p27 Expression in Inflammatory Bowel Disease-Associated Neoplasia

Further Evidence of a Unique Molecular Pathogenesis

Shaun Walsh,* Michael Murphy,*
Mark Silverman,[†] Robert Odze,[‡] Donald Antonioli,*
Harvey Goldman,* and Massimo Loda^{†§}

From the Department of Pathology,* Beth Israel Deaconess Medical Center, Boston; the Department of Pathology,[†] Labey-Hitchcock Medical Center, Burlington; the Department of Pathology,[‡] Brigham and Women's Hospital, Boston; and the Department of Adult Oncology,[§] Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts

The cyclin-dependent kinase inhibitor p27 is a negative regulator of the transition from G1 to S phase of the cell cycle, protects against inflammatory injury and promotes epithelial differentiation. Because p27 protein has been shown to be abnormally expressed both in dysplasia associated with Barrett's esophagus and in sporadic colorectal adenomas, we used immunohistochemistry to evaluate p27 expression in inflammatory bowel disease (IBD)-associated dysplasia and carcinomas. Normal, inflamed, and transitional mucosa, sporadic adenomas, and sporadic colonic carcinomas were studied as controls. In normal colonic epithelium p27 expression was restricted to the superficial, terminally differentiated cells. In colitic and inflamed diverticular mucosa p27 was expressed in the base of the crypts in 86 and 70% of cases, respectively. Similarly, in transitional mucosa adjacent to sporadic carcinomas p27 was expressed in the base of the crypts in all cases. Strong p27 expression extended more frequently from the base of the crypts to superficial cells in IBD-associated dysplasia than in sporadic adenomas ($P < 0.007$). Twenty of 20 (100%) IBD-associated carcinomas showed low p27 expression (<50% nuclei positive) compared to 6 of 20 (30%) stage-matched sporadic colorectal carcinomas ($P < 0.001$). We conclude (i) aberrant p27 protein expression in inflamed and IBD-associated nondysplastic mucosa is indistinguishable from that found in transitional mucosa adjacent to sporadic carcinomas; (ii) p27 is overexpressed in dysplastic lesions, perhaps as an attempt to counterbalance proliferative stimuli; and (iii) IBD-associated colorectal carcinomas have significantly lower p27 expression, commonly associated with poor prognosis, than stage-matched

sporadic colorectal carcinomas. These findings further substantiate the existence of divergent molecular pathogenetic pathways between these types of carcinomas and suggest an intrinsically more aggressive behavior of IBD-associated colon carcinomas compared to sporadic ones. (Am J Pathol 1999, 155:1511-1518)

Patients with inflammatory bowel disease (IBD) are at increased risk for the development of colorectal neoplasia. The risk of carcinoma is higher in patients with longer duration of disease and with greater anatomical extent of disease. Epithelial dysplasia has been recognized as a marker for the development of carcinoma in patients with chronic colitis. As a result, endoscopic biopsy surveillance for dysplasia is being used to monitor these patients, even though its effectiveness has recently been questioned.¹⁻⁴

Although the transition from inflamed epithelium to epithelial dysplasia and, ultimately, to carcinoma superficially parallels the sequence of normal colonic epithelium to sporadic adenoma to carcinoma, several important differences in their molecular pathogeneses have recently been identified. Firstly, p53 gene mutations and deletions occur at an early stage in IBD-associated neoplasia^{5,6} and may in fact precede dysplasia,⁷ but are known to occur later in the sporadic carcinoma sequence.⁸⁻¹⁰ Secondly, APC gene mutations are rare in IBD-associated neoplasia but common in the sporadic sequence.^{11,12} IBD-associated carcinomas rarely overexpress the tumor suppressor gene Bcl-2, whereas this protein is overexpressed in approximately 60% of sporadic colorectal carcinomas.¹³ Finally, K-ras mutations are rare in IBD-associated neoplasms and are a frequent event in adenomas and early sporadic cancers.¹⁴ The role of many other genes, such as p16^{ink-4a}, transforming growth factor- β receptor II (TGF β RII), and mismatch re-

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Address reprint requests to Massimo Loda, M.D., Department of Adult Oncology, Dana Farber Cancer Institute (Dana 740B), 44 Binney Street, Boston, MA 02115. E-mail: massimo_loda@dfci.harvard.edu.

pair genes, important in the development and behavior of sporadic colorectal carcinoma, have been only partially investigated in IBD-associated neoplasia.¹⁵⁻²⁰

Cell cycle progression is regulated by a family of cyclin-dependent kinases (Cdks). Different Cdks in association with different activating subunits known as cyclins are required at various stages of the cell cycle.^{21,22} Cyclin-Cdk activity is, in turn, regulated by Cdk inhibitors, which bind the Cdk-cyclin complexes, inhibit their activity, and block cell cycle progression.²¹ Recently, p27, a member of the Cip/Kip family of Cdk inhibitors, has been shown to be dysregulated in colorectal carcinogenesis. Overexpression of p27 has been demonstrated in sporadic colonic adenomas, and loss of p27 protein expression has been associated with aggressive behavior in sporadic colorectal carcinomas.^{23,24} In human tumors, no structural alterations and only very rare genetic mutations, which do not affect its function, have been identified in the p27/Kip1 gene.²⁵⁻²⁷ Rather, we previously demonstrated that, in sporadic colorectal carcinomas, p27 is eliminated by enhanced degradation via the ubiquitin-proteasome pathway.²⁴

Little is known about Cdk inhibitor activity in chronic inflammatory conditions such as IBD. However, in Barrett's esophagus, p27 is overexpressed in dysplastic epithelium.²⁸ Furthermore, studies of experimental glomerulonephritis induced in p27 gene knockout mice produced increased epithelial cell injury compared to normogenic control mice, suggesting a protective role for p27 in inflammatory conditions.²⁹ Lastly, mutation of the TGF β RII gene, a transducer of signals involved in the regulation of p27 and cyclin D1 activity, has been demonstrated in dysplasia and carcinoma complicating ulcerative colitis.^{16,17} Therefore, we hypothesized that p27 may be dysregulated in neoplasia, complicating IBD. The aim of this study was to characterize the expression of p27 in normal, dysplastic, and neoplastic colon specimens from IBD patients.

Materials and Methods

Patient Population

We performed a retrospective search through the surgical pathology files of the Beth Israel Deaconess Medical Center (Boston MA), the Brigham and Women's Hospital (Boston MA), and the Lahey-Hitchcock Medical Center (Burlington MA) for colectomy specimens from patients with neoplasia complicating chronic ulcerative colitis or Crohn's disease. Forty-three colectomy specimens were identified in which tissue was available for study and yielded 42 dysplastic lesions and 20 invasive adenocarcinomas. Thirty-eight patients (88%) had chronic ulcerative colitis and five (12%) had Crohn's disease. Normal colorectal mucosa from five patients, diverticular mucosa adjacent to pericolic abscess from 10 patients, 28 endoscopically removed sporadic adenomas from 27 patients, and 20 patients with stage-matched sporadic carcinomas were randomly selected and studied as controls.

Pathological Analysis

Hematoxylin and eosin (H&E)-stained tissue sections from the paraffin-embedded tissue block to be used for immunohistochemistry were reviewed for the following pathological features: presence and grade of dysplasia, presence and stage of invasive adenocarcinoma, and degree of differentiation, using previously published criteria.^{30,31} The tumors were staged according to American Joint Committee on Cancer staging criteria.³²

Immunohistochemical Analysis

Five-micrometer-thick tissue sections were cut from the paraffin-embedded tissue blocks, placed on charged slides, deparaffinized in xylene, and rehydrated through graded alcohol solutions. Immunohistochemistry was performed on an automated instrument (Ventana ES, Ventana Medical Systems, Tucson, AZ) as previously described.^{24,28} Briefly, after antigen retrieval by microwave irradiation (10 mmol/L sodium citrate buffer [Biogenex, San Ramon, CA]), pH 6.0, in a pressure cooker at 750W for 30 minutes), a mouse monoclonal antibody against p27 (Transduction Laboratories, Lexington, KY) or Ki67 (Immunotech, Marseilles, France) was applied on the slides at dilutions of 1:400 and 1:500, respectively. Steps performed by the instrument included blocking with normal horse serum, application of a secondary antibody conjugated to the avidin-biotin peroxidase complex, and visualization with 3,3'-diaminobenzidine as a substrate with standardized development times. Identical reaction times permitted accurate comparison of all samples. The slides were counterstained with Mayer's hematoxylin. A negative control in which the primary antibody was omitted was performed with each run. An osteosarcoma cell line, MG-66 (American Type Culture Collection, Manassas, VA), was used as a positive control for p27. After 48 hours of serum starvation, which is necessary to increase levels of p27, cells from two confluent flasks were harvested, fixed in neutral buffered formalin for 8 hours, and embedded in paraffin. In addition, mature lymphocytes in the lamina propria or from the mantle zone of lymphoid aggregates served as internal positive controls on every slide. Reactive tonsil was used as a positive control for Ki-67.

Immunostaining Evaluation

Distribution of p27 positivity in tumors was first assessed at low magnification ($\times 40$). To avoid biased selection of p27-negative clones, areas displaying the strongest nuclear immunostaining for p27 were selected for counting. This was performed at 400 $\times$ magnification. Specifically, at least 10 high-power fields and a minimum of 1000 cells were counted. Slides were graded for percentage of nuclei strongly positive for p27. Cytoplasmic staining was recorded but not included in the statistical analysis. A value of 50% was chosen as a cutoff to separate low and high expressors of p27, as described in previous studies.^{24,28} Tumors with <3% p27-positive cells were con-

sidered negative for p27 (a category previously shown to represent 10% of sporadic colorectal carcinomas²⁴), but were not separated as a group. Slides were reviewed to assess interobserver variability. There was >95% interobserver concordance among the three pathologists who scored the cases.

For all samples of normal, inflamed, and dysplastic mucosa, the extent and subcellular localization of p27 staining within the epithelium was recorded.

Statistics

Results of immunohistochemistry for p27 were compared between IBD-associated carcinomas and sporadic carcinomas and between sporadic adenomas and IBD-associated dysplasia, using Fisher's exact test for categorical data.

Results

Patient Population

The study group had a predominance of men (84%). The patients' age at diagnosis ranged from 29 to 77 years with a mean of 52 years. Twenty-one low grade and 21 high grade dysplastic lesions were identified. These included 13 dysplastic lesions adjacent to carcinoma and 29 lesions separate from carcinoma. Fifteen percent of the invasive adenocarcinomas were well differentiated, 55% moderately differentiated, and the remaining 30% poorly differentiated. The pathological stages of these invasive carcinomas were 1 Stage I (5%), 10 Stage II (50%), and 9 Stage III (45%). Samples of colitic mucosa adjacent to either dysplasia or carcinoma were available for study in 22 cases.

Normal and Inflamed Mucosa (Table 1)

p27 Expression in Normal Mucosa

Normal epithelium (Figure 1a) was studied in five cases. Cells expressing p27 protein were present only in the superficial, terminally differentiated two-thirds of the epithelium in all cases (Figure 1b). The staining was localized to the nuclei in all cases. Ki-67 staining was noted in the basal proliferative zone (lowest one-third of crypts) in all cases.

p27 Expression in Colitic Mucosa Adjacent to IBD-Associated Dysplasia or Carcinoma

Twenty-two samples of colitic mucosa were available for study. Cells expressing p27 were located in the upper two-thirds of the epithelium in 15 of 22 samples (68%). In contrast to normal epithelium, cells in the putative stem cell compartment, in the lowest one-third of crypts, also expressed p27 in 19 of 22 samples (86%; Figure 1d). p27 staining was both nuclear and cytoplasmic in 16 of 19 samples (84%).

Table 1. p27 Expression in Normal, Inflamed, and Dysplastic Colonic Epithelia

Cell type	Location of epithelial nuclear staining within crypts	
	Upper 2/3	Lowest 1/3
Non-IBD		
Normal (5)*	100	0
Diverticulitis (10)	40	70
Transitional (18)	61	100
Total Adenoma (28)	32 [†]	96
Adenoma < 0.5 cm (14)	14 [‡]	93
Adenoma > 0.5 cm (13) [§]	54 [‡]	100
IBD		
Inflamed (22)	68	86
Dysplasia (42)	70*	97

Numbers in last two columns indicate percentage of cells staining positively.

*Number in parentheses is the number of cases in each category.

[†]P = 0.007, expression of p27 in superficial two-thirds of crypts in IBD-associated dysplasia versus sporadic adenoma.

[‡]P = 0.0461, expression of p27 in superficial two-thirds of crypts in adenoma >0.5 cm versus adenoma <0.5 cm.

[§]One case was negative for p27.

Figure 1. **a:** Normal colonic mucosa, H&E stain. **b:** Nuclear p27 protein expression in terminally differentiated epithelial cells in the uppermost one-third of crypts. Lymphocytes in the lamina propria also show nuclear staining. **c:** IBD-associated nondysplastic epithelium, H&E stain. **d:** Strong nuclear and cytoplasmic p27 protein expression at the base of the crypts in the putative stem cells. **Inset:** Transitional mucosa adjacent to a sporadic carcinoma showing p27 protein expression at the base of crypts.

p27 Expression in Diverticular Mucosa Adjacent to Pericolic Abscess

Ten samples of inflamed and regenerative mucosa from diverticula adjacent to pericolic abscesses were studied. Cells expressing p27 protein were located in the superficial two-thirds of the epithelium in 4 of 10 samples (40%). Cells in the lowest one-third of crypts, again in the stem cell compartment, expressed p27 protein in 7 of 10 cases (70%; not shown). p27 expression was both nuclear and cytoplasmic in 8 of 10 samples (80%). Ki-67 staining was detected in the lowest one-third of crypts in all samples and in the upper two-thirds in 3 of 10 samples (30%).

p27 Expression in Transitional Mucosa Adjacent to Sporadic Carcinomas

Eighteen samples were available for study. Superficial epithelial cells expressed p27 in 11 of 18 (61%) cases. All 18 samples showed p27-positive cells in the lowest one-third of crypts in the stem cell compartment (Figure 1d, inset). Ki-67 staining was negative in 9 of 15 (60%) samples analyzed. Four cases (22%) showed Ki-67 expression in the lowest one-third of the epithelium and two cases (11%) showed Ki-67 expression extending to the upper two-thirds.

Dysplastic Lesions (Table 1)

p27 Expression in Dysplastic Epithelium from Patients with IBD

Forty-two dysplastic epithelial lesions were available for study. Positive epithelial staining was detected in 40 of 42 (95%) lesions. Cells in the lowest one-third expressed p27 in 39 of 40 (97%) positive cases. Two lesions (5%) were completely negative for p27. Cells expressing p27 extended to the superficial two-thirds of the epithelium in 28 of 40 (70%) positive cases (Figure 2d). Nuclear staining alone was seen in 5 lesions. Both nuclear and cytoplasmic staining was seen in 35 lesions (87%). No difference in staining pattern, subcellular localization, or intensity was observed between low and high grade epithelial dysplasia or between dysplastic epithelium adjacent to invasive carcinoma and dysplastic lesions separate from carcinoma. Ki-67 expression extended to the superficial cells in 27 of the 28 (96%) samples analyzed.

p27 Expression in Sporadic Adenomas

Twenty-eight sporadic adenomas were stained for p27 protein. Twenty-seven of 28 (96%) showed positive staining in epithelial cells. One adenoma was negative. Importantly, only 9 of 28 (32%) adenomas showed p27 protein expression in the superficial two-thirds of the crypts compared to 70% of IBD-associated dysplasia ($P = 0.007$). Epithelial cells expressing p27 protein were detected in the lowest one-third of crypts in all positive cases (Figure 1b). In 26 of 27 (96%) adenomas, staining

was both nuclear and cytoplasmic. Adenomas were then grouped according to size (<0.5 cm or >0.5 cm) and the location of p27 staining was compared. Only 2 of 14 (14%) adenomas <0.5 cm across in dimension showed p27 expression in the superficial epithelium. In contrast, 7 of 13 (54%) adenomas >0.5 cm across showed p27 expression in the superficial epithelium ($P = 0.0461$).

Carcinomas (Table 2)

p27 Expression in IBD-Associated Carcinomas and Sporadic Colorectal Carcinomas

Twenty of 20 IBD-associated carcinomas (100%) showed low p27 expression ($<50\%$ nuclei positive) compared to 6 of 20 stage-matched sporadic carcinomas (30%) ($P < 0.001$; Figure 3). Among IBD-associated carcinomas, 11 of 20 (55%) showed both nuclear and cytoplasmic staining and 9 of 20 (45%) showed nuclear staining alone. Low p27 expression did not correlate with either tumor stage or degree of differentiation.

Discussion

Cyclin-dependent kinase inhibitors are of fundamental importance in the regulation of transition from G1 to S phase. Failure to control this checkpoint may result in neoplasia.^{21,22} It has also been shown that p27 gene knockout mice have increased body weight and multiple organ hyperplasia, suggesting that p27 protein inhibits proliferation *in vivo*.³³⁻³⁵ Loss of p27 expression, as assessed by immunohistochemistry, is associated with aggressive behavior in a variety of epithelial neoplasms including colorectal carcinomas.³⁶ However, the role of p27 in IBD-associated neoplasia had not previously been investigated.

It is important for epithelial cells to exit the cell cycle as they migrate to the mucosal surface during the process of differentiation. In this and previous studies the terminally differentiated superficial cells of normal colonic epithelium have been shown to strongly express p27 protein in their nuclei.²⁴ In contrast, colitic (both IBD-associated and diverticular) and transitional mucosa adjacent to carcinoma frequently expressed p27 protein in colonic epithelial stem cells located at the base of crypts and not in superficial cells. The finding of strong p27 protein expression in these basal, putative stem cells in morphologically normal mucosa adjacent to inflammation or cancer is intriguing. Prostatic basal stem cells may be both p27-positive and p27-negative.³⁷ In fact, these authors postulated that basal cells lacking p27 may represent a transiently proliferating compartment. In inflamed, non-dysplastic mucosa, expression of p27 by colonic stem cells may reflect a block in proliferation, whereas absence of p27 expression by superficial cells may attest to loss of differentiation, indicating a frozen colonic crypt. In fact, studies have indicated that p27 protein may, in addition to its anti-proliferative action, promote cellular differentiation.³⁸⁻⁴⁹

If the noxious stimuli persist for prolonged periods of time, however, as is the case in IBD, the result is dyspla-

Figure 2. **a:** Sporadic colorectal adenoma, H&E stain. **b:** p27 protein expression limited to the lowest one-third of crypts in this sporadic colorectal adenoma. **c:** IBD-associated dysplasia, H&E stain. **d:** Strong p27 protein expression throughout crypts.

Figure 3. **a:** Sporadic colorectal carcinoma, H&E stain. **b:** Strong nuclear expression of p27 protein in tumor cells. **c:** IBD-associated adenocarcinoma, H&E stain. **d:** Tumor is negative for p27 protein expression. Note positively reactive lymphocytes surrounding tumor cells.

Table 2. Immunohistochemical Analysis of p27 Protein Expression in IBD-Associated and Sporadic Colorectal Carcinomas

IBD-associated carcinomas			Sporadic carcinomas		
Case no.	Stage	p27 level (% of nuclei positive)	Case no.	Stage	p27 level (% of nuclei positive)
1	I	8	1	I	31
2	II	19	2	II	1
3	II	1	3	II	95
4	II	1	4	II	96
5	II	7	5	II	31
6	II	4	6	II	74
7	II	1	7	II	52
8	II	1	8	II	77
9	II	44	9	II	76
10	II	1	10	II	89
11	II	6	11	II	68
12	III	43	12	III	88
13	III	38	13	III	32
14	III	31	14	III	67
15	III	3	15	III	92
16	III	42	16	III	67
17	III	4	17	III	55
18	III	3	18	III	91
19	III	1	19	III	1
20	III	1	20	III	37
Mean $\pm$ SE		12.9 $\pm$ 3.7			61 $\pm$ 6.7

P < 0.001 (IBD-associated carcinomas versus sporadic carcinomas).

Figure 4. Hypothesis of colonic carcinogenesis with respect to p27 expression in normal colonic mucosa and inflammatory bowel disease. p27 is localized in superficially differentiated cells in normal colonic epithelium. Both in transitional mucosa adjacent to tumor and in nondysplastic mucosa from patients with IBD, p27 is expressed in the basal stem cells (and it is both nuclear and cytoplasmic), but not in terminally differentiated superficial colonocytes. In sporadic adenomas and in IBD-associated dysplasia, p27 is overexpressed throughout the crypt epithelium in a transcriptionally controlled manner to counterbalance proliferative stimuli. Invasive adenocarcinomas, which are more frequently exophytic in sporadic cancers, express p27 in >50% of cells in 70% of cases whereas all cases of IBD-associated tumors express low levels of p27. Targeted degradation of p27, which is ubiquitin-proteasome-dependent, appears to be responsible for loss of p27 in tumors. In addition, IBD-associated cancers tend to invade the submucosa earlier, skipping an exophytic growth phase. Finally, the initial molecular alterations in sporadic (APC, ras, bcl-2) and IBD-associated cancers (p53, TGFβRII, p16) appear to be distinctly different. Thus, p27 protein expression appears to underscore the different tumor progression occurring in IBD-associated cancers with respect to sporadic carcinomas.

sia, which is characterized by p27 overexpression throughout the crypts (Figure 4). Expression of p27 protein by epithelial stem cells of the normal mucosa adjacent to carcinoma or in inflamed nondysplastic IBD mucosa may thus function to protect against inflammatory injury or to counterbalance excess proliferative stimuli. In dysplastic epithelium there is a failure of epithelial cells to mature as they extend upward from the crypt base. In this and previous studies, Ki-67 staining, which identifies cycling cells, has been shown to extend to the superficial epithelial cells of dysplastic epithelium, indicating that the dysplastic cells have lost the ability to exit the cell cycle and terminally differentiate.²⁸ In fact, IBD-associated dysplasias, which are considered to be more aggressive lesions than sporadic adenomas, demonstrated more frequent extension of p27 expression to the upper two-thirds of crypts than did sporadic adenomas. In addition, when the sporadic adenoma group was stratified by size, more aggressive adenomas (>0.5 cm in diameter) also showed more frequent p27 expression in the superficial two-thirds of crypts when compared to smaller adenomas. These findings suggest that expression of p27 through the full thickness in dysplasias is associated with more advanced premalignant lesions. Interestingly, in dysplasia associated with Barrett's esophagus both p27 mRNA and protein were found to be up-regulated, suggesting transcriptional control of p27 in *in situ* lesions.²⁸

When compared to sporadic colorectal tumors, carcinomas complicating ulcerative colitis arise in younger subjects, are more often multiple, and are more fre-

quently of the mucinous type.⁴ Recently, molecular genetic differences between the two types of carcinomas have also been identified.⁵⁻²⁰ Specifically, inactivation of the gatekeeper APC gene is a universal early event in sporadic colorectal cancer,¹¹ whereas APC mutations are rare in IBD-associated tumors.¹² Importantly, p53 alterations are associated with late stage disease in a variety of tumors including colorectal cancers.^{8,9} Early inactivation of this gene in IBD-associated neoplasia may indicate aggressive behavior in the early phases of carcinogenesis in this disease.^{5,6} Markedly different expression of antiapoptotic genes such as bcl-2 indicate, again, a difference at the molecular level between sporadic and IBD-associated cancer.¹³ In this study, low nuclear p27 expression was detected in 100% of IBD-associated carcinomas but in only 30% of stage-matched sporadic carcinomas ($P < 0.001$). This difference in p27 expression likely results from tumor-specific, ubiquitin-proteasome-mediated degradation of p27 in the face of unaltered transcription, as previously shown in sporadic adenocarcinomas.²⁴ Targeted degradation of p27 may in fact alter the balance between noxious stimuli and protective response and facilitate progression from dysplasias to adenocarcinomas.

It has been proposed that IBD-associated carcinomas have a worse prognosis than sporadic carcinomas merely as a result of delays in diagnosis, because surveillance programs have led to early detection of IBD-associated carcinomas and improved prognosis.^{49,50} However, to accurately predict the intrinsic biological aggressiveness of IBD-associated neoplasms, the precise molecular events underlying this disease need to be understood. In our study 100% of IBD-associated carcinomas showed low nuclear p27 expression, independent of grade, stage, or histological type. Previously, 10% of sporadic carcinomas were shown to express p27 in <3% of tumor cells, a category characterized as p27-negative.²⁴ These patients were shown to have a dismal prognosis and a 12-fold relative risk of dying of disease in a multivariate analysis. The number of p27-negative cases (defined by having <3% p27-positive cells) in IBD-associated cancers in this series was as high as 45%. In this cohort of patients, the relationship between p27 status and patient survival could not be further assessed, as follow-up data were not available in many cases. However, we have previously shown that low p27 expression is an independent negative prognostic indicator in sporadic colorectal carcinoma.²⁴ Thus, the low nuclear p27 expression among IBD-associated carcinomas suggests aggressive growth in these tumors and further emphasizes the need for early detection. In addition, down-regulation of p27 is associated with the development of metastases,⁵¹ perhaps because of its role in mediating extracellular signals.^{52,53}

Altered expression of p27 protein occurs in both inflamed and dysplastic epithelium in both IBD and non-IBD patients. IBD-associated colorectal carcinomas have significantly lower p27 expression when compared to stage-matched sporadic colorectal carcinomas. These findings further support a distinct molecular pathogenesis for IBD-associated carcinomas when compared to the

sporadic counterpart. We propose (Figure 4) that low p27 status may contribute to more aggressive clinical behavior in IBD-associated carcinomas.

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