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Frequent Inactivation of PTEN by Promoter Hypermethylation in Microsatellite Instability-High Sporadic Colorectal Cancers

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ABSTRACT

Loss of PTEN tumor suppressor function is observed in tumors of breast, prostate, thyroid, and endometrial origin. Allelic losses in the proximity of the *PTEN* locus (10q23) also occur in sporadic colorectal cancers (CRCs), but biallelic inactivation of this site has not been frequently demonstrated. We hypothesized that alternative mechanisms of *PTEN* allelic inactivation, such as promoter hypermethylation, might be operative in CRC and that *PTEN* inactivation may be related to recognized forms of genomic instability. We characterized a cohort of 273 sporadic CRCs by determining their microsatellite instability (MSI) status. Of these, 146 cancers were examined for *PTEN* promoter methylation by methylation-specific PCR. Mutations at the poly(A)$_6$ repeat sequences in *PTEN* exons 7 and 8 and deletions at the 10q23 locus were also identified using microsatellite analysis. The presence of PTEN protein was determined by immunostaining, and the results were correlated with the promoter methylation status. We observed that *PTEN* promoter hypermethylation was a frequent occurrence in MSI-high (MSI-H) tumors (19.1% of MSI-H *versus* 2.2% of MSI-low/microsatellite stable tumors; $P = 0.002$). A *PTEN* mutation or a deletion event was present in 60% of the tumors with promoter region hypermethylation. Hypermethylation of the *PTEN* promoter correlated significantly with either decreased or complete loss of PTEN protein expression ($P = 0.004$). This is the first demonstration of *PTEN* inactivation as a result of promoter hypermethylation in MSI-H sporadic CRCs. These data suggest that this silencing mechanism plays a major role in *PTEN* inactivation and, in colon cancer, may be more important than either allelic losses or inactivating mutations. The significant correlation of *PTEN* hypermethylation with MSI-H tumors further suggests that *PTEN* is an additional important "target" of methylation along with the *hMLH1* gene in the evolution of MSI-H CRCs and also confers the "second hit" in the biallelic inactivation mechanism for some proportion of tumors.

INTRODUCTION

The novel candidate tumor suppressor *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) gene (also known as MMAC/TEP1) has been recently identified and mapped to chromosome 10q23.3 (1, 2). PTEN is a dual phosphatase, acting at both serine-threonine and tyrosine sites. PTEN inhibits the activation of Akt/protein kinase B by phosphatidylinositol 3,4,5-trisphosphate (3–5), thus modulating a major pathway controlling cell proliferation and survival (6). The COOH-terminal region of the PTEN protein is required for its tumor suppressor function (5). Mutations in *PTEN* exons 7, 8, and 9 produce truncated proteins lacking the COOH-terminal region. These truncated products are rapidly degraded, resulting in loss of PTEN protein expression.

Genetic alterations at the *PTEN* locus have been described in a variety of neoplasms, including tumors of the central nervous system, thyroid, breast, prostate, and bladder and of endometrial origin. Germline mutations of *PTEN* have been detected in 80% of patients with Bannayan-Zonana syndrome, Cowden disease, and juvenile polyposis (7, 8), suggesting that *PTEN* is also an inhibitor of intestinal polyposis. *PTEN* mutations in sporadic colorectal cancers (CRCs) are uncommon (9, 10). The coding region of *PTEN* contains several repeat sequences, including two poly(A)$_6$ tracts in exons 7 and 8. Recently, mutations at the (A)$_6$ repeat of *PTEN* were reported in approximately 18% of patients with colorectal tumors showing microsatellite instability (MSI), suggesting that *PTEN* might be a target of defective mismatch repair function in colorectal carcinogenesis (11, 12). Alterations of *PTEN* in the form of allelic losses have also been observed in some colorectal tumors (13). An increasing amount of evidence suggests that *PTEN* may be inactivated by mechanisms other than mutations and/or deletions (14, 15). Studies of prostate cancer (15) and leukemia and lymphoma cell lines (14) suggest that epigenetic mechanisms may account for cases in which PTEN expression is down-regulated or even totally ablated in the absence of a detectable mutation. Recently, *PTEN* promoter methylation was identified as a mechanism of functional loss in some endometrial cancers (16). We hypothesized that alternative mechanisms of allelic inactivation might also be operative during colon carcinogenesis. We determined the frequency of *PTEN* promoter hypermethylation in colon cancer and investigated the relationship between this characteristic and tumor MSI. We also examined the extent to which *PTEN* promoter hypermethylation was either a biallelic event or constituted the "second hit" in a two-hit inactivation mechanism.

MATERIALS AND METHODS

Cohort Description. Tumor specimens were obtained from a total of 273 patients with sporadic CRC treated by physicians associated with either the Cancer and Leukemia Group B (CALGB) or the University of California San Diego. Clinical data were available on 267 of these patients. Institutional review board approval was granted for this study. The CALGB cohort with clinical data consisted of 168 individuals who received chemotherapy for colon cancer as part of CALGB Protocol 8896 (Intergroup 0089). These patients underwent surgical resection of an adenocarcinoma of the colon and were determined to have a high risk of tumor recurrence based on regional nodal disease (127 patients, stage III) or local extension of tumor with obstruction or perforation due to tumor (41 patients, stage II). All of the CALGB patients received 5-fluorouracil-based adjuvant chemotherapy. Median clinical follow-up for the CALGB cohort, by the Kaplan-Meier method, is 8.25 years. The University of California San Diego cohort contained 99 individuals with clinical data treated for colon cancer between January 1983 and November 1993. Of these 99 patients, 41 (41.8%) were stage I, 8 (8.2%) were stage II, and 33 (33.7%) were stage III, and 16 (16.3%) were stage IV. Stage could not be determined for one individual. Adjuvant chemotherapy was administered to 20.4% of the University of California San Diego patients, with one stage II

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(12.5%) and nine stage III (27.3%) patients receiving this treatment. The median clinical follow-up for the University of California San Diego patients is 8.0 years. Available median clinical follow-up for the combined patient population is 8.25 years.

DNA Extraction. Matching tumor and normal tissue specimens were obtained from all patients from a site distant from the target lesion. All specimens were reviewed by a single pathologist, and the histological type and grade of tumors were classified according to the established criteria. Paraffin-embedded primary tissue and control samples were prepared from H&E-stained 5-μm section slides. Genomic DNA from tumor and matched normal tissues was extracted by microdissection. The microdissected tissues were hydrated, digested in proteinase K, and then processed using Wizard DNA Clean-up System (Promega, Madison, WI) using standard protocols described previously (17).

MSI Determination. Microsatellite analysis was performed on tumor and corresponding normal DNA using a panel of five National Cancer Institute workshop-recommended markers including two mononucleotide (*BAT25* and *BAT26*) and three dinucleotide repeat sequences [*D2S123, D5S346,* and *D17S250* (18)]. PCR was performed using ^{32}P-labeled primers and subsequent electrophoresis on 8% polyacrylamide gels (17). Tumors exhibiting shifts in electrophoretic mobility at two or more of the five loci analyzed were classified as MSI-high (MSI-H), whereas those showing a shift in one locus were classified as MSI-low (MSI-L). The remaining tumors, lacking MSI events, were determined to be microsatellite stable (MSS). The presence of mutation of the poly(A)$_{10}$ tract of transforming growth factor β receptor II was investigated by PCR followed by PAGE as described above. The presence of bandshifts or an additional band was interpreted as a mutation.

Bisulfite Modification and Methylation-Specific PCR Assay. Genomic DNA (2 μg) was denatured with NaOH. Bisulfite treatment, during which methylated DNA is protected, and unmethylated cytosine is converted to uracil, was carried out for 16 h at 50°C on denatured genomic DNA as described previously (19). DNA samples were then purified using the Wizard DNA Clean-up System (Promega), followed by treatment with NaOH, ethanol precipitation, and resuspension in water.

The modified DNA was used as a template for methylation-specific PCR using primers specific for either the methylated or modified unmethylated sequences (19). Appropriate negative and positive controls were included in each PCR reaction. Primer sequences used to amplify a 173-bp unmethylated product were 5′-TGGGTTTTGGAGGTTGTTGGT-3′ (sense) and 5′-ACTTAACTCTAAACCACAACCA-3′ (antisense), which amplify a 173-bp product, and primer sequences for the methylated reaction were 5′-GGTTTCGGAGGTCGTCGGC-3′ (sense) and 5′-CAACCGAATAATAACTACTACGACG-3′ (antisense), generating a 155-bp product (20). Step-down PCR reactions were performed in a 25-μl reaction volume containing 1× PCR buffer (Invitrogen Life Technologies, Inc., Carlsbad, CA), 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 0.5 μM of each PCR primer, 0.75 unit of AmpliTaq polymerase, and approximately 25 ng of bisulfite-modified DNA, as described previously (19). Reactions were hot-started at 95°C for 5 min. This was followed by 33 cycles at 95°C for 45 s, 57°C for 30 s, and 72°C for 30 s, followed by a 10-min extension at 72°C in a PTC 200 DNA Engine Thermocyler (MJ Research, Inc., Waltham, MA). Human lymphocytic DNA artificially methylated by *SssI* methylase was used as a positive control for the methylated primer set in each PCR reaction, whereas untreated genomic DNA was used as a positive control for the unmethylated reaction. A water blank was used as a negative control with every PCR amplification. The amplification products were separated on a 3% agarose gel and visualized by ethidium bromide staining and UV transillumination. The results obtained by methylation-specific PCR of PTEN promoter were subsequently confirmed by bisulfite sequencing.

Table 1 *Summary of the frequency of PTEN promoter hypermethylation in sporadic colorectal cancers and its relationship with microsatellite instability status*

MSI[a] status	*PTEN* methylated	*PTEN* unmethylated
MSS	0/46 (0%)	46/46 (100%)
MSI-L	2/44 (4.6%)	42/44 (95.5%)
MSI-H	8/42 (19.1%)[b]	34/42 (81.0%)
Total	10/132 (7.6%)	122/132 (92.4%)

[a] MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, MSI-low; MSI-H, MSI-high.
[b] Significantly different when compared with MSI-L/MSS tumors ($P = 0.002$).

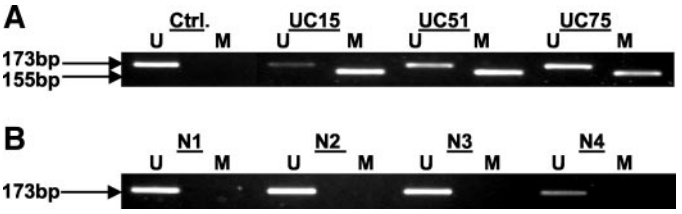

Fig. 1. Methylation-specific PCR of the *PTEN* promoter region in sporadic colorectal cancers. Bisulfite-treated DNA was amplified with methylated and unmethylated specific *PTEN* primers. The 173-bp product is indicative of an unmethylated *PTEN* allele, whereas the 155-bp product indicates a methylated *PTEN* allele. *A* shows products amplified from the sporadic cancers. The *two left lanes* (*Ctrl.*) show a control sample from normal human lymphocytic DNA, amplifying only the unmethylated *PTEN* allele. The *lanes* to the *right* show tumors with methylated products for two of the samples and one tumor with just an unmethylated product. Because the tumor cells contained normal cells, amplification of the unmethylated sequence is seen in all of the cases. *B* shows amplification of DNA from normal matched tissues from patients with sporadic cancer, all having only normal alleles.

Table 2 *Summary of the frequency of PTEN promoter hypermethylation in sporadic colorectal cancers and its relationship with TGFβRII*[a] mutational status

TGFβRII status	*PTEN* methylated	*PTEN* unmethylated
Mutation present	7/10 (70%)[b]	17/119 (14.3%)
Mutation absent	3/10 (30%)	102/119 (85.7%)
Total	10/129 (7.8%)	119/129 (92.3%)

[a] TGFβRII, transforming growth factor β receptor type II.
[b] The presence of *PTEN* promoter methylation significantly differed in tumors with and without TGFβRII mutation (Fisher's exact test, $P = 0.0003$).

Mutations in *PTEN* Coding Sequences. Mutations of *PTEN* at repetitive sequences within its coding region were identified using a PCR-based assay. A 229-bp region encompassing the *PTEN* poly(A)$_6$ repeat beginning at nucleotide 795 of exon 7 and a 200-bp region encompassing the poly(A)$_6$ repeat beginning at position 963 of exon 8 were amplified using previously published primers (21). PCR was carried out for 30 cycles, and each cycle consisted of denaturation for 30 s at 94°C, annealing for 60 s at 58°C, and extension for 45 s at 72°C. PCR products were electrophoresed on 8% polyacrylamide gels, and the shifts in bands were scored for insertion-deletion mutations.

Loss of Heterozygosity (LOH) Determination. Colon cancers in which both tumor and corresponding normal DNA were available were examined to determine the presence of allelic losses at the 10q23 locus. *PTEN* lies between *D10S1765* and *D10S541*, a genetic distance of 1 cM, but a physical distance of only several hundred kbp. *D10S1765* is within 500 kb upstream of the transcriptional start site, and *D10S541* is within 300 kb of the transcriptional stop site. We analyzed the three most commonly used polymorphic markers (*D10S541, D10S215,* and *D10S1765*) flanking and within *PTEN* to study LOH events at the 10q23 locus in all tumors showing *PTEN* promoter methylation or mutations. Additionally, 10q23 LOH status was determined for 16 patients whose tumors contained unmethylated *PTEN*. LOH was defined by ≥50% loss of intensity in one or more bands in the tumor DNA compared with the allelic pattern in the normal DNA. All three markers were screened as documented previously (22). PCR conditions for these markers have been described elsewhere (23, 24).

PTEN Expression in Tumor Specimens. The monoclonal antibody 6H2.1, raised against the last 100 COOH-terminal amino acids of PTEN (Cascade Biosciences, Winchester, MA; Ref. 25), was used to identify the presence of PTEN in tumor samples. Staining was performed on 5-μm, formalin-fixed, paraffin-embedded sections on all cases containing a methylated *PTEN* promoter. Briefly, the sections were deparaffinized and hydrated by passing through xylene and a graded series of ethanol. Microwave antigen retrieval was performed for 20 min at 98°C in 0.01 M sodium citrate buffer (pH 6.4). To block endogenous peroxidase activity, the sections were incubated in 0.3% hydrogen peroxide for 30 min. After blocking for 30 min in 5% normal serum, the sections were incubated with PTEN antibody overnight at 4°C, washed in PBS, and then incubated in a biotinylated second antibody followed by avidin peroxidase using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). The chromogenic reaction was performed with the Vector NovaRED substrate kit, and sections were counterstained with Lerner's hematoxylin. The immunostaining patterns and intensities were evaluated inde-

pendently by two investigators using endometrial stroma and/or normal colonic epithelium as an internal positive control. Cytoplasmic immunostaining intensities for PTEN equal to colonic stroma and/or normal colonic epithelium in a sample were scored as ++; weak or decreased staining intensity was scored as +; and no immunostaining was scored as −, as described previously for colon and other tissues (26).

Statistical Analyses. Associations between *PTEN* promoter methylation and protein expression status and clinical characteristics such as tumor stage, lymph node metastasis, and tumor grade were tested using the χ^2 test or Fisher's exact test, as appropriate. Associations with survival were tested using the log-rank test. Survival was measured from the date of diagnosis until death due to colon cancer (cause-specific death). Patients who died of other causes were censored at the date of death. The Kaplan-Meier method was used to estimate survival curves. Data were analyzed using SAS and S-Plus statistical software.

RESULTS

MSI in Sporadic CRCs. Using a panel of five National Cancer Institute workshop-recommended markers, 267 sporadic CRCs were examined for the presence of MSI (18). Informative results, defined as successful amplification of at least four of the five markers, were obtained for all of the samples studied. Of the 267 CRCs examined, 42 (15.7%) harbored altered allelic lengths in two or more markers, which were defined as being positive for MSI-H. As expected, a majority of these tumors revealed MSI at the *BAT26* marker, which has been suggested to be highly specific for determining the MSI-H phenotype in sporadic colon cancers (27). The remaining 225 tumors showed altered allelic lengths at one marker and were defined as MSI-L (n = 53; 19.8%), or they were not mutated at any of the five microsatellite markers and were classified as MSS (n = 172; 64.4%).

Frequent Hypermethylation of the *PTEN* Promoter in Colon Cancers. The CpG island of *PTEN* spans approximately 406 bp, and the sequences immediately 5′ to the transcription start site contain multiple putative binding sites for transcription factors, including AP2, AP4, E2F, and SP1, which are sensitive to methylation. The region between nucleotides −405 and −104 from the transcription site of *PTEN* has a 72% GC content, which fulfills the criteria for a CpG island (16). Of the 267 cancers included in our study cohort, 146 tumors were selected for *PTEN* promoter methylation analysis. These tumors included all MSI-H and MSI-L cancers, as well as a comparable number of randomly chosen MSS tumors for comparison. Informative results were obtained from 130 cancers. Ten of 132 informative tumors (7.6% of this selected population) showed hypermethylation of the *PTEN* promoter (Table 1). Fig. 1*A* shows the results of amplification using methylated and unmethylated specific primers. The 173-bp band represents unmethylated *PTEN* and is present in all tumors because, despite microdissection, these samples still contain normal cells as well as tumor cells. The 155-bp band represents the methylated *PTEN* promoter. To investigate the possibility that *PTEN* promoter methylation is a cancer-specific (28) event, we studied normal colonic cell DNA from an additional 20 individuals with no evidence of tumor (data not shown). None of these normal samples showed aberrant methylation of the *PTEN* promoter, indicating that the observed methylation is cancer specific and might have played a role in the progression of these neoplasms (Fig. 1*B*).

In this study, we grouped MSI-L and MSS tumors together for

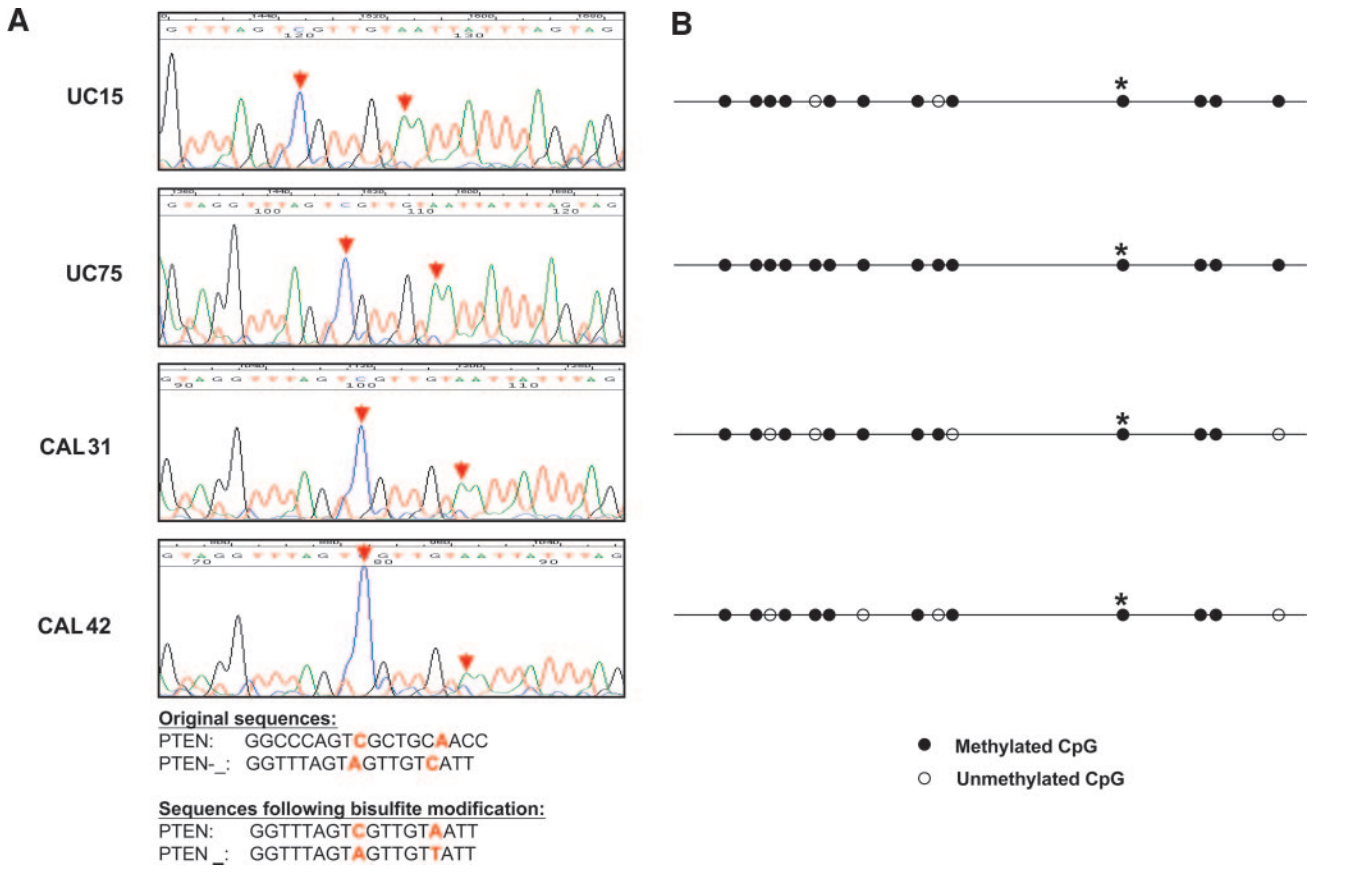

Fig. 2. Bisulfite sequencing data of the PTEN promoter region. Bisulfite sequencing of the PTEN promoter region was performed using Set-III primers described by Zysman *et al.* (20) on the cases that were found to be methylated by methylation-specific PCR assay. *A,* four of the cases that were analyzed for bisulfite sequencing clearly demonstrate that the sequence shown in the electropherograms matched the *PTEN* gene at the two critical nucleotide positions (marked with *arrows*). *B,* methylation profile of each of the 13 individual CpG sites of the PTEN promoter region showing that majority of these sites are methylated (●) and that only a couple of them were unmethylated (○), which was in agreement with the methylation-specific PCR results. The CpG site marked by an *asterisk* represents the same CpG site marked by an *arrow* on the *left* in the electropherograms.

comparison purposes because both have similar molecular and clinical features and do not differ in clinical outcome (29). *PTEN* promoter hypermethylation was significantly associated with MSI-H tumors (19.1% of MSI-H *versus* 2.2% of MSI-L/MSS tumors; Fisher's $P = 0.002$), as depicted in Table 1. In keeping with this, 70% of tumors with *PTEN* promoter hypermethylation also contained a mutation in the poly(A)$_{10}$ tract of transforming growth factor β receptor II, a defect found in only 30% of tumors without *PTEN* promoter hypermethylation (Fisher's $P = 0.0003$; Table 2).

The methylation-specific PCR data were further confirmed by performing bisulfite sequencing on some of the sporadic colon cancers methylated for the PTEN promoter. These data clearly showed that after bisulfite sequencing, each of the four cases showed sequences specific to the *PTEN* gene and not the pseudogene (Fig. 2*A*). As shown in this figure, there are only two critical nucleotides in the *PTEN* gene and the pseudogene that can discriminate between the two sequences, and in each of these cases the sequences obtained were specific for the *PTEN* gene and not the pseudogene. These data also clearly showed that there was heavy methylation of the *PTEN* gene promoter because the majority of the individual CpG sites were methylated in each of these cases (Fig. 2*B*).

***PTEN* Mutations in Sporadic CRCs with Hypermethylation.** *PTEN* mutation is an infrequent finding in colon cancer, but it may be more common in MSI-H tumors because mutations at the (A)$_6$ repeat of *PTEN* were reported recently in 18–21% of colorectal tumors with MSI-H (11, 12). To determine whether *PTEN* promoter hypermethylation might function as a "second hit" in a biallelic inactivation mechanism, mutations at the poly(A)$_6$ repeat sequences in exons 7 and 8 were determined in all tumors ($n = 10$) that were hypermethylated at the *PTEN* promoter (Table 3). For comparison, 16 tumors randomly chosen from a group of 111 tumors with unmethylated *PTEN* promoters were also analyzed (Table 3). Seven of the 26 tumors (26.9%) harbored frameshift mutations in the coding poly(A)$_6$ repeats within *PTEN*, with a predictive effect of early stop codon insertion and a resultant failure to translate the PTEN protein. Mutation rates at the (A)$_6$ repeat of *PTEN* tended to be different between methylated (40%; 4 of 10) and unmethylated (19%; 3 of 16) sporadic cancers (Fisher's $P = 0.37$). The frequency of frameshift mutations was equally distributed between both exons of *PTEN*, with four tumors showing mutations in exon 7, and three tumors bearing mutations in the exon 8. All mutational events were independent in every tumor, and none of the cancers showed evidence of homozygous mutations or the presence of multiple mutations at exon 7 and 8 repeat sequences.

LOH Analysis at the 10q23 Locus in Colon Cancers Methylated at the *PTEN* Promoter. Allelic losses at the 10q23 locus are a possible mechanism of PTEN inactivation in sporadic colon cancers containing *PTEN* promoter methylation but no evidence for mutations in the poly(A)$_6$ coding tract of *PTEN*. LOH at 10q23 was observed in 23% (6 of 26) of the tumors, with at least one LOH event at one of the three markers analyzed (Table 3). We did not detect allelic imbalances in any of the normal specimens using these markers. Of all of the cases showing deletions, we observed LOH events in 83% (5 of 6) of tumors at the *D10S1765* locus, 33.3% (2 of 6) of tumors at the *D10S541* locus, and 16.6% (1 of 6) of tumors at the *D10S215* locus. Two tumors showed allelic losses at both the *D10S541* and *D10S1765* markers. Interestingly, none of the tumors with frameshift mutations at the coding poly(A)$_6$ repeat showed allelic loss at the 10q23 locus, suggesting that either a mutation or deletion might inactivate the second allele in tumors containing promoter region methylation.

PTEN Expression Correlates with the Promoter Methylation. PTEN expression was determined by immunohistochemistry of tissue specimens from all sporadic CRCs whose *PTEN* promoter methylation, *PTEN* mutation, and 10q23 LOH status were known. Many of the tumor sections also contained stroma and/or normal colonic epithelium that showed strong cytoplasmic and nuclear PTEN immunostaining, providing internal positive controls (graded ++; Ref. 26). If blood vessels were present, their endothelium also expressed PTEN strongly (graded ++). In other cases, a separate section of normal tissue from the same patient was used as a control. The majority of the colon cancers with methylated *PTEN* promoters [8 of 10 (80%)] demonstrated weak (+) or absent (−) cytoplasmic PTEN staining (Fig. 3 and Table 3). Of the 10 tumors with methylated *PTEN* promoter regions, 3 tumors (30%) showed no PTEN protein immunoreactivity (−), and 5 tumors (50%) demonstrated weak (+) cyto-

Table 3 *Summary of mutations, allelic losses, and the relationship to protein expression in sporadic colorectal cancer with the methylation status of the PTEN promoter*

Case no.	*PTEN* methylation	MSI[a] status	Mutations at (A)$_6$		LOH at 10q23			PTEN expression[b]
			Exon 7	Exon 8	*D10S215*	*D10S541*	*D10S1765*	
UC15	+	MSI-L	wt	wt	ns	LOH	LOH	−
UC29	+	MSI-H	wt	wt	ns	ns	ns	++
UC51	+	MSI-H	wt	Mut	ns	ns	ns	+
UC75	+	MSI-H	Mut	wt	ns	ns	ns	−
CAL31	+	MSI-H	Mut	wt	ni	ns	ni	+
CAL42	+	MSI-H	wt	wt	ns	ns	ns	+
CAL65	+	MSI-H	wt	wt	ns	ns	ns	++
CAL79	+	MSI-H	wt	wt	ns	ns	ns	+
CAL88	+	MSI-L	wt	wt	ns	ns	LOH	+
CAL97	+	MSI-H	Mut	wt	ni	ns	ns	−
UC9	−	MSI-H	wt	wt	ns	ns	ns	++
UC10	−	MSI-H	wt	wt	ns	ns	ns	++
UC16	−	MSI-L	wt	wt	LOH	ns	ns	+
UC20	−	MSI-L	wt	wt	ns	ns	ns	++
UC26	−	MSI-H	wt	wt	ni	ns	ns	++
UC45	−	MSI-L	Mut	wt	ns	ns	ns	++
UC61	−	MSS	wt	wt	ns	ns	LOH	++
UC62	−	MSS	wt	wt	ns	ns	ns	++
CAL7	−	MSI-H	wt	wt	ns	ns	ni	++
CAL8	−	MSI-H	wt	Mut	ni	ns	ns	+
CAL16	−	MSI-H	wt	wt	ns	ns	ns	++
CAL33	−	MSI-L	wt	wt	ns	ns	LOH	++
CAL46	−	MSI-L	wt	wt	ns	LOH	LOH	+
CAL51	−	MSI-L	wt	Mut	ns	ns	ns	++
CAL103	−	MSI-H	wt	wt	ns	ns	ns	++
CAL163	−	MSI-H	wt	wt	ns	ns	ns	++

[a] MSI, microsatellite instability; LOH, loss of heterozygosity; MSI-L, MSI-low; wt, wild-type allele; ns, no significant loss; MSI-H, MSI-high; Mut, mutated; ni, not informative.
[b] PTEN protein expression: normal staining, ++; weak staining, +; absence of staining, −.

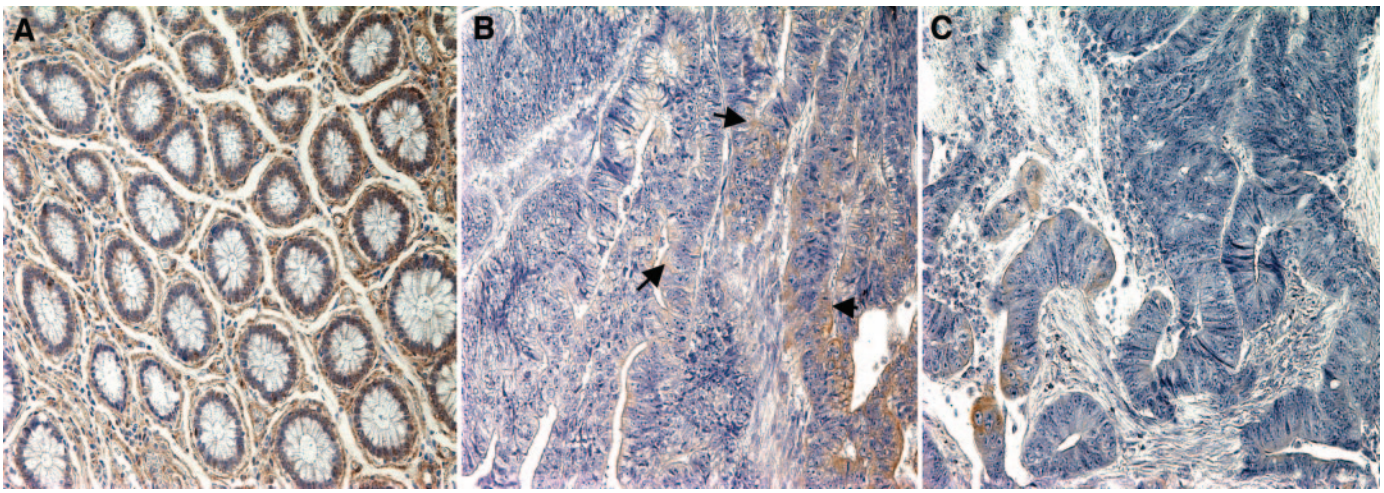

Fig. 3. PTEN immunohistochemistry in sporadic colon cancers. *A,* a sporadic cancer exhibiting positive staining (++) for PTEN in all of the normal epithelial cells. *B,* a cancer exhibiting weak staining (+) in majority of the cells (*arrows*). *C,* a cancer with negative staining (−) for PTEN in all of the tumor cells. Original magnification, ×200.

plasmic PTEN immunostaining. The remaining two tumors (20%) showed either normal (++) cytoplasmic immunostaining or had mixed tumor cell populations showing weak/absent as well as normal immunostaining intensity. None of the unmethylated sporadic colon cancers showed complete loss of PTEN expression, whereas 19% (3 of 16) had weak (+) staining, and the majority (81%) of these cancers showed normal cytoplasmic expression. All three tumors with less than ++ staining were also positive for MSI or LOH, implying that *PTEN* promoter methylation is not a necessary event and that additional genetic events may lead to decreased protein expression.

Association of *PTEN* Promoter Methylation with Clinical and Pathological Data. The relationship of *PTEN* promoter hypermethylation to clinical and histopathological variables was examined (Table 4). There was no significant correlation between *PTEN* status and any of the variables examined, although there was a trend toward a higher incidence of *PTEN* promoter methylation in tumors that were poorly differentiated and arose in a proximal location. Methylation of the *PTEN* promoter region did not influence survival probability in univariate analysis (Fig. 4). Numbers are too small to compare survival within tumor stage, although results are suggestive of poorer prognosis in stage 3 patients with methylated tumors (data not shown).

DISCUSSION

PTEN is a candidate tumor suppressor that may have a multifunctional role in regulation of cell proliferation, migration, and invasion (3, 7). Recent reports suggest that PTEN exerts an important tumor suppressor role in colorectal carcinogenesis (7, 8), but the extent and nature of PTEN loss during CRC development have not been fully characterized. Allelic deletions in *PTEN* in CRCs have been reported (13), but somatic mutations are rarely found when unselected cohorts are examined (30). Frameshift mutations at the $(A)_6$ repeats in the coding sequences of *PTEN* have been described in 20% of patients with MSI-H sporadic CRCs (11, 12). Loss of *PTEN* function has been

Table 4 *Correlation of clinical and pathological variables with PTEN promoter methylation status*

	Cohort		PTEN promoter methylated		PTEN promoter unmethylated		
		%	*n*	%	*n*	%	*P*[a]
Stage	125		9		116		0.29
1	30	24.0	2	22.2	28	24.1	
2	24	19.2	4	44.4	20	17.2	
3	63	50.4	3	33.3	60	51.7	
4	8	6.4	0	0.0	8	6.9	
Age (yrs)	126		9		117		0.47
<50	22	17.5	0	0.0	22	18.8	
50–65	40	31.8	3	33.3	37	31.6	
>65	64	50.8	6	66.7	58	49.6	
Gender	126		9		117		0.49
Male	73	57.9	4	44.4	69	59.0	
Female	53	42.1	5	55.6	48	41.0	
Race	124		9		115		0.20
White	98	79.0	9	100.0	89	77.4	
Nonwhite	26	21.0	0	0.0	26	22.6	
Differentiation	121		9		112		0.10
Well	23	19.0	0	0.0	23	20.5	
Moderate	71	58.7	5	55.6	66	58.9	
Poor	27	22.3	4	44.4	23	20.5	
Lymph nodes	114		8		106		0.28
0 LN+	48	42.1	5	62.5	43	40.6	
>1 LN+	66	57.9	3	37.5	63	59.4	
Tumor site	125		9		116		0.08
Proximal	56	44.8	7	77.8	49	42.2	
Distal	69	55.2	2	22.2	67	57.8	

[a] All *P*s are based on Fisher's exact test.

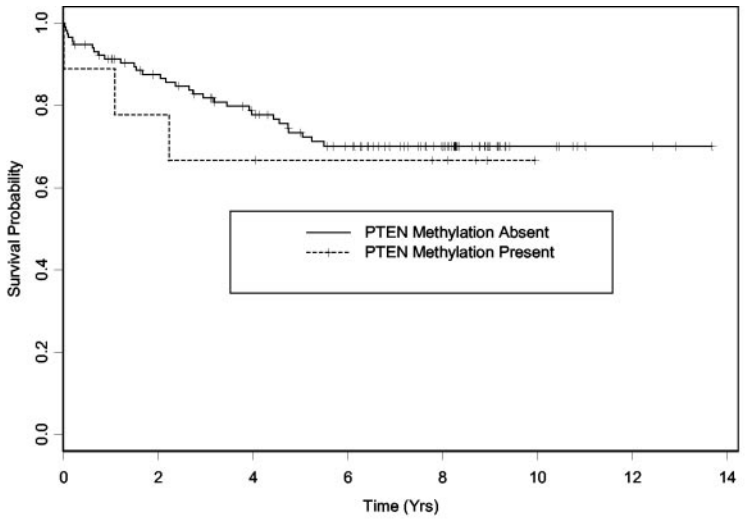

Fig. 4. Kaplan-Meier curves showing overall survival of patients with and without PTEN promoter methylation. As depicted, the presence of PTEN promoter methylation did not influence the overall survival probability in the univariate survival analysis.

Group	Total	Failed	Censored	Percent Censored
PTEN Methylation Absent	116	31	85	73.28
PTEN Methylation Present	9	3	6	66.67

Chi-Sq = 0.2093 P-value = 0.6473

related to weak or absent protein expression, but the majority of patients with this phenotype harbored monoallelic mutations and/or deletions at the *PTEN* locus (31). There has been no evidence to date of biallelic inactivation of *PTEN* during colon carcinogenesis, suggesting that additional mechanisms of gene inactivation might exist during the formation of these tumors.

Epigenetic silencing of *PTEN* by promoter methylation was initially postulated for a subset of prostate cancer cell lines and also identified as a mechanism in melanoma progression (31). The genomic sequence of PTEN is 98% identical to a highly conserved processed PTEN pseudogene (psiPTEN), and this sequence identity extends 841 bp into the promoter region. As a consequence, it was recently emphasized that extreme caution needs to be exercised in selecting primer sets that can exploit the critical nucleotide differences in the sequences between PTEN and its pseudogene when analyzing the PTEN promoter region methylation status (20). Following these recommendations, in the present investigation, we report that *PTEN* promoter methylation is a frequent event in MSI-H sporadic CRCs and may constitute an important epigenetic mechanism of PTEN inactivation. We also found that *PTEN* mutations were predominantly observed in MSI-H cancers that comprise ~10–15% of all sporadic cancers. Methylation of the *hMLH1* promoter is a mechanism of mismatch repair loss in a subset of sporadic MSI-H CRCs (32). The predicted overall frequency of *PTEN* mutations in unselected sporadic

Table 5 *Institutions participating in this study*

Institution name	Location	Principal investigator	Supported by Grant no.
CALGB[a] Statistical Office	Durham, NC	Stephen George, Ph.D.	CA33601
Dana-Farber Cancer Institute	Boston, MA	George P. Canellos, M.D.	CA32291
Dartmouth Medical School-Norris Cotton Cancer Center	Lebanon, NH	Marc Ernstoff, M.D.	CA04326
Massachusetts General Hospital	Boston, MA	Michael L. Grossbard, M.D.	CA12449
Mount Sinai School of Medicine	New York, NY	Lewis Silverman, M.D.	CA04457
Rhode Island Hospital	Providence, RI	William Sikov, M.D.	CA08025
Roswell Park Cancer Institute	Buffalo, NY	Ellis Levine, M.D.	CA02599
Southeast Cancer Control Consortium Inc. CCOP	Goldsboro, NC	James N. Atkins, M.D.	CA45808
SUNY Upstate Medical University	Syracuse, NY	Stephen L. Graziano, M.D.	CA21060
The Ohio State University	Columbus, OH	Clara D. Bloomfield, M.D.	CA77658
University of California at San Diego	San Diego, CA	Stephen Seagren, M.D.	CA11789
University of California at San Francisco	San Francisco, CA	Alan Venook, M.D.	CA60138
University of Chicago Medical Center	Chicago, IL	Gini Fleming, M.D.	CA41287
University of Illinois at Chicago	Chicago, IL	David Gustin, M.D.	CA74811
University of Iowa	Iowa City, IA	Gerald Clamon, M.D.	CA47642
University of Maryland Cancer Center	Baltimore, MD	David Van Echo, M.D.	CA31983
University of Massachusetts Medical Center	Worcester, MA	Mary Ellen Taplin, M.D.	CA37135
University of Minnesota	Minneapolis, MN	Bruce A. Peterson, M.D.	CA16450
University of Missouri/Ellis Fischel Cancer Center	Columbia, MO	Michael C. Perry, M.D.	CA12046
University of North Carolina at Chapel Hill	Chapel Hill, NC	Thomas C. Shea, M.D.	CA47559
University of Tennessee Memphis	Memphis, TN	Harvey B. Niell, M.D.	CA47555
Wake Forest University School of Medicine	Winston-Salem, NC	David D. Hurd, M.D.	CA03927
Walter Reed Army Medical Center	Washington, DC	John C. Byrd, M.D.	CA26806

[a] CALGB, Cancer and Leukemia Group B; CCOP, Community Clinical Oncology Program; SUNY, State University of New York.

cancers is 4–5%, which is less than the rate of *PTEN* promoter methylation observed in MSI-H tumors in our study (11, 12). In addition, we found a high degree of association between *PTEN* promoter hypermethylation and mutation of the $(A)_{10}$ region of transforming growth factor β receptor II. These observations suggest that these a "methylator" phenotype can produce mismatch repair deficiency, transforming growth factor β receptor II loss, and *PTEN* methylation in a subset of sporadic CRCs (28, 32).

Although our data suggest that *PTEN* may be a tumor suppressor gene target in the presence of defective mismatch repair function (11, 12), these tumors also show heterozygous *PTEN* mutations. We therefore questioned whether *PTEN* promoter hypermethylation might constitute the "second hit" in a biallelic inactivation mechanism of colon carcinogenesis. We therefore examined the relationship among *PTEN* promoter hypermethylation, *PTEN* mutations, and *PTEN* LOH events in sporadic colon cancers. Interestingly, 50% of tumors with *PTEN* promoter hypermethylation also showed either frameshift mutations at the poly$(A)_6$ repeat sequences or LOH events at regions flanking or within the 10q23 locus. The observed frequency of *PTEN* somatic frameshift mutations in MSI-H cancers (27%) was in accordance with the existing literature (11, 12). A LOH event in at least one of the two informative markers (*D10S541* and *D10S1765*) was observed in 20% of tumors with *PTEN* promoter hypermethylation. Comparable rates of LOH have been cited for these markers in prostate, ovarian, and breast cancers (33). None of the tumors with *PTEN* promoter hypermethylation contained both a mutation and a deletion. Taken together, these findings imply that, during carcinogenesis in MSI-H tumors, PTEN function is lost through a process whereby one allele is silenced by a methylation event, and the second allele is lost by a mutational event. *PTEN* mutations might also occur in sequences outside the $(A)_6$ repeats investigated in our study and thus are not detected by MSI analysis. This has to be taken into account for tumors with weak PTEN expression and no LOH or MSI events.

PTEN expression has been detected in the majority of human tissues, but its distribution is quite variable (34). The staining pattern of PTEN in the colon has not been well studied, although one report suggests that PTEN expression in the colon is very similar to that found in prostate, with punctuate, granular staining throughout the mucosal and epithelial layers (34). In the present investigation, we found that 80% of the methylated CRCs showed reduced or lost PTEN staining. We observed complete loss of staining in three cancers, all containing *PTEN* mutation and/or deletion along with promoter hypermethylation. In sporadic breast and cervical cancers, the *D10S541* marker, which is located just 3′ of *PTEN*, shows a high rate of LOH and correlates well with the loss of PTEN protein expression (35). In these colon cancers, we observed a somewhat lower rate of LOH at *D10S541* when compared with *D10S1765*, which is located at 5′ of *PTEN*. PTEN protein expression, however, correlated strongly with loss at both *D10S541* and *D10S1765*. Although two colon tumors with methylated *PTEN* promoters showed normal levels of PTEN expression, these tumors did not demonstrate either mutations of the $(A)_6$ regions of *PTEN* exons 7 or 8 or 10q23 LOH, suggesting that probably only one allele may have been methylated and that the remaining wild-type allele was responsible for the normal PTEN expression. All of the sporadic cancers containing unmethylated *PTEN* promoters showed PTEN expression, with 81% of these exhibiting normal (++) staining levels. Our data therefore suggest that loss of PTEN expression occurs primarily as a result of *PTEN* promoter hypermethylation combined with a *PTEN* mutation.

We were unable to identify significant correlations between *PTEN* status and any of the clinical variables studied in this investigation, although our data suggest that CRCs with *PTEN* promoter methylation, such as MSI-H tumors, tend to be proximally located and poorly differentiated. The cohort examined here is too small to allow a definitive conclusion concerning PTEN protein or *PTEN* promoter methylation status and clinical outcome independent of MSI status.

To the best of our knowledge, this is the first report describing epigenetic silencing of *PTEN* through hypermethylation of its promoter in a large group of sporadic CRCs. We found that epigenetic inactivation of *PTEN* strongly correlated with the presence of high levels of MSI, suggesting that *PTEN* may be targeted by the same processes creating the MSI phenotype, such as *hMLH1* inactivation. Whereas many aspects of *PTEN* function and regulation have been identified, how the stability of PTEN protein is regulated in certain tumors without *PTEN* mutations, but with low or absent protein levels, remains to be established. Studies are currently under way to address these issues.

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