Methylation of the 5' CpG Island of the p16/CDKN2 Tumor Suppressor Gene in Normal and Transformed Human Tissues Correlates with Gene Silencing

Mirella Gonzalez-Zulueta, Christina M. Bender, Allen S. Yang, TuDung Nguyen, Robert W. Beart, Jan M. Van Tornout, and Peter A. Jones

Urological Research Laboratory, USC/Norris Comprehensive Cancer Center [M. G.-Z., C. M. B., A. S. Y., T. N., J. M. V., P. A. J.], and Department of Colorectal Surgery [R. W. B.], University of Southern California, School of Medicine, Los Angeles, California 90033

Abstract

Loss of heterozygosity on 9p21, where the p16/CDKN2 tumor suppressor and the p15INK4B cell cycle regulator genes are located, is a common genetic alteration in bladder cancer. However, it has been difficult to demonstrate homozygous deletions and intragenic mutations in either of these two genes in primary transitional cell carcinomas (TCC) of the bladder. Similarly, colon cancer-derived cell lines have shown no homozygous deletions of the p16/CDKN2 locus in contrast to a wide variety of tumor-derived cell lines. We have investigated abnormal methylation of the 5' CpG islands of the p16/CDKN2 and p15INK4B genes as an alternative mechanism of inactivation of these genes in bladder and colon cancers. De novo methylation of the 5' CpG island of p16/CDKN2 was observed in 12 of 18 (67%) uncultured bladder TCCs and in 2 of 3 (67%) bladder cell lines. In contrast, only 1 of 10 (10%) colon carcinomas showed methylation of the 5' CpG island of p16/CDKN2. It was striking to find that this region was extensively methylated and the gene not expressed in the normal colonic mucosa of 6 of 10 (60%) patients with colon cancer, whereas 5 of the corresponding colon tumors showed no methylation and high levels of p16/CDKN2 expression. Our data show a significant correlation (P = 0.00001, two-sided) between the absence of p16/CDKN2 expression and methylation of its 5' CpG island in bladder tumors, cell lines, and normal colon mucosa. In contrast, no association was observed between expression and methylation status of the 5' CpG island of p15INK4B. Our results suggest that the p16/CDKN2 tumor suppressor gene may be inactivated by methylation of its 5' CpG island in TCCs of the bladder. We also present evidence of methylation of the 5' CpG island in this autosomal gene in normal colonic tissue.

Introduction

The p16 protein inhibits CDK4 and CDK6, which are key regulators of the progression of eukaryotic cells through the G1 phase of the cell cycle (1). The p16/CDKN2 gene resides on chromosome band 9p21, a region frequently altered in diverse tumor types (2, 3). The high frequency of p16/CDKN2 alterations initially reported in tumor-derived cell lines (4, 5) and in some tumor types (6, 7), along with the role of p16/CDKN2 in cell cycle control, made this gene an excellent candidate for a tumor suppressor. Emerging evidence suggests that p16/CDKN2 alterations are involved in the progression of certain tumor types (8, 9). Another related cell cycle regulator, the p15INK4B gene, is also located on 9p21, and it has been shown to be up-regulated in human keratinocytes following treatment with TGF-β, suggesting that it may be an effector of TGF-β-mediated cell cycle arrest (10). Although most of the p16/CDKN2 homozygous deletions also include the p15INK4B locus, no intragenic mutations in p15INK4B have been reported to date.

Hemizygous deletions of 9p21 are a frequent genetic alteration in TCC of the bladder (11, 12), which suggests that a tumor suppressor gene for bladder cancer may reside in this area. However, p16/CDKN2 homozygous deletions and intragenic mutations occur at low frequencies in uncultured TCCs (13, 14), although they are common in uncultured squamous cell carcinomas of the bladder (15). No homozygous deletions of the p16/CDKN2 locus have been described in colon cancer-derived lines in contrast to a wide variety of tumor-derived cell lines (4). This relative low rate and absence of p16/CDKN2 alterations in bladder and colon tumors, respectively, may reflect that p16/CDKN2 inactivation occurs in these tumor types by alternative mechanisms. Transcriptional repression by DNA methylation of promoter and 5' regulatory sequences may be a pathway to inactivate the p16/CDKN2 and p15INK4B genes. Global changes in DNA methylation patterns are known to occur during tumorigenesis, and gene silencing has been associated with methylation of CpG islands located in, or near, promoters and 5' regulatory regions (16, 17). CpG islands are G+C rich regions that show a higher frequency of CpG dinucleotides than is normally seen in the vertebrate genome and that are not methylated in the germine (18). With the exception of islands in genes on the inactive X chromosome (19), Alu and L1 sequences (20), and some imprinted genes (21), CpG islands are usually unmethylated in normal somatic cells. In contrast, widespread methylation of CpG islands occurs on autosomal genes during oncogenic transformation (22, 23). Promoters silenced by methylation can be reactivated in many cases by treatment with the drug 5-aza-2'-deoxycytidine, which is a well-established inhibitor of DNA methylation (24).

The exon 1 coding sequences of the p16/CDKN2 and p15INK4B genes reside within 5' CpG islands, and their exon 2 regions are 83% homologous and also constitute CpG islands. We hypothesized that abnormal DNA methylation might be an alternative mechanism of inactivation of the p16/CDKN2 and p15INK4B genes in bladder and colon tumors. In this study, we examined the methylation status and expression levels of the p16/CDKN2 tumor suppressor gene and the p15INK4B cell cycle regulator in normal tissues, cell lines, and bladder and colon cancer. Samples from normal tissues included sperm (n = 1), colon epithelium from individuals without colon cancer (n = 4), bladder urothelium (n = 1), kidney (n = 1), and WBC (n = 1). We also obtained 18 bladder transitional cell carcinoma specimens, as well as normal colonic mucosa (n = 10) and corresponding tumor specimens (n = 10) from patients with colon cancer, and one ulcerative colitis specimen. Our data suggest that expression of the p16/CDKN2 tumor suppressor gene, but not expression of the p15INK4B cell cycle regulator, is controlled by methylation of its 5' CpG island, and that de novo methylation of this island is a mechanism for p16/CDKN2 inactivation in bladder TCCs. In contrast, the
p16/CDKN2 5’ CpG island is sometimes methylated and the gene silenced in normal colonic mucosa.

Materials and Methods

Tissue Samples. Sperm DNA (n = 1) was isolated from a donated sample. Peripheral blood lymphocytes (n = 1) and normal kidney (n = 1) were obtained from a patient with bladder cancer. Normal colon mucosa (n = 4) was obtained from individuals with normal sigmoidoscopy, who had undergone colectomy for a noncancerous process. One ulcerative colitis specimen was obtained from a patient affected with ulcerative colitis. Colon carcinoma specimens (n = 10) and matched normal colonic mucosa (n = 10) were obtained from 10 patients with colon cancer. Colon tumors were graded according to Dukes’ classification (25) and ranged from stage B1 to C2. The collection process of the normal colon mucosas prevented major contamination of epithelial cells with cells from the rest of the colon wall since the epithelial cell layer was dissected from submucosa, muscle, and surrounding adipose tissue. Bladder TCCs (n = 18) were obtained from 18 patients, and their grades ranged from T1 to T4, according to the Berkovist classification (26). DNA was isolated by proteinase K digestion and phenol/chloroform extraction.

Cell Lines. EJ, HT1376 and J82 (bladder TCC-derived cell lines) were obtained from the American Type Culture Collection (Rockville, MD). DNA and total RNA were isolated from confluent, exponentially growing cells.

RT-PCR. Total RNA was isolated as described (27). RNA was reverse transcribed using random hexamers. cDNA was amplified using primers specific for the p16/CDKN2 gene, the p15INK4 gene, or the GAPDH gene, which was used as a control. The primers used for p16/CDKN2 amplifications were located in exon 1 and exon 2 of the gene, flanking intron 1. Their sequences were 5’-AGC CGG CTG ACT GGC TGG-3’ (sense) and 5’-CTG GAT CGG CTG ACT GGC TGG-3’ (antisense) specific for the p16/CDKN2 gene, the p15INK4B gene, or the GAPDH gene, which was used as a control. Primers used for GAPDH amplification were 5’-TGA TGA TGG GCA GCC ACC ATG ACC TGG A-3’ (antisense). Conditions for p16/CDKN2 amplifications were 94°C for 3 min, 22 cycles of 94°C for 1 min, 56°C for 30 s, and 72°C for 40 s, followed by incubation at 72°C for 1 min. Primers for p15INK4B were 5’-TGA TGA TGG GCA GCC ACC ATG ACC TGG A-3’ (antisense) and 5’-TGA TGA TGG GCC ACC GC-3’ (sense). Conditions for p15INK4B amplifications were 94°C for 3 min, 22 cycles of 94°C for 1 min, 56°C for 30 s, and 72°C for 40 s, followed by incubation at 72°C for 2 min. Primers for the GAPDH gene were 5’-CAG CGC ACC AAC ACC TCA TCT C-3’ (sense) and 5’-TGA GCC TTT TCA TCT C-3’ (antisense). Conditions for GAPDH amplifications were 94°C for 1 min, 22 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 45 s, followed by incubation at 72°C for 1 min. Each PCR was performed with an amount of cDNA equivalent to 100 ng of RNA, and PCRs for the p16/CDKN2 and p15INK4B genes contained 10% DMSO. Under the above conditions, all amplifications were in the linear range of the assay (data not shown). PCR products were resolved on 2% agarose gels. DNA was transferred to a nylon membrane (Zetaprobe; Bio-Rad, Richmond, CA) via alkaline transfer. p16/CDKN2 PCR products were hybridized to radiolabeled p16/CDKN2 cDNA. p15INK4B PCR products were hybridized to a radiolabeled or digoxigenin-labeled internal oligonucleotide. GAPDH PCR products were radiolabeled GAPDH cDNA. Quantitation of PCR products was performed by scanning autoradiographs with an LKB UltraScan XL Laser densitometer. The ratio between p16/CDKN2 and GAPDH and p15INK4B and GAPDH signals was obtained for each sample. All reactions were done at least twice, and all were controlled with the omission of reverse transcriptase.

PCR-based Methylation Assay. A PCR assay relying on the inability of some restriction enzymes to cut methylated sequences (28) was used to analyze the methylation status of the first and second exons of the p16/CDKN2 and p15INK4B genes. The sites examined were: one FnuDII, one SacII, one HpaII, and two CfoI sites in exon 1 of p16/CDKN2; and two NaeI sites in exon 1 of p15INK4B. Due to the high sequence similarity, identical restriction sites could be examined in exon 2 of p16/CDKN2 and p15INK4B which included one Smal, four HpaII, and six CfoI sites. DNA digests were performed according to the manufacturer’s directions (Boehringer Mannheim, Indianapolis, IN). DNA (1 μg) was digested for 2 h, with 10 units of enzyme/μg of DNA. Fifty ng of the digested DNA was amplified with primers flanking the restriction sites. The PCR-based methylation assay of p16/CDKN2 exon 1 was 5’-AGC CTG CTG ACT GGC TGG-3’ (sense) and 5’-CTG GAT CGG CCT CCG ACC GTA-3’ (antisense), under the following conditions: 94°C for 3 min, 21 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 40 s, followed by incubation at 72°C for 1 min. Primers used for p16/CDKN2 exon 2 were 5’-CTG CTT GGC GGT GAG GGC G-3’ (sense) and 5’-CTG CAC ATG AGC GAC CCT C-3’ (antisense); conditions were the same as for exon 1, except for an annealing temperature of 57°C. The primer set used for methylation analysis of p15INK4B exon 1 was 5’-AGC TCG CTG ACT GGC TGG-3’ (sense) and 5’-CTG GAT CGG CCT CCG ACC GTA-3’ (antisense). Conditions for p15INK4B were 95°C for 3 min, 21 cycles of 94°C for 1 min 40 s, 58°C for 45 s, and 72°C for 45 s, followed by incubation at 72°C for 2 min. Primers for p15INK4B exon 2 were 5’-TCT TTA AAG GTC CCC ACC T-3’ (sense) and 5’-CTG CAC ATG AGC GAC CCT C-3’ (antisense); conditions were the same as for p15INK4B exon 1 amplifications. Under the indicated PCR conditions, amplifications with each primer set were in the linear range of the assay as determined by cycle curves and DNA concentration curves performed to establish the optimal conditions of the assay (data not shown). PCR products were resolved on 2% agarose gels, transferred to a nylon membrane, and hybridized as described for the RT-PCR products.

PCR-based methylation analysis using restriction enzymes may be subject to variability if the DNA digests are not complete and if amplifications are performed for an excessive number of cycles. To rule out the possibility of incomplete restriction, all samples were digested twice with each of the enzymes in independent experiments. PCR amplifications from each of the duplicate digests were repeated at least twice to ensure reproducibility of the results. We also performed PCR with various sets of primers flanking the same restriction sites in other regions to assess complete digestion. To prevent overcycling, the cycle number was determined for each of the primer sets by performing a cycle curve using undigested template and template digested with MspI restriction enzyme, which is methylation insensitive. The optimal cycle number was determined to be that which produced a detectable band of minimal intensity after hybridization of PCR products from the undigested template and that did not produce any signal from the MspI-digested DNA. An undigested DNA control and a MspI-digested DNA control were included for every site examined.

Results

Methylation and Expression of the p16/CDKN2 and p15INK4B Genes in Primary Bladder TCCs and Cell Lines. The p16/CDKN2 and p15INK4B genes both contain CpG islands within their first and second exons based on the criteria of Gardiner-Garden and Frommer (29), since they have a G+C content over 50%, and an observed/expected CpG dinucleotide ratio greater than 0.6. The methylation status of several CpG sites within the first and second exons of p16/CDKN2 and p15INK4B was examined in normal bladder mucosa, 18 uncultured TCCs of the bladder, and 3 bladder carcinoma-derived cell lines. The sites were analyzed by a PCR-based assay (28) using methylation-sensitive endonucleases for DNA cleavage, followed by PCR amplification of the digested DNAs (Fig. 1). The normal urothelium showed no methylation in either exon of either gene, because no PCR product was generated following digestion with the corresponding enzyme (Fig. 1). In contrast, 2 of 3 (67%) cell lines and 12 of 18 (67%) uncultured TCCs showed de novo methylation of exon 1 of p16/CDKN2, since PCR products were generated after cutting with the methylation-sensitive enzymes FnuDII and SacII (Fig. 1). Fig. 1 also shows that while no methylation was observed in exon 2 of p16/CDKN2 in the normal bladder mucosa, extensive methylation of this CpG island occurred in the bladder tumors. Exon 2 of p16/CDKN2 was found to be de novo methylated in 15 of 18 (83%) uncultured TCCs and in 3 of 3 cell lines (Table 1).

RNA for expression analysis was available from normal urothelium, three of the uncultured tumors, and three bladder cell lines. A good correlation between methylation of exon 1 of p16/CDKN2 and transcriptional inactivity of the gene was found as one of the three tumors (Fig. 1), and two of three cell lines showed methylation of exon 1 with no expression of p16/CDKN2, whereas the normal urothelium (Fig. 1), two of three tumors, and one of three cell lines showed
no methylation of exon 1 and high levels of p16/CDKN2 mRNA. In contrast, no correlation was found between the methylation status of exon 2 and expression levels of p16/CDKN2 or between expression and methylation of either exon of the p15INK4B gene in uncultured TCCs and bladder cell lines.

**Methylation and Expression of the p16/CDKN2 and p15INK4B Genes in Colon Carcinomas and Adjacent Normal Mucosa.** Fig. 1 shows a representative example of the 10 colon cancer cases examined, from which normal colonic epithelium and the corresponding colon tumor were available. In contrast to the lack of methylation seen in the normal bladder mucosa, all five sites examined in exon 1 of p16/CDKN2 were fully methylated in this normal colonic mucosa presented in Fig. 1. In contrast, no PCR product was obtained from the colon tumor DNA of the same individual following enzyme digestion presented in Fig. 1. In the remaining five tumors, p16/CDKN2 expression varied from undetectable (tumors G and H) to low levels (tumors F, I, and J). In cases F, I, and J, in which there was no detectable methylation in the normal mucosa and both normal and tumor tissue expressed p16/CDKN2, it is possible that the low expression levels detected in the tumor were due to contamination with normal cells. Importantly, in no case was exon 1 methylated and the p16/CDKN2 gene expressed. De novo methylation of the CpG island in exon 2 of p16/CDKN2 was observed in 7 of 10 cases (Fig. 2). Fig. 2 shows that in 7 of 10 cases, p16/CDKN2 expression was undetectable in the normal colonic epithelium of patients with colon cancer, and only 3 patients (F, I, and J) showed expression of the gene in their normal mucosa. All samples were found to contain a wild-type p16/CDKN2 gene by single-strand conformation polymorphism analysis (data not shown), and the tumors showed no up-regulation of CDK4 expression (data not shown). Furthermore, the levels of p16/CDKN2 expression were markedly increased in the tumors of 50% (5 of 10) of the patients (A to E) when compared to the normal mucosa (Fig. 2). In the remaining five tumors, p16/CDKN2 expression varied from undetectable (tumors G and H) to low levels (tumors F, I, and J). In cases F, I, and J, in which there was no detectable methylation in the normal mucosa and both normal and tumor tissue expressed p16/CDKN2, it is possible that the low expression levels detected in the tumor were due to contamination with normal cells. Importantly, in no case was exon 1 methylated and the p16/CDKN2 gene expressed. De novo methylation of the CpG island in exon 2 of p16/CDKN2 was observed in 7 of 10 cases (Table 1). Interestingly, an inverse pattern of expression of p16/CDKN2 and p15INK4B was also observed in cases A, C, D, and E, i.e., p16/CDKN2 expression was undetectable in the normal mucosa but significantly up-regulated in the corresponding tumor, whereas p15INK4B expression was higher in the normal tissue when compared to the tumor. In contrast to p16/CDKN2, no clear correlation was observed between methylation of exon 1 of p15INK4B and expression of the gene. In addition, p15INK4B mRNA was detected in most (9 of 10) of the normal colonic mucosas and, in general, decreases in the level of expression were observed in the tumors.

The methylation status and expression levels of the p16/CDKN2 and p15INK4B genes were also examined in several normal tissues including sperm, bladder urothelium, kidney, WBC, and colonic epithelium from patients without colorectal cancer (results not shown). Partial methylation of the two NaI sites within the p15INK4B exon 1
was seen in sperm. Normal bladder epithelium, kidney, and WBC were not methylated at the sites examined in either exon of either gene. However, the normal colonic epithelium from the individuals without colon cancer showed methylation of exon 1 of p16/CDKN2 (4 of 4; data not shown), which correlated with the lack of p16/CDKN2 expression in this tissue. On the other hand, p15INK4b was not methylated in either exon in this normal colon mucosa. The results showing methylation of exon 1 of p16/CDKN2 in the normal colon mucosa of individuals with normal sigmoidoscopy were similar to those obtained in one case of ulcerative colitis (data not shown) as well as in the normal colonic epithelium of 6 of the 10 patients with colon cancer examined (Fig. 2), where exon 1 of p16/CDKN2 was extensively methylated (example shown in Fig. 1; summary of results in Fig. 2).

**Methylation of Exon 1, but not of Exon 2, of p16/CDKN2 Is Associated with Transcriptional Silencing.** Table 2 is a compilation of the analyses of mRNA expression and methylation status of exons 1 and 2 of the p16/CDKN2 gene conducted in a total of 30 specimens from which both RNA and DNA was available, including 4 normal tissues, 10 colon cancer cases (normal and tumor from each case), 3 bladder tumors, and 3 bladder cell lines. Overall, exon 1 of p16/CDKN2 was methylated in 11 of 30 (37%) specimens, and remarkably, no case revealed expression of the gene with methylation of this exon. On the other hand, exon 1 was not methylated in 17 of 30 (57%) specimens that expressed p16/CDKN2, while the remaining 2 specimens did not show methylation of exon 1 nor expression of the gene. The correlation between methylation of both the FnuDII and SacII sites in exon 1 and transcriptional silencing of p16/CDKN2 was statistically significant (P = 0.00001). In contrast, there was not a significant association between methylation of exon 2 and transcriptional repression of p16/CDKN2 (P = 0.340), since 12 of 30 (40%) specimens showed extensive methylation of exon 2 with p16/CDKN2 expression (Table 2).

**Discussion**

Our results show a highly significant correlation (P = 0.00001, two-sided) between methylation of exon 1 of p16/CDKN2 and transcriptional silencing of the gene, since none of the normal and tumor specimens that showed methylation of this exon (11 of 30) expressed p16/CDKN2, and 17 of 30 specimens expressed p16/CDKN2 without evident methylation of exon 1 (Table 2). These data agree with results reported recently (30, 31) and suggest that methylation of the 5' CpG island of p16/CDKN2 might be a mechanism to control expression of this tumor suppressor gene. It remains to be seen whether the methylation is causally related to the silencing of expression; however, there are numerous examples in the literature where methylation of 5' CpG islands strongly suppresses gene activity (16, 17, 22, 23). Additional evidence that methylation of the 5' CpG island is involved in p16/CDKN2 silencing comes from experiments showing the induction of p16/CDKN2 expression in cell lines after treatment with the inhibitor of DNA methylation 5-aza-2'-deoxycytidine.

We are presently examining exon 1 and the upstream region in more detail to investigate the potential for this de novo methylation to silence p16/CDKN2 expression.

Our analysis of uncultured bladder TCCs and bladder cell lines indicates that the 5' CpG island of the p16/CDKN2 tumor suppressor gene is frequently methylated in this tumor type. The high frequency (67%) of uncultured tumors showing de novo methylation of the p16/CDKN2 5' CpG island could explain the low rate at which p16/CDKN2 homozygous deletions and intragenic mutations are found in bladder TCC (13, 14). Thus, our data indicate that, in contrast to squamous cell carcinomas of the bladder in which p16/CDKN2 is frequently deleted (15), de novo methylation of the 5' CpG island of p16/CDKN2 may be a more common mechanism of inactivation of this tumor suppressor gene in TCC.

Our study is the first to show that methylation of the 5' CpG island of p16/CDKN2 occurred not only in bladder tumors and bladder cell lines but also in normal colon mucosa. In contrast to other normal tissues examined, methylation of this region was observed in the normal colonic epithelium from four patients who had segments of the colon removed for reasons other than cancer development and also in the normal colon mucosa of several (6 of 10) patients with colon cancer (Fig. 2). This is striking as methylation of CpG islands in autosomal genes is generally restricted to genes located on the inactive X chromosome (19), imprinting genes (21), or genes that undergo de novo methylation during cell line immortalization and tumorigenesis (22, 23). A study by Herman et al. (31) in this issue of Cancer Research reports the absence of p16/CDKN2 methylation in normal colon mucosa based on Southern analysis. Several possibilities may explain this apparent discrepancy: (a) the CpG sites examined were not the same in both studies, although they are all located within the 5' CpG island of p16/CDKN2; (b) Southern analysis may be less sensitive in detecting methylation changes than the PCR-based assay used in our study; and (c) the normal colon samples examined in our
study were enriched for colon mucosa cells (see “Materials and Methods”). To date, methylation of CpG islands of autosomal genes in normal tissues has only been reported for the estrogen receptor gene in normal colonic mucosa, where methylation of this gene was observed to arise as a direct function of age (17). However, we did not observe any association between methylation of p16/CDKN2 in normal colonic mucosas and the age of the patients in our study. It will be important to determine if the methylation and transcriptional silencing of p16/CDKN2 observed in some colon specimens are involved in normal cell function or are precursors to tumorigenesis.

The results obtained with the normal colon and matched tumor pairs were surprisingly different to those obtained with the bladder TCCs. In 50% of the colon normal/tumor matched cases, p16/CDKN2 was methylated and not expressed in the normal tissue but unmethylated and highly expressed in the tumor tissue. Variable results regarding the expression of the gene in the tumor and the normal mucosa were seen in the other patients; however, in no case was expression seen in samples that demonstrated methylation of the sites examined in exon 1. Furthermore, in four of the five colon carcinomas in which p16/CDKN2 expression was strongly up-regulated, we observed a clear inverse expression pattern of the p16/CDKN2 and p15INK4B genes, with p16/CDKN2 up-regulation and p15INK4B down-regulation in the tumors when compared to the corresponding normal mucosa. p16/CDKN2 appears to accumulate in cells in which the function of the retinoblastoma susceptibility gene product (pRb) has been compromised (1). It would be of interest to examine the status of pRb in our panel of colon carcinomas, although pRb alterations have not been reported in colon cancer. Hannon and Beach (10) have shown a strong up-regulation of p15INK4B expression by TGF-β in human keratinocytes and have proposed a role for p15INK4B as an effector of TGF-β-mediated cell cycle arrest. In normal colonic epithelium, the upper 2/3 of the crypts are composed of quiescent cells that are rapidly replaced by new cells migrating from the lower 1/3 of the crypt (25). The appreciable levels of p15INK4B expression detected in the normal colonic mucosas in our study may indicate that this cell cycle regulator has a role in inducing quiescence in the majority of cells of the normal colonic epithelium. Markowitz et al. (32) have recently reported a high frequency of mutations in the TGF-β type II receptor in colon carcinomas; therefore, it would be important to examine the status of this receptor in our panel of colon carcinomas to determine if the observed p15INK4B down-regulation in a subset of these tumors correlates with TGF-β type II receptor mutations.

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