

Analysis of Adenomatous Polyposis Coli Promoter Hypermethylation in Human Cancer¹

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Abstract

Germ-line mutations in the tumor suppressor gene *APC* are associated with hereditary familial adenomatous polyposis (FAP), and somatic mutations are common in sporadic colorectal tumors. We now report that methylation in the promoter region of this gene constitutes an alternative mechanism for gene inactivation in colon and other tumors of the gastrointestinal tract. The *APC* promoter is hypermethylated in 18% of primary sporadic colorectal carcinomas ($n = 108$) and adenoma ($n = 48$), and neoplasia with *APC* methylation fails to express the *APC* transcript. Methylation affects only wild-type *APC* in 95% of cases and is not observed in tumors from FAP patients who have germ-line *APC* mutations. As with *APC* mutation, aberrant *APC* methylation occurs early in colorectal carcinogenesis. When other tumor types are analyzed ($n = 208$), methylation of the *APC* promoter is not restricted to the colon but is present in tumors originating elsewhere in the gastrointestinal tract but rarely in other tumors. Our data suggest that hypermethylation of *APC* provides an important mechanism for impairing *APC* function and further underscores the importance of the *APC* pathway in gastrointestinal tumorigenesis.

Introduction

Germ-line mutations in the *APC*³ tumor suppressor gene occur in at least 90% of patients with FAP (1–3). FAP is an autosomal dominant disease in which affected individuals develop hundreds to thousands of benign colorectal adenomas. Some of these benign lesions inevitably progress into malignant carcinomas. Although colorectal polyps are the hallmark manifestations of FAP, patients may also present with pigmented ocular fundic lesions or tumors within other regions of the GI tract, the abdominal cavity, or the brain and thyroid gland (4). According to Knudson's two-hit hypothesis for tumor suppressor gene inactivation, tumors developing in FAP patients must have both alleles of *APC* inactivated. Indeed, when tumors from FAP patients with characterized germ-line *APC* mutations are examined, somatic *APC* mutations ("second hits") have been identified in 75% of the cases retaining both alleles (5, 6).

The majority of *APC* mutations causing FAP result in truncation of the *APC* protein (1, 2). The defective *APC* protein produced in these cases is not able to bind and degrade β -catenin, and the resultant

increased β -catenin levels lead to activation of growth-promoting genes such as c-myc via the action of increased β -catenin/Tcf-4 transcription complexes (7, 8). *APC* mutations are emblematic of the common paradigm that genes mutated in cancer families are frequently mutated in sporadic tumors of the same type. Indeed, *APC* somatic mutations are present in 80% (9, 10) of sporadic colorectal carcinomas and appear very early in colorectal tumor progression (9). However, some of these nonfamilial tumors with *APC* mutations lack a second identifiable inactivating event on the remaining allele, and up to 20% of tumors have no *APC* mutation. In addition to colorectal cancer, *APC* somatic mutations have been occasionally described in other gastrointestinal tumors, including gastric, pancreatic, esophageal, and hepatic carcinomas (11–14). However, the rates of mutation of *APC* are much lower than colorectal tumors, despite a high rate of loss of heterozygosity at the *APC* locus in these tumor types (15, 16). One explanation for the failure to detect genetic alterations in these tumors is that the *APC* is a large gene containing 15 exons, and mutational screening is technically laborious.

Alternatively, other mechanisms associated with gene inactivation, such as transcriptional silencing by promoter hypermethylation, could play a role in loss of *APC* function in those tumors in the absence of *APC* mutations or with only one "hit" occurring in the *APC* gene. Methylation is the main epigenetic modification in humans, and changes in methylation patterns play an important role in cancer. In particular, hypermethylation of normally unmethylated CpG islands located in the promoter regions of many tumor suppressor and DNA repair genes, such as *p16*^{INK4a} and *hMLH1*, correlates with loss of expression in cancer cell lines and primary tumors (17). Methylation of *APC* has been described in a subset of colorectal tumors (18, 19), but the precise position and density of this change, its functional consequences, and its tumor distribution are not known. In the present study, we demonstrate that hypermethylation frequently affects the *APC* promoter, is associated with loss of expression of the gene early in colorectal tumorigenesis, and occurs in carcinomas arising from regions of the GI tract other than the colon.

Materials and Methods

Tumor Samples. Initially, 108 colorectal carcinomas and 48 colorectal adenomas were obtained from surgical patients at the Hospital Sant Pau in Barcelona and The Johns Hopkins Hospital in Baltimore, respectively. This set of samples has been characterized previously for clinicopathological parameters and the *p14*^{ARF} and *MGMT* promoter methylation status (20). Subsequently, 66 additional colorectal carcinomas where the *APC* and β -catenin mutational status had been studied previously (7, 21) and 37 colorectal tumors from six FAP families obtained from the Hospital Sant Pau were included in the study. The samples of the remaining tumor types were obtained at the time of the surgery from the Johns Hopkins Hospital and The Greenbaum Cancer

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³ The abbreviations used are: *APC*, adenomatous polyposis coli; FAP, familial adenomatous polyposis; GI, gastrointestinal tract; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; MSI, microsatellite instability.

Center in Baltimore. The Ethics Committee in each institution approved specimen collection procedures. All of the samples were frozen in liquid nitrogen immediately after resection and stored at -70°C until processing. DNA was extracted by standard methods.

Bisulfite Genomic Sequencing of Individual Alleles. DNA was modified by sodium bisulfite as described previously (22). Using $3\ \mu\text{l}$ of resuspended, sodium bisulfite-treated DNA, PCR was performed in a $50\text{-}\mu\text{l}$ reaction (22). Reactions were hot-started at 95°C for 5 min and held at 80°C before addition of 1.25 units of Taq (Sigma). Temperature conditions for PCR were as follows: 39 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by 1 cycle of 72°C for 5 min. Primers used were 5'-ATT TAT TGT AAT TTA TTT AAT ATT ATT GTT-3' (sense) and 5'-AAC TAC ACC AAT ACA ACC ACA TAT C-3' (antisense), which amplify a 356-bp product. The 5' position of the sense and antisense primers corresponds to bp 440 and 826 of GenBank U02509, respectively. Our sequenced products include 25 CpG sites between positions -366 and -42 relative to the APC major transcription start site (23). PCR products were cloned into the TA vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and transformed into bacteria. Clones were single-colony purified. Plasmid DNA from isolated clones was purified using Wizard mini-prep (Promega Corp., Madison, WI) and analyzed by automated DNA sequencing (ABI).

MSP. DNA methylation patterns in the CpG islands of the APC gene were determined by MSP (22). The primer sequences designed for the promoters 1A and 1B of APC spanned 7 and 6 CpGs, respectively. Primer sequences of APC promoter 1A for the unmethylated reaction were 5'-GTG TTT TAT TGT GGA GTG TGG GTT-3' (sense) and 5'-CCA ATC AAC AAA CTC CCA ACA A-3' (antisense), which amplify a 108-bp product; and for the methylated reaction, 5'-TAT TGC GGA GTG CGG GTC-3' (sense) and 5'-TCG ACG AAC TCC CGA CGA-3' (antisense), which amplify a 98-bp product. The 5' position of the sense unmethylated and methylated primers corresponds to bp 696 and 702 of GenBank sequence no. U02509, respectively. Both antisense primers originate from bp 782 of this sequence. Primer sequences of APC promoter 1B for the unmethylated reaction were 5'-GAT AGA ATA GTG AAT GAG TGT TT-3' (sense) and 5'-CTT CCA ACA ACC ACA CCC CA-3' (antisense), which amplify a 195-bp product; and for the methylated reaction 5'-TAG AAT AGC GAA CGA GTG TTC-3' (sense) and 5'-TCC GAC GAC CAC ACC CCG-3' (antisense), which amplify a 190-bp product. The 5' position of the sense unmethylated and methylated primers corresponds to bp 77 and 75 of GenBank sequence no. D13981. The unmethylated and methylated antisense primers originate from bp 257 and 259 of this sequence, respectively. The annealing temperature for both the unmethylated and methylated reactions of promoters 1A and 1B was 55°C . Placental DNA treated *in vitro* with SssI methyltransferase was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as negative control for methylated genes. PCR products were analyzed as described (22).

Restriction Enzyme Analysis after Bisulfite Modification. To check for a specific cytosine methylation within the APC promoter region studied, MSP products were digested with the restriction enzymes SfaNI and AclI as described (22). The SfaNI and AclI recognition sites will remain only if the CpGs are methylated after bisulfite treatment and amplification but will be lost if the CpGs are unmethylated. *In vitro* methylated DNA was used as positive control for APC promoter hypermethylation.

RT-PCR. RT-PCR was performed as described previously (24) using 3 mg of total cellular RNA to generate cDNA. One hundred ng of this cDNA were amplified by PCR with primers for exon 1A, 5'-GAG ACA GAA TGG AGG TGC TGC-3' (sense), and exon 2, 5'-GTA AGA TGA TTG GAA TTA TCT TCT A-3' (antisense) of APC, which amplify a 170-bp product. Glyceraldehyde-3-phosphate dehydrogenase served as a positive control (24). Ten ml of each PCR reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Mutational Analysis of APC and β -Catenin. Samples with full-length expression or without prior evidence of APC mutation were analyzed for mutations in codons 680-1693. This region of APC was amplified by PCR from genomic DNA in two segments (2 and 3) as described previously (1). Segment 2 was analyzed for mutations by the *in vitro*-synthesized protein assay (1), whereas segment 3 was analyzed for mutations by DNA sequencing (9). For β -catenin, a genomic PCR fragment including codon 1 in exon 2 to codon 90 in exon 4 and encompassing the NH_2 -terminal regulatory region was

amplified as described previously (21). PCR products were gel purified and sequenced directly with internal primers using ThermoSequenase (Amersham Corp.) and ^{32}P -labeled ddNTPs (Amersham) according to the manufacturer. Each mutation was verified in both the sense and antisense directions.

Loss of Heterozygosity Analysis at the APC Gene. Allelic loss at the APC gene was determined using the D5S346 microsatellite (or D5S429 in those patients homozygous for the D5S346). PCR was performed on 20 ng each lymphocyte and tumor DNA. Before amplification, 200 ng of one primer from each pair were end labeled with [^{32}P]ATP (20 mCi/ml; Amersham Life Science, Inc., Arlington Heights, IL) and bacteriophage T4 kinase (New England Biolabs, Inc., Beverly, MA) in a total volume of $50\ \mu\text{l}$. PCR reactions were carried out in a total volume of $10\ \mu\text{l}$ containing 2 ng of labeled primer and 60 ng of each unlabeled primer. The PCR buffer included 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM β -mercaptoethanol, and 1% DMSO to which were added 1.5 mM deoxynucleotide triphosphates and 1.0 unit of Taq DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN). PCR amplifications of each primer set were performed for 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. One-third of the PCR product was separated on 8% urea-formamide-polyacrylamide gels and exposed to X-ray film for 4 to 48 h. For informative cases, LOH was scored if one allele was decreased by $>40\%$ in tumor DNA when compared with the same allele in normal DNA.

Results

Methylation of the 5'-Region of APC in Normal Cells. To dissect the methylation patterns of the 5'-region of the APC gene, we first analyzed the major APC promoter, denoted as promoter 1A, from which the main APC transcript is initiated (23). A detailed methylation pattern of the desired area can be obtained by the use of bisulfite genomic sequencing. Primers suitable for bisulfite genomic sequencing spanned 25 CpG from positions -366 to -42 bp in the promoter 1A of APC. Sequencing of DNA from normal lymphocytes revealed a virtual absence of methylation in any of 25 CpG sites included in this fragment from nine different clones (Fig. 1A), as expected for a CpG island. To extend the study to additional normal tissues, MSP primers were designed in this area and used to screen other normal tissues, including colon, stomach, lung, breast, brain, esophagus, and kidney ($n = 16$). No APC promoter hypermethylation was observed in any case (Fig. 1B).

We also studied the methylation status of promoter 1B, located 3' to the major APC promoter. A minor APC transcript initiates from this region (23), and promoter 1B is also CpG rich and resides far enough from the promoter 1A that it can be considered a separate CpG island. The methylation status at promoter 1B was studied using a set of MSP primers covering six CpG sites. All normal tissues analyzed, including normal colon, stomach, lung, breast, brain, esophagus, kidney, and lymphocytes, were unmethylated in this region.

APC Promoter Hypermethylation in Primary Colorectal Tumors. DNA from 108 primary colorectal carcinomas was evaluated for APC promoter 1A methylation using MSP. APC promoter hypermethylation was present in 20 of 108 (18%) of these tumors (Fig. 2A). In 28 patients where DNA from normal adjacent colorectal mucosa was available, no methylation of the APC promoter was observed in any matched case, even among 17 cases where the adjacent tumor had aberrant APC methylation (Fig. 2B). The presence of methylation was confirmed by bisulfite-restriction cut analysis using two different enzymes, as outlined above. For two of the tumors designated as hypermethylated by MSP, we also performed bisulfite genomic sequencing of individual alleles. Most allelic clones demonstrated a dense methylation pattern, with almost every CpG methylated (Fig. 3, B and C). In both tumors, a few clones were found completely unmethylated, and these were most likely derived from contaminating normal cells such as normal colon epithelium, fibroblasts, or lympho-

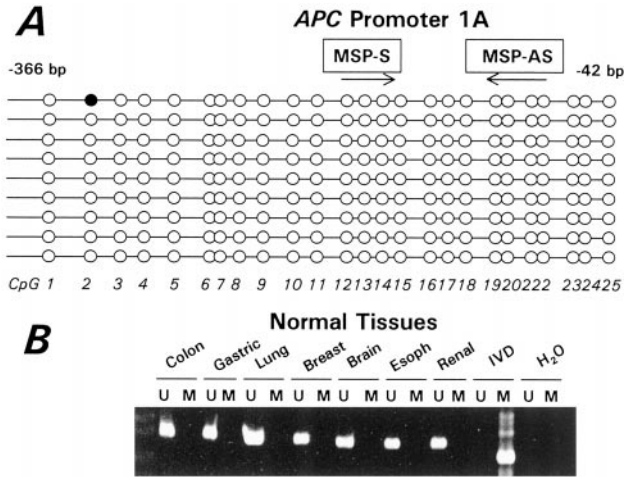


Fig. 1. A, schematic representation of the sodium bisulfite genomic sequencing of the CpG island in the APC promoter 1A. The localization of the MSP primers sense (MSP-S) and antisense (MSP-AS) are indicated. Each row represents an individual cloned and sequenced allele after sodium bisulfite DNA modification. CpG sites are marked as circles and drawn to accurately reflect CpG density of the region. ○, unmethylated CpG; ●, methylated CpG. The CpG island located in the APC promoter 1A is unmethylated in normal lymphocytes. B, methylation-specific PCR of APC promoter 1A in normal tissues [colon, stomach, lung, breast, brain, esophagus (Esoph), and kidney (Renal)]. PBR322/Msp digest are shown at left as molecular weight markers. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes; the presence of product in those lanes marked M indicates the presence of methylated genes. *In vitro* methylated DNA (IVD) was used as positive control for methylation, normal lymphocytes were used as negative control for methylation, and water was used as negative PCR control. All of the normal tissues analyzed are unmethylated at the APC promoter 1A.

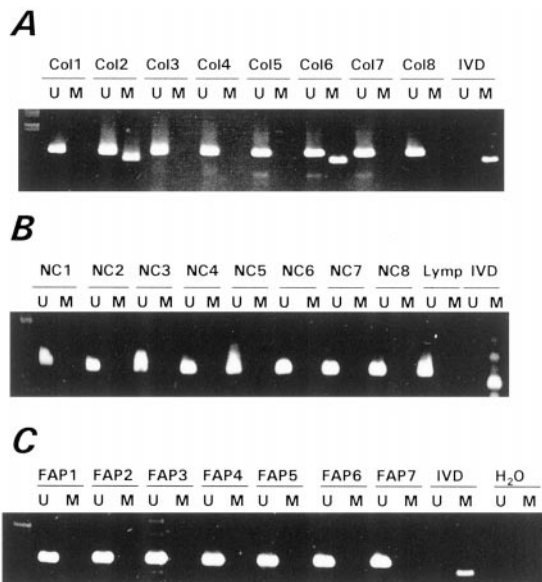


Fig. 2. A, MSP of APC promoter 1A in primary colorectal tumors. Samples in columns 2 and 6 (Col2 and Col6) show APC promoter hypermethylation. B, MSP of APC promoter 1A in the corresponding normal colorectal mucosa. All samples are unmethylated at the APC locus. C, MSP of APC promoter 1A in primary colorectal tumors from FAP families. All of these have an unmethylated APC promoter.

cytes. Indeed, bisulfite genomic sequencing of APC in the normal colorectal mucosa from one of the two patients with hypermethylated tumors revealed an almost completely unmethylated CpG island in all of the alleles studied (Fig. 3A). Bisulfite genomic sequencing for one colon cancer that was unmethylated at APC by MSP revealed a completely unmethylated CpG island (Fig. 3D). Thus, APC promoter hypermethylation is a tumor-specific change that densely affects the APC promoter 1A in some primary colorectal carcinomas.

We also assessed whether the APC 1B promoter was aberrantly methylated in tumors. We analyzed 20 primary tumors by MSP, of which 10 were hypermethylated at the 1A promoter and 10 were unmethylated. No aberrant methylation at the 1B promoter was observed, suggesting a minor role, if any, of epigenetic lesions at this promoter.

Correlates of APC Methylation. Abnormal methylation of the APC 1A promoter region in the colorectal carcinomas was not associated with significant differences of gender, age of onset, clinical status, Duke's stage, DNA ploidy, or the presence of residual disease after initial surgical excision of primary tumor (Fisher's exact test two-tailed, $P > 0.05$). Furthermore, APC promoter hypermethylation was also not associated with aberrant methylation of other genes analyzed previously in some of these primary colorectal carcinomas (24, 25), including the cell cycle inhibitor *p16^{INK4a}*, the *mdm2* regulator *p14^{ARF}*, and the DNA repair gene *MGMT*. Finally, APC methylation was not more common in tumors with MSI than in MSI stable neoplasms (1 methylated of 6 MSI+ tumors versus 19 methylated of 102 MSI- tumors).

APC Hypermethylation Occurs Early during Colon Neoplasia Progression. The above data, notably the lack of association of higher Duke's stage with methylation, suggested that aberrant APC methylation might not arise in the progression of established colon carcinomas but might represent an early change, as do APC coding mutations. We examined this possibility by studying aberrant APC methylation in benign colorectal adenomas, a precursor lesion to invasive colorectal tumors. Nine of 48 adenomas (18%) demonstrated hypermethylation of APC (Fig. 4A), a frequency similar to invasive colorectal carcinomas. Furthermore, APC hypermethylation was present in both small (≤ 15 mm) and large adenomas (> 15 mm), 3 of 18 (17%) versus 6 of 30 (20%), respectively. These data point to methylation of APC as an early event in colorectal tumorigenesis.

Hypermethylation of APC and Allelic Loss. The early appearance of aberrant APC methylation in colon tumor progression suggested that promoter methylation might have important functional

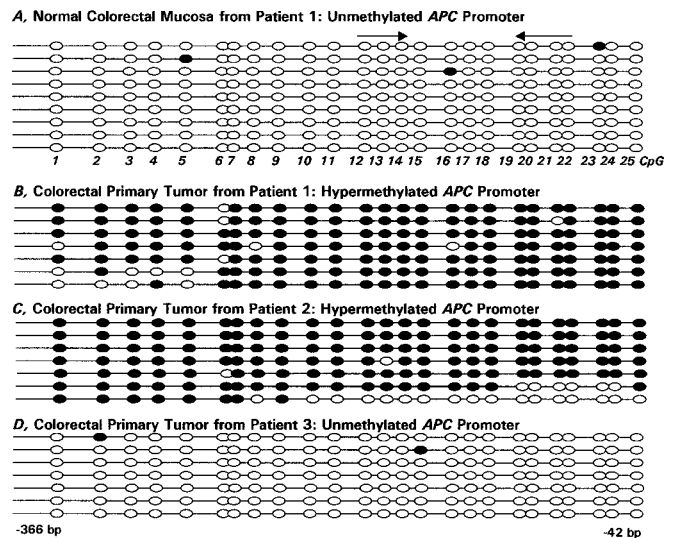


Fig. 3. Schematic representation of the sodium bisulfite genomic sequencing of the CpG island in the APC promoter 1A. A, normal colon mucosa adjacent to the tumor in B. C, a second colorectal tumor methylated at APC by MSP. D, a colorectal tumor unmethylated at APC by MSP. The localization of the MSP primers sense (→) and antisense (←) is indicated. Each row represents an individual cloned and sequenced allele after sodium bisulfite DNA modification. CpG sites are marked as circles and drawn to accurately reflect CpG density of the region. ○, unmethylated CpG; ●, methylated CpG. The CpG island located in the APC promoter 1A is unmethylated in the normal mucosa (A) adjacent to the extensively methylated tumor in B. Dense methylation is also observed in the tumor in C, whereas the tumor in D is almost devoid of methylation.

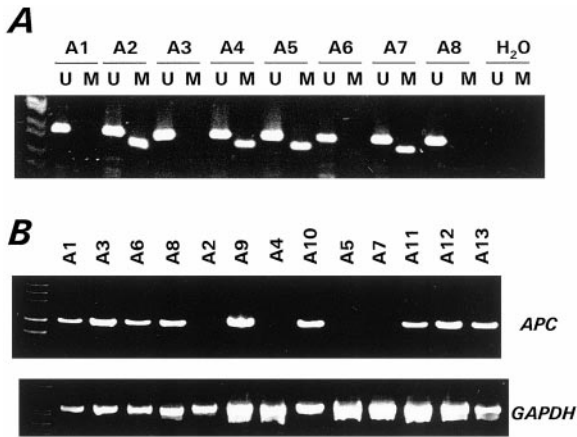


Fig. 4. A, MSP of APC promoter 1A in colorectal adenomas. Samples A2, A4, A5, and A7 show APC promoter hypermethylation. B, expression of the APC transcript determined by reverse transcription PCR in colorectal adenomas. Adenomas with APC promoter hypermethylation show complete lack of APC expression (A2, A4, A5, and A7), whereas colorectal adenomas unmethylated at APC (A1, A3, A6, A9, A10, A11, A12, and A13) show expression of the transcript. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression demonstrates that mRNA and cDNA are intact.

consequences for the tumorigenic process. To address this, we compared APC promoter hypermethylation to the allelic status of APC in a subset of these primary colorectal carcinomas ($n = 37$) using two microsatellite markers in the vicinity of the APC gene. Two tumors were homozygous at both microsatellite markers used, and four demonstrated microsatellite instability. Among the 31 informative samples, LOH at the APC locus was found in 7 of 14 (50%) colorectal tumors with APC promoter hypermethylation but was present in only 4 of 17 (24%) tumors without APC aberrant methylation. Thus, methylation of APC was not always associated with loss of the remaining allele. In hypermethylated tumors with LOH, it is possible, as will be addressed below, that both copies of APC are functionally lost with one being deleted and the other methylated. Alternatively, biallelic methylation of APC could be present in the tumors without LOH, as demonstrated for the mismatch repair gene *hMLH1* in colon tumors (26).

Functional and Mutational Context of Aberrant APC Methylation in Colon Tumorigenesis. To further determine the precise relationship in colon cancers between APC coding region mutations, allelic status, and hypermethylation, we studied the APC methylation status in an additional 66 colorectal carcinomas where the APC mutational status had been characterized previously (7, 21). It would be expected that if hypermethylation is functionally important as a loss of function event, then this change should be less frequent in those tumors with coding region mutations of the gene. APC promoter hypermethylation was present in 8 of 66 (12%) samples. When the samples were decoded for their APC mutational status, APC was hypermethylated in 5 of 19 (26%) colorectal tumors with wild-type APC but only in 3 of 47 (6%) tumors with a mutant APC (Fisher's exact test, two-tailed $P = 0.04$). Thus, APC promoter hypermethylation is biased toward tumors with genetically intact APC.

In these three tumors with APC methylation and mutation, only mutant APC protein is produced, as demonstrated by the *in vitro*-synthesized protein assay (7, 21). One possibility for this is methylation-mediated silencing of a second retained wild-type allele. LOH studies of the APC locus using microsatellite analysis demonstrated retention of both APC alleles in two of these three cases. Genomic sequencing also showed a heterozygous APC mutation, thus suggesting methylation and silencing of the wild-type allele. Methylation of

a transcriptionally silenced wild-type allele and mutation of an expressed unmethylated allele has been reported previously for *p16^{INK4a}* in the colorectal cancer cell line HCT-116 (27). In the third case, the presence of only one mutant allele was demonstrated by deletion and sequencing analysis, and it must be assumed that this allele is also methylated in the APC promoter. However, when an APC allele harbors a mutation, in 98% of the cases (46 of 47) that APC promoter remains unmethylated.

To further address the relevance of hypermethylation changes in tumor suppressor genes, one may examine the incidence of this change in tumors arising in a familial setting, where a germ-line mutation is already present. Hypermethylation should be very infrequent in this setting, because most often the second allele is deleted or inactivated by a somatic mutation in the tumor (1–3, 28). However, aberrant methylation of the remaining allele as a "second hit" for gene inactivation can occasionally occur for genes such as *VHL* and *LKB1/STK11* (17). However, in 37 colorectal neoplasms obtained from 6 FAP families, we found that none demonstrated APC hypermethylation, even in tumors where both alleles of APC were retained (Fig. 2C).

The functional consequences of APC methylation may also be correlated with the status of other components of the APC pathway. As reported previously, β -catenin is a critical downstream component of the APC pathway. The role of APC in modulating β -catenin activation would predict the diminishing need of β -catenin mutations in tumors with APC promoter hypermethylation, assuming that only one "hit" in the same pathway would be necessary during transformation. Indeed, β -catenin mutations are usually only found in colorectal tumors containing a wild-type APC (7, 21, 29). However, APC was hypermethylated in colorectal tumors with wild-type β -catenin (6 of 53; 11%) and tumors with mutant β -catenin (1 of 7; 14%). In this regard, although infrequent, β -catenin and APC mutations have been found in the same tumor sample (29). Furthermore, β -catenin mutations have been found in tumor types without frequent APC mutations, suggesting that β -catenin and APC mutations are not completely exchangeable genetic lesions.

One of the most critical tests of the functional significance of APC hypermethylation in colon tumors is the relationship of this promoter change to expression of the gene. To address this question, we examined the expression of the APC transcript in colorectal adenomas using RT-PCR. All 20 colorectal adenomas without APC methylation showed APC mRNA expression, whereas none of four adenomas with APC methylation had detectable APC mRNA transcripts (Fig. 4B). Thus, as demonstrated for other genes (17), promoter hypermethylation was associated with transcriptional silencing.

Pattern of APC Promoter Hypermethylation in Human Cancer. Although the mutational data from sporadic tumors pinpoints the colon as the main target for APC inactivation, tumors from other organs develop in the FAP families and LOH in the APC region is common in other tumor types. APC promoter hypermethylation was examined in 208 primary human tumors of multiple origins and was only relatively common in other gastrointestinal neoplasia, including stomach (13 of 38; 34%), pancreas (6 of 18; 33%), liver (6 of 18; 33%), and esophagus (4 of 27; 15%; Fig. 5). In contrast, among other solid malignancies, APC methylation was less frequent, including tumors derived from the bladder (2 of 19; 10%), kidney (1 of 12; 8%), or breast (1 of 19; 5%) or was not observed at all: brain (0 of 10), lung (0 of 17), head and neck (0 of 10), or ovary (0 of 20). Examples are shown in Fig. 5. Thus, APC promoter hypermethylation was not restricted to colorectal tumors but included extracolonic carcinomas particularly within the GI tract.

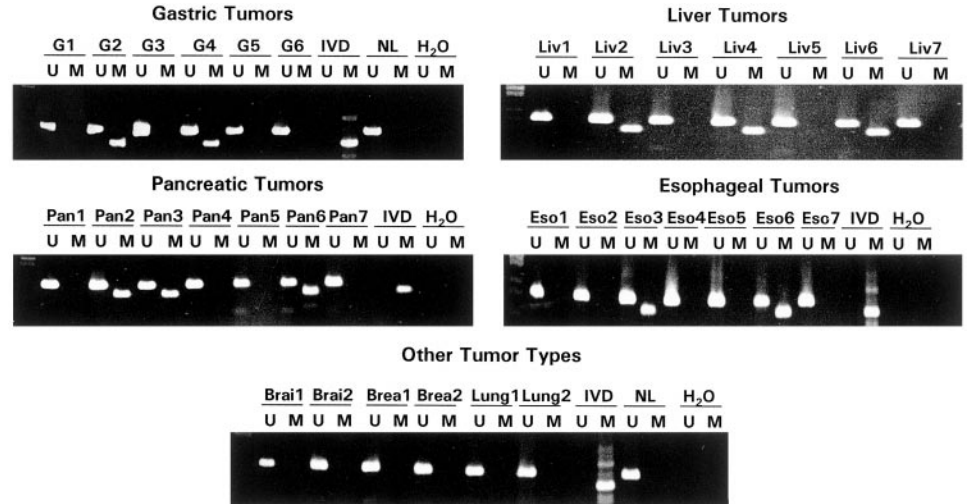


Fig. 5. APC promoter hypermethylation in human cancer using MSP of APC promoter 1A. A, primary gastric tumors. Samples G2 and G4 are hypermethylated at APC. B, primary liver tumors. Samples Liv2, Liv4, and Liv6 are hypermethylated at APC. C, primary pancreatic tumors. Samples Pan2, Pan3, and Pan6 are hypermethylated at APC. D, primary esophageal tumors. Samples Eso3 and Eso6 are hypermethylated at APC. E, other tumor types: brain (Brai1 and Brai2), breast (Brea1 and Brea2), and lung (Lung1 and Lung2). All of these are unmethylated at the APC locus. U, presence of unmethylated genes; M, presence of methylated genes.

Discussion

Mutation is the most common and principal cause of APC inactivation, particularly in colorectal tumors. Most sporadic colorectal neoplasia have a somatic mutation in APC leading to a truncated protein. In addition, LOH at the APC locus is frequent in colorectal tumors. Is there any "room" for another alteration in APC? In this work, we present evidence that APC promoter hypermethylation can complete APC loss of function in cases without apparent APC genetic alteration and as a second hit in tumors with only a "hit" in one allele. The nearly mutually exclusive hypermethylation and coding region mutations of APC in both somatic and inherited colon tumors and the correlation of hypermethylation with transcriptional loss underscore the importance of promoter methylation of this gene. Like coding region mutations of APC, hypermethylation of the promoter appears with equal frequency in both preinvasive and invasive colon neoplasia. The epigenetic event, then, appears to occupy the same "gatekeeper" position in colorectal tumorigenesis, as do genetic lesions in APC.

Although APC gene mutations are confined mostly to the colon, they may occur occasionally in other tumor types, most frequently those originating in other regions of the GI tract (11–14). APC promoter hypermethylation has a very similar distribution, although this epigenetic change is more frequent in extracolonic GI tract tumors. One possible explanation for the paucity of extracolonic mutations in APC is that mutations have been missed or not yet studied. The recent introduction of improved techniques for detection of APC mutations, such as the monoallelic mutation analysis (3) or the mass spectrometric approach (30), and the search for APC mutations in unexplored regions such as the promoter (3) will help to clarify this issue.

An alternative explanation for the observed frequencies of APC methylation is the apparent predilection for epigenetic versus genetic lesions in various tumor types. The tumor suppressor genes *p16^{INK4a}* and *p14^{ARF}* provide us with an illustrative example. Both genes are located at the 9p21 region and are common targets of homozygous deletions. However, this loss has marked variation among different tumor types. For example, lung and breast carcinomas commonly undergo homozygous deletion of this *INK4a/ARF* locus, which is uncommon in colorectal carcinoma (31). Colorectal neoplasia, however, exhibit frequent promoter hypermethylation of *p16^{INK4a}* and *p14^{ARF}* (24). Similarly, it appears that APC promoter hypermethylation is more common in gastric tumors, whereas mutation of APC is the principle mechanism for APC inactivation in colorectal neoplasia.

The presence of frequent LOH at the APC locus in GI tumors with APC methylation, *i.e.*, gastric, pancreas, liver, and esophagus (15, 16), supports a role for APC in these tumors. The current finding of APC promoter hypermethylation as a tumor-specific epigenetic inactivation further underscore the importance of the APC pathway as a critical event in neoplasia arising from other locations in the GI tract.

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