Short Communication

Down-Regulation of p27 Is Associated with Development of Colorectal Adenocarcinoma Metastases

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The cyclin-dependent kinase inhibitor p27 is a negative regulator of the cell cycle and a potential tumor suppressor gene. Because we had previously demonstrated that loss of p27 protein is associated with aggressive behavior in colorectal adenocarcinomas, we used immunohistochemistry and *in situ* hybridization to evaluate the potential role of alterations in p27 expression in primary and metastatic colorectal adenocarcinomas. Parallel immunostaining was performed for Ki-67 and p53. We evaluated 13 cases of metachronous and 23 cases of synchronous primary and metastatic colorectal tumor pairs. In the synchronous subgroup (Stage IV tumors), 57% of the primary tumor and metastases pairs did not express p27 protein and the remainder were low expressors. In the metachronous subgroup, 54% of the primary tumors were low expressors and the remainder high expressors of p27 protein. There was a significant reduction in the expression of p27 in the metachronous metastases (mean positive cells: 14.5%) when compared to the corresponding primary tumors (mean positive cells: 41.8%), $P = 0.0023$. All the primary and metastatic tumors in the metachronous subgroup showed high levels of p27 mRNA expression. There was no association between loss of p27 and either Ki-67 count or p53 expression. Because p27 is known to be up-regulated when epithelial cells are grown in suspension, the down-regulation of p27 in circulating tumor cells may confer the ability to grow in an environment of altered extracellular matrix or intercellular adhesion properties, two situations which may facilitate metastases. (*Am J Pathol* 1998, 153:681–687)

Progression through the cell cycle involves sequential activation and inactivation of cyclin-dependent kinases (CDK). CDKs are activated through association with positive regulators called cyclins and inactivated by cyclin-CDK inhibitors (CKI).^{1,2} CKIs can be divided into two groups, the ink4 family and the cip/kip family.¹ The ink4 proteins (p15, p16, p18, and p19) specifically inhibit the cyclin D-CDK4/6 complexes. Point mutations and deletions or inactivation by methylation of *p16* and *p15* genes have been reported in various human malignancies and transformed cell lines.^{1,3}

The cip/kip proteins (p21, p27, and p57) share partial structural homology, although p27 seems to target CDK2 preferentially.^{1,3} Overexpression of p27 protein in mammalian cells induces a G₁ block of the cell cycle.^{4,5} Furthermore, the high levels of p27 found in quiescent cells suggests that it also plays a role in maintaining cells in G₀.^{6–8} It is of interest that no homozygous deletions and only rare mutations of the *p27* gene have been found in human tumors.^{9–12} We and other investigators have shown that absent or low p27 protein expression is a powerful negative prognostic marker in breast,^{13–15} colorectal,¹⁶ gastric,¹⁷ prostate,^{18,19} esophageal,²⁰ and pulmonary carcinomas.^{21,22} Furthermore, in both colorectal and non-small-cell lung carcinomas, we demonstrated that low levels of p27 in tumor cells were due to tumor-specific, enhanced, proteasome-mediated protein degradation.^{16,21}

The aim of this study was to characterize the expression of p27 in primary and metastatic (both synchronous and metachronous) colorectal adenocarcinomas and to determine whether alterations in levels of p27 protein expression confer metastatic potential on tumors. Since

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Table 1. Immunohistochemical Analysis of p27 Protein Expression in Primary Tumor-Metachronous Metastases Pairs

Patient no.	Stage	Chemotherapy	Interval to metastases (months)	p27 level (%)	
				in primary tumor	in metastases
1	III	+	35	67	3
2	III	—	36	60	2
3	III	+	60	0	7
4	I	—	35	38	13
5	II	+	36	17	32
6	II	+	18	54	28
7	II	+	21	0	0
8	III	+	17	63	7
9	II	+	19	48	11
10	II	—	6	69	26
11	III	+	21	67	34
12	III	+	20	18	14
13	III	+	27	41	11
Mean $\pm$ SEM				41.7 $\pm$ 7.0	14.5 $\pm$ 3.2 ($p = 0.0023$)

p53 mutation is a late event in colorectal carcinogenesis and may be associated with the development of metastases from colorectal cancers, we also evaluated the expression of its gene product.^{23,24} In addition, we compared p27 expression to that of Ki-67, a marker of proliferation.

Materials and Methods

Patient Population

We performed a retrospective search through the surgical pathology files of the Beth Israel Deaconess Medical Center (West Campus) from 1974 to 1997 to identify patients who had undergone colon resections for Stage I-III adenocarcinomas and subsequently underwent liver ($n = 12$) or pulmonary ($n = 1$) resection for metastatic colon adenocarcinomas.^{25,26} Another group of patients with synchronous metastases (Stage IV) was used as a control. Paraffin-embedded tissue was available from 13 patients with metachronous and 23 patients with synchronous metastases. Metachronous metastases were defined as metastases occurring more than 6 months after resection/treatment of the primary tumor. Synchronous metastases were defined as metastases found at the time of resection of the primary tumor.

Age at diagnosis of the primary tumor, sex, pathological stage (using the American Joint Committee on Cancer Staging protocol),²⁷ histopathological grade, and interval until development of metastases were recorded.

The demographic data for patients in the metachronous and synchronous subgroups were as follows, respectively: mean age, 62.5 and 61.5 years; male: female ratio, 6:7 and 15:8. In the metachronous subgroup there were 7 Stage III, 5 Stage II, and 1 Stage I primary tumors. Mean interval to metastases was 27.7 months (range, 6 to 60 months). Eleven primary tumors were moderately differentiated and 2 were poorly differentiated. One patient (Table 1, patient 4) presented with pulmonary metastasis 35 months after resection of the primary colorectal tumor. Another patient (Table 1, patient 3) initially developed

hepatic metastasis that was resected and subsequently presented with brain metastasis, which was also resected. Ten patients received chemotherapy (5-fluorouracil alone or with levamisole). In the synchronous subgroup all tumors were by definition Stage IV. All patients in this subgroup already had liver metastases at the time of resection of their primary colorectal tumors. In this group, 21 tumors were moderately differentiated and 2 were poorly differentiated. Thirteen patients received chemotherapy (5-fluorouracil alone or with levamisole), 3 declined treatment, and follow-up was unavailable on 7 patients.

Immunohistochemistry

Tissue sections 5 μ m thick were cut from paraffin-embedded blocks, placed on charged glass slides, deparaffinized in xylene, and rehydrated through graded alcohol. Immunohistochemistry was performed as previously described.^{13,16}

Briefly, after antigen retrieval by microwave irradiation (10 mmol/L sodium citrate buffer, pH 6.0, (Biogenex, San Ramos, CA)) in a pressure cooker at 750 W for 30 minutes, mouse monoclonal antibodies against p27 (Transduction Laboratories, Lexington, KY), anti-Ki-67 (Immunotech, Westbrook, ME), and anti-p53 (Calbiochem, Cambridge, MA) were applied on the slides at dilutions of 1:400, 1:100, and 1:500, respectively, in phosphate-buffered saline (PBS). Immunohistochemistry was performed by an automated processor (Ventana ES, Ventana Medical Systems, Tucson, AZ). Steps performed by the instrument included blocking with normal horse serum, application of 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 lightly counterstained with hematoxylin. A mixture containing antibodies with no known human recognition site was used as a negative control. An osteosarcoma cell line MG-63 (obtained from the American Type Culture

Collection, Rockville, MD) was used as a positive control for p27. After 48 hours of serum starvation (necessary to increase the levels of p27), cells from two confluent flasks were harvested, fixed in neutral buffered formalin for 8 hours, and paraffin-embedded. Five-micrometer-thick sections were used in each immunohistochemistry run as a positive control.

In Situ Hybridization

In situ hybridization for p27 mRNA was performed in all primary and corresponding metastatic tumors of the metachronous subgroup. One μ g of recombinant plasmid pCRTMII (Invitrogen, San Diego, CA), containing the full-length human p27 gene was linearized using *Bam*HI and *Xba*I to generate sense and antisense transcripts, respectively. Digoxigenin-labeled riboprobes were made with T7 and SP6 polymerase for 1 hour at 37°C in 1 $\times$ transcription buffer (Promega Corp., Madison, WI), 10 mmol/L dithiothreitol, 40 U of ribonuclease inhibitor, adenosine, cytosine, and guanosine triphosphates (1 mmol/L each) and a mixture of cold uridine triphosphate and digoxigenin-uridine triphosphate (6.5 and 3.5 mmol/L, respectively, for a total concentration of 1 mmol/L) (Boehringer Mannheim, Indianapolis, IN)). Slide sections were digested with proteinase K (20 μ g/ml) in 1 mol/L Tris-EDTA buffer, pH 8.0, for 8 minutes at 37°C and

then washed in PBS. Hybridization was performed at 42°C for 3 hours with the application of 10 pmol/L digoxigenin-labeled riboprobe in 100 μ L of hybridization buffer (50% deionized formamide, 2 $\times$ sodium chloride/sodium citrate (SSC), 50% dextran sulfate, 10% SDS and denatured herring sperm DNA 10 mg/ml) per slide under liquid coverslip (Ventana Medical Systems, Tuscon, AZ). Four washes of SSC at 55°C, the most stringent of which was at 0.1 $\times$ SSC, followed hybridization. Anti-digoxigenin antibody (1:500) was applied for 28 minutes at 37°C followed by detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 12 minutes. The slides were counterstained with methyl green. Sense probes were used as controls. Duration and temperature of all steps were standardized by the automated *in situ* hybridization instrument.

Immunohistochemical (p27, p53, Ki-67) Scoring for Statistical Evaluation

Two pathologists without knowledge of the clinical and pathological parameters of the cases independently evaluated and scored them for percentage of cells expressing strong nuclear staining for p27. At least 10 high-power fields were chosen at random and 1000 cells were counted. Of these cases, 25% were chosen at

Figure 1. Nuclear localization of p27 by immunohistochemistry in a primary colorectal adenocarcinoma (a) with subsequent down-regulation/loss of expression in the corresponding metachronous metastasis (b). In the synchronous subgroup, there is low expression in both the primary tumor (c) and synchronous metastasis (d). Stromal lymphocytes are strongly stained in both subgroups. Hematoxylin counterstain. Magnification, $\times$ 250.

Figure 2. Primary colorectal adenocarcinoma from the metachronous subgroup showing high levels of p27 mRNA by *in situ* hybridization (a) and p27 protein expression by immunohistochemistry (b). The subsequent metachronous metastasis expresses high levels of p27 mRNA (c) but corresponding immunohistochemistry demonstrates lack of p27 protein expression (d). Magnification, $\times 250$.

random and scored a third time by another pathologist (ML). There was $>98\%$ concordance among observers' scores. The percentage of p27 positive cells was expressed as a ratio of positive cells to the total number of cells counted. The percentage of p53 and Ki-67 cells per total population was obtained similarly. Based on prior reports of p27 expression in colorectal and breast cancers, cases were classified as high or low p27 expressors.^{15,16} Patients with low p27 expression had $\leq 50\%$ of the nuclei in the specimen staining positive, while high expressors had $>50\%$ p27 positive nuclei. For p53 and Ki-67 the cutoff used was $\leq 20\%$ for nonexpressors and $>20\%$ for expressors.

Statistical Methods

The difference in p27, p53, and Ki-67 expression was assessed by the paired *t*-test using absolute percentage of positive cells for the individual tumor/metastases pairs in both the synchronous and metachronous subgroups. In addition p27, p53, and Ki-67 expression was compared in the same groups using the cutoffs described above and McNemar's paired comparison test for binary data. In the metachronous subgroup, the effect of chemotherapy on p27 expression was analyzed using the

Mann-Whitney rank sum test (for two categories). The Pearson correlation coefficient was used to test the strength of association between continuous variables. A *P* value ≤ 0.05 was required for significance.

Results

Primary tumors with metachronous metastases were available in 13 cases. Fifty-four percent of the primary tumors in this subgroup were low expressors of p27 ($\leq 50\%$ of tumor cells expressing p27), while the remainder were high expressors ($>50\%$ of cells expressing p27). The distribution of p27 was consistent with our previous analysis of a larger database.¹⁶ There was a striking reduction in p27 protein nuclear expression in the metastases (mean = 14.5%) compared to the primary tumors (mean = 41.8%); (*P* = 0.0023 by paired *t*-test or *P* = 0.03 by McNemar's paired comparison test for binary data). (Table 1 and Figure 1, A and B). In contrast to the protein level, p27 mRNA as assessed by *in situ* hybridization (ISH) was expressed in all of the primary tumor/metastasis pairs in the metachronous subgroup (Figure 2).

In the metachronous subgroup, the mean value of percentage of positive cells in the primary tumors and

Table 2. Immunohistochemical Analysis of p27 Protein Expression in Primary Tumor-Synchronous Metastases Pairs

Patient no.	Stage	Chemo-therapy	p27 level (%)	
			in primary	in metastases
1	IV	+	0	0
2	IV	+	0	0
3	IV	+	0	0
4	IV	+	0	0
5	IV	—	0	0
6	IV	+	30	27
7	IV	N/A	0	0
8	IV	+	0	0
9	IV	+	0	0
10	IV	—	14	35
11	IV	+	11	2
12	IV	+	49	36
13	IV	N/A	0	0
14	IV	+	0	0
15	IV	N/A	0	0
16	IV	+	23	29
17	IV	N/A	0	0
18	IV	+	14	19
19	IV	N/A	9	30
20	IV	N/A	16	4
21	IV	—	12	17
22	IV	+	0	0
23	IV	N/A	12	8
Mean $\pm$ SEM			8.3 $\pm$ 2.6	9.0 $\pm$ 2.8 ($p = 0.6852$)

N/A, chemotherapy data not available.

metastases for p53 was 61 ± 10.6 and 50.6 ± 10.7 , respectively ($P = 0.41$ by paired t -test or $P = 0.75$ by McNemar's paired comparison test for binary data). There was no correlation between p53 and p27 in the primary tumors ($r^2 = -0.09$, $P = 0.77$) or in the metastases ($r^2 = 0.12$, $P = 0.69$). As for the proliferation index, as assessed by Ki-67, the mean value of percentage of positive cells in the primary tumors and metastases was 65.9 ± 8.7 and 55.9 ± 8.0 , respectively ($P = 0.07$ by paired t -test or $P = 1.00$ by McNemar's paired comparison test for binary data). Similarly, there was no correlation between p27 and Ki-67 in the primary tumors ($r^2 = 0.11$, $P = 0.73$) or the metastases ($r^2 = 0.18$, $P = 0.55$).

Ten patients received chemotherapy after resection of their primary tumors. There was no significant difference in the percentage of positive cells expressing p27 in the metachronous metastases of treated and untreated patients. ($P = 0.9$, Mann-Whitney rank sum test).

In the set of tumor/synchronous metastasis pairs, there was no significant difference in p27 protein expression between the primary tumors (mean = 8.3%) and metastases (mean = 9%) ($P = 0.6582$). As expected these patients were all low p27 expressors in the primary tumor (Table 2: Figure 1, C and D). Of note, we also identified distinct cytoplasmic expression in 7 pairs of primary tumors and their corresponding synchronous metastases. In the synchronous subgroup, the mean value of percentage of positive cells in the primary tumors and metastases for p53 was 79 ± 6.0 and 79.1 ± 5.9 , respectively ($P = 0.11$). The mean value of percentage of positive cells in the primary tumors and metastases for Ki-67 in the synchronous subgroup was 64.2 ± 4.8 and 60.0 ± 5.0 , respectively ($P = 0.31$). Similarly, comparison of p27, p53, and Ki-67 in the primary tumor/synchronous metastasis pairs was not significantly different when the McNemar's comparison test for binary data was used ($P = 1.00$ for all three markers) (Table 3).

Discussion

The CKI p27 plays a significant role in the regulation of the transition from G₁ to S phase in both normal and neoplastic cells.^{1,3} Defective regulation of this important checkpoint may result in uncontrolled cellular proliferation.¹ In fact, inactivation of several CKIs has been associated with neoplastic transformation in a variety of tissues.¹⁻³ The development of multiple organ hyperplasia and pituitary tumors in p27 knockout mice demonstrates that p27 plays an important role in repressing tumor growth *in vivo*.²⁸⁻³⁰

We have previously demonstrated the role of p27 loss in determining prognosis in colorectal cancer.¹⁶ Here we evaluated the potential role of p27 loss in the process of metastasis. All of the primary and subsequent metastatic tumors tested in the metachronous subgroup expressed high levels of p27 mRNA via *in situ* hybridization. In con-

Table 3. Summary of p27, p53, and Ki-67 Percentage Positive Tumor Cells and P Values in the Metachronous and Synchronous Subgroups

Subgroup	p27	p53	Ki-67
Metachronous			
Primary tumor	41.7 $\pm$ 7.0	61.0 $\pm$ 10.6	65.9 $\pm$ 8.7
Metastases	14.5 $\pm$ 3.2	50.6 $\pm$ 10.7	55.9 $\pm$ 8.0
P value (paired t -test)	0.0023	0.41	0.07
P value (McNemar's paired test)	0.03	0.75	1.00
Synchronous			
Primary tumor	8.3 $\pm$ 2.6	79.5 $\pm$ 6.0	64.2 $\pm$ 4.8
Metastases	9.0 $\pm$ 2.8	79.1 $\pm$ 5.9	60.0 $\pm$ 5.0
P value (paired t -test)	0.69	0.11	0.31
P value (McNemar's paired test)	1.00	1.00	1.00

The difference in p27, p53, and ki-67 expression was assessed by the paired t -test when comparing absolute numbers and the McNemar's paired test for binary data when cutoffs were used (see Statistical Methods) for individual tumor/metastasis pairs in both the synchronous and metachronous subgroups.

Values are mean $\pm$ SEM.

trast, all metachronous metastases were low expressors of p27 protein by immunohistochemistry, suggesting that loss of p27 resulted from a posttranslational target-specific enhanced proteasome-mediated degradation as shown previously.^{16,21,31} This may be a mechanism used by aggressive tumors to eliminate p27. Although the database is small, loss of p27 appears to be independent of the effects of chemotherapy.

There was no direct inverse or positive correlation between p27 and Ki-67 expression in the metachronous subgroup. Proliferative index has not been shown to have a prognostic significance in colorectal cancer.^{32,33} Furthermore, we and others have previously demonstrated a lack of correlation between p27 and Ki-67 in colorectal adenocarcinoma^{16,34} as well as in breast cancer.¹³ It is becoming progressively clearer that CKIs have functions other than cell cycle inhibition. In fact, CKIs are expressed in postmitotic cells in the absence of any CDK activity. For instance, p27 has been shown to be involved in apoptosis,³⁵ whereas p21 appears to have a role in differentiation.³⁶ Both of these effects are independent of those involving cell cycle kinetics.

Our study indicates yet another potential mechanism of action of p27, namely its involvement in the process of cell adhesion. Cell adhesion has an essential role in regulating proliferation during the G₁ phase and loss of this adhesion capacity is a feature of oncogenic transformation. Cell-cell contact such as that occurring at the confluence of epithelial or mesenchymal cells in culture and loss of cell adhesion, eg, when cells are grown in suspension, up-regulate p27 levels.^{3,37-41} In cells growing in suspension, p27 up-regulation is due to an increase in protein stability.³⁹ Enforced expression of G₁ cyclins allows cell proliferation in suspension.^{40,41} Increased expression of cyclin D1 in both colon tumor cell lines and human colonic tumors has been shown to contribute to tumorigenicity.^{42,43} Cyclin overexpression or loss of p27 protein via enhanced tumor-specific degradation may give tumor cells the ability to grow in the presence of altered extracellular matrix properties and altered intercellular adhesion, two conditions that might facilitate metastasis.

p53 mutations are a late event in colorectal carcinogenesis⁴⁴ and have been shown to be associated with the development of metastases in colorectal adenocarcinomas.^{23,24} However, no difference in p53 overexpression (indicative of mutation in the gene) was found in the primary or metastatic tumors of either the metachronous or synchronous subgroups. In addition, there was no correlation between p27 and p53 expression, suggesting an independent pathway for p53 in the development of metastases.

Altered cellular compartmentalization may also play a role in the down-regulation of p27 in anchorage-independent cells.⁴⁵ Although p27 normally is localized to the nucleus, we noted distinct cytoplasmic localization of p27 in both the primary tumor and its corresponding synchronous metastasis in a small subset of patients. Previously we showed that confinement of p27 to the cytoplasm, confirmed by subcellular fractionation, was associated with a poor prognosis in Barrett's associated esophageal

adenocarcinoma.²⁰ The mechanism for nuclear exclusion is unclear at present. It remains to be determined whether the nuclear exclusion of p27 is involved in the metastatic process.

We have shown here that p27 protein expression is down-regulated or lost in metachronous metastases in patients with primary colorectal adenocarcinomas. This novel finding implies that p27 protein down-regulation in colorectal adenocarcinomas is associated with tumor progression and may be an important factor in the development of metastases.

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