not reach significance (p = 0.26, χ^2 test). There was also no significant correlation between the numbers of apoptotic cells and the percentage of cyclin D1 positive cells (R = −0.874, p = 0.29). Positivity of cyclin D1 in pRb positive tumours also showed no correlation with the numbers of apoptotic cells (p = 0.81).

Of the 51 tumours with a low apoptotic count, 23 showed loss of pRb and 28 did not. Also, of the 46 tumours with a high apoptotic count, 20 showed loss of pRb and 26 tumours did not (table 2). This was not significant (p = 0.87). As to histological type and pRb expression (table 3), the tubular, mucinous, invasive cribriform, and lobular tumour types (the more well differentiated types) showed loss of Rb expression in only 20% of cases, whereas the ductal and medullary (more poorly differentiated) tumour types showed loss of Rb expression in 51% of cases (p = 0.014). Apart from correlation with tumour type, loss of pRb correlated positively with histological grade, as shown in table 4. Loss of pRb was seen in 33% (13 of 40) of the grade I specimens, in 44% (14 of 32) of the grade II cases, and in 64% (16 of 25) of the grade III cases (p = 0.015).

As described in a previous study, overexpression of cyclin D1 was found mainly in the more well differentiated tumour types (table 3).[25] There was a negative correlation between overexpression of cyclin D1 and histological grade (table 4). Overexpression of cyclin D1 was seen in 73% (29 of 40) of the grade I specimens, in 44% (14 of 32) of the grade II cases, and in 20% (five of 25) of the grade III cases (p < 0.001).

Discussion

The aim of this study was to correlate cyclin D1 gene overexpression and loss of Rb expression with the numbers of apoptotic cells in order to evaluate the possible anti-apoptotic effects of cyclin D1 and pRb in a group of 97 invasive breast cancer patients. Cyclin D1 was overexpressed in 49% of cases. This compares well with the results of Bartkova and colleagues,[39] Gillett and colleagues,[26] and Zhang and colleagues,[42] but is somewhat higher than that found in the study of Michalides and colleagues.[20] Because it is also higher than the amplification found in 19% of cases in a previous study,[23] there may be overexpression of cyclin D1 in the absence of amplification.[19 23 31 43] This could result from a mutation induced longer half-life of the protein, a translocation, or increased hormone sensitivity, as has been suggested previously.[20 25]

The average numbers of apoptotic cells/mm^2 were nine (median (SD), 6 (8.5); range 1–62). There were no significant correlations between cyclin D1 overexpression and either the percentage of cyclin D1 positive cells or the number of apoptotic cells. Therefore, no evidence has been found for an anti-apoptotic effect of cyclin D1 expression in invasive breast cancer.

The role of cyclin D1 in the process of apoptosis in breast cancer is still to be determined. Cyclin D1 can accelerate the progress of cells through the G_1 phase by binding to cyclin dependent kinases and subsequently phosphorylating pRb, thereby releasing the E2F transcription factor. Overexpression of cyclin D1 is shown to shorten the G_1 phase in mouse fibroblasts,[44] rat cells,[45] and breast cancer cells,[21] thereby theoretically suppressing apoptotic processes. In contrast, cyclin D1 overexpression in late G_1 can prolong the S phase,[46] and inhibit DNA replication,[47] indicating a growth restricting function and perhaps an apoptosis inducing effect of cyclin D1. Indeed, cyclin D1 has been shown to stimulate apoptosis in neurons in the developing nervous system.[32 33] The present study provides no evidence for a relation between cyclin D1 overexpression and apoptosis in breast cancer. Perhaps the possible apoptosis stimulating and suppressing mechanisms balance out in this tumour.

Loss of Rb expression was found in 44% of cases. This is in accord with the results of Trudel and colleagues[48] and Pietilainen and colleagues.[49] However, the figures are higher than those of Borg *et al* who, using western blot analysis, only found low concentrations or an absence of pRb in 15% of cases.[50]

There were also no correlations between loss of Rb expression and the number of apoptotic cells. pRb has been shown previously to inhibit apoptosis induced by various stimuli, such as ionising radiation,[3] TGFβ1,[35] IFNγ,[36] and wild-type p53 overexpression.[37] We found no evidence for any apoptosis inhibiting effects of pRb in invasive breast cancer in the present study. As to the possible reasons for the lack of an apoptosis inhibiting effect of pRb, one has to bear in mind that the apoptosis in breast cancer studied here is not induced by therapy, but concerns "spontaneous" apoptosis. Also, wild-type p53 overexpression in breast cancer is probably rare, because in most cases with positive p53 staining, a mutation is found. TGF β 1 is expressed frequently in breast cancer,[51 52] but it could be that its apoptosis inducing effect may be receptor or cell type dependent.

We found a positive correlation between the expression patterns of cyclin D1 and Rb, whereby tumours with loss of Rb expression were correlated with low or no overexpression of cyclin D1. This is in accordance with the study of Gjetting *et al*, who showed that pRb can induce cyclin D1.[53] The loss of Rb expression correlated with a more poorly differentiated tumour type. In a previous study, we also found a strong correlation between cyclin D1 overexpression and well differentiated tumour types.[25] These results point to a role for pRb and cyclin D1 in differentiation rather than in apoptosis in invasive breast cancer.

Several studies have been devoted to the mechanisms of apoptosis in general. The p53 protein is involved in apoptosis, as it halts cells with DNA damage in G_1, enabling DNA to be repaired; cells that do not successfully repair their DNA undergo apoptosis.[54] The bcl-2 protein family plays an important role in apoptosis. The bcl-2[13–15] and bcl-x_L proteins[8 16] inhibit apoptosis. The bax protein promotes apoptosis in its homodimeric form, but after heterodimerising with bcl-2 it prevents apoptosis.[8] bcl-2 is expressed in almost half of invasive breast cancers, and bcl-2 expression correlates with prognosis,[55] but there are few studies of bcl-x and bax in human breast cancer. The c-myc protein increases the sensitivity of cells to undergo apoptosis.[54 56] Study of the clinical usefulness of c-myc is hampered by the fact that few antibodies are available. Further studies are necessary to elucidate which mechanisms of apoptosis play a role in breast cancer and the complexity of their relations.

In conclusion, cyclin D1 overexpression and loss of pRb do not correlate with the numbers of apoptotic cells in invasive breast cancer and, therefore, no evidence has been found for an (anti) apoptotic effect of cyclin D1 and pRb. Cyclin D1 and pRb are both found mainly in well differentiated tumour types and, therefore, might be related to the differentiation of invasive breast cancers. Further studies on mechanisms of apoptosis in invasive breast cancer are required.

This work was partly supported by grants 28–1814 and 28–2015 of the Praeventiefonds, and grant 92–51 of The Netherlands Cancer Foundation.

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