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## Original Paper

# Expression of Cyclin D1 and Retinoblastoma Protein in Colorectal Cancer

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**Abnormal expression of the retinoblastoma protein (pRb) and cyclin D1 have been reported in a variety of malignancies, but the frequencies of these deregulations and their relation to prognosis in colorectal cancer has not been clarified. We characterised 90 colorectal cancers with respect to immunohistochemical expression of cyclin D1, pRb and Ki-67. Two of 90 (2%) tumours lacked nuclear pRb staining, indicating inactivation of the protein, while 10 (11%) expressed high levels of pRb. Abnormal expression of pRb was significantly correlated to low levels of nuclear cyclin D1 observed in 32% of the tumours. Strong nuclear cyclin D1 expression was detected in 12% of the tumours. Cytoplasmic staining of cyclin D1 was observed in 17% of the tumours, showing an inverse relationship ($P = 0.006$) to the Ki-67 labelling index. Eight of 11 tumours with high nuclear overexpression of cyclin D1 and both tumours with pRb defects were located in the right colon in comparison with zero of 25 in the rectum ($P = 0.009$). Regarding prognosis, neither pRb nor cyclin D1 expression correlated with patient survival.** 

**Key words: cell cycle control, cyclin D1, pRb, colorectal cancer**
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## INTRODUCTION

COLORECTAL CANCER is one of the most common malignancies in the Western world with a poor prognosis for approximately 50% of patients. The prognosis for colorectal cancer has not changed significantly over the last 30 years and treatment is still based on surgical removal, even though new adjuvant treatments have shown promising results [1]. In order to guide clinicians in selecting patients for further treatments such as chemo- and radiotherapy, prognostic tools are needed and tumour stage according to Dukes' has so far been one of the most important prognostic factors for colorectal cancer. Proliferation is central to tumour progression and has been shown to be an independent prognostic marker in several malignant tumours. In colorectal cancer the prognostic impact of proliferation is nevertheless more uncertain and conflicting results have been published, indicating that further studies are needed in order to clarify the clinical importance of proliferation in colorectal cancer [2–4].

The cell cycle consists of a complex cascade of events controlled and accomplished by limited sets of key proteins, including cyclins, cyclin dependent kinases (CDKs), CDK inhibitors (e.g. p16, p21 and p27) and target substrates, such as the retinoblastoma protein (pRb) [5]. Cyclin D1 is essential for progression through G1 by forming complexes with CDK4 and CDK6. This enables CDK activating-kinases (CAK) to activate the cyclin/CDK complex initiating phosphorylation of pRb and release of bound transcription factors, such as E2F, which activates genes required for S phase entry. Apart from the cyclin D/CDK complex, cyclin E activates CDKs which phosphorylate pRb and other target substrates during the G1–S transition, securing further advancement through the cell cycle.

Deregulation of cell cycle control is one evident alteration in tumour growth which has been proposed to be obligatory in cancer development [6]. Cyclin D1 expression may be deregulated by genetic lesions such as chromosomal translocations and gene amplification, or conceivably, by alterations in cyclin D1 regulating genes leading to abnormal expression of the cyclin D1 protein [7]. Hypothetically, overexpression of cyclin D1 will cause increased phosphorylation/inactivation of pRb and partial loss of the G1–S check-point resulting in increased cell proliferation, even though studies in breast cancer have reported lower proliferation in cyclin D1 overexpressing tumours [8]. Limited studies of colorectal cancers

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have found overexpression of cyclin D1 in 14–30% of tumours [9–11], but the role for cyclin D1 deregulation in relation to prognosis in colorectal cancer has not been fully clarified. In breast cancer, overexpression of cyclin D1 relates to a less aggressive phenotype lacking lymph node involvement and showing a better prognosis [12].

The retinoblastoma (Rb) gene is an archetypal tumour-suppressor gene, and its product (pRb) is known to act as a central negative regulator in the control of the cell cycle [13]. Inactivation of the *Rb* gene has been observed in different cancers [14–16], but in colorectal cancer loss or inactivation of the Rb gene seems to be rare [17–19]. Further studies are nevertheless needed to clarify the degree of pRb inactivation in colorectal cancer and especially in relation to other cell cycle defects. In contrast to the general idea of loss of pRb in tumours, some studies have observed increased levels of *Rb* transcripts in colorectal cancer, suggesting a potential oncogenic role for pRb, a hypothesis which needs to be substantiated [20, 21].

In order to clarify potential aberrations and explore the prognostic significance of these regulators in colorectal cancer, we investigated the expression of cyclin D1 and pRb using immunohistochemistry in colorectal cancer samples.

## PATIENTS AND METHODS

*Patient and tumour data*

97 patients with primary colorectal cancer were retrospectively included in this study. 7 of the 97 were excluded due to lack of positive staining in non-tumour cells, serving as internal controls, or due to repetitive loss of tumour tissue during the antigen retrieval procedures, leaving 90 patients for investigation. For survival analyses, only patients treated with potential curative surgery were included, and 6 patients not eligible for radically surgery due to known distant tumour spread were, therefore, excluded from the survival analyses. 47 patients (52%) were men and 43 (48%) were women. Of 90 tumours, 11 were classified as Dukes' stage A, 44 as Dukes' B and 35 as Dukes' C (including six tumours not available for potential curative surgery—five of six were classified as Dukes' C, while one was classified as Dukes' B). The median age of the patients was 71.5 years (range 37–88). Clinical follow-up data were available for all included patients and the follow-up time of surviving patients ranged from 14 to 106 months (median 42 months). Each colorectal cancer was further classified according to grade (well, moderately or poorly differentiated according to WHO classification), growth pattern at the invasive margin (pushing or infiltrating) and the amount of lymphocytes at the invasive margin. The tumour location within the bowel was classified as either right colon (defined as caecum, colon ascendens and colon transversum), left colon (colon descendens and colon sigmoideum) or rectum. Of 90 evaluable tumours 38 (42%) were located in the right colon, 27 (30%) in the left colon and 25 (28%) in the rectum.

*Immunohistochemical procedures*

Colorectal cancer specimens were fixed in formalin and embedded in paraffin according to routine procedures. Sections (4 μm) were prepared, dried, dewaxed and rehydrated before microwave treatment in citrate buffer (pH 6.0) for 4×5 min. In order to obtain optimal conditions in the immunohistochemical procedure, all stainings were performed using a semiautomatic staining machine (Ventana ES, Ventana Inc., Tucson, Arizona, U.S.A.). Three different antibodies were used: (1) polyclonal anti-pRb (C15, Santa Cruz Inc, Santa Cruz, California, U.S.A.) at a dilution of 1:100; (2) monoclonal anti-cyclin D I (DCS-6, a kind gift from Dr Jiri Bartek, Copenhagen, Denmark) at a dilution of 1:200; and (3) anti-Ki67 (A0047, Dako A/S, Denmark) at a dilution of 1:75. Antibody visualisation was performed according to the Ventana-program. Subsequently, the slides were manually counterstained with Mayer's haematoxylin for 1.5 min. From each tissue block sections were also taken for routine histological evaluations.

*Evaluation of immunohistochemical staining*

Slides were interpreted by one of the investigators, unaware of the results of the other analyses. All slides were independently reviewed twice and intra-observer disagreements (< 10%) were reviewed a third time followed by a conclusive judgement. Evaluation of the expression of the three antibodies was performed using different semiquantitative scales. The entire tissue section for each tumour was scanned using a 20× objective magnification to estimate clearly the subjective mean level for each tumour. The expression of pRb was evaluated according to the staining intensity of the tumour cells. The pRb scale included three intensity grades: (−) low intensity level compared with non-tumour cells, (±) equal to non-tumour cells, and (+) high intensity compared with non-tumour cells. The intracellular staining pattern was almost exclusively nuclear and for pRb only nuclear staining was taken into consideration.

The intensity of nuclear cyclin D1 expression in tumour cells was assessed in relation to normal cells present in the tissue section: (−) lower intensity than non-tumour cells *or* presence of less than 5% positively stained tumour cells, (±) equal to non-tumour cells, (+) higher than non-tumour cells; cytoplasmic cyclin D1 expression was divided into: (+) low intensity, (++) medium intensity, (+++) high intensity (Figure 1). The fractions of cyclin D1 positive nuclei were evaluated using a four graded scale: (−), (+), (++) and (+++), approximately representing labelling indices (LIs) of 0–5%, 6–25%, 26–50% and > 50%, respectively. The fraction of Ki-67 positive cells was evaluated similarly.

*Statistics*

A linear association between two ordinal scale variables was performed by the exact linear-by-linear association test. When at least one variable contained nominal data Fisher's exact test was performed. The Kaplan–Meier method was used to estimate cancer specific survival, and comparison between groups was performed with the log-rank test. The time measured from surgery to death was recorded as the survival time and death with known locoregional or distant metastases was processed as a death event. If no event occurred, the patient was censored at the time of the last clinical follow-up or death from other causes. A significance level of 0.05 was used. Statistical analyses were performed using SPSS version 7.0 (SPSS Inc, Illinois, U.S.A.).

## RESULTS

*pRb and cyclin D1 expression*

Table 1 shows the results of the immunohistochemistry. For pRb expression, two (2%) tumours showed absent or low nuclear staining intensity compared with non-tumour cells, 78 (87%) showed equal levels and 10 (11%) higher levels of

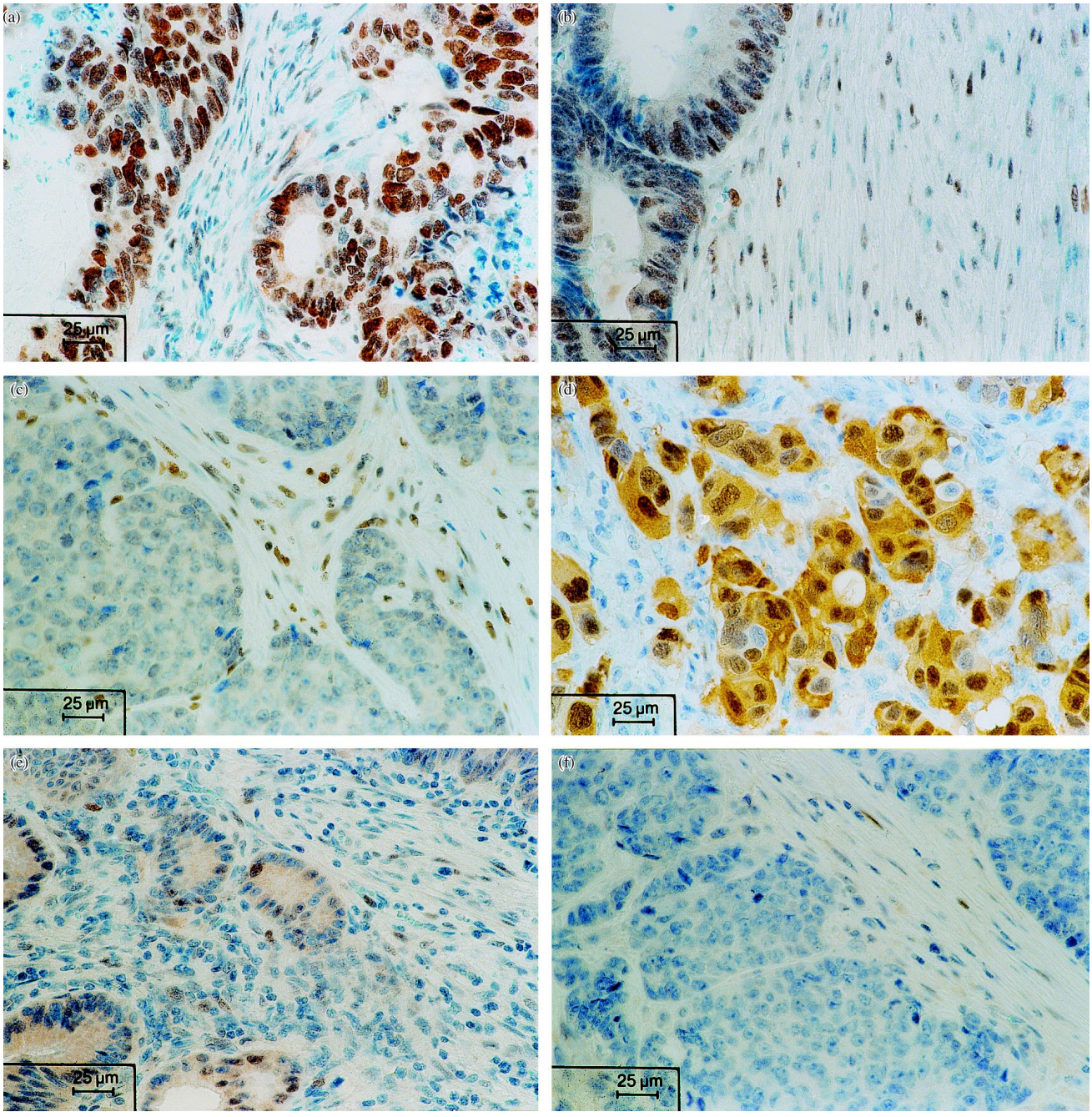


**Figure 1. Immunohistochemical stained sections for retinoblastoma protein (pRb) and cyclin D1. (a) Overexpression of pRb in tumour cells; (b) pRb in normal tissue; and (c) inactivation of pRb in tumour cells. (d) Overexpression of nuclear cyclin D1 and cytoplasmic cyclin D1 in tumour cells; (e) nuclear cyclin D1 and moderate expression of cytoplasmic cyclin D1 in normal tissue; and (f) low expression of both nuclear and cytoplasmic cyclin D1 in tumour cells.**

pRb in tumour cells. For cyclin D1, 29 (32%) tumours showed faint nuclear staining, whereas 11 (12%) expressed high levels of nuclear cyclin D1. Cytoplasmic expression of cyclin D1 was nearly exclusively found in tumour cells and it was not possible to relate the intensity to an internal control population consisting of normal cells as described for pRb and nuclear cyclin D1. If cyclin D1 expression was observed in the cytoplasm there was no variation between tumour cells and we, therefore, only determined the staining intensity and not the LI. Forty tumours (44%) expressed low levels, 35 (39%) moderate levels and 15 (17%) high levels. Examples of immunohistochemical staining patterns are shown in Figure 1. No obvious association was found between nuclear and cytoplasmic expression of cyclin D1 ($P = 0.575$). However, the LI of nuclear cyclin D1 was significantly related to nuclear cyclin D1 staining intensity levels ($P < 0.001$), but not to cytoplasmic cyclin D1 levels ($P = 0.320$).

The two tumours with pRb inactivation both expressed low nuclear cyclin D1 levels. In addition, abnormal expression of pRb, defined as either under- or overexpression, was significantly associated with low levels of nuclear cyclin D1 staining ($P = 0.007$). No such trend was observed for cytoplasmic cyclin D1 staining in relation to pRb expression ($P = 0.656$).

*Table* 1. *Immunohistochemical expression of retinoblastoma protein (pRb) and the proliferative activity (Ki-67) in relation to nuclear expression of cyclin D1, the cytoplasmic expression of cyclin D1 and the fraction of nuclear cyclin D1 positive cells (labelling index)*

| | pRb intensity | | | Ki-67 labelling index | | | |
|---|---|---|---|---|---|---|---|
| | – | ± | + | + | ++ | +++ | Total *n* |
| Cyclin D1 nuclear intensity | | | | | | | |
| – | 2 | 21 | 6 | 4 | 16 | 9 | 29 |
| ± | 0 | 48 | 2 | 14 | 31 | 5 | 50 |
| + | 0 | 9 | 2 | 3 | 6 | 2 | 11 |
| Cyclin D1 cytoplasm | | | | | | | |
| + | 1 | 36 | 3 | 5 | 25 | 10 | 40 |
| ++ | 1 | 29 | 5 | 9 | 21 | 5 | 35 |
| +++ | 0 | 13 | 2 | 7 | 7 | 1 | 15 |
| Cyclin D1 nuclear | | | | | | | |
| – | 1 | 15 | 4 | 2 | 12 | 6 | 20 |
| + | 1 | 23 | 4 | 7 | 17 | 4 | 28 |
| ++ | 0 | 31 | 2 | 10 | 18 | 5 | 33 |
| +++ | 0 | 9 | 0 | 2 | 6 | 1 | 9 |
| Ki-67 labelling index | | | | | | | |
| + | 0 | 20 | 1 | | | | 21 |
| ++ | 2 | 45 | 6 | | | | 53 |
| +++ | 0 | 13 | 3 | | | | 16 |
| Total *n* | 2 | 78 | 10 | 21 | 53 | 16 | 90 |

An inverse linear relationship was observed between the proliferative activity (Ki-67) and the cytoplasmic expression of cyclin D1 ($P = 0.006$).

*Ki-67 LI*

To determine the proliferative capacity in colorectal cancer with abnormal expression of pRb and/or cyclin D1, the percentage of Ki-67 positive cells was analysed (Table 1). No significant association was observed between proliferative activity (LI for Ki-67) and nuclear cyclin D1 or pRb staining intensities, whereas a statistically significant inverse relationship was observed between cytoplasmic cyclin D1 levels and Ki-67 ($P = 0.006$). Sixteen tumours were classified as highly proliferative, and of these, 10 expressed low levels of cytoplasmic cyclin D1. Of 15 tumours with high levels of cytoplasmic cyclin D1, seven had a low Ki-67 LI whilst only one had a high Ki-67 LI. Furthermore, no significant relationship was seen between LI for Ki-67 and LI for nuclear cyclin D1 ($P = 0.114$).

*Tumour stage and grade*

pRb expression, cytoplasmic or nuclear expression of cyclin D1 or Ki-67 LI, was not significantly related to tumour stage or grade, although a trend towards a relationship between nuclear expression of cyclin D1 and grade ($P = 0.096$) was observed. Three of three tumours classified as well differentiated expressed normal levels of nuclear cyclin D1, while downregulation or overexpression of cyclin D1 was observed in six of eight poorly differentiated tumours. Tumours with high levels of cytoplasmic cyclin D1 had less lymphocytic infiltration at the invasive margin ($P = 0.014$), and vice versa. No relationship was observed between pRb expression, nuclear or cytoplasmic cyclin D1 expression and the tumour growth pattern at the invasive margin.

*Tumour localisation*

Interestingly, the only two colorectal cancers with low/absent nuclear pRb staining as well as eight of 11 tumours with overexpression of nuclear cyclin D1 were located in the right colon in comparison with tumours in the rectum, where these defects were never observed (Figure 2; $P = 0.036$ for cyclin D1 and $P = 0.009$ analysing cyclin D1 and pRb defects together). Overexpression of pRb, Ki-67 expression or cytoplasmic cyclin D1 levels was not correlated with any specific part of the large bowel.

*Survival analysis*

In the 84 colorectal cancer patients included for prognostic considerations, the expression of pRb did not predict for survival ($P = 0.64$), although one should consider that there were few observations with pRb alterations and further statistical analyses were, therefore, not meaningful. It should be noted that the 2 patients with inactivated pRb tumours both survived during the total observation period. The groups with normal expression and overexpression of pRb did not differ in survival (Figure 3a). No obvious difference concerning survival was observed among Dukes' B or Dukes' C patients with respect to pRb expression (data not shown).

Nuclear cyclin D1 expression did not correlate with patient survival, neither generally (Figure 3b) nor when specific Dukes' stages were analysed separately (data not shown). For the cytoplasmic expression of cyclin D1, there was a trend, although not statistically significant, towards impaired prognosis for Dukes' C patients with low cytoplasmic cyclin D1 staining ($P = 0.077$) (Figure 3c). For Dukes' B patients and the entire series, no obvious difference in survival was observed with respect to cytoplasmic cyclin D1 expression ($P = 0.80$ and $P = 0.46$, respectively).

## DISCUSSION

In the present study, we showed that pRb inactivation was rare (only 2%) in colorectal carcinomas, a result which is consistent with previous reports studying allelic loss of the *Rb1* locus [17, 18]. However, a recent report of 250 colorectal cancers, based on immunohistochemical evaluation of pRb, but without relating the nuclear intensity in tumour cells with

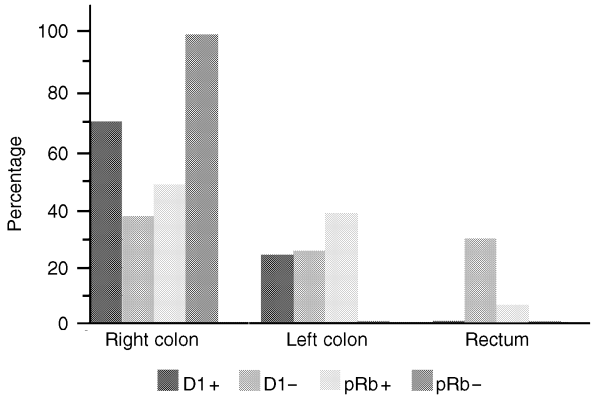


**Figure 2. Localisation in the large bowel compared with abnormal expression of nuclear cyclin D1 and the retinoblastoma protein (pRb) expressed as a percentage of the total number of abnormalities, respectively. D1+ denotes overexpression of cyclin D1 ($n$=11) and D1– denotes downregulation of cyclin D1 ($n$=29), Rb+ denotes overexpression of pRb ($n$=10) and pRb– denotes inactivation of pRb ($n$=2).**

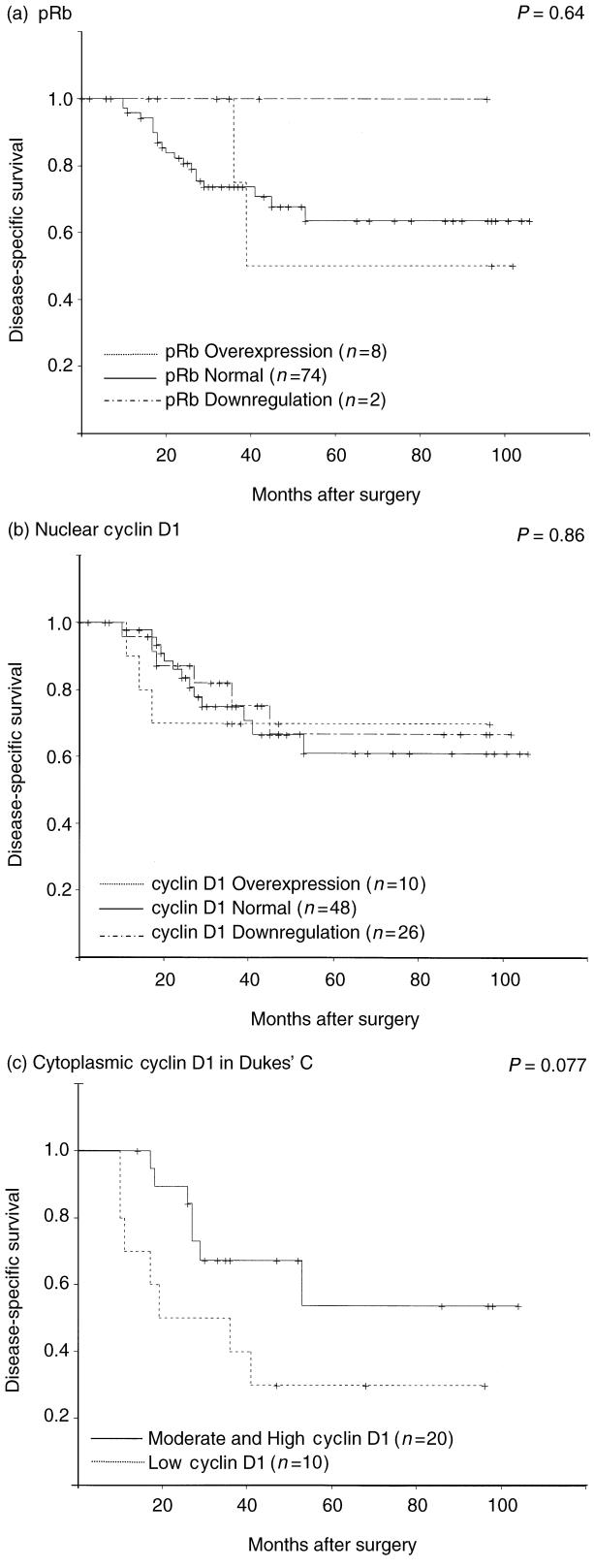


**Figure 3. Kaplan–Meier cancer specific survival curves for 84 patients with colorectal cancer. (a) Retinoblastoma protein (pRb) expression; (b) nuclear cyclin D1 expression. No significant difference between the three groups (high, normal and low) was observed; (c) cytoplasmic expression of cyclin D1 in the group of Dukes' C tumours. Tumours with low expression levels were compared with the group including both medium and high levels.**

that of normal adjacent cells, suggested that as many as 17% of colorectal cancers had inactivated pRb [22]. According to our experience and other reports [23–25], it is important to interpret pRb staining in tumour cells in relation to an internal control, and if this is overlooked, there is a risk of judging pRb normal tumours as being inactivated, due to technical shortcomings. Consequently, the reported frequency of 17% inactivation of pRb in colorectal cancer might be an overestimation and as shown here and in earlier publications, pRb inactivation is probably rare in colorectal cancer.

In this study, overexpression of pRb was substantially more frequent (11%) than inactivation of pRb, although it has to be stated that overexpression of pRb was less apparent and more difficult to evaluate than the distinct pRb inactivation. The frequency of immunohistochemical pRb overexpression is in agreement with earlier observations that approximately one-third of colorectal cancers have increased copy numbers of one *Rb1* allele [20, 21] a 2- to 5-fold increase in pRb mRNA levels compared with adjacent normal mucosa [18], as well as a 2-fold increase of the abundance of pRb and its phosphorylation status compared with normal mucosa [26]. However, the *Rb* gene is a tumour-suppressor gene and it is, therefore, difficult to explain the overexpression of pRb in colorectal cancer. Speculatively, it cannot be excluded that the *Rb* gene might contain genetic abnormalities which could lead to a functional inactivated protein with a subsequent longer degradation resulting in high protein levels [20]. A similar scenario has been described for the overexpression of mutated *p53*. Interestingly, tumours with both high and low levels of pRb expressed low levels of cyclin D1, supporting the idea that pRb overexpressing tumours might represent tumours with functionally inactivated pRb with a secondary downregulation of cyclin D1 due to an imbalance in the CDK4, p16 and cyclin D1 complex. Cyclin D1 downregulation in pRb defect cells is well established and has been observed in several tumours [27]. There are also reports suggesting that pRb has an oncogene-like effect in colorectal cancer, especially valid for aneuploid tumours [21].

The differences in nuclear cyclin D1 overexpression for colorectal cancer (11–30%), reported in the present study and by others [9–11], might be explained by the antibodies used and/or variations in threshold definitions for overexpression between the laboratories. Cytoplasmic cyclin D1 expression has been shown to be common in non-small lung cancer [28]. In colorectal cancer, a frequency of 26% has been reported, which is close to the 17% we observed, despite using different semiquantitative scales. Arber and colleagues [9] stipulated that cytoplasmic localisation of cyclin D1 is probably not caused by leakage of protein from the nucleus, since cytoplasmic staining was observed in the total absence of nucleus staining. In support of this, we detected tumours with strong cytoplasmic and low nuclear staining intensity of cyclin D1. The intracellular localisation of cyclin D1 and D2 is changed during progress through the cell cycle and from the G1–S transition the protein becomes more soluble, reflecting the loss of nuclear anchorage as part of its regulation [29]. This functional redistribution of cyclin D1 does not by itself explain why several tumours have increased levels of cytoplasmic cyclin D1, especially when considering the lack of correlation between cytoplasmic and nuclear staining patterns and the inverse correlation between cytoplasmic cyclin D1 staining and proliferative activity, as presented in this study.

Overexpression of cyclin D1, as well as pRb inactivation, showed strong specificity to the right colon. This emphasises the possibility that cancers developing in the right colon have a different spectrum of genetic damage and biological behaviour than cancers in the left colon and rectum. Our results contrast with the study of Arber and colleagues [9], reporting an overabundance of overexpression of cyclin D1 in tumours of the left colon and rectum. Nevertheless, our results encourage further and larger studies of cell cycle and other genetic defects in relation to cancer localisation, in order to understand the molecular mechanisms behind cancer development in different parts of the colon and rectum.

Surprisingly, the proliferative activity in colorectal cancer did not correlate with cyclin D1 levels or the fraction of cyclin D1 positive cells (LI). The concept that cyclin D1 overexpression contributes to an increased proliferative potential due to unscheduled activation of CDK4 and CDK6 might, therefore, be inadequate in colorectal cancer. Similar results have also been found in hypopharyngeal carcinoma [30] and breast cancer [23], and cyclin D1 might be important in the mechanisms behind malignancies, but does not confer any growth advantage, despite the obvious function in the G1–S transition. The heterogeneity observed for several parameters within colorectal cancer [31] might also confound any comparison between proliferation and expression of cyclin D1.

This is one of the first studies analysing the relationship between cyclin D1 expression and prognosis in colorectal cancer, together with a recent publication indicating a shorter disease-free survival for colorectal cancer with overexpression of cyclin D1 [11]. In contrast, our results suggest that cyclin D1 overexpression did not add prognostic information for patients with colorectal cancer. The reason for the discrepancy concerning survival is not clear, but selection of materials or geographical differences might have influenced the results.

Similar results to those shown in this study have been observed in non-small lung cancer [28] and Mantel cell lymphoma [32]. For breast cancer, an improved relapse-free survival for patients with cyclin D1 overexpressing tumours has been reported [12] and we have observed an improved survival for breast cancer patients with concurrent cyclin D1 overexpressing tumours with low cyclin E levels [23]. However, for head and neck, hypopharyngeal and oesophageal carcinomas, cyclin D1 overexpression seems to be significantly associated with an impaired survival [30, 33, 34]. Thus, the importance of cyclin D1 overexpression seems complex and probably differs between tumours.

A trend towards decreased survival for Dukes' C patients with low cytoplasmic cyclin D1 expression was observed, but because of the lack of correlation in the entire material or in the Dukes' B group, no conclusions can be made concerning prognostic considerations and cytoplasmic cyclin D1 expression in colorectal cancer. Similar remarks can be made concerning survival analysis and pRb status, which lacked prognostic impact in this study, probably partly because of the few patients with pRb abnormalities. The fact that the 2 patients with pRb inactivated tumours both survived during the observation period is intriguing, but larger studies are needed to verify the observation.

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