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ORIGINAL ARTICLE

Expression of Cell Cycle Regulators P21 and P27 as Predictors of Disease Outcome in Colorectal Carcinoma

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Abstract
Background Recent studies suggest that aberrations in cell cycle checkpoint controllers are a common feature in human malignancies and predict prognosis independent of stage.
Objectives This study correlated two cell cycle regulators (p27 and p21) with clinical and pathological variables in colorectal cancer (CRC) patients to assess their role as prognostic factors.
Patients and Methods A series of 65 CRC patients were analyzed for p27 and p21 expression in their tumors using immunohistochemistry.
Results Forty-six percent of tumors showed positive nuclear p27 expression, whereas 72% of cases were completely p21 negative. There were no significant correlations between p27 and p21 expression and gender, age, lymph node involvement, stage, and grade. However, p27 (but not p21) expression revealed highly significant correlation with tumor location ($p<0.01$), depth of invasion ($p<0.03$), and lympho-vascular invasion ($p<0.02$). Tumors with high p27 expression showed a higher recurrence rate than tumors with no expression ($p<0.03$). In Kaplan–Meier survival analysis, there was a significant ($p=0.046$) difference in disease-free survival (DFS) between p27-positive and p27-negative tumors in favor of the latter. p21 did not show any predictive value of DFS ($p<0.7$). Neither p27 nor p21 did predict disease-specific survival (DSS) in Kaplan–Meier analysis, but DSS time was much shorter for p27-positive tumors. In multivariate (Cox) model, p27 lost its value as independent predictor of DFS, and none of the covariates were independent predictors of DSS.
Conclusion p27 expression seems to be more powerful than p21 expression in providing useful prognostic information in CRC, particularly in predicting the patients at high risk for recurrent disease. Larger cohort and longer follow-up are needed to fully elucidate the value of p27 (and p21) as independent predictors of disease outcome.

Keywords CRC · p27 · p21 · IHC · Disease outcome · Adjuvant therapy

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Introduction

Colorectal carcinoma (CRC) is among the most common neoplasms affecting the industrialized nations. It is the third most frequently diagnosed cancer and the third most common cause of death in the USA among both men and women [1]. In Saudi Arabia, CRC is considered the first most common cancer among men representing 5.3% of all

tumors, and the third most common cancer among women representing 4% of all tumors [2]. The prognosis of CRC is affected by a number of clinicopathological factors among which the stage of the tumor at the time of diagnosis is the most powerful indicator of tumor aggressiveness, although not a perfect predictor of clinical outcome [3, 4]. Recent studies implicate that molecular markers such as DNA content (diploid or aneuploid) of tumor cells, markers of angiogenesis, invasion and metastasis, apoptotic pathways, tumor-suppressor genes and oncogenes, as well as proliferative activity markers, are associated with the aggressiveness of CRC and may predict prognosis independent of the stage [5–8].

Aberrations in proteins controlling cell cycle checkpoints are a common feature of many human malignancies including CRC [9–11]. The p27 (cyclin-dependent kinase inhibitor 1B, CDKN1B or $p27^{KIP-1}$) is one of the cyclin–cyclin-dependent kinase (CDK) inhibitors and plays a key role in preventing progression from G1 into S-phase of the cell cycle by binding and inhibiting cyclin/CDKs [12, 13]. p27 expression is peaking in G0 and G1 cells, and its levels decrease when the cells enter the cell cycle. Recent studies recognized p27 as an important determinant of the biological behavior of invasive tumors [14–16]. Thus, decreased expression of $p27^{kip-1}$ has been correlated with advanced tumor stage and short survival in several human cancers, including breast [17], prostate [18], and lung [19].

The WAF1/CIP1 gene product, p21, is an inhibitor of cyclin-dependent kinases that blocks cell cycle progression and seems to be related to cell proliferation and differentiation [20, 21]. Previous studies found a strong association between reduced/absent p21 and progression of CRC, Hodgkin lymphoma, and breast cancer [22, 23]. The present study was undertaken to assess the expression levels of p21 and p27 in a series of CRC patents from Saudi Arabia, with particular reference to their associations with various clinicopathological features as well as disease-free survival (DFS) and disease-specific survival (DSS).

Patients and Methods

The cohort of the present series consists of CRC specimens from 65 patients, retrospectively collected from the archives of Anatomical Pathology Laboratory in King Abdul Aziz University, Jeddah, covering the period from January 2005 to December 2009. Serial sections were cut from paraffin blocks, stained with hematoxylin and eosin for routine histological examination, classification, grading, and staging following the AJCC staging system [24]. The pertinent clinicopathological features (gender, age, stage, grade, and lymph node status), and the follow-up data were retrieved from the patients' records after obtaining all the relevant ethical approvals (Tables 1 and 2). The mean age in the present cohort was 58 years, with a median of 59 years (range, 24–90 years).

Immunohistochemical Procedures

Four-micrometer tissue sections were cut from the paraffin blocks (containing both tumor and benign tissues), mounted on charged poly-L-lysine-coated slides, and subjected to immunohistochemical (IHC) procedure using Avidin Biotin detection system, following manufacturer kits' manual. The antibodies used were monoclonal mouse Anti-Human $p27^{kip1}$ (clone SX53G8, isotype IgG1; Dako Cytomation Norden A/S, Glostrup, Denmark, dilution 1:50), and monoclonal mouse Anti-Human $p21^{WAF1\text{-}Cip1}$ (clone SX118, isotype IgG1; Dako Cytomation Norden A/S, Glostrup, Denmark, dilution 1:10) IHC procedure was carried out by an automatic immunostainer (Ventana Bench Mark XT, Ventana Inc., Tucson, AZ, USA). In each analysis, positive controls consisted of CRC samples previously shown to stain with the antibodies used. Staining in lymphocytes and uninvolved colonic epithelium within the tissue sections served as internal positive controls for the expression of p27 and p21. Tris-buffered saline in place of the primary antibody was used as a negative control.

Interpretation of Immunohistochemical Staining

Cells were considered positive for p27 and p21 only when distinct nuclear staining was identified. The percentage of immunoreactive nuclei in carcinomas was semi-quantitatively evaluated by counting at least 500 cells in the most representative areas. Positivity of p27 nuclear staining was unequivocally categorized into three groups: score +1, p27 positive in 10–20% of tumor cells stained; score +2, p27 positive in 20–30% of tumor cells stained; score +3, p27 positive in >30% of tumor cells stained, and p27 negative (none or ≤10% of tumor cells stained). Positivity to p21 was scored following this same scoring system.

Statistical Analysis

Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, IL, USA) and STATA (StataCorp., Texas, USA) software packages (PASW Statistics for Windows, version 18.0.2 and STATA/SE 11.1). Frequency tables were analyzed using the chi-square test, with likelihood ratio or Fischer's exact test being used to assess the significance of the correlation between the categorical

Table 1 p27 expression as related to clinicopathological variables

Parameter	Number of cases (%)	p27 staining		*p* Value
		Negative	Positive	
Gender				
Male	32 (49)	16	16	0.54
Female	33 (51)	14	19	
Age group (years)				
<60	32 (51)	18	14	0.70
>60	33 (49)	16	17	
Tumor type				
Adenocarcinoma	59 (91)	32	37	0.15
Mucoid	6 (9)	3	3	
LN involvement				
Yes	40 (61)	22	18	0.66
No	18 (28)	11	7	
Unknown	7 (11)			
Tumor stage				
I/II	16 (25)	11	5	0.50
III/IV	40 (61)	21	19	
Unknown	9 (14)			
Tumor grade				
Well	14 (22)	7	7	0.66
Moderate	36 (55)	21	15	
Poor	4 (6)	3	1	
Unknown	11 (17)			
Tumor location				
Right colon	27 (42)	16	11	0.01
Left colon	17 (26)	13	4	
Rectum	21 (32)	6	15	
Lymphovascular invasion				
Yes	6 (9)	3	3	0.02
No	17 (26)	16	1	
Unknown	42 (65)			
Free margins				
Yes	25 (38)	20	5	0.39
No	3 (5)	3	0	
Unknown	37 (57)			
Recurrence during follow-up				
Yes	26 (40)	13	13	0.03
No	20 (31)	13	7	
Unknown	19 (29)			
Response to treatment				
Objective response	17 (26)	12	5	0.56
No response	33 (51)	17	16	
Unknown	15 (23)			
Status at end point				
A live	48 (74)	21	27	0.743
Died of disease	8 (12)	4	4	
Missing data	9 Cases			

variables. Odds ratios and their 95% confidence intervals (95%CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann–Whitney or Kruskal–Wallis) for two and multiple independent samples, respectively. Analysis of variance was only used

Table 2 p21 expression as related to clinicopathological variables

Parameter	Number of cases (%)	p21 staining		*p* Value
		Negative	Positive	
Gender				
Male	32 (49)	25	7	0.30
Female	33 (51)	22	11	
Age group (years)				
<60	32 (51)	23	9	0.94
>60	33 (49)	24	9	
Tumor type				
Adenocarcinoma	59 (91)	42	17	0.26
Mucoid	6 (9)	5	1	
LN involvement				
Yes	40 (61)	29	11	0.67
No	18 (28)	14	4	
Unknown	7 (11)			
Tumor stage				
I/II	16 (25)	12	4	0.98
III/IV	40 (61)	29	11	
Unknown	9 (14)			
Tumor grade				
Well	14 (22)	9	5	0.22
Moderate	36 (55)	26	10	
Poor	4 (6)	4	0	
Unknown	11 (17)			
Tumor location				
Right colon	27 (42)	20	7	0.10
Left colon	17 (26)	15	2	
Rectum	21 (32)	12	9	
Lympho-vascular invasion				
Yes	6 (9)	5	1	0.95
No	17 (26)	14	3	
Unknown	42 (65)			
Free margins				
Yes	25 (38)	20	5	0.39
No	3 (5)	3	0	
Unknown	37 (57)			
Recurrence during follow up				
Yes	26 (40)	20	6	0.07
No	20 (31)	14	6	
Unknown	19 (29)			
Response to treatment				
Objective response	17 (26)	12	5	0.92
No response	33 (51)	25	9	
Unknown	15 (23)			
Status at end point				
A live	48 (74)	36	12	0.473
Died of disease	8 (12)	5	3	
Missing data	9 (14)			

for deriving the mean values (and their 95%CI) of each individual stratum. Univariate survival analysis for the outcome measure (DSS, DFS) was based on Kaplan–Meier method, with log-rank (Mantel–Cox) comparison test. To assess the value of p27 as an independent predictor, multivariate survival analysis was performed, using the

Cox proportional hazards regression model controlling for the confounding by the following variables: age, sex, tumor localization, T, grade, (for DFS), and recurrence as additional variable (for DSS). In all tests, the values $p<0.05$ were regarded statistically significant.

Results

General Description of p27 Expression

In normal colonic mucosa, p27 immunoreactivity was detected in the nuclei of the lower third of the glands and crypts, while it was absent from nuclei in the middle and superficial parts of the crypts. Of the 65 cases analyzed, 30 (46%) showed positive strong nuclear positivity, while 35 tumors (54%) tested p27 negative. p27 immunoreactivity was usually heterogeneous being stronger in areas of more pronounced glandular differentiation (Fig. 1). Correlation of p27expression to clinicopathological variables is shown in Table 1.

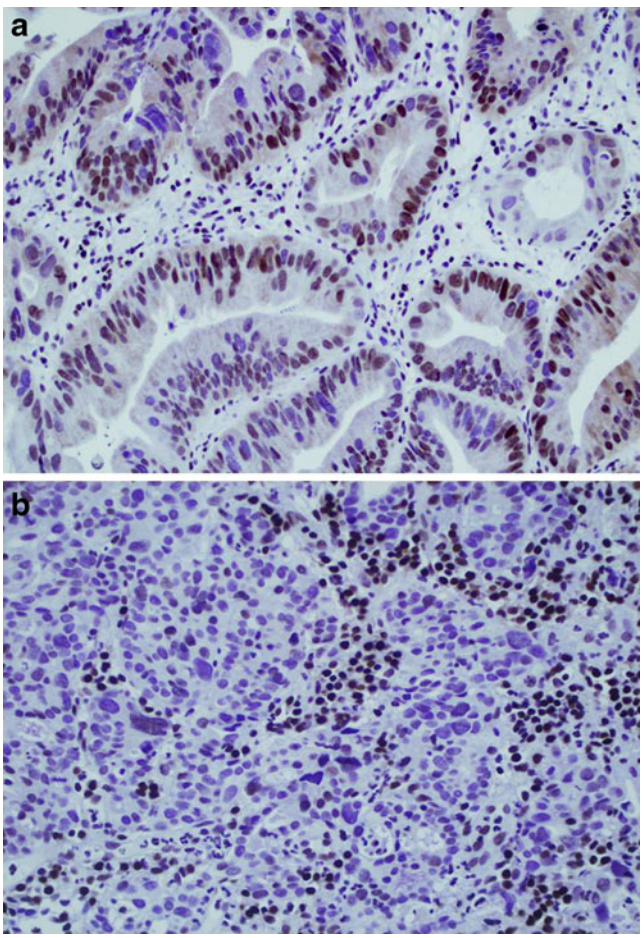

Fig. 1 **a** A case of well differentiated colon carcinoma revealing positive nuclear staining for p27. **b** A case of poorly differentiated colon carcinoma revealing negative staining for p27. Positive nuclear staining is seen in the inflammatory cells

General Description of p21 Expression

In normal colonic mucosa, p21 immunoreactivity was confined to the nuclei in the upper third of the glands and to the surface epithelium, while it was absent from nuclei in the middle and lower parts of the crypts. p21 immunoreactivity was usually heterogeneous; higher percentage of p21-reactive cells were seen in the superficial areas of the tumors or in areas of more pronounced glandular differentiation. Forty-seven cases (72%) showed complete loss of p21 staining, whereas 18 cases showed positive nuclear p21 immunoreactivity (28%; Fig. 2). Correlation between p21 positivity and negativity to clinicopathological variables are shown in Table 2.

Correlation of p27 and p21 Expression with the Clinicopathological Features

Gender, age, grade, or tumor stage had no significant relationship with the expression of p27 (Table 1). However,

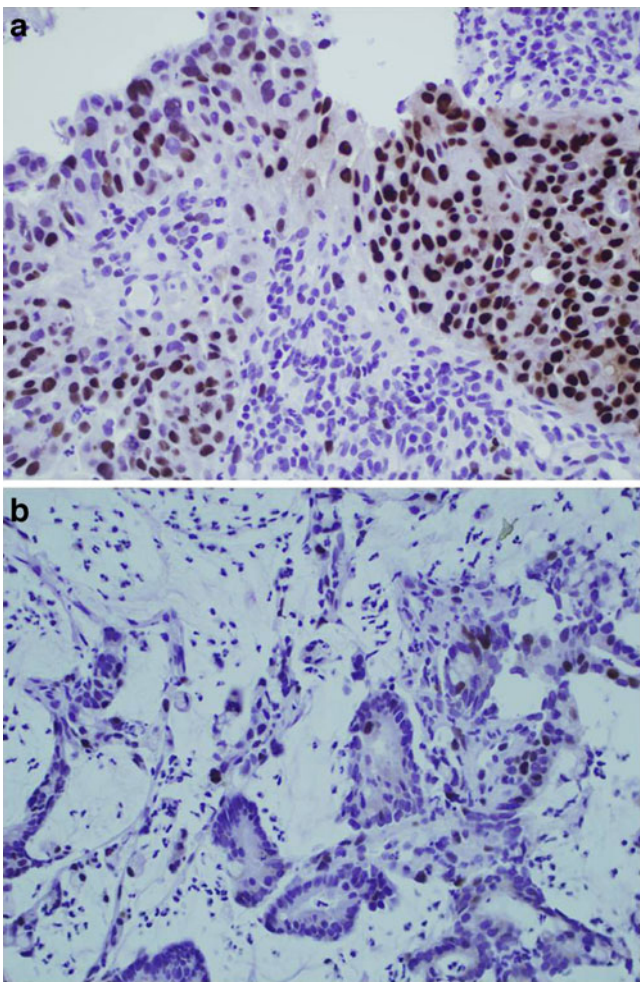

Fig. 2 **a** A case of poorly differentiated colon carcinoma revealing positive nuclear staining for p21. **b** A case of mucinous colon carcinoma revealing negative staining for p21. Only very few cells are immunoreactive (<10%)

the deepness of tumor invasion and lymphovascular invasion (LVI) were significantly associated with p27 expression, in that loss of p27 expression is more common in deeply invaded tumors than in less invaded tumors ($p<0.03$, data not shown), and while less LVI was seen in tumors with negative expression of p27 ($p<0.02$). Of interest is the association between the site of tumor and expression of p27. There was a clear difference between tumors situated in colon or in rectum. Tumors located in colon were mainly less expressing p27 than tumors located in rectum ($p<0.01$).

Similarly, p27 expression showed a clear association with disease recurrence during the follow-up, in that the patients who developed early tumor recurrence (16 months) had positive p27 expression, as contrasted to patients with no expression of p27 who developed recurrence much later (30 months; $p=0.03$). The same trend was observed in patients who died of disease, patients with p27 negative tumors living significantly longer ($p=0.025$). In contrast, p21 expression did not show any correlation with the clinicopathological features in this cohort. Only in univariate analysis, a borderline significant correlation was observed between the p21 expression and disease recurrence; patients who developed early tumor recurrence (15 months) had positive p21 expression, as contrasted to patients with no expression of p21 who developed recurrence markedly later (26 months; $p=0.07$).

In Kaplan–Meier survival analysis, there was a significant ($p=0.046$) difference in DFS between patients with p27-negative tumors (longer DFS) and those with p27-positive tumors (Fig. 3). For p21, this difference was not statistically significant ($p=0.6$; Fig. 4). As to DSS, p21 and p27 did not show any predictive power in Kaplan–Meier analysis.

To assess the value of p27 as an independent predictor, a multivariate survival analysis was done, using the Cox proportional hazards regression model controlling for confounding by the following covariates: gender, age, tumor localization, T, grade, (for DFS), and recurrence as additional variable (for DSS). Because not significant in univariate analysis, this multivariate model was not tested for p21. In the Cox model for DFS, the two independent predictors were: Tumor invasion (T), with HR=6.8, 95% CI 2.14–21.75 ($p=0.001$) as well as tumor grade (G) with HR=5.54, 95% CI 1.91–16.12 ($p=0.002$). In this model, p27 lost its value as an independent predictor. In a similar model for DSS (including disease recurrence as an additional covariate), none of the covariates proved to be an independent predictor of DSS.

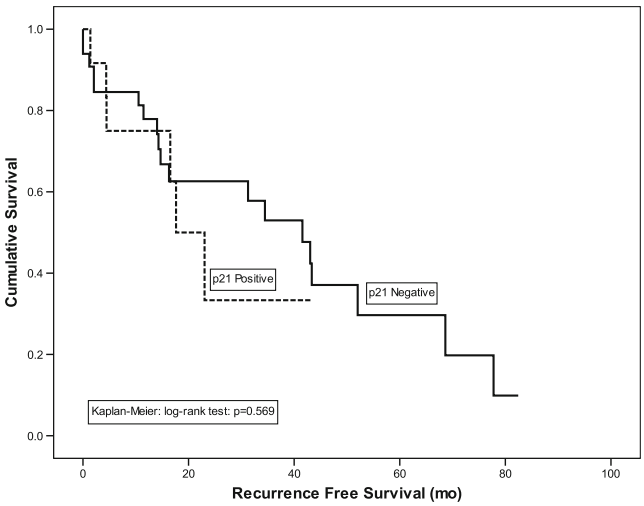

Fig. 3 The impact of p21 expression on disease-free survival (DFS) in univariate (Kaplan–Meier) survival analysis

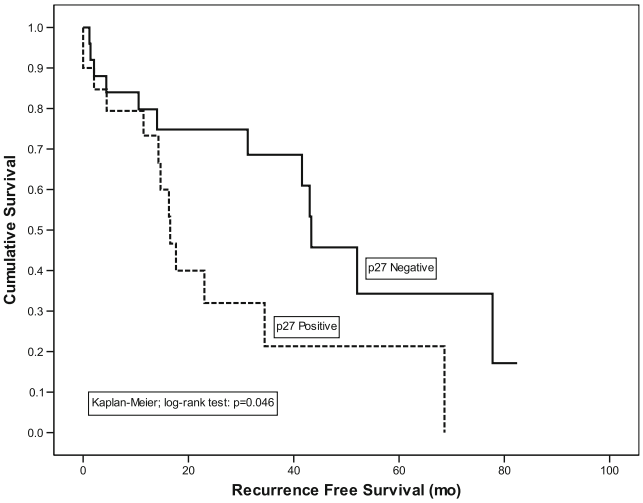

Fig. 4 The impact of p27 expression on disease-free survival (DFS) in univariate (Kaplan–Meier) survival analysis

Discussion

This study is a continuation of our efforts to further elucidate the biology of CRC and to identify more effective prognostic factors than the traditional staging system to aid therapeutic decision making. The aim of the present study was to cast further light on the issues related to prognostication of CRC, while assessing the value of quantitative cell cycle regulators; p27 and p21 expression profiles as independent prognostic factors. In this study, we focused on stage II–IV disease, where molecular and other markers may help pinpointing a subgroup of patients, who would eventually benefit from the use of adjuvant therapy for their disease. This important decision involves a careful weighing of the risks of toxicity and complications against the potential curability of the disease [25]. It is well established that early CRCs can be cured with radical surgical resection alone [26]. Unfortunately, however, some 30% of all patients who undergo curative resection subsequently

present with relapse and eventually die of their disease [27]. Prediction of disease outcome in individual patients after curative resection is still far from reliable [28]. However, there is some hope and our results already suggest that p27 expression studied by IHC could be used to help in this decision. In addition, more rational decisions can be done as soon as we learn more of markers and diagnostic tools in accurate prediction of the disease outcome in individual patients [29, 30].

On the basis of the present results, we do believe that this detection can be improved using the assessment of p27 expression in the primary tumors. In this assessment, quantifying the p27 expression seems to be very important. In this study, we compared different methods of grading this expression and found the dichotomized negative/positive grading to provide the most consistent and meaningful correlations to the clinicopathological variables. Thus, we suggest that this grading system classifying CRCs as p27 positive/negative is the clinically most relevant approach.

In the present cohort, several interesting and important observations were made, all implicating that the quantitatively measurable p27 expression in tumor cells could provide significant prognostic information in CRC. First, a significant correlation was shown between p27 expression and tumor invasion; deep invasion is associated with negative expression of p27 as compared with tumors showing more superficial invasion.

This observation is in agreement with the report of Palmqvist et al. [31] who notices that p27 downregulation correlated significantly with the depth of tumor invasion, but not with age, gender, or tumor type. Interestingly, in contrast to deep invasion of tumors, LVI was more pronounced in tumors with positive p27 expression. Altogether, these observations implicate p27 as a biological factor that might affect the behavior of tumor cell populations. Some studies have shown that in cancers where both invasive and in situ tumors coexist, loss of p27 is observed in both components [32].

Interestingly, we observed a close association between p27 expression and tumor localization; negative p27 expression was significantly more frequent in tumors of the colon than in those of the rectum. This is in alignment with Palmqvist et al. [31] who described that low p27 expression was more commonly in right colon cancers than in other locations of colon or rectum. This finding was also confirmed by Zhang et al. [33]. This suggests that there may be differences between normal right and left colonic segments that could favor malignant transformation through different molecular pathways. Such differences are probably related to different molecular profiles of the tumors, microsatellite instability and methylator phenotypes being associated with right-sided tumors, and chromosomal instability with the left-sided tumors [34]. We suggest that the higher levels of p27 expression associated with distal tumors may be due to these divergent genetic pathways present in the left- and right-sided tumors. However, this remains only speculative at this stage and future molecular studies are necessary to confirm this hypothesis [35–37].

Obviously, one of the most important observations of the present study is the one linking p27 expression with the disease outcome, i.e., appearance of recurrence and the length of DFS. This is clinically relevant for several reasons. Because a number of CRC patients of different stages are at high risk of recurrence, it would be of paramount importance to develop reliable markers that would accurately predict those patients to become considered for adjuvant therapy.

In the present series, 40% of the patients eventually developed a recurrence within the median follow-up period of 14.7 months (mean 21.9; range, 1–78). This is a substantially high rate particularly for a group of Lymph node - negative (stage II) CRC patients. Importantly, our data showed that the patients who developed an early tumor recurrence (mean DFS 16 months) had positive p27 expression as contrasted to patients with negative expression of p27 who developed recurrence significantly later (mean DFS 30 months).

As shown in Fig. 3, at 40 months of follow-up, only 25% of the patients with negative p27 expression had recurrence, as compared to 80% of those with tumors expressing p27, respectively. This is in contrast to the study by Loda et al. [38] who showed that p27 expression was significantly associated with survival by actuarial analysis, with a median survival of 241 months in patients whose tumors displayed more cells with positive p27 expression, 149 months in tumors expressing less p27, and 69 months in patients with tumor cells not expressing p27. Similarly, Palmqvist et al. [31] also reported that tumors expressing less than 50% of p27 staining conferred impaired prognosis as compared to highly p27 expressing tumors.

In most of the previous studies that evaluated the expression of p27 expression human cancer, there was a correlation between loss of p27 and poorer prognosis and shorter survival time; however, many other studies showed no correlation or even association with better prognosis and longer survival. So the significant of p27 expression is still controversial. Chen et al. demonstrated that overexpression of p27 may expect good prognosis for patients with hepatocellular carcinoma [39]. No association between survival and prognosis and p27 expression was observed in colorectal cancer [40, 41], gallbladder cancer [42], and gastric cancer [43, 44]. Fan et al. [45] showed that low p27 expression was significantly associated with low tumor grade in colorectal carcinoma. Moore et al. [46] demonstrated that positive p27 expression in rectal cancer after preoperative chemoradiation is an independent negative predictor of recurrence free survival. Our results are contradicting some of the previous

studies that correlate loss of p27 with poorer prognosis and shorter survival. So, some of these apparently discrepant observations might be explained by differences in the technical aspects of recording p27 expression or by differences in interpretation of the expression. The ethnic factor also cannot be omitted from this contradictory. Therefore, we would need a larger cohort and a substantially longer follow-up time to shed light on this dilemma.

As to DSS, p27 expression was positive equally often in patients, who eventually died of their disease as in those who were alive at the completion of the follow-up period (p= 0.743). However, the difference in the length of DSS time between p27-positive and -negative tumors was significant (p=0.025), being shorter among the former. These observations suggest that CRC tumors with positive p27 expression are at high risk for local or distant recurrence and because of the strong adverse impact of the latter on survival (i.e., none of the patients without recurrence died of disease in our cohort); these patients are also more likely to eventually die of their disease. These patients should be appropriate candidates for close and frequent post-operative follow-up for prescribing the appropriate adjuvant therapy.

Taken together, p27 (and even less p21) expression did not correlate with the gender, age, or tumor type, as also confirmed by other studies [31]. However, p27 expression showed significant associations with tumor invasion (T) and LVI as well. Furthermore, positive p27 staining in the tumors was related to a higher recurrence rate as compared to p27-negative tumors, suggesting a link towards development of a metastatic phenotype. Finally, positive p27 expression in our cohort was associated with shorter DSS times, as compared with p27-negative CRCs, possibly implicating some differences in the inherent malignancy of CRC that become manifest after reasonable follow-up period. To fully elucidate this, however, we would need a larger cohort and a substantially longer follow-up time.

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