

# Hypermethylation of the *p14<sup>ARF</sup>* Gene in Ulcerative Colitis-associated Colorectal Carcinogenesis<sup>1</sup>

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## ABSTRACT

The *p14<sup>ARF</sup>* protein directly inhibits the MDM-2 oncoprotein, which mediates degradation of the p53 protein. It has been shown that *p14<sup>ARF</sup>* expression is frequently down-regulated by *p14<sup>ARF</sup>* gene hypermethylation in colorectal cancer. To determine whether *p14<sup>ARF</sup>* inactivation was involved in ulcerative colitis (UC)-associated carcinogenesis, the frequency and timing of *p14<sup>ARF</sup>* methylation was investigated in four different histological stages of UC-associated carcinogenesis. Methylation-specific PCR and bisulfite sequencing were used to determine the prevalence of *p14<sup>ARF</sup>* gene methylation. *p14<sup>ARF</sup>* methylation was observed in 19 of 38 (50%) adenocarcinomas, 4 of 12 (33%) dysplasias, and 3 of the 5 (60%) nonneoplastic UC mucosae. In contrast, 3 of 40 (3.7%) normal tissues showed *p14<sup>ARF</sup>* methylation ( $\chi^2$  test:  $P = 0.0003$ ). Bisulfite sequencing was used to analyze 28 CpGs of *p14<sup>ARF</sup>* gene in 20 samples. The number of methylated CpGs ranged from 0 to 4, 0 to 20, and 0 to 28 in the normal, dysplastic, and carcinomatous samples, respectively (Kruskall-Wallis test:  $P = 0.0005$ ). Densely methylated alleles were detected only in carcinomas by bisulfite sequencing. In conclusion, our data suggest that methylation of *p14<sup>ARF</sup>* is a relatively common early event in UC-associated carcinogenesis. *p14<sup>ARF</sup>* offers potential as a biomarker for the early detection of cancer or dysplasia in UC. Finally, analyses of *p14<sup>ARF</sup>* methylation in other organs should explore not only frank cancers but other premalignant lesions.

## INTRODUCTION

UC<sup>3</sup> is a chronic disease characterized by inflammation of the mucosa and submucosa of the large intestine. The duration and extent to which a patient suffers from UC correlate directly with an increased propensity to develop colorectal carcinoma (1, 2). For patients who have had UC for >20 years, the incidence of colorectal cancer is 10–20-fold greater than that of the general population, and the average age of onset is 20 years earlier (3). UC-associated colorectal carcinoma is different from sporadic carcinoma; unlike sporadic colorectal carcinoma, which arises from adenomatous polyps, UC-associated colorectal carcinoma progresses from areas of dysplastic mucosa. Although the molecular events that facilitate the progression of adenoma to carcinoma in sporadic colorectal cancer have been well investigated (4), much remains to be learned regarding molecular events underlying the progression of UC mucosa to dysplasia and carcinoma.

The *p53* tumor suppressor gene is frequently inactivated in both sporadic and UC-associated colorectal carcinoma (4–6). Although point mutation and loss of heterozygosity are the most commonly reported mechanisms resulting in *p53* inactivation, other genetic

and epigenetic factors have been shown to modify *p53* activity as well. Amplification of the *MDM-2* gene (the protein product of which tags *p53* for degradation) and expression of viral oncoproteins (which sequester *p53*) are two such examples (7).

Recently, *p14<sup>ARF</sup>* has been ascribed a role in modulating the cellular amounts of *p53* through direct interaction with MDM-2. MDM-2, which tags *p53* for degradation through the ubiquitin/proteasome pathway, is inhibited by *p14<sup>ARF</sup>* (8). Homozygous deletion of the *p14<sup>ARF</sup>* locus has been reported in a variety of cancers (9–13), and gene knockout of *p14<sup>ARF</sup>* correlates with tumorigenesis (14). In addition to mutation, the *p14<sup>ARF</sup>* gene can also be epigenetically inactivated through hypermethylation of its normally unmethylated CpG island. Esteller *et al.* (15) demonstrated that *p14<sup>ARF</sup>* hypermethylation occurs frequently in sporadic colorectal cancer. To determine whether *p14<sup>ARF</sup>* hypermethylation occurs during the progression of UC mucosa to carcinoma, the frequency and timing of *p14<sup>ARF</sup>* hypermethylation were investigated in clinical samples ranging from nonneoplastic UC mucosa to colorectal carcinoma.

## MATERIALS AND METHODS

**Tissue Samples.** Matching normal and tumor tissues were obtained at the time of surgical resection from 40 patients with one or more UC-associated colorectal neoplasms. The UC-associated neoplasms consisted of 38 adenocarcinomas and 12 dysplasias. Normal control samples consisted of 30 ileal mucosae, 2 smooth muscle tissues, and 3 nonmetastatic lymph nodes. All tissues were grossly dissected free of normal surrounding tissue, and parallel sections were used for histological characterization. Microdissection was not performed; however, the tissues were selected to include only those tumors containing 70% or more tumor cells by H&E staining.

**DNA Extraction.** Genomic normal and tumor DNAs were extracted using published protocols (16, 17).

**MSP.** DNA methylation of *p14<sup>ARF</sup>* was determined by MSP (18). MSP distinguishes unmethylated alleles of a given gene based on DNA sequence alterations after bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracils. Subsequent PCR using primers specific to sequences corresponding to either methylated or unmethylated DNA sequences is then performed. We slightly shifted the MSP primer location used by Esteller *et al.* (Ref. 15; Fig. 1). Primers for MSP were designed using the GenBank L41934 sequence for *p14<sup>ARF</sup>*. Primer sequences of *p14<sup>ARF</sup>* for the unmethylated reaction were forward (5'-GTTTTTGGTGATTTTTTGGATTTGGT-3') and reverse (5'-TAC-CCACTCCCCATAAACACAA-3'), which amplify a 94-bp product. Primer sequences for the methylated reaction were forward (5'-GGT-GATTTTTCGGATTCGGC-3') and reverse (5'-CACTCCCCGTAAAC-CGCGA-3'), which amplify an 84-bp product. Briefly, 2  $\mu$ g of genomic DNA were denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega Corp., Madison, WI), treated with NaOH, precipitated with ethanol, and resuspended in 20  $\mu$ l of water. Two  $\mu$ l of modified DNAs were PCR amplified in a total volume of 50  $\mu$ l. The annealing temperature for both unmethylated and methylated reactions was 58°C. PCR was performed in a thermal cycler (Biometa T-Gradient Thermoblock, Goettingen, Ger-

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<sup>3</sup> The abbreviations used are: UC, ulcerative colitis; MSP, methylation-specific PCR.

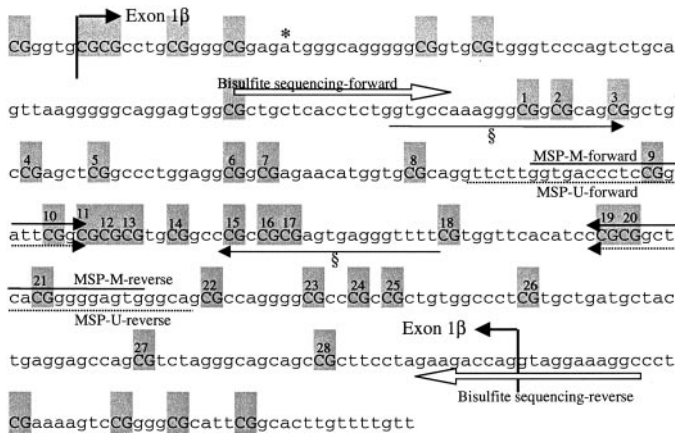


Fig. 1. Primer design for MSP and bisulfite sequencing. The original genomic sequence of *p14<sup>ARF</sup>* exon 1β, containing 6 bp of additional upstream sequence, is shown. *High-lighted large capital CGs* indicate potential methylation sites. Our MSP produced 94- and 84-bp amplicons from the unmethylated and methylated reactions, respectively (*MSP-U-forward*, *MSP-U-reverse*, *MSP-M-forward*, and *MSP-M-reverse*). Primers for bisulfite sequencing (*open arrows*) were placed in areas completely lacking CpGs to amplify both methylated and unmethylated alleles with equal efficiency. The PCR product for bisulfite sequencing contained 28 CpGs between the two primers. The MSP primers used by Esteller *et al.* (15) were also in exon 1β (§). \*, translational start site.

many) for 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, followed by a final 4-min extension at 72°C for all primer sets. PCR products were then loaded directly onto a nondenaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination. CpGenome Universal Methylated DNA (Intergen, NY) was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated genes. The sensitivity of this MSP was established by using totally methylated, positive control DNA serially diluted by normal lymphocyte DNA (18). MSPs with 1:10, 1:100, and 1:1000 diluted positive control DNA produced detectable methylated bands (data not shown).

**Bisulfite Sequencing.** To verify MSP data and investigate the distribution and density of methylated CpG sites, bisulfite sequencing was performed in 20 samples from eight patients with a range of MSP results. Primer sequences of *p14<sup>ARF</sup>* for bisulfite sequencing were forward (5'-GTTGTTTATTTTGGTGTTA-3') and reverse (5'-ACCTTTCCTACTAATCTTC-3'), which amplify a 272-bp product (Fig. 1). This area contains 28 CpG sites, 6 of which were detected by the MSP primers (forward, 9th–11th sites; reverse, 19th–21th sites). Amplified PCR products were gel purified (Gel Extraction kit; Qiagen) and ligated into the pCR4-TOPO plasmid vector using the TA-cloning system (Invitrogen). Plasmid-transformed *Escherichia coli* were cultured, and plasmid DNA was isolated (QIAprep 96; Qiagen). Purified plasmid DNA containing the *p14<sup>ARF</sup>* sequence was sequenced using an ABI 377 automated sequencer with BigDye Terminator chemistry and the M13 reverse primer. Samples that had clones with >50% methylation of CpGs were designated as partially methylated. Samples containing clones with methylation at all 6 MSP-recognition CpGs sites were deemed methylation positive. All other samples were designated as being methylation negative.

**Statistical Analysis.** A proportional analysis between the sample groups was performed using Fisher's exact probability test in 2 × 2 contingency tables and with the  $\chi^2$  or Kruskal-Wallis tests in  $n \times n$  contingency tables. All tests were two sided, and the results were considered to be significant when  $P < 0.05$ .

**RESULTS**

**MSP of *p14<sup>ARF</sup>*.** DNAs obtained from 40 patients were analyzed by MSP. *p14<sup>ARF</sup>* hypermethylation was present in 19 of 38 (50%) adenocarcinomas, 4 of 12 (33%) dysplasias, and 3 of 5 (60%) nonneoplastic UC mucosae. Conversely, 3 of 40 (7.5%) normal tissues showed *p14<sup>ARF</sup>* hypermethylation ( $\chi^2$  test:  $P = 0.0003$ ). No other correlations were found between hypermethylation status of *p14<sup>ARF</sup>* and clinicopathological features such as age, gender, histology, anatomical location of sample (right *versus* left colon), or Dukes' stage. All three methylated bands obtained from normal tissue were faint. Examples are shown in Fig. 2.

**Bisulfite Sequencing of *p14<sup>ARF</sup>*.** Two hundred thirty-two clones from 20 samples (>10 clones from each sample) were investigated using bisulfite sequencing. Bisulfite sequencing showed 3 negative, 2 partial, and 3 positive results among 8 MSP-positive samples, whereas all MSP-negative samples were sequence negative (Table 1). As shown in Table 2, no densely methylated clones were found in normal samples. An example is shown in Fig. 3. Partially methylated clones were observed in dysplastic samples, and densely methylated clones were detected only in carcinoma samples (Kruskall-Wallis test:  $P = 0.0005$ ) In 4 samples, we detected 12 clones with methylation at all 6 MSP primer-recognized CpGs.

Table 1 Comparison between MSP and bisulfite sequencing

Sample	Patient	Histology	Bisulfite sequencing <sup>a</sup>	MSP <sup>b</sup>
1	H24	Normal tissue	Negative	Negative
2		Carcinoma	Negative	Negative
3	H81	Normal tissue	Negative	Negative
4		Carcinoma	Negative	Positive
5	H82	Normal tissue	Negative	Negative
6		Carcinoma	Negative	Positive
7	H92	Normal tissue	Negative	Positive
8		Carcinoma	Partial	Positive
9	H89	Normal tissue	Negative	Negative
10		Dysplasia	Partial	Positive
11	H73	Normal tissue	Negative	Negative
12		Dysplasia	Negative	Negative
13		Carcinoma	Negative	Negative
14	H69	Normal tissue	Negative	Negative
15		Dysplasia	Negative	Negative
16		Carcinoma 1	Positive	Positive
17		Carcinoma 2	Positive	Positive
18	H26	Normal tissue	Negative	Negative
19		Dysplasia	Positive	Positive
20		Carcinoma	Positive	Positive

<sup>a</sup> Negative, very low density of methylation; partial, presence of clones, more than half of which CpGs were methylated; positive, presence of clones with all six MSP-recognition CpGs positive.

<sup>b</sup> Negative and positive, absent and present of methylated band, respectively.

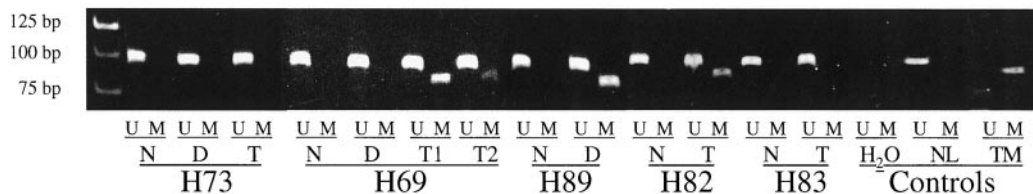


Fig. 2. MSP analysis of the *p14<sup>ARF</sup>* gene. A visible PCR product in *Lane U* indicates the presence of unmethylated *p14<sup>ARF</sup>*; a visible product in *Lane M* indicates the presence of methylated *p14<sup>ARF</sup>*. CpGenome Universal Methylated DNA (Intergen, NY) was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated genes. Water controls for the PCR reaction are also shown. Lesions T1 and T2 of H69, D of H89, and T of H82 were methylation positive, whereas all others were methylation negative. N, normal tissue; D, dysplasia; T, tumor; NL, normal lymph node; TM, totally methylated control DNA.

Table 2 The density of methylated CpGs in the *p14<sup>ARF</sup>* gene

No. of methylated CpGs	0-4	5-9	10-14	15-19	20-24	25-28	Total
Normal	93	0	0	0	0	0	93
Dysplasia	43	1	1	1	2	0	48
Carcinoma	77	0	2	0	4	8	91
Total	213	1	3	1	6	8	232

In each sample, more than 10 clones of *p14<sup>ARF</sup>* PCR product were examined by bisulfite sequencing. Kruskal-Wallis test:  $P = 0.0005$ .

The number of methylated CpGs in these 12 clones ranged from 20 to 28 (mean, 25.3) of 28 CpGs. No particular pattern could be observed in the distribution of methylated CpGs in different samples. We confirmed that the methylated and unmethylated control DNAs used in MSP were totally methylated and unmethylated, respectively, by bisulfite sequencing.

### DISCUSSION

The purpose of this study was to determine the frequency and timing of *p14<sup>ARF</sup>* hypermethylation in UC-associated colorectal carcinogenesis. In this study, hypermethylation of *p14<sup>ARF</sup>* was present in 19 of 38 (50%) carcinomas and in 4 of 12 (33%) dysplasias. Esteller *et al.* (15) and Burri *et al.* (19) reported that the frequency of *p14<sup>ARF</sup>* hypermethylation is 28–33% in sporadic colorectal cancers and 32% in colon adenomas. In comparison with these data, our hypermethylation frequencies are somewhat higher.

Although several groups have reported genetic abnormalities in nonneoplastic UC mucosa, such as microsatellite instability (20, 21) and *p53* mutation (22–25), hypermethylation of cancer-related genes has not been reported in this setting. Hypermethylation of tumor suppressor genes has been demonstrated in other precancerous lesions associated with chronic inflammation. Hypermethylation of *E-cadherin* has been reported in chronic gastritis (26), and hypermethylation of *APC*, *p16<sup>INK2a</sup>*, and *E-cadherin* has been demonstrated in the esophagitis-associated lesion, Barrett’s esophagus (27). Previously, Esteller *et al.* (15) found no evidence of

*p14<sup>ARF</sup>* hypermethylation in normal uninflamed colonic mucosa; however, we have noted *p14<sup>ARF</sup>* hypermethylation in 3 (60%) of 5 (60%) nonneoplastic inflamed UC mucosae. Therefore, it is possible that longstanding chronic inflammation induces aberrant methylation of the *p14<sup>ARF</sup>* gene in colonic mucosa. It is also possible that *p14<sup>ARF</sup>* methylation varies according to location within the intestinal tract (*e.g.*, absent in the ileum but present in the colon). However, our study suggests that *p14<sup>ARF</sup>* methylation is a potential marker of early carcinogenesis in the setting of UC. In this regard, *p14<sup>ARF</sup>* may resemble the *p53* tumor suppressor gene, in that alterations of *p53* are regarded as relatively early events in UC-associated carcinogenesis (5, 22–25).

In this study, 3 of 20 samples studied by bisulfite sequencing showed results different from MSP. Because our samples had varying degrees of contamination by normal and stromal cells, our observed ratios of methylated CpGs could underrepresent actual methylation ratios in tumor cells. However, according to our bisulfite sequencing data, the density of methylated CpGs increased during the progression from dysplasia to carcinoma. These findings suggest that aberrant methylation of the *p14<sup>ARF</sup>* gene may be progressive during the dysplasia-carcinoma sequence of UC.

Regarding the distribution pattern of methylated CpGs, no significant pattern was found in this study. Zheng *et al.* (28) described that the 3’ region of exon 1β was more densely methylated than the promoter and 5’ region of exon 1β. However, their primers for bisulfite sequencing were situated on several CpGs, and the forward primer sequence appeared to be based on the unmethylated sequence, whereas the reverse primer was based on the methylated sequence. Primers for bisulfite sequencing are generally placed in regions lacking the sequence CpG.

In conclusion, our data suggest that hypermethylation of *p14<sup>ARF</sup>* is a relatively common and early event in UC-associated carcinogenesis. Thus, *p14<sup>ARF</sup>* is worthy of study to explore its potential as a biomarker for the early detection of cancer or dysplasia in UC. Furthermore, future analyses of *p14<sup>ARF</sup>* methylation in other or-

### Patient: H26

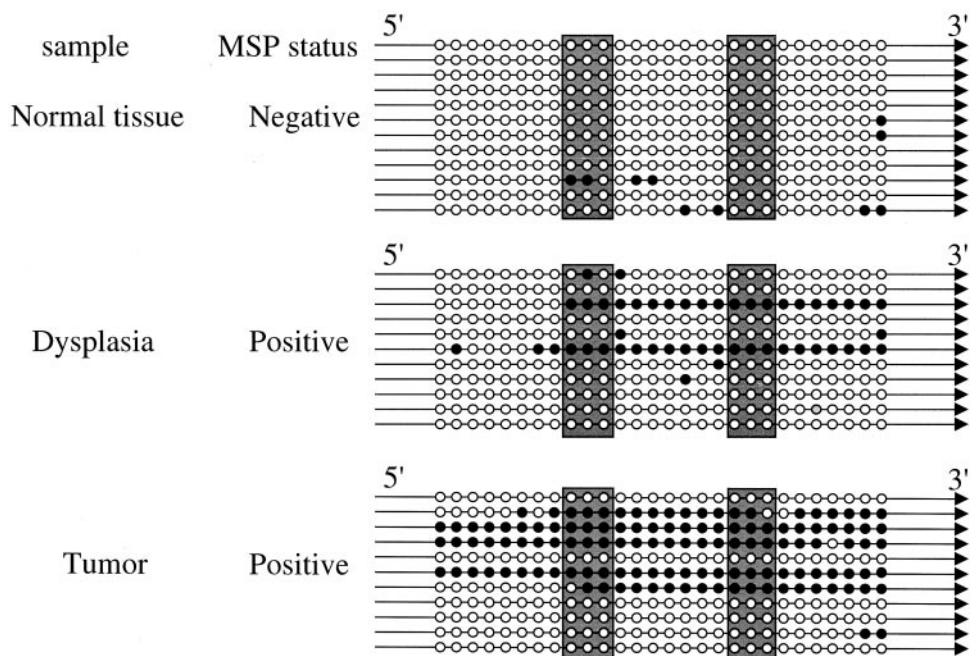


Fig. 3. Distribution of methylated CpGs in the *p14<sup>ARF</sup>* gene. Long arrows containing 28 circles indicate discrete clones of *p14<sup>ARF</sup>* PCR product. ○, unmethylated CpGs; ●, methylated CpGs; circles in gray rectangles, CpGs recognized by MSP primers.

gans should focus not only on frank cancers but on other pre-malignant lesions as well.

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