Loss of $p21^{\text{WAF1/Cip1}}$ Protein Expression Accompanies Progression of Sporadic Colorectal Neoplasms but not Hereditary Nonpolyposis Colorectal Cancers

Frank A. Sinicrope, Gardiner Roddey, Michael Lemoine, Sanbao Ruan, L. Clifton Stephens, Marsha L. Frazier, Yu Shen, and Wei Zhang

Departments of Gastrointestinal Medical Oncology & Digestive Diseases [F. A. S., G. R., M. L., M. L. F.], Neuro-oncology [S. R., W. Z.], Veterinary Medicine & Surgery [L. C. S.], and Biomathematics [Y. S.]. The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

$p21$ ($p21^{\text{WAF1/Cip1}}$), a cyclin-dependent kinase inhibitor, induces $G_1$ arrest and can inhibit the activity of the proliferating cell nuclear antigen (PCNA). We analyzed $p21$ expression during colorectal tumorigenesis, its association with its transcriptional regulator $p53$, and its relationship to rates of cell proliferation and apoptosis. $p21$ and $p53$ protein expression were examined in sporadic tumors and hereditary nonpolyposis colorectal cancers (HNPCCs) by immunohistochemistry (IHC) and immunoblotting. Apoptosis was examined using a DNA nick end-labeling assay, and cell proliferation was examined by PCNA staining. In normal colorectal epithelia, nuclear $p21$ staining was uniformly detected in crypt cells of the superficial compartment (upper one-third) that stained negatively for PCNA. $p21$ and PCNA expression were, therefore, mutually exclusive. In sporadic cases, a decrease in the frequency of $p21$ expression accompanied adenoma development and progression to carcinoma. Specifically, $p21$ was detected in 12 of 16 (75%) adenomas and 10 of 32 (31%) carcinomas. In contrast to sporadic cases, HNPCCs with known mutations in DNA mismatch repair genes expressed $p21$ in 12 of 15 (80%) carcinomas. An inverse relationship between $p21$ and $p53$ was observed wherein mutant $p53$ proteins were detected in 4 of 15 (27%) HNPCCs versus 22 of 32 (69%) sporadic carcinomas. Although $p21^{+}$ carcinoma cells were generally negative for $p53$, IHC revealed that some carcinoma cells expressed both $p21$ and $p53$ proteins. Furthermore, $p53$-mutated SW480 colon carcinoma cells were found to coexpress $p21$ and $p53$, suggesting that $p21$ can also be activated by a $p53$-independent mechanism. No association was found between $p21$ or PCNA and apoptotic labeling indices in adenomas or carcinomas. In conclusion, a decrease in $p21$ expression accompanies neoplastic progression in sporadic cases but not in HNPCCs. This finding appears related to $p53$ status in that the frequency of $p53$ expression was significantly reduced in HNPCCs compared to sporadic cases, suggesting a difference in their molecular pathways of tumorigenesis.

INTRODUCTION

The $p21$ ($p21^{\text{WAF1/Cip1}}$) gene is located on chromosome 6p and encodes the $p21$ protein, which functions as a potent inhibitor of cdk activity (1, 2). Members of a family of cdks function as key positive regulators of the cell cycle that promote cell proliferation. $p21$ induces $G_1$ arrest and blocks entry into S phase by inhibiting cdks (1, 2) or by binding to the PCNA, resulting in a blockade in DNA replication (3, 4). $p21$ is a downstream target effector of the wt $p53$ protein, which transcriptionally activates $p21$ (5, 6). Wt $p53$ proteins accumulate as a consequence of DNA damage and can bind to specific sites on the $p21$ promoter, resulting in induction of $p21$ expression (5). Mutational inactivation of $p53$, a frequent event in colorectal cancers (7), with resultant failure to transactivate $p21$, may thereby produce uncontrolled cell proliferation.

Transfection of the $p21$ cDNA and overexpression of the $p21$ protein have been shown to inhibit the growth of cultured colon, brain, and lung cancer cells, as well as leukemia cells (5, 8, 9). Furthermore, $p21$ overexpression reduced the tumorigenicity of colon carcinoma cells when injected into nude mice (9). Studies in colon cancer cells (6) and in $p21$-deficient murine fibroblasts (10) have shown that $p21$ is necessary for $p53$-mediated $G_1$ arrest induced by DNA damage. The requirement for $p21$ in $p53$-mediated growth arrest suggests that $p21$ plays a critical role in $p53$-mediated tumor suppression (6, 11). Suppression of tumor growth may occur secondary to cell cycle arrest or, alternatively, by induction of apoptosis (12). Interestingly, loss of $p53$ function has been associated with attenuated apoptotic rates in vivo (12). Although $p53$ appears to be a key regulator of $p21$, abundant evidence indicates that $p21$ can also be induced independently of $p53$ (13–16). In most normal tis-
sues of the adult mouse, including the intestinal epithelium, p21 expression is p53 independent (14, 15). p53-independent expression of p21 has also been reported in human leukemia (16), non-small cell lung carcinoma (17), and ovarian carcinoma (18).

The p21 protein has been detected in a variety of normal tissues, including colorectal epithelia, where its expression has been associated with terminally differentiated or senescent cells that have ceased to divide (14, 19). These observations indicate that p21 is involved in regulating epithelial cell turnover. Potentially, alterations in p21 expression may deregulate growth and contribute to neoplastic development and/or progression. To address this issue, we examined p21 and PCNA expression at the cellular and tissue level in colorectal carcinomas.

To date, p21 expression has not been examined in HNPCCs. HNPCC is a dominantly inherited disorder characterized by multiple cases of colorectal cancer within a family (21). HNPCC accounts for up to 5% of all colorectal cancer cases and is caused by germ-line mutations in at least four DNA MMR genes (21). Two MMR genes, hMLH1 and hMSH2, account for the majority of MMR mutations that result in genomic instability and early onset of colorectal cancer. MMR-deficient tumors display microsatellite instability as a consequence of an accumulation of mutations at short tandem repeats of DNA, known as microsatellites, which are distributed throughout the genome (22). Some of these mutated genes contribute to tumorigenesis in HNPCC, and studies are ongoing to identify these specific mutational targets. To date, p21 expression has not been examined in HNPCCs.

MATERIALS AND METHODS

Tissue Specimens. Formalin-fixed, paraffin-embedded tissue blocks were obtained from the Surgical Pathology Laboratory at the University of Texas M. D. Anderson Cancer Center and from referring institutions. Normal colorectal epithelia (n = 11) were obtained from the resection margins of colorectal cancer cases. Hyperplastic (n = 5) and adenomatous (n = 16) polyps were obtained from both surgical and endoscopic biopsy specimens. All adenomas had tubular or tubulovillous histology, with the exception of one villous tumor. Three adenomas were from patients with synchronous colorectal carcinomas, and another three adenomas contained foci of severe dysplasia. All nonneoplastic tissues were obtained from patients without clinical evidence of a familial colon cancer syndrome, i.e., sporadic cases. Primary, untreated colorectal adenocarcinomas were from both sporadic (29 colon and 3 rectal (n = 32)) and HNPCC [colon (n = 15)] patients. Ten of 15 HNPCC cases had known germ-line mutations in hMLH1 or hMSH2 MMR genes as previously determined by sequencing of genomic DNA (23). The remaining five also came from families meeting the Amsterdam criteria (21), and four of these had loss of hMSH2 protein expression by IHC. Specifically, tumor cells lacked nuclear hMSH2 staining in contrast to adjacent normal epithelium. Absence of hMLH2 immunoreactivity is consistent with either germ-line or somatic hMSH2 mutation (24). In the seven cases with hMSH2 mutations detected by DNA sequencing, concordant results were found by IHC in all but one case. This discordant case (patient 4; Table 1) had a missense mutation that did not result in a loss of immunoreactivity. Tumor stage of HNPCCs was not available. For sporadic cancers and using the tumor-node-metastasis classification, there were 4 stage II, 22 stage III, and 6 stage IV tumors. Degree of tumor differentiation for sporadic cases was as follows: 3 well, 24 moderately, and 5 poorly differentiated. Eight of 15 HNPCCs were poorly differentiated, and 7 were moderately differentiated. Consecutive 4–6-μm tissue sections were cut from each tumor block for subsequent analysis. In addition to archival materials, we also analyzed fresh tissue from sporadic colorectal carcinomas (n = 5) and histologically normal tissue from the resection margins (distance of >10 cm from primary tumor). Tissue specimens were immediately frozen on dry ice and ethanol and stored at −75°C until use. To ensure that tumor tissue was being analyzed, H&E sections were prepared and examined from the cut margin of the tumor and found to be highly tumor enriched. Furthermore, formalin-fixed, paraffin-embedded sections from these cases were examined for diagnosis, and additional sections were cut for a comparison of p21 and p53 expression by IHC versus immunoblotting.

Cell Culture. The SW480, HT29, and RKO human colon carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD) and grown in DMEM:F-12 medium (Life Technologies, Inc.) supplemented with heat-inactivated 10% FCS and 10 units/ml penicillin and streptomycin. Cultures were maintained in a humidified environment at 37°C with 5% CO2. The LN-Z308 human glioblastoma cell line expresses low levels of p21 endogenously and lacks wt p53. As previously reported (25), wt p53 transfected into LN-Z308 cells, which activated p21 expression and inhibited cell growth. LN-

Table 1: Analysis of HNPCC cases for mutations in hMLH1 and hMSH2 genes determined by DNA sequencing (23) or hMSH2 protein expression. p21 and p53 expression were also examined by IHC

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gene</th>
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<th>p53β</th>
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<tr>
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α Absence of staining (−) is consistent with germline or somatic mutation (24).
β Percent tumor cell positivity (0, ≤5; 1, 6–25; 2, 26–50; 3, 51–75; 4, ≥75%)
γ ND, not determined.
Z308 cells were maintained in DMEM:F-12 medium supplemented with 10% FCS and maintained as described above.

**Immunobots.** Frozen tissue samples and cultured cell lines were processed per standard procedures and a cell lysate was prepared (26). Protein extraction was performed on tissue and cell lysates and the proteins were analyzed on a SDS-polyacrylamide gel as described previously (26). Proteins were then transferred to an Immobilon membrane (Millipore, Bedford, MA) and incubated overnight with MAbs against p21, p53, and actin (Oncogene Science, Uniondale, NY). The level of protein expression was measured using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) per the manufacturer's instructions. In an attempt to quantitate the level of p21 expression in immunoblot, autoradiography films were scanned using a densitometer. Density values for p21 were calculated in carcinomas relative to normal tissue, in which mean density was designated as 1.0. Actin levels were analyzed as controls for protein loading.

**IHC.** An immunoperoxidase method using an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC) was used to stain all slides. p21 staining was performed as follows. Slides were placed in xylene and then rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by incubating slides for 30 min in 3% H₂O₂ at RT, followed by rinsing in deionized water. Sections were then placed in 1 M sodium citrate buffer (pH 6) and microwaved on high for 10 min. After rinsing in PBS, sections were treated with a nonsenspr protein block (Dako Corp., Carpinteria, CA) for 10 min followed by normal horse serum for 20 min. Slides were then incubated overnight at RT with the WAF1 murine IgG1 MAb (Ab-1; Oncogene Science, Uniondale, NY) to p21 at a dilution of 1:75. Following washing in PBS for 5 min, the secondary antibody was applied for 30 min at RT. Slides were again rinsed in PBS, and then the ABC reagent was applied for 45 min at RT. The chromogen DAB (Research Genetics, Huntsville, AL) was subsequently applied to each section, and the color reaction was observed using light microscopy. The reaction was stopped by immersing slides in deionized water, and a hematoxylin counterstain was applied. Slides were then covered slipped using a nonaqueous mounting medium.

The procedure to detect hMSH2 proteins was as outlined for p21 above. Sections were incubated overnight with the hMSH2 MAb (Ab-2; Oncogene Science) at a dilution of 1:80 at RT. For p53 (Ab-6; Oncogene Science), the procedure was again as above except that tissue sections were placed in PBS and microwaved on high for 4 min, as described previously (25). The staining method used for PCNA was identical to that reported for p21, excepting that (a) microwaving of slides was not performed, and (b) slides were incubated with the primary antibody at a dilution of 1:100 for 1 h at RT (27). The anti-PCNA MAb (PC10; Signet Laboratories, Dedham, MA) is a murine antirat subclass IgG2a, known to recognize the DNA polymerase ε accessory protein in all vertebrate species.

For double immunostaining, anti-p21 was the first MAb applied, and the procedure was performed as outlined for p21 above excepting that nickel chloride was added to DAB (0.5 mg in 2 ml), and the color reaction was developed. The second primary MAb, anti-p53, was then applied, and the remainder of the procedure was identical to that described for p53. For p21, normal human epidermis was used as a positive control (Fig. 1A). Appropriate positive and negative tissue controls used for p53 and PCNA were described previously (27). In negative control slides, the primary antibody was omitted, and no immunostaining was observed. In colorectal neoplasms, the percentage of tumor cells expressing p21 was analyzed in each section at light microscopy and arbitrarily categorized as ≤5%, 6–25%, or >25%. Scoring for p53 is shown in Table 1. Topographical distribution of staining was also assessed. Tumors with ≤5% immunoreactive cells were considered negative (25). A PCNA LI was calculated as the ratio of the number of PCNA positive cells to 500 total cells examined, expressed as a percentage (27).

**Labeling of Apoptotic Cells by TUNEL Assay.** A method has been described that allows in situ detection of apoptotic cells using terminal deoxynucleotidyl transferase in incorporation of labeled nucleotides into the 3' end of DNA strand breaks (20). Apoptotic cells are then identified using an immunoperoxidase detection system. After deparaffinization and rehydration, tissue sections were incubated in 20 µg/ml of proteinase K (Sigma Chemical Co., St. Louis, MO) for 15 min at RT. After rinsing in PBS for 5 min, endogenous peroxidase activity was blocked by incubating the sections in 3% H₂O₂ for 30 min at RT. Following rinsing in PBS, the slides were covered with terminal deoxynucleotidyl transferase plus nucleotide mixture (TUNEL reagent; In Situ Cell Death Detection kit; Boehringer Mannheim, Indianapolis, IN) at a 1:35 dilution and incubated for 60 min in a humid chamber at 37°C. The reaction was terminated by immersing the slides in PBS containing 2% BSA for 10 min at RT. The sections were then covered with a nonsenspr protein block (Dako Corp.) for 10 min at RT. Following rinsing in PBS with 2% BSA for 5 min, slides were covered with an anti fluoroscein antibody conjugated with horseradish peroxidase for 15 min at RT. Following rinsing in PBS plus 2% BSA, DAB (Research Genetics) was applied, and the reaction was observed under light microscopy. Positive control slides consisted of a tissue section exposed to DNase I and an irradiated murine lymphoma known to be radiation sensitive. Exposure to DNase I produced staining of all nuclei in the section. In the irradiated lymphoma, numerous TUNEL-stained apoptotic cells and bodies were detected. For negative controls, the TUNEL reagent was omitted, and no immunoreactive cells were observed.

**Quantitation of Apoptotic Cells in Histological Sections.** Using light microscopy, TUNEL-stained cells were identified in tissue sections by a brown reaction product confined to the nucleus or nuclear fragments (Fig. 5; Ref. 27). In addition to TUNEL staining, morphological features of apoptosis were also required given that apoptosis can occur in the absence of DNA strand breaks (28) and that TUNEL positivity may not always indicate impending cell death (29). The number of apoptotic nuclei per 100 cells in five high-power fields per slide was determined. An AI was then calculated as follows:

\[
AI = \frac{\text{number of apoptotic cells}}{\text{total number of cells}} \times 100
\]

**Statistics.** The relationship between p21 and p53 protein expression was analyzed using the nonparametric Kruskal-
Fig. 1 Immunohistochemical analysis of p21 expression in normal and neoplastic colorectal epithelia. Dark staining indicates a positive signal. A, p21 staining is seen in epithelial cell nuclei in the suprabasal layer of normal epidermis (positive control). B, normal colorectal epithelium demonstrates nuclear p21 staining in differentiated crypt cells of the superficial compartment. These p21-positive cells stained negatively for PCNA (see Fig. 2). C, transition of normal and adenomatous colorectal epithelium. p21 staining in the adenoma localizes to the superficial region. D, adenoma displaying p21 staining in superficial crypt cells. E, p21 staining in tumor cell nuclei in a well-differentiated colon carcinoma. F, p21 staining in a poorly differentiated colon carcinoma.
Wallis statistic. Relationships between p21 and AI and PCNA LI values in neoplasms were assessed by the Spearman’s correlation test and the Kruskal-Wallis test. The χ² test was used to assess the relationship or dependence of the categorical variables between two groups. Statistical significance was defined as a two-sided P of <0.05.

RESULTS

p21 and PCNA protein expression were examined in four histological types of colorectal epithelia representing histological stages in the evolution of colorectal cancer. p21-positive cells were identified by dark brown nuclear staining. In normal human epidermis that served as a positive control, p21 expression was detected in epithelial cells in the suprabasal layer (Fig. 1A). In normal and hyperplastic colorectal epithelia from sporadic cases, p21 was uniformly expressed in crypt cell nuclei in the superficial one-third of the mucosa, including luminal epithelial cells. p21 was therefore topographically restricted to nonproliferating and differentiated crypt cells (Fig. 1B). In contrast to p21, PCNA immunoreactive cells were confined to the lower one-third of normal mucosa, corresponding to the proliferative compartment (Fig. 2A). Thus, PCNA and p21 staining were inversely distributed and nonoverlapping in their topography (Figs. 1B and 2A). This finding is consistent with another study in colonic epithelia, in which double staining for p21 and Ki-67 proteins revealed that their expression was mutually exclusive (19).

Whereas p21 was expressed in all specimens of normal and hyperplastic mucosa, p21 staining was found in 12 of 16 (75%) sporadic adenomas (Table 2). Hence, a reduction in the fre-
frequency of p21 expression was associated with neoplastic development in sporadic patients. p21 staining was found predominantly in crypt cells in superficial regions of adenomas and tended to occur in clusters (Fig. 1, C and D). In the majority of adenomas (10 of 16 (62%]), staining was found in ≤25% of the total tumor cell populations (Table 3, Fig. 1D). However, a subset of adenomas with >25% p21-positive cells was found in which p21 staining extended deep into the tubular glands (Table 3). Staining intensity was medium to strong in all adenomas and did not differ appreciably from p21 staining intensity in non-neoplastic epithelia (Fig. 1). Whereas p21 staining was preferentially expressed in superficial versus basal regions of adenomas (Fig. 1, C and D), no specific pattern of PCNA expression was observed (Fig. 2C). Hence, the strict compartmentalization of p21 and its inverse relationship with PCNA that were seen in normal epithelia became disorganized in the adenomas.

A further reduction in the frequency of p21 expression occurred during progression from adenoma to carcinoma in sporadic patients. Specifically, 10 of 32 (31%) carcinomas expressed p21 proteins (Table 2). p21 nuclear staining was detected in clusters of tumor cells, but no spatial distribution of p21 staining was discernable, given the architectural disorganization of carcinomas. As with adenomas, a subset of carcinomas (3 cases) expressed p21 in >25% of tumor cells, consistent with a high level of expression (Table 3). Thus, the level of p21 expression in adenomas and carcinomas was heterogeneous. Of all p21+ carcinomas, one-third had strong p21 staining, and the remainder had staining that was medium in intensity. In contrast to sporadic cases, 12 of 15 (80%) HNPCCs were found to express p21 proteins (Table 2). At least 25% p21+ tumor cells were detected in 3 (20%) cases (Table 3), and the pattern of p21 staining in HNPCCs was similar to that in sporadic cases. Importantly, these results pertain to HNPCC cases with known hMLH1 or hMSH2 mutations (n = 14), as determined by DNA sequencing or analysis of protein expression (Table 1; see "Materials and Methods"). Absence of hMSH2 immunoreactivity is consistent with either germline or somatic hMSH2 mutation (Fig. 3).

Nuclear p53 expression, consistent with the accumulation of mutant p53 proteins (30), was detected in 5 of 16 (31%) adenomas and 22 of 32 (69%) carcinomas from sporadic patients (Table 2; Ref. 25). p53 staining was focally expressed in dysplastic crypt cells but was not detected in either normal or hyperplastic colorectal epithelium. In HNPCCs, p53 expression was detected in 4 of 15 (27%) carcinomas (Table 2), suggesting a significant decrease in the frequency of p53 mutation in these tumors. Furthermore, 3 of 4 p53+ HNPCCs had <25% p53+ tumor cells (Table 3). This is in contrast to sporadic carcinomas, in which 13 of 32 (41%) tumors had >75% p53+ tumor cells. Given that wt p53 transcriptionally activates p21, we hypothesized that p53 expression would be inversely related to p21. Therefore, we compared the frequency of p21 expression in p53+ versus p53− neoplasms. An inverse association between p53 and p21 was found in HNPCCs. Specifically, p53− HNPCCs expressed p21 in 9 of 15 (60%) cases, and p53+ tumors had detectable p21 in 3 of 15 (20%) cases. These data suggest that the low rate of mutant p53 expression compared to sporadic cases can explain the increased frequency of p21 expression. In sporadic cases, p21 staining was detected in 8 of 11 p53− versus 4 of 5 p53+ adenomas (P = 0.75). In carcinomas, 5 of 11 p53− versus 10 of 21 p53+ tumors expressed p21 (P = 0.80).

Thirteen of 32 (41%) sporadic carcinomas had diffuse p53 staining and were found to express p21 in 6 (46%) cases, suggesting that some tumor cells may coexpress p53 and p21 proteins. To address this issue, we performed double immunostaining and found that p53 and p21 were rarely detected within the same malignant gland. In some malignant glands, however, tumor cell nuclei appeared to coexpress p53 and p21, and within this subset, both proteins were clearly detected in adjacent tumor cells. By immunoblotting, frozen tissues from 3 of 5 sporadic carcinomas were found to coexpress p53 and p21 proteins (Table 4). Furthermore, SW480 colon carcinoma cells, known to carry a p53 mutation (31, 32), overexpressed p53 and p21. These findings suggest that p21 can be induced by a p53-independent mechanism. Overexpression of p53 was detected in p53 mutated SW480 and HT29 colon carcinoma cells (Fig. 4; Refs. 31 and 32) but not in wt p53-containing RKO cells (32). Taken together, our data support p53-dependent and p53-independent induction of p21. Moreover, our results and those of others (33) indicate that p53-dependent p21 induction is the predominant mechanism in colorectal carcinomas.

We attempted to quantitate the level of p21 expression in carcinomas relative to histologically normal mucosa from the same patients. Frozen tissue from five sporadic colon cancers was analyzed by immunoblotting, and autoradiographs were subjected to densitometry. The p53-deficient human glioblastoma cell line LN-Z308 was included as a positive control (34). LN-Z308 cells were previously transfected with a wt p53 expression vector (34), and immunoblotting detected a protein band of 21 kilodaltons (Fig. 4). Colon carcinoma cell lines were also examined, and a high level of p21 expression was detected in SW480 and RKO cells (Fig. 4); however, p53 mutated HT-29 cells were observed to lack p21 expression. In carcinoma spec-

### Table 2 p21 and p53 protein expression during colorectal tumorigenesis

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<th>Normal (n = 11)</th>
<th>Hyperplastic (n = 5)</th>
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### Table 3 Percentage of p21+ tumor cells detected by IHC in colorectal neoplasms

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<td>6 (37%)</td>
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<td>Carcinoma</td>
<td>12 (75%)</td>
<td>10 (62%)</td>
<td>10 (31%)</td>
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**a** Number and percentage of specimens that overexpress p21 or p53 as detected by IHC.

**b** See Table 1.
imms, p21 expression was detected in 5 of 5 tumors, and a 1.5–8-fold increase in the level of p21 was found relative to normal mucosa (Fig. 4, Table 4). In paraffin sections from these same tumors, the percentage of p21+ cells did not predict the relative level of p21 (Table 4). However, normal mucosa was not present on these sections, and therefore, the absence of a denominator precluded a direct comparison.

As shown in this study and in others (19, 33), p21 and PCNA expression in normal colorectal epithelia were inversely distributed and mutually exclusive in their topography. This inverse relationship was maintained in hyperplastic polyps. In sporadic adenomas and carcinomas, significantly fewer p21-reactive cells were seen compared to PCNA (Figs. 1B and 2A). Furthermore, the percentage of PCNA-positive tumor cells was greater in neoplastic than in nonneoplastic mucosa (Fig. 2). We then examined the PCNA LI stratified by p21 staining. The mean PCNA LI of p21-positive versus p21-negative adenomas was 43.3 and 32.4, respectively (P = 0.11; Table 5). p21-positive carcinomas had a mean PCNA LI of 44.3 versus 43.2 for p21-negative cancers (n = 21; P = 0.85). Thus, p21 expression was not significantly related to overall proliferative activity in colorectal neoplasms. To determine whether an association existed between p21 and rates of spontaneous apoptosis, we quantified apoptotic cells in paraffin sections using the TUNEL assay in addition to morphological criteria (Fig. 5), as described previously (27). We found that the mean AI in p21-positive versus p21-negative adenomas was 2.7 and 1.9 (P = 0.18) and for carcinomas was 2.5 and 2.3, respectively (P = 0.66; Table 5). The above results did not change significantly when the p53 status of these neoplasms was considered. Thus, no relationship between p21 expression and in situ apoptotic rates was observed.

**DISCUSSION**

Our results demonstrate a significant difference in p21 expression in sporadic colorectal carcinomas versus HNPCCs with known mutations in MMR genes. In sporadic patients, we found a reduction in the frequency of p21 expression during adenoma development and progression to carcinoma. Complete
1258 p53 and p21 proteins, respectively. Lane 2, though p21 acts as a tumor suppressor in vitro contribute to malignant transformation and progression. Alp21 in colon carcinoma cells has been shown to suppress their expression using specific antibodies. The levels of p21 proteins in tumors relative to normal tissue were determined by densitometry (see Table 4). IHC results for p21 and p53 in these same tissue specimens are shown in Table 4.

Table 5: Relationship of p21 to PCNA expression and apoptotic indices in sporadic colorectal neoplasms

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<th>Tumor Type</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>p21+</td>
<td>43.3</td>
<td>2.7</td>
</tr>
<tr>
<td>P = 0.11</td>
<td>P = 0.18</td>
<td></td>
</tr>
<tr>
<td>Carcinoma (n = 32)</td>
<td>43.2</td>
<td>2.3</td>
</tr>
<tr>
<td>p21−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21+</td>
<td>44.3</td>
<td>2.5</td>
</tr>
<tr>
<td>P = 0.85</td>
<td>P = 0.66</td>
<td></td>
</tr>
</tbody>
</table>

PCNA labeling index (27). Al, apoptotic index (see “Materials and Methods”).

Additionally, Cottu et al. (37) found that 4 MMR-deficient human colon cancer cell lines had wt p53, whereas 15 of 17 MMR-proficient lines contained p53 mutations. Although patients with HNPCC show early onset of carcinoma, intact p53 function and overexpression of p21 may contribute to the better prognosis seen in HNPCCs versus sporadic colorectal cancers (21). Taken together, these findings suggest that HNPCCs, unlike sporadic cases, frequently develop through a pathway that is independent of p53 mutation.

Our results suggest that loss of p21 expression in sporadic colorectal cancers may be a baseline event, and those tumors retaining p21 expression can be considered a high p21 group. In this regard, we found that the level of p21 in sporadic carcinomas was increased 1.5–8-fold relative to normal mucosa. Furthermore, HNPCCs were found to overexpress p21 in 80% of cases, reflecting an increased level of expression. These results are consistent with data in human gliomas, in which the level of p21 expression was markedly elevated compared to normal brain tissue (26). Similarly, p21 RNA and protein were expressed at higher levels in non-small cell lung carcinomas than in normal respiratory epithelia (17). Increased p21 expression has also been reported in breast (38) and ovarian (18) carcinomas, as well as in acute myelogenous leukemia (39). The heterogeneity of p21 expression found in colorectal and brain tumors is unlikely to be related to p21 mutations because somatic mutations in the p21 gene have not been found in either tumor type, although polymorphisms were detected (40, 41). At present, the biological and clinical implications of p21 overexpression in human cancers are poorly understood. However, a recent study found that a high level of p21 in acute myelogenous leukemia cells was associated with resistance to chemotherapy (39). Additionally, data in breast carcinomas indicate that p21 overexpression in untreated tumors is associated with adverse prognostic features and shorter disease-free survival (38). These findings suggest that cell cycle inhibition by p21 may adversely affect treatment response and/or clinical outcome.
A comparison of p21 expression in normal and p53-null mice revealed a similar pattern of expression in intestinal epithelia (14, 19). Thus, p21 is constitutively expressed in normal colorectal epithelia and is therefore p53 independent. In a subset of sporadic colorectal carcinomas, some tumor cells were found to coexpress p21 and p53 proteins, suggesting p53-independent activation of p21. Furthermore, we detected coexpression of p21 and p53 in p53-mutated SW480 colon cancer cells. Similarly, studies in Caco-2 colon cancer cells have shown that p21 increases during spontaneous differentiation in the absence of functional wt p53 protein (42). Our findings, as shown by IHC and immunoblotting, are in agreement with in vitro data (13–16), as well as data in human brain tumors (26), non small-cell lung carcinomas (17), and ovarian carcinomas (18), in which p53-independent expression of p21 has been demonstrated. Although it is theoretically possible that some p53 mutations may retain transcriptional activity allowing induction of p21, Matsui et al. (31) showed that mutated p53 in SW480 cells failed to activate p21. Additionally, p53 expression may not always indicate p53 mutation, although expression by IHC is strongly correlated with accumulation of mutant p53 proteins (30). Of note, we found complete concordance between results for p53 using IHC and immunoblotting. Our data also support p53-dependent activation of p21, because p53 and p21 staining were rarely detected within the same tumor cells or malignant glands. Furthermore, RKO colon cancer cells containing wt p53 (31) expressed p21. These data are consistent with the findings of Doglioni et al. (33), in which an inverse relationship between p21 and p53 staining was generally observed in colorectal carcinomas. Furthermore, this inverse relationship was clearly seen in our HNPCC cases. Our results and those of others (33) suggest that p53-dependent expression of p21 is the predominant mechanism of p21 induction in colorectal cancers and may, therefore, contribute to the heterogeneity of p21 expression found in these tumors.

Treatment of colon carcinoma cells by TGF-β has been shown to transcriptionally induce p21 through a p53-independent mechanism (43). The increase in p21 mRNA is due to transcriptional activation of the p21 promoter by TGF-β. Induction of p21, may therefore, mediate the growth inhibitory effects of TGF-β against colon cancer cells. The growth inhibitory signal of TGF-β is transduced by a transmembrane type II receptor. Interestingly, studies indicate that the TGF-β type II receptor gene is frequently mutated in HNPCCs (44). Inactivation of the type II receptor leads to resistance to growth inhibition by TGF-β and may contribute to accelerated tumorigenesis in HNPCC. Our finding of frequent expression of p21 in HNPCCs, in contrast to sporadic colorectal cancers, suggests that p21-mediated cell cycle inhibition may represent an alternative mechanism of growth control in the presence of TGF-β resistance.

In addition to inhibition of cdk activity, p21 binds PCNA, resulting in a direct interference with DNA replication and, hence, cell proliferation (1, 3, 4). In normal epithelia, p21 expression was restricted to crypt cells in the superficial compartment, including terminally differentiated cells at the luminal surface that stained negatively for PCNA. This finding suggests that p21 activation accompanies migration and differentiation of epithelial cells from the proliferative (PCNA+) to the nonproliferative (PCNA−) compartment and is responsible for their cell cycle arrest (14, 42, 45). Thus, p21 appears to be involved in regulating growth in normal mucosa. This inverse relationship between p21 and PCNA was maintained in hyperplastic epithelia. In sporadic adenomas and carcinomas, however, the PCNA LI did not differ significantly between p21− and p21+ neoplasms. Thus, adenoma development and progression to carcinoma are associated with a loss of p21-mediated growth suppression. These results are consistent with studies in human gliomas (26) and ovarian carcinomas (46), in which no relationship was found between p21 and proliferative activity. There-
fore, p21 expression was not sufficient to suppress overall proliferative activity. It is reported that the growth inhibitory effects of p21 can be overcome by activated oncoproteins, including c-erbB-2 (47) or B-raf (48). Furthermore, p53 mutations may potentially counteract p21 function by elevation of positive cell cycle regulators (34).

The correlation between p21 and the AI in untreated, sporadic colorectal carcinomas was examined. Importantly, the spontaneous AI in experimental murine tumors has been shown to predict susceptibility to chemotherapy-induced (49) and radiation-induced (50) apoptosis. Controlling for p53 status, no association between p21 and AIs was found. This result is consistent with data indicating that p21 induction alone is insufficient to promote apoptosis (51). However, p21 has been shown to influence susceptibility to apoptosis induction in colon carcinoma cells (52). Specifically, p21-deleted HCT 116 colon carcinoma cells were shown to undergo apoptosis in response to radiation or chemotherapeutic drugs, whereas parental cells expressing p21 underwent growth arrest (52). HCT 116 cells contain a mutated hMLH1 gene and are MMR deficient (53). p21 deficiency in HCT 116 cells was associated with defective DNA repair, which may account for their increased sensitivity to DNA damage (54). These observations were confirmed in vivo in xenografts established from HCT 116 cell lines differing only in their p21 status (55). Following irradiation, a cure fraction was seen in p21-deficient tumors, in contrast to tumors expressing p21. Extrapolation of these findings to our results would suggest that HNPPCs, and potentially the subset of colorectal cancers overexpressing p21, may exhibit reduced sensitivity to anticancer treatments. Studies are warranted to further address the role of p21 in treatment resistance in colorectal cancer.

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Loss of p21WAF1/Cip1 protein expression accompanies progression of sporadic colorectal neoplasms but not hereditary nonpolyposis colorectal cancers.

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