Prognostic Significance of $p27^{kip-1}$ Expression in Colorectal Adenocarcinomas Is Associated with Tumor Stage

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ABSTRACT

Purpose: **Although the decreased expression of $p27^{kip-1}$, a cyclin-dependent kinase inhibitor, has been correlated with advanced tumor stage and short survival of patients with colorectal adenocarcinomas (CRCs), its prognostic value based on the tumor site, tumor stage, and patient ethnicity was not assessed. Therefore, in this study, we investigated whether the prognostic value of $p27^{kip-1}$ expression varies with the tumor site, tumor stage and patient ethnicity.**

Experimental Design: **We evaluated 206 (85 African Americans and 121 Caucasians) archival tissue specimens of first primary CRCs for immunohistochemical expression of $p27^{kip-1}$, and its prognostic significance was analyzed using univariate Kaplan-Meier and multivariate Cox regression survival methods.**

Results: **Although, similar proportion of CRCs with decreased $p27^{kip-1}$ expression was observed in all stages (range, 26–36%), the decreased $p27^{kip-1}$ expression has been shown as a marker of poor prognosis only for patients with stage III tumors both in univariate (log-rank test, $P = 0.014$) and multivariate (hazard ratio = 3.2, 95% confidence interval = 1.3–7.7; $P = 0.01$) survival analyses. The decreased expression of $p27^{kip-1}$ was associated with a high histologic grade ($P = 0.016$) in stage II CRCs, and with distal tumors ($P = 0.001$), tumor invasion ($P = 0.044$), and with local recurrence ($P = 0.008$) in stage III CRCs.**

Conclusions: **No prognostic significance was found for $p27^{kip-1}$ expression in stages I, II, or IV CRCs, and its prognostic value was not associated with either ethnicity or tumor location. These studies suggest that decreased expression of $p27^{kip-1}$ is an indicator of poor prognosis and aids in identifying a subset of patients with aggressive forms of stage III CRCs.**

INTRODUCTION

Colorectal adenocarcinoma (CRC) is the third leading cause of cancer associated deaths in both men and women in the United States (1). The stage of the tumor at the time of diagnosis remains one of the powerful indicators of aggressiveness of this disease and aids in predicting the survival of patients. However, pathological stage of CRC may not be the best indicator of clinical outcome because groups of patients with tumors of identical stage have different treatment responses and clinical outcomes. Recent studies, however, have reported that the molecular changes that are associated with the aggressiveness of CRC have different biological consequences based on patient age, race, and ethnicity, as well as the anatomical location of the tumor in the colorectum even after controlling for the tumor stage (2–8).

Aberrations in the molecular components of cell cycle checkpoints are a common feature of many human malignancies, and several of these molecules are known to have prognostic significance in CRC. Of these, $p27^{kip-1}$, a cyclin-dependent kinase inhibitor that regulates progression of cells from G_1 into S phase in a cell cycle is being increasingly recognized as an important factor for determining the biological behavior of invasive tumors. Decreased expression of $p27^{kip-1}$ has been correlated with advanced tumor stage and short patient survival in several human cancers, including CRC (9–11). Both biochemical and immunohistochemical evaluation of $p27^{kip-1}$ expression in human CRCs have shown diverse results in relation to its prognostic value (Table 1).

In a recent study, it was noted that both benign (histologically normal) and neoplastic tissues expressed variable amounts of the $p27^{kip-1}$ protein, as assessed by Western blot analyses; however, these differences were not statistically significant (12). In another study, no relationships were found between $p27^{kip-1}$ expression and gender, age, tumor location, growth pattern, and expression of other molecular markers, including p53, p73, and DCC (13); however, in a subset of patients, it was reported that the lack of $p27^{kip-1}$ expression is an indicator of short survival for patients with early-stage CRCs (Duke Stage B) located in the proximal colon (13, 14). Additional larger studies are required to evaluate the biological consequences of expression of $p27^{kip-1}$ in CRCs in relation to tumor stage, anatomical location of the tumor, and patient ethnicity.

In this study, we evaluated a large CRC patient population [total = 206 (85 African Americans and 121 Caucasians)] for phenotypic expression of $p27^{kip-1}$ to assess its prognostic significance based on tumor stage, anatomical location, and patient ethnicity.

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Table 1 Review of description of $p27^{kip-1}$ expression and its prognostic value in colorectal adenocarcinomas

Reference/country of study population	Tumor stages and size of study population	Anatomic location of tumor	Tissue/ fixative	AR[a]	Monoclonal antibody clone analyzed & dilution	Staining localization & cut-off
Present study United States (AL)	$n = 206$ All stages	Colorectal	Paraffin/ Formalin	Yes	1B4 1:30	Nu 0.7 ISS+ ≥50%
M. Loda et al (1997) United States (MA)	$n = 149$ All stages	Colorectal	Paraffin/ Formalin	Yes	57 1:200	Nu & Cy >50% = High ≤50% = Low 0% = NE Present = >0%
J.D. Cheng et al (1999) United States (NY)	$n = 66$ All stages	Colorectal	Paraffin/ Formalin	Yes	57 1:400	Nu 6–100% = positive 0–5% = negative
R. Palmqvist et al (1999) Sweden (Umea)	$n = 89$ Duke's A, B, C	Colorectal	Paraffin/ Formalin	Yes	57 1:200	Nu <50% = +, ++ ≥50% = +++, ++++
C. Belluco et al (1999) Italy (Padova)	$n = 124$ Stages I, II, III	Colorectal	Paraffin/ Formalin	Yes	57 1:600	Nu >50% = High ≤50% = Low 0% = Absent
K. Gunther et al (2000) Germany (Magdeburg)	$n = 164$ All stages	Rectal	Paraffin/ Formalin	Yes	57 1:200	Nu & Cy −, +, ++ = NE-moderate +++ = strong
J. Yao et al (2000) Singapore	$n = 136$ All stages	Colorectal	Paraffin/ Formalin	Yes	57 1:200	Nu & Cy 0–3 = No/Week 4–6 = strong positive
T. Tenjo et al (2000) Japan (Osaka)	$n = 171$ All stages	Colorectal	Paraffin/ Formalin	Yes	57 1:200	Nu ≥46.8% = positive <46.8% = negative
H. Zhang et al (2001) Sweden (Linkoping)	$n = 178$ All stages	Colorectal	Paraffin/ Formalin	Yes	SX53G8 1:50	Cy >10% = positive
M. Kobayashi et al (2002) Japan (Mie)	$n = 221$ All stages	Colorectal	Paraffin/ Formalin	Yes	SX53G8 1:200	Cy >11.7% = High ≤11.7% = Low
H.A. Rossi et al (2002) United States (MA)	$n = 187$ Stages I, II, III	Colorectal	Paraffin/ Formalin	Yes	57 1:50	Nu & Cy <25% = Low ≥25% = High
J.A. McKay et al (2002) UK (Scotland)	$n = 249$ All stages	Colorectal	Paraffin/ Formalin	Yes	1B4 1:40	Nu & Cy >50% = High ≤50% = Low
O. Schwandner et al (2002) Germany (Lubeck)	$n = 160$ Stages I, II, III	Rectal	Paraffin/ Formalin	Yes	SX53G8 1:35	Nu >10% = positive
Hoos et al (2002) United States (NY)	$n = 97$ T2 = 48 T3 = 49	Rectal	Paraffin/ Formalin	Yes	DCS72 1:500	Nu >20% = positive <20% = negative Cyto Normal, abnormal

MATERIALS AND METHODS

Formalin-fixed archival tissue blocks of CRCs from 504 patients (204 African Americans and 300 non-Hispanic Caucasians) were collected randomly from archives of the University of Alabama at Birmingham and the Veterans Administration Hospitals. All these patients underwent surgery for adenocarcinoma of the colorectum with curative or palliative intent. The selection of archival tissues was restricted to patients with first primary CRCs resected between 1981 and 1993. Because of limitations of the resources, for the current study, subsets of 206 patients with CRCs (85 African Americans and 121 Caucasians) were selected randomly from the initial group of 504 without the knowledge of clinical outcome. The pathological characteristics of CRCs, patient demographics, and patient follow-up information were collected

Table 1 Continued

Percent positivity (considered for survival analysis)	Follow-up	Prognostic value of $p27^{Kip-1}$		Independent prognostic markers
		Univariate	Multivariate	
I = 64 II = 72 III = 70 IV = 74	4.7 yrs (median)	All P = NS III P = 0.014	All P = 0.058 III P = 0.010	$p27^{Kip-1}$, pT, pN, Distant metastasis, Differentiation, Ethnicity, age,
All-II >50% = 29 7 Present = 34 32	9 yrs (median)	All P = 0.0048 II P = 0.0002 All P = 0.0014 II P = 0.0017	P = 0.003	$p27^{Kip-1}$
Positive = 51	3.2 yrs (median)	NS	NP	
≥50% = 56	NA	P = 0.0069	P = 0.01	$p27^{Kip-1}$, gender, Tumor type, Location, Growth pattern
Present = 86.3	4.6 yrs (median)	DFS All P < 0.000 I–II P = 0.014	P = 0.005	$p27^{Kip-1}$, Tumor-Node-Metastasis stage
Cy +++ = 25.6 Nu +++ = 51.8	6.2 yrs (median)	Cy P = 0.0185 Nu-NS	Cy P = 0.0552	Cytoplasmic $p27^{Kip-1}$
I/II = 85.4 III/IV = 76.1	5 Years	All P = 0.04 (5-yr Overall) III/IV-NS	NP	
0 = 57.7[b] I = 48.1[b] II = 47.2[b] III = 42.9[b] IV = 41.6[b]	5.6 yrs (median)	P < 0.0001 (5-yr Overall)	All P = 0.0146 II,III P = 0.0375	$p27^{Kip-1}$, Tumor stage
Normal = 35 Primary = 49 Metastatic = 32	5.3 yrs (mean)	All P = 0.08 Duke's B P = 0.03 Proximal colon P = 0.05	NP	
High = 52	3.4 yrs (median)	P = 0.0577	NP	
High = 33	3.9 yrs (median)	P = 0.02	P = 0.01	$p27^{Kip-1}$, p53, Tumor stage, Location, Post-op chemotherapy
High = 21.3	2.9 yrs (median)	NS	NS	p53, Dukes Stage
Positive = 44	3.2 yrs (mean)	NS (Overall) P = 0.0016 (5-Yr DFS)	NS	$p21^{Waf1/Cip1}$, p53, Union International Contre Cancer stage
Nu positive = 34.1 Cy positive = 23.3	6.2 yrs (median)	Nu-NS Cy-NS	NS NS	

[a] AR, antigen retrieval; ISS, immunostaining score; Nu, nucleus; Cy, cytoplasm; DFS, disease-free survival; OS, overall survival; *n*, sample size; NE, nonexpressors; NP, not performed; NS, not significant; NA, not available.

[b] Presented as mean expression of $p27^{Kip-1}$/tumor stage.

from the pathology reports, patient charts, and tumor registries, respectively. Tissue sections stained with H&E were evaluated to determine the grades of the tumors, which were categorized as either well, moderately, or poorly differentiated as suggested by WHO (15). Of these CRCs, 41 were classified as well, 134 as moderately, and 31 as poorly differentiated. Tumor stage was based on the classification of the American Joint Committee on Cancer classification (16, 17). There were 42 stage I, 69 stage II, 53 stage III, and 42 stage IV patients in this study. The anatomical locations of the tumors and their grouping into proximal and distal colonic and the rectal tumors were performed as described in earlier studies (18, 19).

During our initial process of selection, patients with multiple primaries, with multiple malignancies, or with family or personal histories of cancer were excluded; therefore, our study population consists only patients with sporadic first primary

CRCs. We included patients who have undergone surgery alone as a therapeutic intervention, but we excluded patients who received pre- or postsurgical chemo- or radiation therapy in our study to control for treatment bias.

Immunohistochemical Staining. Paraffin sections (5-μm thickness) of blocks representative of both tumor and benign tissues of each case were mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). The immunohistochemical staining procedure was carried out as described earlier (2–4). Specifically, the tissues were incubated for 1 h at room temperature with monoclonal antibody to $p27^{kip-1}$ (clone 1B4; Novacostra, Newcastle upon Tyne, United Kingdom) after antigen recovery by microwave boiling in citrate buffer (pH 7.4) for 10 min. The remainder of the staining procedure and the protocol for the antigen retrieval were described in detail elsewhere (2–4). Hematoxylin was used as a counterstain. A multitissue control block with colonic lymph nodes and benign colonic epithelium was used as the positive control.

Assessment of $p27^{kip-1}$ Staining. The staining assessment was performed by two authors (U. M. and N. J.) independently but together to ensure consistency in evaluation. The proportion of $p27^{kip-1}$-expressing cells varied from 0 to 100%, and the intensity of nuclear staining also varied from weak to strong. Therefore, the percentage of cells at each intensity of staining were recorded on a scale of 0 (no staining) to +4 (strongest staining) by two authors independently. If there was a disagreement in their assessment, they resolved before combining the individual scores. The immunostaining scores of the two authors were combined to obtain an average percent positive score as well as staining intensity for each case. Using this method of staining assessment, we evaluated expression of $p27^{kip-1}$ in benign colonic epithelium away and adjacent to invasive malignant lesion. A cutoff value of immunostaining staining score of $\geq$0.7 plus at least 50% of malignant cells immunostaining was used to classify tumors with increased expression ($\geq$50% cells positive plus $\geq$0.7 staining intensity score) or with decreased expression ($<$50% positivity or $<$0.7 intensity score) of $p27^{kip-1}$ antigen. This cutoff value was the median value of $p27^{kip-1}$ staining in the benign epithelium (average of away and adjacent to invasive lesion). Staining in lymphocytes and uninvolved colonic epithelium within the tissue sections served as internal positive controls for the expression of $p27^{kip-1}$.

Statistical Analyses. The association between $p27^{kip-1}$ and clinicopathological or biological characteristics was analyzed using the χ^2 test (20). *P*s were calculated, and significance was assessed at an α level of 0.05. The median follow-up period of the complete study population of 206 patients was 4.7 years (range, $<$1–18 years). The period from the date of resection to the date of death or last contact (if alive) was used for survival analyses. Outcome analyses were based on patients who were alive or had died of CRC as described previously (2–6). Univariate overall survival was obtained using Kaplan-Meier estimates (21). The log-rank test was used to compare Kaplan-Meier survival curves based on the status of $p27^{kip-1}$ expression. Separate multivariate Cox regression models (22) were built for patients with stages I, II, III, and IV CRCs, and the survival of patients was compared with and without $p27^{kip-1}$ expression after adjustments for confounding variables. The clinical confounding variables of CRC used in these analyses were pT, pN, and M components of Tumor-Node-Metastasis stage, age, sex, ethnicity, tumor location, tumor size, and differentiation. A stepwise model-building procedure was used to determine the significant factors in predicting survival related to CRC. The interactions between $p27^{kip-1}$ expression with other significant variables were tested for significance. Hazard ratios and 95% confidence intervals were calculated to identify the risk factors.

RESULTS

Demographic and Clinicopathological Characteristics of the Patient Population. The clinical, pathological, and biological features of the 206 patients are reported in Table 2. Patients with different tumor stages (I, II, III, and IV) were evenly distributed in this population. The distribution of CRCs in the colorectum was 43, 37, and 20% in the proximal colon, the distal colon, and the rectum, respectively. Mean age at the time of surgery was 65.4 years (range, 26.0–70.0 years), and the median survival was 5.58 years (95% confidence interval = 3.59–9.0). There was a predominance of males in our study (124 of 206, 60%) because the majority of patients treated at the Veterans Administration Hospital of Birmingham were males. At the last follow-up, the proportion of patients alive was 49% (101 of 206), dead because of colorectal neoplasia was 32% (66 of 206), and died because of other causes was 19% (Table 2).

$p27^{kip-1}$ Expression and Its Association with Different Clinicopathological Parameters. As observed in several other studies (Table 1), the expression of $p27^{kip-1}$ was predominantly in the nucleus; however, in a small proportion of cells (~20%) in a tumor tissue section $p27^{kip-1}$ was localized in the cytoplasm. For this study, we considered only distinct nuclear expression of $p27^{kip-1}$. Increased phenotypic expression of $p27^{kip-1}$ was detected in 70% (145 of 206) of CRCs (Table 2).

The analysis of correlation between the proportions of CRCs with increased expression of $p27^{kip-1}$ with different clinicopathological parameters in each tumor stage group was shown in Table 3. The incidence of CRCs with decreased $p27^{kip-1}$ expression was 36, 28, 30, and 26% in stages I, II, III, and IV, respectively. There was a significant association between $p27^{kip-1}$ expression and the degree of histological tumor differentiation in patients with stage II CRCs, which is that its expression was gradually decreased from well to poorly differentiated CRCs (χ^2, $P = 0.016$; Table 3). In stage III patient group, the expression of $p27^{kip-1}$ was significantly associated with the anatomical location of CRCs, the increased $p27^{kip-1}$ was observed in CRCs located in the proximal colon (91%), and in the rectum (77%) as compared with the distal colon CRCs (39%; χ^2, $P = 0.001$). In this patient group, the extent of expression of $p27^{kip-1}$ decreased as the extent of invasion of tumor into the bowel increased (pT_1 to pT_4, χ^2, $P = 0.044$). Also in the stage III cases, the decreased expression of $p27^{kip-1}$ was significantly associated with an increasing incidence of recurrence (χ^2 $P = 0.008$) and with clinical outcome; specifically, a higher proportion of patients with increased $p27^{kip-1}$ expression were alive at the time of last follow-up (χ^2, $P = 0.018$; Table 3).

We also used a multiple logistic regression model to si-

Table 2 Clinicopathological characteristics and expression of $p27^{Kip-1}$ in colorectal adenocarcinomas

	No.	(%)
Sex		
Male	124	(60)
Female	82	(39)
Age (years)		
<65	90	(44)
≥65	116	(56)
Ethnicity		
African Americans	85	(59)
Caucasians	121	(41)
Tumor location		
Proximal colon	89	(43)
Distal colon	76	(37)
Rectum	41	(20)
Tumor size (cm)[a]		
≤5	128	(63)
>5	74	(37)
Tumor differentiation		
Well	41	(20)
Moderate	134	(65)
Poor	31	(15)
Tumor stage (Union International Contre Cancer)		
I	42	(20)
II	69	(34)
III	53	(26)
IV	42	(20)
Tumor type		
Mucinous	25	(12)
Nonmucinous	181	(88)
pT component of stage (bowel wall invasion)		
pT_x	1	(<1)
pT_1	12	(6)
pT_2	44	(21)
pT_3	111	(54)
pT_4	38	(18)
pN component of stage (regional lymph node invasion)		
pN_x	7	(4)
pN_0	113	(55)
pN_1	50	(24)
pN_2	25	(12)
pN_3	11	(5)
M component of stage (distant metastasis)		
M_0	164	(80)
M_1	42	(20)
Cancer relapse (at follow-up)		
Absent	68	(33)
Present	94	(46)
Unknown	44	(21)
Vital status (at follow-up)		
Alive	101	(49)
Deaths because of colorectal cancer	66	(32)
Deaths because of unknown cause or other than colorectal cancer	39	(19)
Mean age at surgery	65.4 ± 11.9	
Years ± SD (range)	(26.0–70.0)	
Median follow-up time in years (range)	4.7 (<1.0–18.0)	
Median survival in years	5.58	
(95% confidence interval)	(3.59–9.0)	
Expression of $p27^{kip-1}$		
Negative	61	(30)
Positive	145	(70)

[a] Four tumors were not evaluated for tumor size because of nonavailability of tumor measurements in the surgical pathology report.

multaneously evaluate the association of each parameter with $p27^{kip-1}$ in all four tumor stage groups (stages I, II, III, and IV). This multivariate logistic analysis will address the issue of multiple testing for each individual parameter, and only one overall model is used to evaluate the clinical parameters simultaneously. In this analysis of association of variables with $p27^{kip-1}$ positivity, we found similar results as reported in Table 3; specifically, tumor differentiation ($P = 0.008$) remained significantly associated with increased expression of $p27^{kip-1}$ in patients with stage II CRCs (data not shown). Despite a small number of patients in stage III group, we still observed significance or an indication of trend of the association of $p27^{kip-1}$ expression with patient outcome ($P = 0.008$), tumor invasion (pT; $P = 0.062$), and tumor location ($P = 0.095$; data not shown).

Survival Analyses. Kaplan-Meier univariate survival analysis on the complete patient population ($n = 206$) demonstrated no significant differences in their overall survival between the patient groups with decreased or increased levels of phenotypic $p27^{kip-1}$ expression (log rank, $P = 0.152$; data not shown). Because, this study population has a considerable number of African American patients, we also conducted survival analyses to assess the significance of $p27^{kip-1}$ expression based on patient race; the analyses of clinical outcome demonstrated that in either of the racial groups, the cumulative survival curves of low and increased levels of $p27^{kip-1}$ expression did not show statistically significant differences (log rank, $P = 0.273$ and $P = 0.183$, for African Americans or Caucasians, respectively; data not shown). Univariate survival analyses based on $p27^{kip-1}$ expression and on tumor location also did not show significant differences for all three anatomical sites (log rank, $P = 0.377$, $P = 0.248$ and $P = 0.631$, for proximal, distal, and rectal tumors, respectively; data not shown).

We also analyzed the significance of $p27^{kip-1}$ expression in the complete study population based on the tumor stage and found that patients with tumors exhibiting decreased $p27^{kip-1}$ expression had significantly shorter overall survival only in the stage III patient-group (log rank, $P = 0.014$; Fig. 1*C*). Similar analyses in stage I, stage II, and stage IV groups did not show statistically significant differences between patients with decreased or increased levels of $p27^{kip-1}$ expression (Fig. 1*A*, *B*, and *D*).

The multivariate regression models built separately for each tumor stage demonstrated that decreased expression of $p27^{kip-1}$ was an independent indicator of poor prognosis only for patients with stage III CRCs. The hazard ratios and 95% confidence interval for stage III was 3.2 and 1.3–7.7 ($P = 0.01$), respectively (Table 4). No prognostic significance was found for $p27^{kip-1}$ expression in stages I, II, or IV CRCs (Table 4). In multivariate regression model, which included all groups of patients (stage I–IV together), the decreased $p27^{kip-1}$ expression was an independent marker of poor overall survival; however, it was only marginally significant (hazard ratio 0.7, 95% confidence interval, 0.4–1.0; $P = 0.058$). In this overall model, older age, African American race, high tumor grade, bowel wall invasion, regional lymph node invasion, and distant metastasis were independent predictors of overall survival (Table 4).

Table 3 Association of $p27^{kip-1}$ expression with selected clinicopathological characteristics of patients with colorectal adenocarcinomas in relation to their tumor stage

	Stage I (n = 42)			Stage II (n = 67)			Stage III (n = 49)			Stage IV (n = 35)		
	No. of tumors positive (27)	% (64%)	P	No. of tumors positive (50)	% (72%)	P	No. of tumors positive (37)	% (70%)	P	No. of tumors positive (31)	% (74%)	P
Age (yrs)												
<65	12	57		14	61		21	81		15	75	
≥65	15	71	0.334	36	78	0.127	16	59	0.088	16	73	0.867
Sex												
Male	17	57		28	72		24	75		15	68	
Female	10	83	0.080	22	73	0.887	13	62	0.309	16	80	0.384
Ethnicity												
Caucasians	20	71		28	70		23	74		18	75	
African Americans	7	53	0.076	22	76	0.590	14	64	0.409	13	72	0.839
Tumor location												
Proximal colon	10	63		22	71		20	91		14	70	
Distal colon	9	64		18	69		7	39		14	78	
Rectum	8	67	0.974	10	83	0.644	10	77	0.001	3	75	0.861
Tumor size (cm)												
≤5	24	64		23	72		24	72		18	69	
>5	3	60	0.361[a]	25	71	0.967	11	61	0.393	13	81	0.390
Tumor type												
Mucinous	0	0		8	80		5	71		2	50	
Nonmucinous	27	71	0.012[a]	42	71	0.564	32	70	0.920	29	76	0.255
Tumor differentiation												
Well	1	25		9	100		12	78		8	61	
Moderate	16	73		38	73		25	69		19	83	
Poor	10	63	0.183	3	38	0.016	0	0	0.240	4	67	0.351
pT component of stage (bowel wall invasion)												
pT_1	8	73					0	0		0	0	
pT_2	19	63	0.341				8	80		1	50	
pT_3				41	71		25	78		15	68	
pT_4				9	82	0.473	4	40	0.044	14	82	0.459
pN component of stage (lymph node invasion)												
pN_0	27	64		50	72					1	50	
pN_1							26	70		9	62	
pN_2							7	70		11	73	
pN_3							4	67	0.984	5	100	0.470
M. (component of stage distant metastasis)												
M_0	27	64		50	72		37	70				
M_1										31	74	
Cancer status (at follow-up)												
Absent	17	61		19	73		12	86		0	0	
Present	5	63		16	67		13	50		25	70	
Unknown	5	83	0.573	15	79	0.667	12	92	0.008	6	100	0.115
Outcome												
Alive	8	67		13	57		15	83		27	71	
Colorectal cancer deaths	13	68		23	79		15	54		0	0	
Death because of unknown cause or other than cancer	6	56	0.731	14	82	0.123	7	100	0.018	4	100	0.210
$p53^{nac}$												
Negative	17	68		25	66		22	69		13	65	
Positive	10	59	0.542	25	81	0.169	15	71	0.835	18	82	0.216

[a] Fisher's exact test P.

DISCUSSION

Our study population had similar proportions of CRCs with the decreased levels of $p27^{kip-1}$ expression in all stages (stages I–IV); however, decreased expression of $p27^{kip-1}$ was associated significantly with short patient survival of patients with stage III tumors only. Our findings also demonstrated that stage III CRCs, which were located in the distal colon, are less likely to express $p27^{kip-1}$ at high levels and that low levels of $p27^{kip-1}$ expression were correlated significantly with the extent of invasion of tumor to other adjacent organs (pT_4) and with local recurrence. These results suggest that $p27^{kip-1}$ expression may be used to identify more aggressive phenotypes of stage III CRCs.

Several abnormalities in the molecular features of cell cycle checkpoints have been implicated in several human malignancies, including CRC; however, the prognostic values of

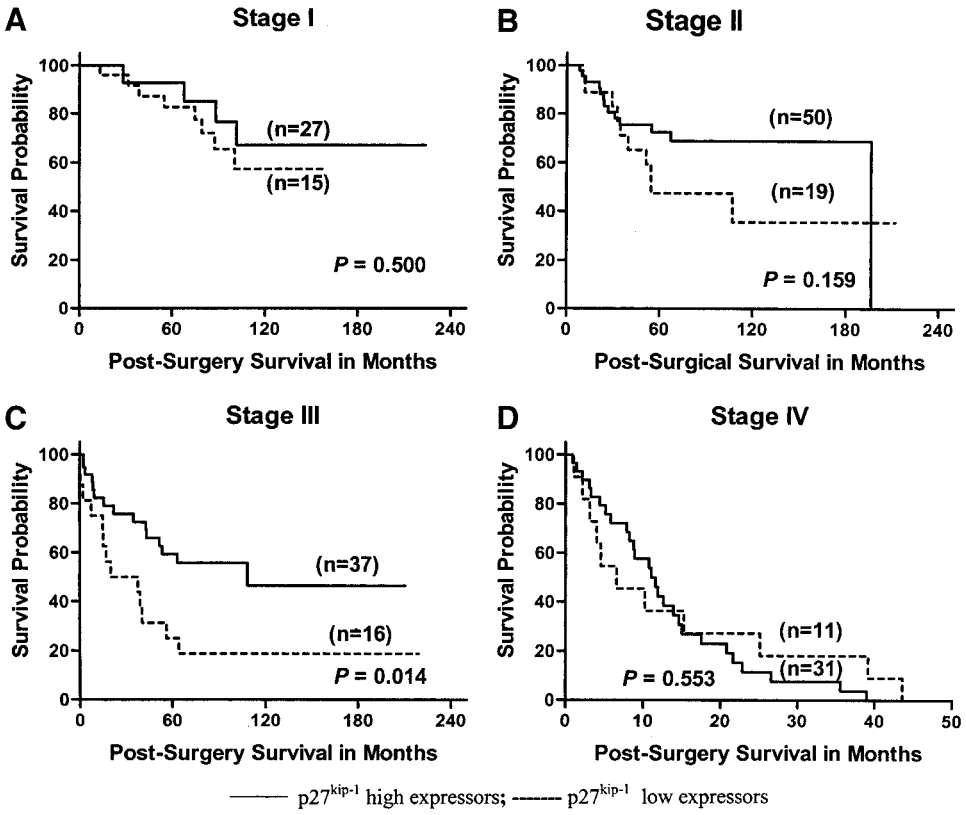

Fig. 1 Kaplan-Meier analysis showing the overall survival of colorectal adenocarcinoma patients categorized according to the stage of the tumor and status of $p27^{kip-1}$ expression. The statistical significance of the difference between curves of $p27^{kip-1}$ high expressors (increased expression) $p27^{kip-1}$ low expressors (decreased expression) was compared in stage I (*A*), stage II (*B*), stage III (*C*), and stage IV (*D*) patient groups. *P*s were calculated by the log-rank test.

many of these cell cycle antigens are controversial in CRC. For example, wild-type p53 eliminates genetically unstable cells that enter the cell cycle, and a mutant p53 will allow these cells to progress to a malignant phenotype. The role of p53 in colorectal neoplasia has been well studied; however, its value in predicting the clinical outcome has been controversial. Nevertheless, some recent studies from others and our laboratories evaluated its prognostic value based on tumor location and ethnicity and have demonstrated that nuclear accumulation of p53 is an independent prognostic indicator of patients with proximal tumors (23–25), specifically, for Caucasian patients (3, 7, 8). These novel strategies of assessing the predictive value of a molecular marker of colorectal neoplasia would yield valuable information, which will be useful in identifying aggressive phenotypes.

Increased expression of $p27^{kip-1}$in mammalian cells induces a G_1 block of the cell cycle (26, 27) and high levels of $p27^{kip-1}$ found in quiescent cells suggest that expression of $p27^{kip-1}$ also plays a role in maintaining cells in G_0 (28, 29). As with other Kip proteins, $p27^{kip-1}$ has a nuclear localization signal in its -COOH terminus. Loss of $p27^{kip-1}$ expression may result in the development and/or progression of tumors; however, this loss of expression does not appear to result from gene mutation (30–32). $p27^{kip-1}$ is posttranslationally regulated by an ubiquitin-proteasome-dependent degradation pathway, and $p27^{kip-1}$ also is regulated by cell-cell contacts (33). $p27^{kip-1}$ expression can also be regulated by transcriptional mechanism. In CRCs, reduced expression of $p27^{kip-1}$ in the metachronous metastases compared with the corresponding primary tumor suggests that the down-regulation of $p27^{kip-1}$ in circulating malignant cells may accentuate the ability of these cells to metastasize to liver (34). Also, accumulation of $p27^{kip-1}$ has been implicated in the differentiation of different varieties of cells (35, 36). In CRCs, Loda *et al.* (9) demonstrated that absent or low levels of $p27^{kip-1}$ are caused by increased protease-mediated degradation rather than from altered gene expression.

Variable levels of $p27^{kip-1}$ expression have been observed in benign and malignant epithelial components of the colorectum (9). The role of this molecular marker in assessing the aggressiveness of CRCs and in predicting the clinical outcome of patients with colorectal neoplasia has been examined by several investigators and the majority of these studies, which are cited in the Medline or PubMed (until the end of July 2003) are listed in Table 1. This review table clearly indicates discrepancy of prognostic significance of $p27^{kip-1}$ expression in colorectal neoplasia. Several studies reported that the lack of $p27^{kip-1}$ expression was associated with short patient survival (9, 11, 13, 14, 37–40); however, such an association was not found in some other studies (41–45). In some studies, it was suggested that $p27^{kip-1}$ was an independent predictor of patients specifically with early stage CRCs (stages I and II; Refs. 9, 13, 39), whereas, studies by Tenjo *et al.* (11) observed that $p27^{kip-1}$ expression as an independent prognostic marker for patients with stage III CRCs.

In our study, all patients have received a uniform treatment of surgical resection either with curative or palliative intents and none of them were treated with chemotherapeutic agents such as

Table 4 Cox regression multivariate analysis to evaluate independent prognostic value of $p27^{kip-1}$ expression in colorectal adenocarcinomas in relation to tumor stage

Variable	Indicator of poor prognosis	Hazard ratio (95% confidence interval)	*P*
Overall			
$p27^{kip-1}$ expression (positive *versus* negative)	$p27^{kip-1}$ negative	0.7 (0.4–1.0)	0.058
Age (<65 *versus* ≥65 years)	≥65 years	1.8 (1.2–2.8)	0.002
Ethnicity			
(African American *versus* Caucasian)	African American	1.8 (1.2–2.6)	0.003
Tumor differentiation			
(Moderate *versus* well)	Moderate	1.7 (1.1–2.0)	
(Poor *versus* well)	Poor	2.4 (1.3–4.8)	0.009
pT component of stage (bowel wall invasion)			
(pT_2 *versus* pT_1)	pT_2	1.4 (1.2–1.9)	
(pT_3 *versus* pT_1)	pT_3	1.9 (1.0–3.4)	
(pT_4 *versus* pT_1)	pT_4	2.6 (1.1–6.3)	0.039
pN component of stage (regional nodal invasion)			
(pN_1 *versus* pN_0)	pN_1	1.9 (1.3–2.9)	
(pN_2 *versus* pN_0)	pN_2	3.8 (2.6–5.1)	
(pN_3 *versus* pN_0)	pN_3	5.9 (3.4–9.6)	0.0001
Distant metastasis			
(M_1 *versus* M_0)	M_1	8.9 (5.4–14.7)	<0.0001
Stage I			
$p27^{kip-1}$ expression (positive *versus* negative)	$p27^{kip-1}$ negative	1.3 (0.4–4.5)	0.680
Age (<65 *versus* ≥65 years)	≥65 years	5.7 (1.5–21.5)	0.011
Stage II			
$p27^{kip-1}$ expression (positive *versus* negative)	$p27^{kip-1}$ negative	0.6 (0.2–1.3)	0.180
Ethnicity (African American *versus* Caucasian)	African American	3.1 (1.3–7.4)	0.012
Stage III			
$p27^{kip-1}$ expression (positive *versus* negative)	$p27^{kip-1}$ negative	3.2 (1.3–7.7)	0.010
Tumor differentiation			
(Moderate *versus* well)	Moderate	3.0 (1.2–7.4)	
(Poor *versus* well)	Poor	9.1 (1.5–55.1)	0.028
Stage IV			
$p27^{kip-1}$ expression (positive *versus* negative)	$p27^{kip-1}$ negative	1.3 (0.6–2.7)	0.555

5-fluorouracil or with any other neoadjuvant or adjuvant therapies. Adjuvant chemotherapies were not common practices at the beginning of our study in 1981, but use of adjuvant therapy for CRC increased in 1988. Although, the Food and Drug Administration has approved leucovorin for use in combination with 5-fluorouracil to prolong survival in the palliative treatment of patients only with advanced CRC (stage IV) in 1952, the adjuvant treatment in combination with 5-fluorouracil after surgical resection in patients with Dukes' Stage C colon cancer was approved only in 1990.[3] Therefore, we did not identify many patients who received chemotherapy during our study period and as stated in methods section, we excluded patients who received pre- or postsurgical treatment. Thus, using our study population, we evaluated the prognostic value of $p27^{kip-1}$ expression without treatment biases. Also, we assessed its prognostic value based on race, the tumor stage, and tumor site.

In this study, similar proportions of CRCs with the decreased levels of $p27^{kip-1}$ expression were noted in all stages (26–36%). Similar to studies by Palmqvist *et al.* (14), we also did not see a significant association between $p27^{kip-1}$ expression and the tumor differentiation; however, when stratified by tumor stage, our study found that in stage II CRCs, the decreased expression of $p27^{kip-1}$ was associated with poorer histological differentiation. This association, however, did not impact overall survival of patients with stage II CRCs. In stage III CRCs, $p27^{kip-1}$ expression was significantly correlated with tumor location, *i.e.*, distal tumors exhibited decreased levels of $p27^{kip-1}$ expression and the extent of $p27^{kip-1}$ expression decreased as the invasion of tumor into the lower layers of the bowel wall increased (pT_1 to pT_4). Similar to our findings, Ciaparrone *et al.* (46) also found a significant correlation between $p27^{kip-1}$ expression and tumor grade with well and moderately differentiated CRCs expressing higher $p27^{kip-1}$, whereas the poorly differentiated CRCs had significantly lower $p27^{kip-1}$ expression. Furthermore, we observed that tumors, which exhibited decreased expression of $p27^{kip-1}$, had higher incidence of local recurrence in patients with stage III CRCs, and such an association also was reported in prostatic carcinoma (47).

[3] Internet address: http://www.fda.gov/cder/cancer/druglistframe.htm.

Our study demonstrated in both univariate and multivariate analyses that the increased expression of $p27^{kip-1}$ was associated with improved survival of patients with stage III CRCs but not with stages I, II, or IV tumors. These results are in contrast to some of the previously published studies where it was shown that decreased $p27^{kip-1}$ expression was associated with poor prognosis of patients with stage II tumors (9, 13) tumors without nodal involvement (stage I plus II; Ref. 39). However, similar to our findings, studies by Tenjo *et al.* (11) observed that $p27^{kip-1}$ is an independent prognostic marker for patients with stage III CRCs. Although, the exact reasons for these contradictory results are not known, the admixture of our patient population for race and for the tumor site may be affecting these findings. Also, it is possible that in our study, we used monoclonal antibody clone 1B4 and established rigorous standards of evaluation as well as cutoff values ($\geq$50% cells with immunostaining score value $\geq$ 0.7), and only nuclear $p27^{kip-1}$ expression was considered in staining evaluation. Whereas, studies by Zhang *et al.* (13) used a different antibody clone (SX53G8), different cutoff values for categorizing tumors as high expressors of $p27^{kip-1}$ ($>$10%), considered only cytoplasmic $p27^{kip-1}$ staining for survival analyses and suggested that decreased expression of $p27^{kip-1}$ was associated with poor survival of patients with Dukes B tumors in univariate analysis. Unlike studies of Zhang *et al.* (13) and Kobayashi *et al.* (42), ours as well as several other studies (Table 1) have used nuclear $p27^{kip-1}$ staining for survival analyses. The higher cutoff values used in our study to categorize CRCs into high and low expressors are similar to other investigators; additionally, we used more rigorous standards of staining evaluation that included two independent observers to reduce individual bias. Besides these technical variations, other confounders of patient survival, including the adjuvant treatment therapies received and racial admixture specifically in studies of the United States, were not clearly described in the majority of earlier studies. Moreover, these contradictory findings also suggest that the future larger studies should consider the tumor stage and the anatomical location of tumor in the colorectum, race, and ethnicity in evaluating the prognostic value of $p27^{kip-1}$ expression in colorectal neoplasia. This has led us to propose that molecular and demographic features should be added to the staging of colorectal tumors (7, 8).

It is well established in the literature that patients with higher tumor stage (stages III and IV) have a poorer prognosis than stage I or II and staging is used in clinical practice for therapeutic decision making. To add to this concept, multivariate survival analyses of our study also noted significant differences in patient outcome based on age ($\geq$65 years), tumor differentiation (poor differentiation), and ethnicity (African Americans) even after controlling for pathological stage of the tumor (Table 4).

In this study, we have shown both in univariate as well as in multivariate analyses that decreased expression of $p27^{kip-1}$ is a significant predictor of poor clinical outcome of stage III patients who received surgery alone as a treatment regimen. Our findings also suggest that in stage III tumors, $p27^{kip-1}$ expression will determine local recurrence. Also, these higher stage tumors exhibit underlying molecular heterogeneity that might determine the behavior of selected tumors. In our studies, the prognostic value of $p27^{kip-1}$ expression was not associated with race or the anatomical location of the tumor. As demonstrated in our prior studies (2–8), the use of multiple molecular markers, including $p27^{kip-1}$ expression, may help in identifying more aggressive tumors and in predicting the clinical outcome, thus, might help clinical oncologists in designing or selecting more aggressive therapies for subgroups of patients with colorectal cancer.

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