Tissue Microarray Molecular Profiling of Early, Node-negative Adenocarcinoma of the Rectum: A Comprehensive Analysis

Axel Hoos,1,2 Aviram Nissan,1 Alexander Stojadinovic, Jinru Shia, Cyrus V. Hedvat, Denis H. Y. Leung,3 Philip B. Paty, David Klimstra, Carlos Cordon-Cardo, and W. Douglas Wong4


ABSTRACT

Purpose: Early-stage adenocarcinoma of the rectum treated with curative intent has a favorable overall prognosis; however, 20%–30% of the patients recur, and the majority ultimately die of disease. Recurrence and tumor-related mortality may be attributable to molecular abnormalities in primary tumors accounting for their more aggressive biological behavior. This study evaluates such molecular phenotypes with regard to cell cycle regulation and proliferation and determines their significance for patient outcome.

Experimental Design: One hundred patients with primary T2-3, N0 adenocarcinoma of the rectum uniformly treated by surgery alone were studied. Core biopsies of pathological specimens were assembled on tissue microarrays, and expression of p53, mdm-2, p21, Bcl-2, p27, cyclin D1, and Ki-67 was analyzed by immunohistochemistry. Molecular profiles were correlated with disease-free (DFS) and disease-specific survival (DSS).

Results: Despite previously described prognostic relevance of some of the investigated molecules in analyses where different stages of colorectal cancer were included, none of the cell cycle-regulatory or proliferation-related markers was associated with recurrence or survival. However, patients with tumors demonstrating down-regulation of p27, a cyclin-dependent kinase inhibitor and tumor suppressor gene associated with development of metastases, showed a trend toward reduced DFS and DSS (P = 0.06 and P = 0.07, respectively).

Conclusions: In this homogeneous group of patients with early-stage, node-negative adenocarcinoma of the rectum uniformly treated by surgery alone, the investigated cell cycle-regulatory and proliferation-associated proteins appear to have no prognostic significance. However, down-regulation of p27 appears to be associated with a trend toward reduced DFS and DSS, which suggests further investigation of other p27-related pathways potentially relevant for metastatic disease.

INTRODUCTION

Adenocarcinoma of the rectum is a common human cancer for which resection remains the mainstay of treatment. Therapeutic approaches have evolved over the past two decades, providing patients with improved chances of sphincter-saving procedures. Adjunct chemotherapy and radiation as well as improved surgical techniques have significantly reduced local recurrence and improved overall survival (1). Treatment selection for the individual patient with adenocarcinoma of the rectum depends mainly on clinical and histological variables (2).

However, early-stage adenocarcinoma of the rectum (AJCC5/UICC stages I and II) treated by complete (R0) resection is associated with 20–30% incidence of recurrence and tumor-related mortality that cannot be predicted by clinical and pathological variables alone (3, 4). Because rectal adenocarcinomas may express multiple abnormalities in cell cycle-regulatory and proliferation-associated molecules, studies of single molecular markers have not been successful in defining the biology of this disease. This has prompted investigators to explore multiple molecules regulating the cell cycle in an effort to identify biologically aggressive tumors and appropriately select patients for adjuvant systemic or targeted therapies (5).

A number of tumorigenic cell cycle-regulatory proteins have been connected to human carcinogenesis and may be relevant to rectal adenocarcinoma. The p53 tumor suppressor plays a pivotal role in cell cycle regulation and apoptosis. Mutations in the p53 gene are among the most common mutations encountered in human malignancy (6, 7). p53-mediated programmed cell death is regulated through the Bcl-2/BAX apoptotic pathway (8). p53 inhibits Bcl-2 gene expression by transcriptional activation of the proapoptotic BAX gene. Murine double min (mdm-2) overexpression appears to be a common mechanism of p53 inactivation in human cancers (9). One
indirect indicator of p53 activity is the CDKI p21. Wild-type p53, along with other cellular growth factors, activates p21 gene expression; the corresponding p21 protein triggers cell cycle arrest in the G1 phase (10, 11). Other components of the complex cell cycle-regulatory machinery such as the CDKI p27 or cyclin D1 have also been implicated in colorectal tumorigenesis (12–15). Besides its cell cycle-regulatory role, p27 was reported to function as a tumor suppressor gene potentially associated with the development of metastatic disease (16–18). Some studies have analyzed the expression patterns of these molecules by IHC in colorectal cancer and compared their findings with clinico-pathological variables and patient outcome. A recent study of patients with stage I-II rectal adenocarcinoma identified p53 and Bcl-2 to be significantly associated with relapse-free survival (19). A second study of Dukes’ A-D colorectal cancers found p53 and Bcl-2 to be independent predictors of DFS and overall survival; this prognostic value was maintained when only Dukes’ B cancers were analyzed independently (20). Both studies identified a particularly malignant molecular phenotype in the form of p53(+)/Bcl-2(−) for colon and rectal cancers. In Dukes’ B colorectal cancers, contradictory data exist on the prognostic significance of cell cycle-regulatory molecules as p53 (21, 22). The functional end point of these growth-regulatory pathways is cancer cellular proliferation. Ki-67 proliferative index appears to be associated with survival in patients with various malignancies (23–26), but results are conflicting for colorectal cancer (27, 28).

Comprehensive expression profiles of p53, Bcl-2, mdm-2, p21, p27, cyclin D1, and Ki-67 in identically staged and treated colorectal cancer are difficult to carry out, in large part due to the complexity of these analyses if done by conventional full tissue section IHC. In the present study we use tissue microarray technology (29, 30) combined with IHC analysis to define the clinical significance of altered expression of these cell cycle-related molecules as well as Ki-67 proliferative index in a cohort of patients with primary T2–3, N0 adenocarcinoma of the rectum uniformly treated and followed at a single institution. Molecular profiles in these rectal adenocarcinomas, adenomas, and normal tissues from the same patients were correlated with outcome to determine their prognostic value.

PATIENTS AND METHODS

Patients. In 1987, the Memorial Sloan-Kettering Cancer Center Colorectal Cancer Database was initiated to prospectively record patient, tumor, treatment, recurrence, and mortality data on all patients admitted for treatment of colon or rectal carcinoma. Written informed consent was obtained from all patients. This database contained 2149 patients operated for rectal cancer. Of those, 1468 patients did not receive preoperative therapy. Four hundred and ninety-four of these 1468 patients had T2–3, N0 tumors. Of these, 166 were treated between 1992 and 1995. Sixty-six patients were excluded from the analysis for the following reasons: 14 patients died of non-cancer-related causes; and 52 patients were lost to follow-up. The remaining 100 patients, for whom full clinical and pathological data were available comprised the study population.

Those 100 patients formed a homogeneous group of localized primary pT2 and pT3, pN0 rectal adenocarcinomas treated with surgery alone at our institution from 1992–1995. All patients underwent total mesorectal excision and were curatively resected (AJCC/UICC R0). All patients were followed prospectively, and only patients who died of disease or had at least 5 relapse-free years of follow-up were included in the study. No patient had recurrence before 3 months. Patient follow-up was complete and obtained mainly from the Colorectal Cancer Database and was updated from clinical chart review, tumor registry information and physician records, patient correspondence, and telephone interviews.

Pathology reports and all available histological slides were critically reviewed for all patients to confirm the diagnosis of rectal adenocarcinoma. Material of three patients was not available, and those patients were also excluded from the study. Permanent section H&E-stained slides were reviewed without knowledge of clinical characteristics or outcome. A tumor was considered a primary rectal lesion if it was located 0–12 cm above the anal verge by rigid proctoscopic examination. Macroscopic margins were assessed at the time of surgery, and microscopic margins were assessed histopathologically. All surgical specimens were routinely examined for both distal and circumferential microscopic margins. Only patients with microscopically negative resection margins (R0) were included in the study.

Primary end points included time to first local recurrence, distant metastasis, and disease-related mortality. Clinico-pathological factors were correlated with study end points. Clinical and pathological covariates investigated in this study included patient age, gender, mural penetration (pT2, pT3), lymphovascular invasion, and immunohistochemical expression profiles as described below.

Tissues, Array Construction, and IHC. Tissue microarray analysis allows for high-throughput molecular profiling of cancer specimens via IHC. The technique was recently validated for use in clinico-pathological analyses in cancer (31), which identified the conditions to obtain concordant results between tissue microarrays and full section analysis by IHC: one block representative for the tumor specimen is chosen, a H&E stain is prepared, and three histomorphologically representative areas are identified, from which 0.6-mm tissue biopsies are taken and arrayed on a tissue microarray. All IHC analyses included in the present study were evaluated following the established criteria, thus reducing the problem of tumor heterogeneity and nonconcordance between tissue microarray and full section analysis potentially below 5% (31).

In the present study, normal rectal tissue, tissue from rectal adenomas, and invasive adenocarcinomas were formalin-fixed and embedded in paraffin. Five-μm sections stained with H&E were obtained to confirm the diagnosis and to identify viable, representative areas of the specimen. From these defined areas, core biopsies were taken with a precision instrument (Beecher Instruments, Silver Spring, MD), as described previously (30, 31). Triplicate tissue cores with a diameter of 0.6 mm from each specimen were punched and arrayed on a recipient paraffin block (31). Five-μm sections of these tissue array blocks were cut and placed on charged polylsine-coated slides. These sections were used for immunohistochemical analysis. Tissues and cell lines known to express the antigens under study were used.
as positive controls. Arrayed normal tissues served as baseline controls. All normal tissue samples demonstrated physiological expression patterns of the analyzed markers.

Sections from tissue arrays were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 15 min in 0.01 mM citrate buffer at pH 6.0. This procedure was performed for all antibodies under study. For Ki-67 antibody, an additional step of incubation in preheated 0.05% trypsin, 0.05% CaCl2 in Tris-HCl (pH 7.6) for 5 min at 37°C before microwave treatment was carried out. Slides were subsequently incubated in 10% normal horse serum for 30 min. The slides were then incubated overnight at 4°C in appropriately diluted primary antibody. Mouse antihuman monoclonal antibodies to p53 (Ab-2, clone 1801; 1:500; Calbiochem, Cambridge, MA), mdm-2 (clone 2A10; 1:500; kindly provided by Dr. A. Levine, Rockefeller University, New York, NY), p21 (Ab-1, clone EA10; 1:100; Calbiochem), cyclin D1 (clone DCS-6; 1:100; Calbiochem), p27 (Ab-2, clone DCS72; 1:500; Oncogene Research Products, Cambridge, MA), Ki-67 (Mib-1; 1:50; Immunotech, Marseille, France), and Bcl-2 (clone 124; 1:72; Dako, Glostrup, Denmark) were used. The anti-p53 antibody detects wild-type and mutated p53. Samples were then incubated with biotinylated antimouse immunoglobulins at 1:500 dilution (Vector Laboratories, Inc., Burlingame, CA) followed by avidin-biotin peroxidase complexes (1:25; Vector Laboratories, Inc.) for 30 min. Diaminobenzidine was used as the chromogen, and hematoxylin was used as the nuclear counterstain.

Immunoreactivity was classified as continuum data (undetectable levels or 0% to homogeneous staining or 100%) for all markers. Several investigators (J. S., A. H., C. V. H., A. N., and A. S.) reviewed and scored slides by estimating the percentage of tumor cells showing characteristic staining. A consensus between the investigators was obtained at the time of the reading. The cutoff values for tumor cell staining used in this study were defined as follows: (a) high Ki-67 proliferative index if >30% tumor nuclei stained; (b) p53 overexpression if >10% tumor nuclei stained; (c) mdm-2 overexpression if >30% tumor nuclei stained; (d) cyclin D1 overexpression if >5% of tumor nuclei stained; (e) Bcl-2 overexpression if >30% of tumor cells demonstrated cytoplasmic staining; (f) p21 positivity if >5% of tumor nuclei stained; and (g) p27 positivity if 20% of tumor nuclei stained. For the determination of cutoff values, previously established cutoffs for the same reagents in other cancer studies were used (31). These served as baseline and were modified for some markers according to clinicopathological correlations.

The presence or absence of p27 cytoplasmic staining was also recorded. Tumors were then grouped into two categories defined as follows: (a) normal expression (neoplasms below defined cutoff value of immunoreactivity in normal, benign, and tumor cells); and (b) abnormal expression (normal and neoplastic tissues above defined cutoff values of immunoreactivity).

Tissue damage during the cutting and staining procedures is a phenomenon associated with the tissue microarray technique (31). Tissue damage was recorded, and damaged tissues were excluded from clinicopathological analyses of the respective markers.

Statistics. Summary statistics were obtained using established methods. Associations between categorical factors were studied with Fisher’s exact test or chi-square test, as appropriate. Local recurrence was defined as the first clinically, radiologically, or pathologically evident tumor of the same histological type, within or contiguous to the previously treated tumor bed, 3 or more months after primary therapy. Nodal recurrence was defined by pathologically confirmed metastases to regional draining lymph nodes. Distant metastasis was defined by clinical or radiological evidence of systemic disease spread outside the primary tumor basin. Outcome was classified according to site of first disease recurrence as defined above (LR and/or DR). The clinical outcomes studied were LR, DR, DFS, and DSS. Time to recurrence and DSS were calculated from the date of primary operation. Patients whose deaths resulted from rectal cancer were considered to have died of disease and were considered in disease-related mortality calculations. Those who died of other causes free of disease were considered to have been alive without evidence of disease and lost to follow-up; thus, the status at last follow-up was regarded as a censored event for DSS estimates. Follow-up was calculated from the time of first operation for the primary rectal cancer to date of last follow-up.

The rate of recurrence or death was estimated using the Kaplan-Meier product limit method (32). Univariate influence of prognostic factors on study end points was analyzed using the log-rank test (33). Multivariate analysis was based on Cox’s proportional hazards regression model. Cutoff values for immunohistochemical staining were determined based on previously established cutoff values and, for some markers, were modified according to their relative frequency in studied rectal cancers by performing multiple comparisons. Multivariate analysis was conducted to assess the independent prognostic effect of the analyzed variables. To have adequate power for multivariate analysis, multiple analyses were conducted, in which molecular markers were analyzed in sets of three. Statistical analysis was performed using SPSS statistical package (SPSS Inc., Chicago, IL), and JMP software (SAS Institute, Cary, NC), P < 0.05 was considered significant.

RESULTS

Clinicopathological Data. Complete histological and clinical data were available for 97 of the 100 patients studied. The median age for the study population was 67 years (age range, 28–85 years). There was a male predominance in this cohort (male:female ratio, 2:1).

Review of the histology slides confirmed the initial T stage in all T2 (N = 48; 49.5%) and T3 (N = 49; 50.5%) patients. All tumors were graded as moderately differentiated, with the exception of 4 patients with well-differentiated tumors. All patients included in the study were node negative (N0). The median number of lymph nodes removed at time of surgery was 12 (range, 2–56 nodes). Lymphovascular invasion was present in 9 of 97 (9.3%) of the tumors studied. Lymphovascular invasion was associated with both higher recurrence (5-year DFS, 33% versus 74%; P = 0.003) and tumor-related mortality (5-year DSS, 33% versus 76%; P = 0.001).

Complete follow-up was available for all study patients. Median follow-up was 79.5 months (range, 57.7–105.9 months).
During this time period, 30 (30.9%) patients have died of disease, and 67 (69.1%) are alive and disease free. The cumulative 10-year risk of local recurrence was 8%. The median DSS for the entire study group was not reached. Five-year DSS was 73% (Table 1).

**Molecular Profiling.** A significant variation was observed in the expression of the individual molecular markers in tumor tissues. The highest rate of positivity was present for Ki-67 proliferative index evident in 61.0% of the tumors. The lowest rate of expression was observed with cyclin D1 evident in 8.0% of the cases. Samples from six rectal adenomas and four normal rectal tissue specimens were stained and used as reference tissue. The expression profiles of all molecular markers in normal tissues, adenomas, and adenocarcinomas are presented in Table 2, and representative images are shown in Fig. 1.

Tissue damage is a significant factor for tissue microarray-based analysis, with loss rates exceeding 30% in some studies (31). The median number of samples lost during sectioning and staining of tissue arrays in this study was 12 samples/marker (12.4%) [range, 3 (8.2%) to 16 (16.5%) samples/marker].

**Cell Proliferation.** High Ki-67 proliferative index was observed in 50 of 82 (61.0%) patients with adenocarcinoma. In addition, one of four normal rectal tissues and one of six adenomas demonstrated this phenotype. High Ki-67 proliferative index did not stratify patients with regard to DFS or DSS. Patient outcome according to Ki-67 phenotype is summarized in Tables 3 and 4.

**Expression of Cell Cycle-Regulatory Proteins.** p53 protein half-life is short, and expression levels are low in normal cells; thus, IHC cannot detect these wild-type p53 levels. In cancer cells, most p53 mutations lead to products that accumulate in the nuclei and can be demonstrated by IHC. Positive immunostaining most commonly represents accumulation of the stable protein product of a mutated p53 gene that has lost its cell cycle-regulatory function. Results of p53 immunostaining can be variably associated with p53 mutation profile, depending on the antibody clone used for analysis. Overexpression of p53 was identified in 52 of 89 (58%) adenocarcinomas and 1 of 6 adenomas but was absent in normal rectal mucosa. There was no difference in DFS or DSS between the p52-positive and -negative patients (Tables 3 and 4; Fig. 2).

Forty-seven of 81 (58.0%) tumors overexpressed mdm-2 protein compared with 2 of 5 (40%) adenomas and no normal rectal mucosa. The relation between mdm-2 and p53 expression was heterogeneous and did not show any clinically significant pattern. There was no difference in DFS or DSS between the mdm-2-positive and -negative carcinoma groups (Tables 3 and 4). The p21-positive phenotype was not identified in normal rectal or adenoma tissue and was balanced among carcinomas: 41 of 85 (48.2%) were p21 positive, and 44 of 85 (51.8%) were p21 negative. There was no difference in DFS or DSS between the p21-positive and -negative groups (Tables 3 and 4). No relational pattern could be identified for p21 and mdm-2 overexpression in the same tumors studied. In summary, no significant correlation was evident between patterns of p53 expression and those of important components in its pathway and patient outcome.

Expression of the cell cycle regulator cyclin D1 was evident in 7 of 87 (8%) cases, but not in normal or adenoma tissues. There was no difference in DFS or DSS between the cyclin D1-positive and -negative carcinomas (Tables 3 and 4). In contrast, the CDK1 p27 was positive in 29 of 85 (34%) carcinomas and negative in 56 of 85 (66%) carcinomas. There was a trend toward improved DFS and DSS in patients with p27-positive tumors (Tables 3 and 4), but it did not reach statistical significance ($P = 0.06$ and 0.07, respectively). Five-year DFS for p27-positive and -negative cancers was 83% and 64%, respectively (Fig. 2). Corresponding 5-year DSS was 80% and 66%. The risk of local recurrence at 5 years was 8.3% and 3.8% in the p27-negative and -positive groups, respectively. In addition, p27 staining was associated with mural penetration ($P = 0.05$; Table 5) but not with lymphovascular invasion. Some investigators have emphasized the importance of cytoplasmic staining of p27. As such, we evaluated cytoplasmic staining of p27 and found overexpression in 20 of 86 (23%) carcinomas and no expression in both adenoma and normal rectal samples. Cytoplasmic staining of p27 did not correlate with nuclear staining patterns, DFS, or DSS. Rates of recurrence and death as well as mural penetration are correlated with molecular expression patterns and are tabulated in Tables 3 and 4.

**Bcl-2 Expression.** Expression of the antiapoptotic Bcl-2 protein was heterogeneous in the investigated carcinomas as well as in adenomas and normal rectal mucosa (Table 2). Cytoplasmic overexpression of Bcl-2 was found in 21 of 84 (25.0%) adenocarcinomas. There was no difference in DFS or DSS between patients with Bcl-2-positive and -negative tumors.
Combined analysis of p53 and Bcl-2 expression identified 33 p53+/Bcl-2(−) and 13 p53(−)/Bcl-2(+) carcinomas. Seven (21.2%) and two (15.4%) patients died of disease in the p53+/Bcl-2(−) and p53(−)/Bcl-2(+) groups, respectively. These molecular phenotypes had no significant influence over clinical outcome.

**Multimolecular Phenotypes.** The differential expression of cell growth promoters and inhibitors in the same tissues was analyzed in these neoplasms according to their multimarker phenotypes. Various molecular marker combinations were analyzed, such as p53+/Bcl-2−/p27+. Expression patterns were heterogeneous, and none of the analyzed molecular clusters stratified patients according to clinical outcome.

**DISCUSSION**

Early-stage colorectal cancer (AJCC/UICC stages I and II) has a favorable prognosis because the majority of patients that are curatively resected remain free of disease. However, 20–30% of patients recur and die of disease despite resection with negative surgical margins (4). For such patients, it is hypothesized that recurrence may be attributable to aggressive cancer phenotypes characterized by a number of molecular abnormalities. The identification of molecular phenotypes in primary tumors that are associated with recurrent disease and tumor-related mortality may prove useful for stratification of patients and for treatment selection.

The cell cycle-regulatory machinery is a complex system of proteins regulating each other’s activity and controlling the division of cells (5). Some of the alterations in expression of cell cycle regulators have been demonstrated to be predictive for outcome in analyses where all stages of colorectal cancers were included. In stage II disease, contradicting data exist on the prognostic significance of these molecular alterations (21, 22). A possible explanation for this is that the sum of molecular alterations in early-stage disease may be much lower than that in later stages and may contain more irrelevant abnormalities that are not yet sufficient to create tumors with the ability to spread aggressively, as seen in advanced disease. Therefore, individual markers may be less likely to be significant predictors of outcome in early-stage disease. This study was undertaken with the aim of determining the clinical significance of expression profiles of various cell cycle-regulatory molecules and Ki-67 proliferative index in a cohort of patients with early, node-negative primary adenocarcinoma of the rectum.

Many investigators and clinicians consider cancer of the colon and rectum to be two distinct diseases; thus, we chose to...
evaluate only patients with T2N0 and early T1N0 rectal cancer treated with surgery alone in an effort to optimize the homogeneity of the study population. To demonstrate the study’s power to detect differences in cancer outcome, lymphovascular invasion, a well-described predictor of poor prognosis even in early-stage rectal cancer (34), was also analyzed. Lymphovascular invasion was found to be an independent predictor of recurrence and tumor-related mortality.

p53 mutations are among the most frequently detected mutations in human cancers, including colorectal adenocarcinoma (6, 7, 35). However, the prognostic value of p53 and the role of IHC in detecting p53 mutations remain controversial in the realm of colorectal cancer (36–39). In the present study, p53 was undetectable in normal rectal tissue and overexpressed in only one adenoma and 58.4% of the adenocarcinomas. p53 overexpression was not predictive of outcome. Similarly, the mdm-2 protein, which binds p53 and induces its degradation, did not correlate with p53 expression, DFS, or DSS.

p53 is a mediator of apoptosis via the Bcl-2/BAX pathway (8). The antiapoptotic Bcl-2 protein has been implicated as a prognostic factor in colorectal cancer. Leahy et al. (40) investigated 66 patients with Dukes’ A–C colorectal cancer and found Bcl-2 expression to correlate with improved survival. Manne et al. (41) studied the correlation between Bcl-2 expression and survival in 107 Caucasian and 146 African-American patients with colorectal cancer. In the Caucasian group, advanced colorectal cancer (hence, adverse outcome) was associated with low expression of Bcl-2. We have found no difference in DFS or DSS among patients with Bcl-2-positive and Bcl-2-negative early-stage rectal cancers.

The combination of p53(−)/Bcl-2(+) has been correlated with improved survival in patients with colorectal cancer. Schwandner et al. (19) reported 5 year-DFS of 97% and 42% for patients with p53(−)/Bcl-2(+) and p53(+/+)Bcl-2(−) adenocarcinomas of the colon and rectum, respectively. However, Bcl-2 was not found to be an independent predictor of survival by multivariate analysis in that study. In the studies of Manne et al. (41) and Schwandner et al. (19), the rate of Bcl-2 expression was disease stage dependent, thereby accounting for the observed differences in survival. In our selected group of homogeneous patients with early-stage adenocarcinoma of the rectum, Bcl-2 alone or in combination with p53 did not correlate with either DFS or DSS.

Another group of investigators (42) described higher rates of p53 expression in locally recurrent rectal cancers compared with primary tumors, but no difference in Ki-67 expression between the two study groups. Ki-67 has been shown to be a prognostic variable for a number of solid organ malignancies (23, 26, 43, 44) but shows conflicting results in colorectal cancer (27, 28, 42). Although high Ki-67 proliferative index was observed in 67% of rectal cancers in the present study, no difference in rates of recurrence or tumor-related death was evident between patients with high Ki-67 proliferative index and those with low Ki-67 proliferative index. Similarly, our findings that cyclin D1 is infrequently expressed in early rectal cancer and does not correlate with outcome are consistent with those of McKay et al. (45), who studied cyclin D1 expression in Dukes’ A–C adenocarcinoma of the colon.

p21/waf1/cip1 has been shown to be an adverse prognostic factor for gastric and bladder carcinoma (46, 47). In colorectal cancer cells, mutated p21 neither suppressed apoptosis nor affected cell survival (48). p21 was found to correlate with advanced-stage colorectal cancer, but this observation did not translate into a survival difference (49). In our study, p21 did not have prognostic significance.

The p27 gene product is a CDKI that regulates the progression of cells from late G1 phase into S phase. It was found to be an independent predictor of survival in a mixed population of 171 stage I–IV primary and recurrent colorectal cancers (42). Other investigators found an association between reduced p27 expression, tumor grade, and stage (12) and poor outcome in stage III–IV colorectal cancers (14). These data are consistent with our findings, showing a trend toward adverse DFS and DSS for patients with down-regulated nuclear p27. Also, an association with mural penetration was identified. However, these data should be further validated in a larger cohort of patients. Based on recent findings, it has been suggested that the down-regulation of p27 may provide circulating micrometastatic tumor cells with the ability to grow in an environment of altered extracellular matrix or intercellular adhesion properties and therefore may facilitate metastatic disease (16). In addition, it was demonstrated that p27 has other functions besides cell cycle control

**Table 4** Outcome according to molecular expression profiles

<table>
<thead>
<tr>
<th>Marker</th>
<th>n (%</th>
<th>DR n (%)</th>
<th>5-yr DFS</th>
<th>95% CI</th>
<th>Log-rank (P)</th>
<th>MVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>±</td>
<td>52 4 (7.7) 11 (21.2) 73% 73.7–93.4 0.69</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>±</td>
<td>37 1 (2.7) 8 (17.1) 76% 70.9–93.2 0.44</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mdm-2</td>
<td>±</td>
<td>50 4 (8.0) 8 (16.0) 78% 77.5–96.6 0.30</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>±</td>
<td>7 1 (14.3) 1 (14.3) 71% 42.2–85.8 0.93</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>±</td>
<td>80 5 (6.2) 20 (25.0) 70% 72.3–89.1 0.76</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>±</td>
<td>41 2 9 76% 77.1–96.9 0.93</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27</td>
<td>±</td>
<td>29 1 4 83% 80.6–101.2 0.06</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

For CI, confidence interval; MVA, multivariate analysis; NS, not significant.
is degraded in a tumor-specific enhanced proteasome-mediated fashion in colorectal cancer (17), and was suggested to function as a tumor suppressor gene (18).

The majority of studies demonstrating correlations between molecular markers and cancer outcome have included patients across all disease stages, suggesting that the molecular differences detected were reflective of differences in tumor biology between patients with disparate prognoses. Based on our data, most molecular factors involved in cell cycle regulation and proliferation do not appear to be relevant for prognostic stratification among patients with early-stage, node-negative rectal cancer. In this context, it needs to be noted that the number of patients involved in this study may still not be enough to detect less significant correlations of molecular factors with patient outcome. These findings suggest that other mechanisms independent of cellular proliferation may account for progression of this early-stage malignant disease. Cellular proliferation and its control through cell cycle regulation vary significantly between colon and rectal cancers of different size and with different extents of invasion and can be correlated with outcome. We have tried to eliminate the influence of established clinical and pathological prognostic factors by selecting patients with similar histopathological characteristics but disparate outcome to define the potential independent prognostic influence of the investigated molecular markers. According to our results, T2–T3, N0 rectal cancers appear to form a group of cancers with heterogeneous molecular expression profiles but balanced abnormalities of various cell cycle regulators that, overall, provide these tumors with similar growth properties. The potential prognostic value of p27 down-regulation observed in this study is interesting because p27 has recently been implicated in cell-cell adhesion interactions and metastatic disease and was significantly correlated with patient prognosis in these studies (15–18). Our results indicate that the cell cycle-regulatory pathway is not a relevant prognostic pathway in this patient subset. However, it is suggested to redirect our focus on investigating pathways of cell-cell adhesion and metastatic disease to identify molecular abnormalities significant for prognosis in this disease.

In summary, our study demonstrates that early-stage rectal cancers have heterogeneous expression of cell cycle-regulatory proteins that have no significant prognostic value in this selected group of patients. However, down-regulation of p27 is associated with a trend toward reduced DFS and DSS. These findings warrant additional molecular and clinicopathological studies of p27 and other molecules involved in p27-related pathways potentially relevant for metastatic disease to further define the biology of rectal cancer and the relevance of such molecules for patient prognosis.

Table 5 Mural penetration according to molecular expression profiles

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mural penetration</th>
<th>T2</th>
<th>T3</th>
<th>( \chi^2 \ P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67+</td>
<td>40</td>
<td>37</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Ki-67-</td>
<td>19</td>
<td>17</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>p53+</td>
<td>26</td>
<td>27</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>p53-</td>
<td>27</td>
<td>19</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>mdm-2+</td>
<td>40</td>
<td>41</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>mdm-2-</td>
<td>5</td>
<td>2</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>CyclinD1-</td>
<td>25</td>
<td>26</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>CyclinD1+</td>
<td>18</td>
<td>16</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Bcl-2+</td>
<td>40</td>
<td>41</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Bcl-2-</td>
<td>19</td>
<td>27</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Bcl-2-</td>
<td>26</td>
<td>16</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 DSS of the entire cohort (N = 97) and according to p27 (N = 85) and p53 (N = 89) expression.
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