Advances in Brief

Analysis of *Adenomatous Polyposis Coli* Promoter Hypermethylation in Human Cancer[1]

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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

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 μl of resuspended, sodium bisulfite-treated DNA, PCR was performed in a 50-μl 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); 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). 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 *Sss*I 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 *Sfa*NI and *Aci*I as described (22). The *Sfa*NI and *Aci*I 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 ^{33}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[rsqbATP(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 μl. PCR reactions were carried out in a total volume of 10 μl 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. 1*A*), 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. 1*B*).

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. 2*A*). 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. 2*B*). 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-
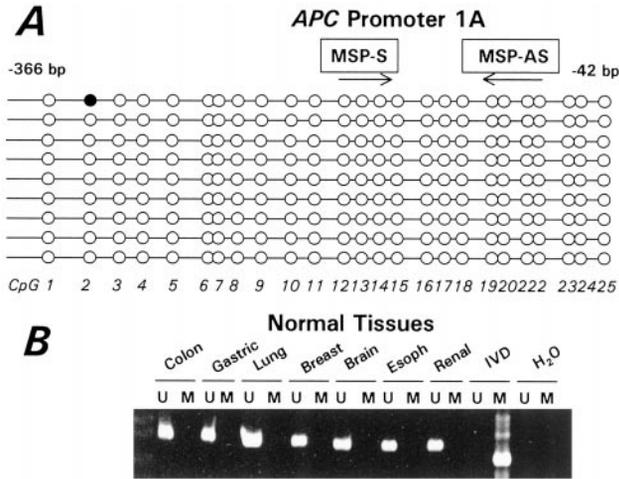
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Fig. 1. *A*, schematic representation of the sodium bisulfite genomic sequencing of the CpG island in the *APC* promoter 1A. The localization of the MSP primers sense (*MSP-S*) and antisense (*MSP-AS*) are indicated. Each *row* represents an individual 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.
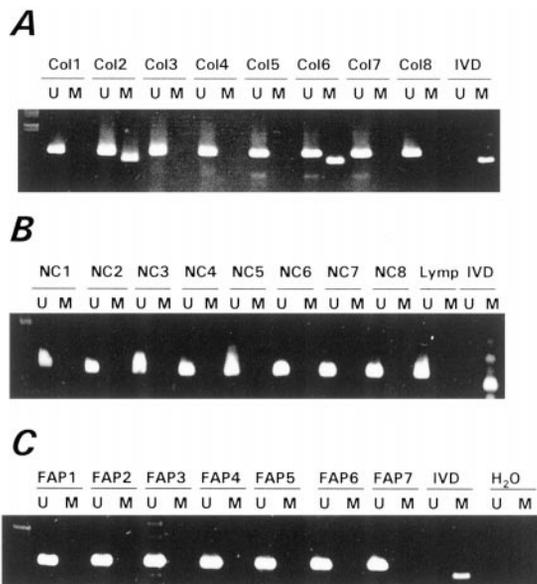

Fig. 2. *A*, MSP of *APC* promoter 1A in primary colorectal tumors. Samples in *columns* 2 and 6 (*Col2* and *Col6*) show *APC* promoter hypermethylation. *B*, MSP of *APC* promoter 1A in the corresponding normal colorectal mucosa. All samples are unmethylated at the *APC* locus. *C*, MSP of *APC* promoter 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. 3*A*). Bisulfite genomic sequencing for one colon cancer that was unmethylated at *APC* by MSP revealed a completely unmethylated CpG island (Fig. 3*D*). 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 MS 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. 4*A*), a frequency similar to invasive colon 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
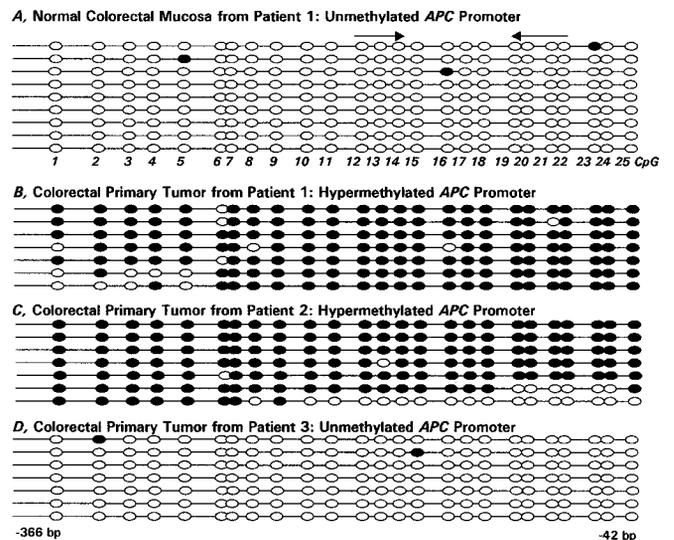

Fig. 3. Schematic representation of the sodium bisulfite genomic sequencing of the CpG island in the *APC* promoter 1A. *A*, normal colon mucosa adjacent to the tumor in *B*. *C*, a second colorectal tumor methylated at *APC* by MSP. *D*, a colorectal tumor unmethylated at *APC* by MSP. The localization 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.
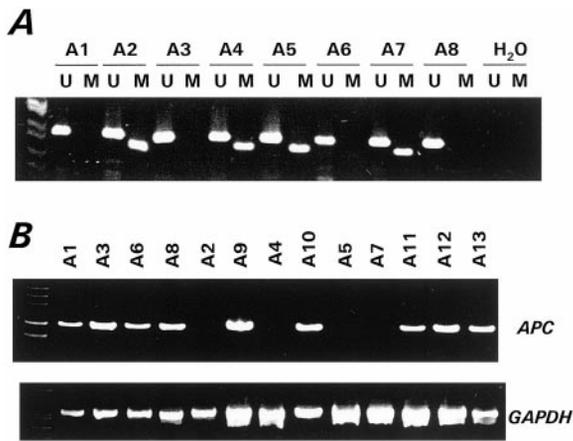
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Fig. 4. *A*, MSP of *APC* promoter 1A in colorectal adenomas. Samples *A2, A4, A5,* and *A7* show *APC* promoter hypermethylation. *B*, expression of the *APC* transcript determined by reverse transcription PCR in colorectal adenomas. Adenomas with *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* where retained (Fig. 2*C*).

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. 4*B*). 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.
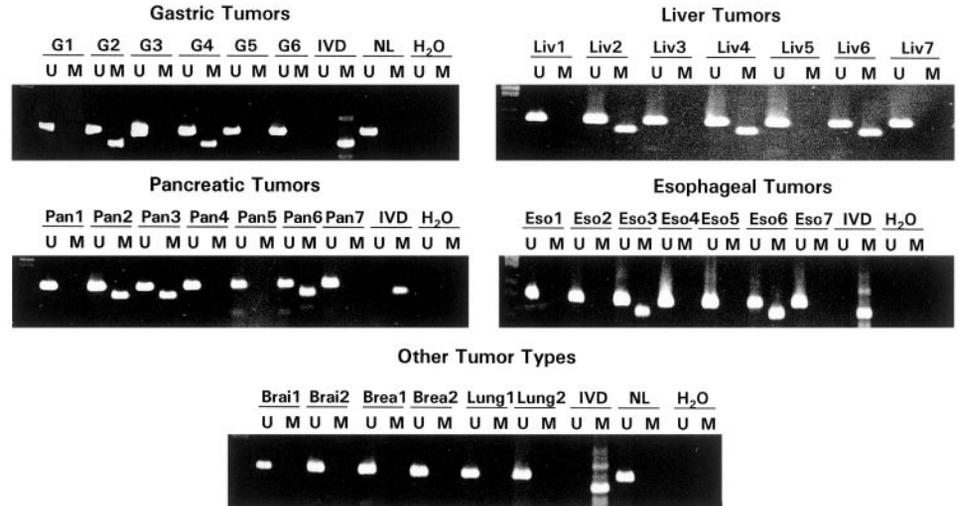
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Fig. 5. APC promoter hypermethylation in human cancer using MSP of *APC* promoter 1A. *A*, primary gastric tumors. Samples *G2* and *G4* are hypermethylated at *APC*. *B*, primary liver tumors. Samples *Liv2, Liv4,* and *Liv6* are hypermethylated at *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 "gate-keeper" 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.

Acknowledgments

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