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p27 Cell-Cycle Inhibitor Is Inversely Correlated With Lymph Node Metastases in Right-Sided Colon Cancer

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p27, a cyclin-dependent kinase inhibitor, suppresses proliferation of normal and neoplastic cells. Expression of p27 is correlated with survival in colon cancer. To some degree, right-sided colon cancers differ biologically and clinically from left-sided colon cancers. We analyzed 41 patients with right-sided colon cancers, including 18 cases with regional lymph node metastases and 23 cases with negative lymph nodes. Immunostaining for p27 was performed on histologic sections of primary cancers and scored. Correlation of p27 protein expression with histologic parameters was performed by *t*-test and multivariate analysis. Decreased p27 protein expression was associated with large tumor size. As percentages of positively stained tumor cells decreased from 70 to 29%, the mean tumor size increased from 1.9 to 7.3 cm. p27 protein expression significantly decreased in primary cancers with angiolymphatic invasion or with positive lymph nodes in comparison with those without angiolymphatic invasion (26 ± 6 vs. $44 \pm 5\%$, $P < 0.03$) or with negative lymph nodes (23 ± 4 vs. $47 \pm 6\%$, $P < 0.003$). p27 expression was not statistically different in terms of depth of tumor invasion (T1/T2 vs. T3/T4), tumor type or tumor differentiation. Multivariate analysis revealed that low p27 expression in primary cancers was correlated with lymph node metastases ($P = 0.01$). However, it did not correlate with any other histologic parameters. In summary, decreased p27 expression was associated with an increased likelihood of lymph node metastases in colon cancers, independent of depth of tumor invasion. This implies that p27 is a potentially important predictor for tumor metastasis and patient's prognosis in right-sided colon cancers. J. Clin. Lab. Anal. 13:291–295, 1999.

Key words: colon cancer; p27; immunostaining; lymph node metastasis

INTRODUCTION

Colorectal cancer is the fourth most prevalent carcinoma and the second most frequent cause of death from cancer in the United States, with an estimated 129,400 new cases and 56,600 deaths in 1999 (1). Histologic features of colorectal cancer that have been found to be of prognostic significance include depth of tumor invasion, tumor grade, extramural large vessel invasion and peritumoral lymphocytic response (2–5). Lymph node involvement and the number of positive nodes have also been found to be independent prognostic indicators (6,7). Studies have showed that 5-year survival rates decrease from approximately 70 to 50% if one lymph node is involved with metastatic carcinoma and rates further decrease to 30% if multiple lymph nodes are involved with metastatic carcinoma (6–9). Because of this, lymph node status is an important determinant of adjuvant therapy (10,11).

p27, a cyclin-dependent kinase inhibitor, inhibits the G1 to S transition of the cell cycle (12,13). p27 protein binds to cyclin D-Cdk4, cyclin E-Cdk2 and cyclin A-Cdk2 complex inhibiting their activities during the cell cycle (14–16). The protein appears to play a role in both cell proliferation and differentiation (16–18). Recently, altered expression of p27 has been described in a variety of malignancies including esophageal (19,20), gastric (21), prostatic (22–24), breast (25–27), lung (28) and thyroid cancers (29). An inverse correlation of p27 expression to a patient's outcome was found in the patients with colorectal cancer (26,30). Carcinoma of the right colon occurs in approximately 30% of patients with colorectal cancer (31). Right-sided cancers have more fre-

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quent polypoid/exophytic growth, larger size, poor differentiation, extracellular mucin production, and peritumoral Crohn's-like lymphoid reaction (31–33). Biologically, cancers from the right colon are more likely to be associated with microsatellite instability and mutations in transforming growth factor-beta type II receptor (34–37). This study was designed to determine p27 protein expression in primary cancers in relation to histologic parameters including tumor type, differentiation, angiolymphatic invasion, depth of tumor invasion, and regional lymph node status. In order to eliminate site-specific variables of p27 protein expression, we selectively investigated right-sided colon cancers.

MATERIALS AND METHODS

Patient and Study Design

A total of 41 patients with right-sided colon adenocarcinoma were randomly selected by retrospective review of the pathology archives at University Hospitals of Cleveland. Right-sided colon cancer is defined as carcinoma occurring in the colon proximal to the splenic flexure (38). These patients included 18 males and 23 females with a mean age of 72 years (ranging from 41 to 92 years). All patients had undergone colonic resection as an initial curative treatment in the previous nine years. Resected specimens were grossed by a standard method for pathological diagnosis. The specimens were fixed in formalin and processed in a routine histologic protocol. Representative sections of specimens were submitted for paraffin embedding and H&E staining. Histologic slides were reviewed by two pathologists and diagnoses were classified using WHO and TNM nomenclature (39). Pathologic parameters including histologic type, differentiation (grading), depth of tumor invasion, angiolymphatic invasion, lymph node involvement, and tumor size were evaluated. After review of all histologic sections, a representative block of each primary tumor with well-preserved histologic features diagnostic for adenocarcinoma, appropriate orientations, and normal colonic mucosa adjacent to the cancers was selected for p27 immunostaining. Histologic sections were cut at 4-μm thickness.

Immunohistologic Staining

Immunostaining for p27 was carried out using a horse-radish peroxidase (HRP) labeled streptavidin biotin (LSAB 2, Dako) system on the Dako Autoimmunostainer (Dako Corporation, Carpenteria, CA). Briefly, sections were treated with 3% H_2O_2/H_2O to eliminate endogenous peroxidase activity, then H_2O rinsed. Following standard microwave epitope enhancement techniques (26,40,41), sections were incubated with a commercially available monoclonal antibody to p27 (Transduction Laboratories, Lexington, KY) at a 1:500 dilution. Following rinse steps with Tris buffered saline, pH 7.60, detection was performed by incubating first with biotinylated anti-mouse immunoglobulins for twenty minutes and then HRP-labeled streptavidin for twenty minutes. Visualization was achieved with application of 3′-3-diaminobenzidine (DAB) for 5 minutes. Slides were counterstained with Harris' hematoxylin, dehydrated to xylene and coverslipped. Optimal dilution of the primary antibody was determined by serial dilution on a number of primary breast carcinomas, a source well established in the literature (25–27). The optimal dilution was determined to be the titer yielding the strongest signal with the least amount of background. In addition, positively stained lymphocytes and negatively stained endothelial and smooth muscle cells within the patient samples also served as internal control elements. Negative antiserum controls were performed on each patient specimen by substitution of the primary antibody with non-immune mouse immunoglobulin in relatively the same concentration.

The specificity of the antibody was validated by Western blot analysis of protein lysates of colon cancer cell lines: FET and Vaco-400. Western blotting demonstrated only one band at 27 kilodaltons size.

Analysis of Immunohistologic Staining

Immunostaining for p27 was independently assessed by two pathologists without knowledge of the histologic and clinical parameters. Discrepancies were resolved with a combined review. Immunoreactivity to p27 occurring to cell nuclei was considered positive. All negative stained tumors were classified as such if lymphocytes in the lamina propria or surrounding the tumors stained positive for p27 protein. Cytoplasmic staining alone was considered negative. Each section contains 5–10 low-power fields (each field containing at least 2,000 tumor cells). Tumor cells positively stained for p27 were estimated using 10× or 20× fields and expressed as ratios of microscopic positive fields to negative fields in continuous variables (40). No cell counts were attempted. Inter-observer variability was low after appropriate training.

Statistics

Comparison of staining scores for p27 was performed using a *t*-test with differences of $P < 0.05$ considered to be statistically significant. p27 expression was correlated with histologic parameters using multivariate logistic regression analysis.

RESULTS

Immunoreactivity to p27 was defined as immunostaining of tumor cell nuclei. All cases showed a positive internal control in which lymphocytes in the lamina propria or reactive peritumoral lymphocytes stained positive with p27. No consistent pattern of staining in terms of geographic location—central vs. peripheral and superficial vs. deep—was noted. The age of the blocks did not affect p27 staining patterns.

The mean of tumor size was 6.3 ± 0.5 cm with a range

from 1 cm to 15 cm. Percentages of tumor cells stained positive were 37 ± 4% (Mean ± Standard Error) ranging from 5 to 90%. An inverse correlation by univariate linear regression was noted between tumor size and percentages of p27 positive tumor cells (Table 1). As positive tumor cells decreased from 70 to 29%, the mean tumor size increased from 1.9 cm to 7.3 cm (r = –0.3, $P = 0.06$). The ratios of percentages of positive cells to tumor size showed a rapid decrease from 37 to 3 as tumor size increased from 1.9 cm to 10.7 cm (r = –0.6, $P = 0.01$).

p27 protein expression showed no statistically significant differences in terms of tumor type (adenocarcinomas vs. mucinous adenocarcinomas), tumor differentiation (moderate vs. poor differentiated adenocarcinomas), or depth of tumor invasion (T1/T2 vs. T3/T4) (Table 2).

Angiolymphatic invasion was identified in 17 cases. There was an almost twofold decrease in p27 protein expression in the adenocarcinomas with angiolymphatic invasion in comparison with those adenocarcinomas with no angiolymphatic invasion (26 ± 6 vs. 44 ± 6%, $P < 0.03$, Table 2). Mean number of lymph nodes dissected was 15.8 per case. Of 41 adenocarcinomas, 17 cases had lymph node metastases with mean numbers of 4 (ranging from 1 to 18) positive lymph nodes. p27 protein expression in the primary carcinomas with positive lymph nodes was significantly lower than that in those with negative lymph nodes (23 ± 4 vs. 47 ± 6%, $P < 0.003$, Table 2). Multivariate logistic regression analysis showed that lymph node metastases were correlated with lower p27 expression ($P = 0.01$). No correlation with other histologic parameters was found by multivariate analysis.

TABLE 2. p27 Protein expression and histopathologic parameters[a]

Histolopathologic parameters	Number of cases	Percentage of positive tumor cells[b]
Histologic types		
Adeocarcinoma	30	37 ± 5
Mucinous adenocarcinoma	11	36 ± 10
		$P = 0.85$, NS[c]
Differentiation		
Moderate	34	38 ± 5
Poor	7	31 ± 13
		$P = 0.56$, NS[c]
Depth of tumor invasion		
T1 and T2	15	40 ± 8
T3 and T4	26	35 ± 5
		$P = 0.58$, NS[c]
Angiolymphatic invasion		
With angiolymphatic invasion	17	26 ± 6
No angiolyphatic invasion	24	44 ± 5
		$P < 0.03$[d]
Lymph node status		
With lymph node metastasis	17	23 ± 4
No lymph node metastasis	24	47 ± 6
		$P < 0.003$[d]

[a]Statistical analyses were performed by *t*-test. $P < 0.05$ was considered significant.
[b]Mean ± Standard Error.
[c]NS, Not statistically significant.
[d]Statistically significant.

DISCUSSION

Lymph node metastasis and depth of tumor invasion are considered the most important predictors of patient outcome in colorectal cancers (5–7). Five-year survival rates decline from 90% in T1/T2 lesions to 70% in T3/T4 lesions without lymph node metastases, but decrease sharply to 30% for patients with node involvement (5). Clinically and biologically, right-sided colon cancers appear different from left-sided colon and rectal cancers (31,32,34,43–45). We investigated p27 expression in this subgroup of colonic carcinomas in correlation with pathologic prognostic parameters. Interestingly no differences were found in expression of p27 between T1/T2 and T3/T4 lesions when controlling for the presence or absence of lymph node metastases. When lymph node status was considered regardless of the depth of tumor invasion, significant differences existed between the lymph node positive and negative tumors (Table 2). An inverse correlation of p27 expression to lymph node metastases but not the depth of tumor invasion was found by multivariate analysis. The fact that low expression of p27 protein in the primary carcinomas is more likely to develop lymph node metastases, independent of depth of tumor invasion, indicates that lower p27 protein expression in primary tumors may be an important predictor for lymph node metastases.

TABLE 1. Inverse correlation of p27 protein expression to tumor size

Number of cases	p27 Positive tumor cells (%)	Mean tumor size (cm)	Ratios (T of positive tumor cells to tumor size)
3	70	1.9 (< 3)	37
8	40	3.3 (3–4)	12
13	35	5.2 (4.1–6)	7
8	29	7.3 (6.1–8)	4
9	33	10.7 (> 8)	3

An association of high p27 expression with small tumor size was noted (Table 1). These findings are consistent with the known inhibitory effects of p27 during the cell cycle (12–14). As a negative regulator for cell proliferation, p27 forms a functional complex with Cdk2 and cyclin E, resulting in inactivation of the Cdk2 and cell proliferation arrest at G1/S transition during the cell cycle (14–17).

As well as the inhibitory role of p27 in cell proliferation, there is some evidence that p27 plays a role in cell differentiation (16). Croix et al. found that decreased p27 protein levels were associated with reduced expression of E-cadherin on the surfaces of colon, breast, and lung carcinoma cells (46). They found that reduced E-cadherin expression not only

promotes cell proliferation but also facilitates tumor cell invasion (46). In their study it appears that low p27 expression fosters aggressive tumor cell behavior. The present study demonstrated diminished p27 expression in the primary tumors with angiolymphatic invasion but not in those without angiolymphatic invasion (Table 2). The finding may reflect the role of p27 in cell differentiation. However, multivariate analysis did not reveal that angiolymphatic invasion was correlated with decreased p27 expression. Other pathways involving transforming growth factor (35), steroid hormone (47), prostaglandin (25), or interleukin (15) have been found linked to p27 expression in normal or tumor cells. Thus, accumulating evidence indicates that p27 is involved in multiple mechanisms that control biological behaviors of tumor cells.

In the present study, tumor differentiation or type showed no significant association with p27 protein expression (Table 2). It is known that poorly differentiated colonic carcinomas carry a poorer prognosis than moderately differentiated carcinomas. p27 expression was lower in poorly differentiated carcinomas than in moderately differentiated carcinomas (31 vs. 38%); but the levels did not reach those of statistical significance (Table 2). This may be due to a limited number of poorly differentiated carcinomas in our study. However, an inverse correlation of p27 expression to differentiation in breast and colorectal cancers has been reported in other studies (26,27,30). A similar finding was noted in tumor type (adenocarcinomas vs. mucinous adenocarcinomas) in relation to p27 expression.

In summary, this study demonstrated that p27 protein expression in right-sided colon adenocarcinomas is inversely correlated with regional lymph node metastases. Primary colonic carcinomas with large tumor size or angiolymphatic invasion have a predilection of low p27 protein expression. No significant correlation was observed to other histologic parameters including histologic types, tumor differentiation, and depth of tumor invasion. This may relate to the role of p27 in cell proliferation and differentiation involving multiple pathways of tumor progression. These findings might provide additional prognostic information for patients with right-sided colon adenocarcinomas.

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