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# p16, cyclin D1 and Rb expression in colorectal carcinomas: Correlations with clinico-pathological parameters and prognosis

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Received October 23, 2007; Accepted November 30, 2007

**Abstract.** In an immunohistochemical study, 200 colorectal carcinomas were stained with monoclonal antibodies recognizing the cell cycle regulators p16, cyclin D1 and Rb, in order to study their expression and prognostic impact. Cyclin D1 and Rb were generally expressed in a nuclear pattern, whereas p16 also exhibited cytoplasmic reactivity. Immunoreactivity was observed in 94% (p16), 75% (cyclin D1) or 91% (Rb) of the carcinomas. Cytoplasmic p16 and cyclin D1 correlated with the extent of the mucinous tumor component. The cytoplasmic expression of p16 was reduced in advanced pN stages. At a cut-off point of 20%, cyclin D1 was significantly upregulated in tumors of the right colon. Further correlations with gender, growth pattern, staging, grading or prognosis were not revealed. The three cell cycle regulators do not represent useful markers of prognosis or predictors of colorectal carcinoma.

## Introduction

The G1/S-phase controlling mechanism known as the p16/CDK4/cyclin D1/Rb pathway is commonly deregulated in human malignancies. Normally, cyclin D1 binds CDK4 and CDK6 leading to phosphorylation and inactivation of the retinoblastoma protein (1-3). The phosphorylation of Rb results in the detachment of the transcription factor E2F and therefore genes involved in progression of the S-phase, for example cyclin A, can be expressed. Thus, the stimulation of growth is an important aspect of cyclin D1 function and underlines its role as an oncogene. The tumor suppressor p16 was identified as an inhibitor of both CDK4 and 6. Its transient expression leads to hypophosphorylation of pRb and inhibition of DNA-synthesis caused by binding to CDK4 (4-7).

Generally, aberrant or missing expression of p16 can be found in a wide variety of carcinomas, such as breast (8) and lung cancer (9) or mesothelioma (10). In colon cancer, p16 expression is mostly elevated, whereas normal tissues exhibit only little or no protein expression (7). Recently, many studies concentrated on the inactivating mechanisms of the tumor suppressor gene, such as methylation of promoter or 5' regulator regions. In some cases, methylation of p16 correlated with shorter survival or worse prognosis (11,12). Additionally, the concurrent occurence of K-ras mutations seems to play a role, even resulting in shorter survival (13,14). These investigations have been carried out mostly on the basis of methylation-specific PCR. Some immunohistochemical studies exploring the association with clinico-pathological parameters and prognosis were also performed. However, the results of these studies are inconsistent.

Aberrant expression of cyclin D1 can also be found in a wide variety of carcinomas, such as breast (15) and lung cancer (16) or tumors of the gastrointestinal tract (17) or central nervous system (18). Protein expression in colorectal cancer seems to resemble p16. Normal tissues showed only little or no cyclin D1 expression (19-21), whereas the highest levels were found in colorectal carcinomas (22-24).

Finally, aberrant Rb is widely expressed in the majority of human cancers, for example in carcinoma of the breast, small-cell lung cancer or tumors of the central nervous system (25-27). In most cases, a high Rb expression was discovered in normal (28,29) and neoplastic (29,30) colon tissues. The prognostic significance of Rb protein and its association to clinico-pathological parameters in colorectal carcinoma have rarely been investigated.

Previous results regarding correlations between the three important cell cycle regulators in colorectal cancer with clinico-pathological parameters and prognosis remain contradictory and have not, especially in the case of cyclin D1 and Rb, been frequently investigated. We therefore studied the immunohistochemical expression of these three molecules in a series of 200 patients suffering from colorectal adenocarcinoma. The staining results were correlated with various clinical and pathological features as well as with survival data.

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*Key words:* p16, cyclin D1, Rb, monoclonal antibody, colorectal carcinoma, prognosis

## Materials and methods

*Patients*. The study comprised 200 patients who underwent different potentially curative procedures, such as hemicolectomy, transversecolectomy, perianal exstirpation of the rectum or deep anterior amputation of the rectum. Of the patients, 104 were male and 96 female. The mean age was 64.8 years (SD 11.7) with a median of 65.2 years. Patients who died four weeks after the surgical intervention were excluded (post-operative mortality). (Neo-)adjuvant radio- or chemotherapy was not performed. Surviving patients were followed-up for at least five years. All procedures were in accordance with the Helsinki declaration of 1975.

*Tissue preparation, monoclonal antibodies and immunohistochemical method*. Tumor samples were routinely fixed in 5% formalin and embedded in paraffin. The sections were cut (3-5 $\mu$m) and de-paraffinized according to standard pathological procedures, then each immunohistochemical method was performed as described below.

*p16 immunohistochemistry*. Paraffin sections were incubated in a microwave oven at 750 W (2x2 min in citrate buffer pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by 0.3% $H_2O_2$ methanol for 10 min at room temperature (RT), followed by washing twice with Tris-buffered saline (TBS), pH 7.6. The primary antibody (Ab-7, NeoMarkers, Fremont, USA) was diluted 1:5 (v/v) in a mixture of 20% normal rabbit serum and 2% casein-PBS at a dilution of 1:5 (v/v) and was incubated overnight at 4˚C. Normal mouse serum and TBS were used as negative controls. As secondary bridging antibody, a biotinylated rabbit anti-mouse antibody (E354, Dako, Hamburg, Germany) was applied for 30 min at RT. Subsequently, the slides were incubated with the peroxidase-coupled streptavidin-biotin-complex (K355, Dako) for 30 min at RT followed by the biotinyltyramine solution for 10 min at RT. This primary solution was incubated 72 h at 4˚C, readjusted to pH 8.0 and stored at -80˚C. Before the primary solution was used, it was diluted (v/v 1:50) in TBS with an addition of 30% $H_2O_2$. Washing steps in TBS were performed between each step of the procedure. Finally, the slides were immersed in a solution with covalent complexes of alkaline phosphatase and streptavidin (K391, Dako). As a chromogen, AS-BI-phosphate (Sigma, Munich, Germany) and new fuchsin (Chroma, Koengen, Germany) were applied.

*Cyclin D1 and Rb immunohistochemistry*. After microwave pre-treatment (cyclin D1 5x4 min at 600 W in EDTA pH 8.0 and Rb 2x4 min at 600 W in citrate buffer pH 6.0), the sections were washed in cold water for about 15 min. Endogenous peroxidase activity was blocked by 0.3% $H_2O_2$ methanol for 20 min at RT, followed by washing once with water and twice with Tris-buffered saline (TBS), pH 7.6. The incubation with monoclonal antibodies directed against cyclin D1 (NCL-CYCLIN D1-GM; Novocastra, Newcastle, UK) and Rb (NCL-RB-358; Novocastra) followed. Both were incubated in a dilution of 1:40 (v/v) in an antibody dilution buffer (Zymed, San Francisco, USA) overnight at 4˚C.

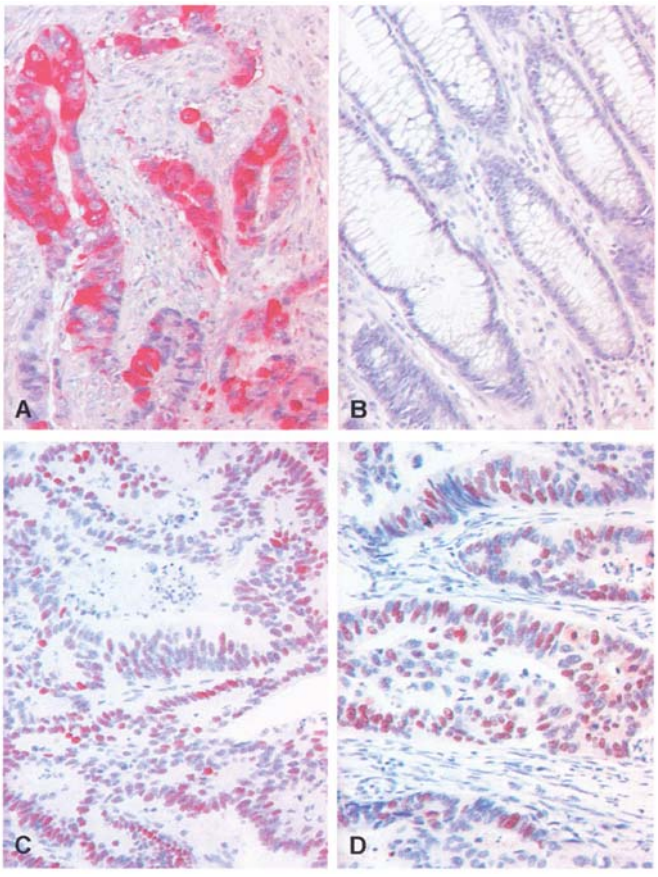


Figure 1. Typical nuclear and cytoplasmic staining pattern of p16 in a colorectal carcinoma (A) and lack of p16 expression in normal tissue. Typical nuclear staining pattern of cyclin D1 (C) and Rb (D).

Normal mouse serum and TBS were used as negative controls. The sections were washed twice with TBS, followed by incubation with EnVision™ + HRP (Dako) for 30 min at RT. After washing twice again with TBS, the reaction was visualized by 3-amino-9-ethylcarbazol solution (Dako) for 30 min at RT, and the samples were counter-stained with haematoxylin and embedded in glycerol jelly.

*Microscopic scoring and statistical analysis*. To estimate the degree of the staining reaction determined as a percentage of positive tumor nuclei and, in the case of p16, of cytoplasm, a semi-quantitative scoring system was performed as follows: 0, 0% positive tumor cells; 1, >0-5%; 2, >5-20%; 3, >20-50%; 4, >50%. Cases with a score of 0 were regarded as negative and cases with a score of 1-4 as positive.

For statistical analysis, StatView (version 4.57) software for Windows (Abacus, Berkeley, CA, USA) was used. The relationship between p16, cyclin D1 and Rb immunoreactivity and their clinico-pathological parameters were evaluated by applying the $\chi^2$ tests at a significance level of 5%. Univariate analysis was performed according to Kaplan-Meier, applying the log-rank (Mantel Cox) test. Additionally, statistical analysis of all antibodies was applied at the following cut-off points: p16 ≤20/>20%, cyclin D1 ≤5/>5% and ≤20/>20%, Rb ≤20/>20% and ≤50/>50%. Nuclear and cytoplasmic p16 were analyzed separately.

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*Immunohistochemical staining patterns of p16, cyclin D1 and Rb in colorectal carcinomas*. The 200 colorectal carcinomas displayed a positive staining reaction for the tumor suppressor gene p16 in 188 (94%) cases while 12 (6%) specimens were negative. All positive tumor samples showed a nuclear and cytoplasmic staining pattern (Fig. 1A and B). One hundred and fifty (75%) colorectal carcinomas exhibited nuclear cyclin D1 immunostaining (Fig. 1C), 50 (25%) specimens were negative. The tumor suppressor gene Rb showed a similar staining pattern. Among the cohort, 182 (91%) samples showed positive immunostaining within the nucleus (Fig. 1D), while 18 (9%) tumor samples were negative.

*Correlation of p16, cyclin D1 and Rb immunoreactivity with clinico-pathological parameters*. Positivity or negativity of the three molecules was correlated with various clinico-pathological parameters by applying the $\chi^2$ test. Whereas the status of nuclear p16 (Table I) and Rb (Table IV) did not show any association with these variables, cytoplasmic p16 (Table II) and cyclin D1 (Table III) were associated with the extent of the mucinous tumor component. With an increase of the mucin content, the expression of both genes was reduced. Additionally, cytoplasmic p16 exhibited a significant association with pN staging (Table II). At a cut-off point of 20%, cyclin D1 correlated with the localisation of the carcinomas, since a significantly stronger expression in the right colon was observed (Table V). No significant correlation with gender, growth pattern, grading or TNM staging was shown.

*Analysis of the prognostic relevance of p16, cyclin D1 and Rb*. The possible prognostic importance of the three cell cycle regulators was tested applying the univariate survival analysis according to Kaplan-Meier. Cyclin D1 and Rb did not show any significant association with patient survival probability (data not shown). At a cut-off point of 5%, a tendency of cyclin D1 to correlate with a better prognosis was observed (Fig. 2), but did not reach statistical significance.

Table I. Correlation of nuclear p16 with clinico-pathological parameters.

| Parameter | Score 0 | 1 | 2 | 3 | 4 | $\chi^2$-p | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| Gender | | | | | | | |
| Male | 4 | 37 | 10 | 28 | 25 | 0.187 | 6.173 |
| Female | 8 | 31 | 11 | 15 | 31 | | |
| Localisation | | | | | | | |
| Caecum/Ascendens | 3 | 14 | 4 | 8 | 6 | 0.4 | 12.585 |
| Transversum | 0 | 4 | 0 | 2 | 1 | | |
| Sigma/Descendens | 2 | 25 | 7 | 18 | 16 | | |
| Rectum | 7 | 25 | 10 | 15 | 33 | | |
| Growth pattern | | | | | | | |
| Exulcerative | 6 | 40 | 16 | 28 | 36 | 0.714 | 5.403 |
| Flat | 0 | 6 | 1 | 2 | 3 | | |
| Polypoid | 6 | 22 | 4 | 13 | 17 | | |
| Mucin content | | | | | | | |
| 0 (<5%) | 6 | 43 | 15 | 31 | 41 | 0.574 | 6.659 |
| 1 (5-50%) | 3 | 19 | 5 | 9 | 11 | | |
| 2 (>50%) | 3 | 6 | 1 | 3 | 4 | | |
| pN | | | | | | | |
| pN0 | 7 | 37 | 12 | 21 | 28 | 0.952 | 2.687 |
| pN1 | 2 | 16 | 6 | 9 | 14 | | |
| pN2 | 3 | 15 | 3 | 13 | 14 | | |
| Staging-TNM | | | | | | | |
| T1-2, N0, M0-Mx | 4 | 10 | 5 | 8 | 10 | 0.875 | 6.726 |
| T3-4, N0, M0-Mx | 3 | 26 | 7 | 12 | 18 | | |
| T1-4, N1-2, M0-Mx | 5 | 28 | 9 | 19 | 23 | | |
| T1-4, N0-2, M1 | 0 | 4 | 0 | 4 | 5 | | |
| Grading | | | | | | | |
| 1&2 (G1&2) | 9 | 64 | 20 | 39 | 48 | 0.193 | 6.087 |
| 3 (G3) | 3 | 4 | 1 | 4 | 8 | | |

## Discussion

In the present study, the majority of colorectal tumors expressed p16, cyclin D1 and Rb. With the exception of cyclin D1, our results confirm previous data exhibiting high expression rates of these proteins in colon cancer (7,24,31,32). Regarding cyclin D1, other studies have revealed values ranging between 8.6% (30) and 68.3% (33), while the median expression seems to be placed between 50 and 64% (20,23,24,34,35). The EnVision$^{TM+}$ technique was employed in our study, since it facilitates the detection of even low protein concentrations. Consequently, the relatively high incidence of cyclin D1$^+$ can be explained.

The majority of previous investigations concentrated on the inactivating mechanisms of p16, such as the methylation of the promoter or 5'-regulator regions. In some cases, methylated p16 seemed to be associated with clinico-pathological parameters, such as grading and prognosis (11,12,36-39). Results from immunohistochemical studies are inconsistent. Correlations with various clinico-pathological parameters, such as grading (40), lymph node metastasis (41,42) and prognosis (40,42,43), remain unconfirmed by others (24,36,43). Our study and those of other authors (24,31,36,42,43) could not reveal a clinico-pathological relevance of the tumor suppressor gene. However, the cytoplasmic expression of the cell cycle regulator was observed to be reduced at advanced pN stages. Analogously, Kim *et al* (41) demonstrated that decreased p16 expression was associated with lymph node metastasis and increased tumor size, while increased expression correlated with a low incidence of metastasis. Furthermore, Tada *et al* (42) revealed that the average rate of lymph node metastases was significantly higher in tumors exhibiting low levels of the tumor suppressor gene. Considering these results, it is tempting to speculate that the reduction of p16 expression could lead to tumor progression as decreased levels result in an advanced pN stage. Additionally, in our study cytoplasmic p16 was

Table II. Correlation of cytoplasmic p16 with clinico-pathological parameters.

| Parameter | Score 0 | 1 | 2 | 3 | 4 | $\chi^2$-p | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| Gender | | | | | | | |
| Male | 6 | 26 | 16 | 17 | 39 | 0.736 | 1.999 |
| Female | 6 | 17 | 17 | 14 | 42 | | |
| Localisation | | | | | | | |
| Caecum/Ascendens | 6 | 8 | 3 | 5 | 13 | 0.069 | 19.887 |
| Transversum | 0 | 2 | 0 | 3 | 2 | | |
| Sigma/Descendens | 1 | 18 | 14 | 10 | 25 | | |
| Rectum | 5 | 15 | 16 | 13 | 41 | | |
| Growth pattern | | | | | | | |
| Exulcerative | 5 | 29 | 19 | 18 | 55 | 0.393 | 8.426 |
| Flat | 0 | 3 | 1 | 2 | 6 | | |
| Polypoid | 7 | 11 | 13 | 11 | 20 | | |
| Mucin content | | | | | | | |
| 0 (<5%) | 7 | 27 | 21 | 25 | 56 | 0.042 | 16.018 |
| 1 (5-50%) | 1 | 13 | 10 | 3 | 20 | | |
| 2 (>50%) | 4 | 3 | 2 | 3 | 5 | | |
| pN | | | | | | | |
| pN0 | 8 | 28 | 13 | 18 | 38 | 0.031 | 16.916 |
| pN1 | 1 | 8 | 15 | 4 | 19 | | |
| pN2 | 3 | 7 | 5 | 9 | 24 | | |
| Staging-TNM | | | | | | | |
| T1-2, N0, M0-Mx | 2 | 10 | 5 | 8 | 12 | 0.270 | 14.459 |
| T3-4, N0, M0-Mx | 6 | 18 | 8 | 9 | 25 | | |
| T1-4, N1-2, M0-Mx | 4 | 12 | 17 | 10 | 41 | | |
| T1-4, N0-2, M1 | 0 | 3 | 3 | 4 | 3 | | |
| Grading | | | | | | | |
| 1&2 (G1&2) | 10 | 38 | 32 | 28 | 72 | 0.624 | 2.615 |
| 3 (G3) | 2 | 5 | 1 | 3 | 9 | | |

Table III. Correlation of cyclin D1 with clinico-pathological parameters.

| Parameter | Score 0 | 1 | 2 | 3 | 4 | $\chi^2$-p | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| Gender | | | | | | | |
| Male | 26 | 37 | 13 | 17 | 11 | 0.980 | 0.431 |
| Female | 24 | 37 | 11 | 16 | 8 | | |
| Localisation | | | | | | | |
| Caecum/Ascendens | 7 | 10 | 6 | 7 | 5 | 0.295 | 14.091 |
| Transversum | 2 | 0 | 0 | 3 | 2 | | |
| Sigma/Descendens | 16 | 28 | 8 | 11 | 5 | | |
| Rectum | 25 | 36 | 10 | 12 | 7 | | |
| Growth pattern | | | | | | | |
| Exulcerative | 30 | 43 | 15 | 23 | 15 | 0.604 | 6.386 |
| Flat | 5 | 3 | 1 | 2 | 1 | | |
| Polypoid | 15 | 28 | 8 | 8 | 3 | | |
| Mucin content | | | | | | | |
| 0 (<5%) | 32 | 51 | 20 | 25 | 8 | 0.002 | 24.986 |
| 1 (5-50%) | 16 | 12 | 3 | 5 | 11 | | |
| 2 (>50%) | 2 | 11 | 1 | 3 | 0 | | |
| pN | | | | | | | |
| pN0 | 26 | 42 | 11 | 14 | 12 | 0.319 | 9.289 |
| pN1 | 13 | 12 | 5 | 11 | 6 | | |
| pN2 | 11 | 20 | 8 | 8 | 1 | | |
| Staging | | | | | | | |
| T1-2, N0, M0-Mx | 10 | 15 | 4 | 6 | 2 | 0.808 | 7.706 |
| T3-4, pN0, pM0-Mx | 15 | 26 | 7 | 8 | 10 | | |
| T1-4, N1-2, M0-Mx | 22 | 27 | 12 | 16 | 7 | | |
| T1-4, pN0-2, pM1 | 3 | 6 | 1 | 3 | 0 | | |
| Grading | | | | | | | |
| 1&2 (G1&2) | 43 | 69 | 22 | 29 | 17 | 0.736 | 1.999 |
| 3 (G3) | 7 | 5 | 2 | 4 | 2 | | |

associated with the extent of the mucinous tumor component as an increased mucin content was related to reduced antigen expression. Similar observations were made by Tada *et al* (42). In their study, decreased p16 expression was observed in mucinous tumors.

In the present study, cyclin D1 was correlated with the localisation of the carcinomas at a cut-off point of 20%. Our result is in accordance with the observations of Holland *et al* (20), reporting on increased nuclear expression of cyclin D1 in the right colon. However, this did not apply to cytoplasmic expression. Controversially, data neglecting associations between the oncogene and localisation were also reported (21,23,24,31). In these studies, different monoclonal antibodies and immunohistochemical detection systems were applied. As described earlier (20,21,24,33), cyclin D1 seems to correlate with prognosis. Thus, McKay *et al* (24) reported cyclin D1 positivity as implying survival advantage for the patients, although oncogene expression was not an independent prognostic factor in the Cox regression analysis. Additionally, both nuclear and cytoplasmic expression of the cyclin were observed to be correlated with longer survival (20). Our data confirm these results in part, but the tendency of cyclin D1 to correlate with a better prognosis at a cut-off point of 5% did not reach statistical significance. Other results remain controversial. On the one hand, cyclin D1 turned out to be an independent prognostic factor in multivariate analysis (33) and overexpression of the oncogene was associated with worse prognosis (21). On the other hand, the prognostic relevance of cyclin D1 was not found by others (30,34,44-46). These differences reflect the heterogeneity of methods and procedures. Furthermore, a distinct definition of cyclin D1 overexpression seems necessary, as each group investigates either cyclin D1 expression or its overexpression according to their individual point of view. Previous data, neglecting the relevance of cyclin D1 expression in mucinous tumors (22) or extracellular mucin (23), are not confirmed by the present study.

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. Correlation of Rb with clinico-pathological s.

| Parameter | Score 0 | 1 | 2 | 3 | 4 | $\chi^2$-p | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| Gender | | | | | | | |
| Male | 11 | 13 | 10 | 12 | 58 | 0.385 | 4.160 |
| Female | 7 | 16 | 9 | 19 | 45 | | |
| Localisation | | | | | | | |
| Caecum/Ascendens | 5 | 7 | 2 | 1 | 20 | 0.616 | 9.996 |
| Transversum | 1 | 1 | 0 | 1 | 4 | | |
| Sigma/Descendens | 7 | 8 | 7 | 12 | 34 | | |
| Rectum | 5 | 13 | 10 | 17 | 45 | | |
| Growth pattern | | | | | | | |
| Exulcerative | 9 | 16 | 12 | 18 | 71 | 0.700 | 5.528 |
| Flat | 1 | 3 | 1 | 3 | 4 | | |
| Polypoid | 8 | 10 | 6 | 10 | 28 | | |
| Mucin content | | | | | | | |
| 0 (<5%) | 13 | 18 | 13 | 19 | 73 | 0.822 | 4.370 |
| 1 (5-50%) | 4 | 9 | 3 | 8 | 23 | | |
| 2 (>50%) | 1 | 2 | 3 | 4 | 7 | | |
| pN | | | | | | | |
| pN0 | 7 | 16 | 11 | 18 | 53 | 0.839 | 4.199 |
| pN1 | 5 | 8 | 3 | 8 | 23 | | |
| pN2 | 6 | 5 | 5 | 5 | 27 | | |
| Staging | | | | | | | |
| T1-2, N0, M0-Mx | 3 | 7 | 5 | 3 | 19 | 0.770 | 8.192 |
| T3-4, N0, M0-Mx | 3 | 9 | 6 | 14 | 34 | | |
| T1-4, N1-2, M0-Mx | 10 | 12 | 7 | 11 | 44 | | |
| T1-4, N0-2, M1 | 2 | 1 | 1 | 3 | 6 | | |
| Grading | | | | | | | |
| 1&2 (G1&2) | 15 | 27 | 17 | 29 | 92 | 0.792 | 1.692 |
| 3 (G3) | 3 | 2 | 2 | 2 | 11 | | |

Table V. Correlation of cyclin D1 with the localisation at a cut-off point of 20%.

| Localisation | n | ≤20% | >20% |
|---|---|---|---|
| Caecum/Ascendens | 35 | 23 (65.7) | 12 (34.3) |
| Transversum | 7 | 2 (28.6) | 5 (71.4) |
| Sigma/Descendens | 68 | 52 (76.5) | 6 (23.5) |
| Rectum | 90 | 71 (78.8) | 19 (21.1) |
| P-value | 0.02 | | |

Until now, little information is available on the possible correlations between the retinoblastoma protein, clinico-pathological parameters and survival probability in colorectal carcinoma patients. In most cases, no significant association regarding gender, localisation, lymph node metastasis, mucin content, grading or staging could be demonstrated (24,43,46). Analogously, our data did not reveal any clinical relevance of Rb expression. Regarding prognosis, we and other groups (24,46) could not observe a pivotal role for the tumor suppressor gene, whereas few contradictory results were reported. Thus, the loss or increase of Rb appeared to be an independent predictor of death from relapse (43), while its expression in liver metastasis was related to a survival advantage for the patients (47).

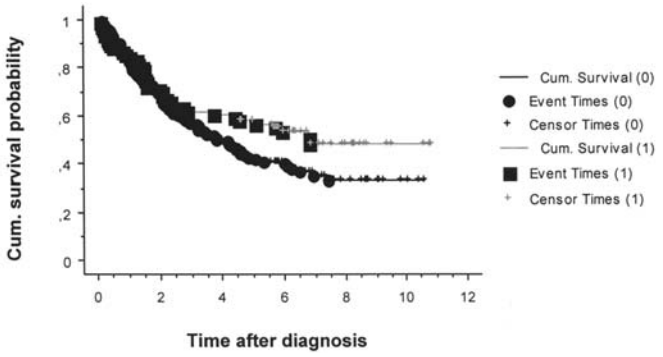


Figure 2. At a cut-off point of 5%, cyclin D1 positive tumors showed a tendency to correlate with a better prognosis.

In conclusion, our data confirm that the three cell cycle regulators do not represent useful markers for prognosis and other clinico-pathological parameters of colorectal carcinoma. However, cyclin D1 seems to be associated with tumor localisation. Additionally, the correlations of p16 (cytoplasmic) and cyclin D1 with the mucinous tumor component, as well as the association of cytoplasmic p16 with pN stage, reveal interesting aspects of colorectal carcinogenesis which warrant further investigation.

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