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Advances in Brief

Aberrant Methylation of the *CDH13* (H-cadherin) Promoter Region in Colorectal Cancers and Adenomas[1]

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Abstract

Expression of the cadherin family member *CDH13* (H-cadherin) is reduced in several human tumors, and it has been hypothesized that this gene functions as a tumor suppressor gene. Previously, we reported that the 5′ region of *CDH13* is frequently methylated in breast and lung cancers. Here we confirmed the promoter activity of 5′ region of *CDH13* by luciferase assay and examined its aberrant methylation in colorectal cancers, cell lines, and adenomas. Methylation status was investigated by methylation-specific PCR (MSP) and by bisulfite DNA sequencing of cloned DNA of PCR amplicons. In cell lines, we examined the correlation between methylation status and mRNA expression by reverse transcription-PCR. Aberrant methylation of *CDH13* was present in 7 of 13 (54%) cell lines, and expression was absent in 6 of 13 (46%) cell lines. *CDH13* expression was present in six cell lines that showed only the unmethylated form by MSP and in one cell line that showed both the methylated and unmethylated forms. Treatment with 5-aza-2′-deoxycytidine restored *CDH13* expression in methylated cell lines. In surgically resected samples, 17 of 35 (49%) cases of primary colorectal cancer, 2 of 33 (6%) cases of corresponding nonmalignant colorectal mucosa, and 8 of 19 (42%) adenomas were methylated. Sequence data after bisulfite treatment indicated that primary cancers and two cell lines with loss of expression were highly methylated compared with nonmalignant colorectal epithelial cells, especially at the attachment sites of primers for MSP, although there was heterogeneity in methylation status. Our results suggest that *CDH13* expression is frequently silenced by aberrant methylation in colorectal cancers and adenomas and that methylation of *CDH13* commences at an early stage of multistep colorectal tumorigenesis.

Introduction

The cadherins are a family of cell surface glycoproteins responsible for selective cell recognition and adhesion (1). Several family members, including *CDH1* (E-cadherin) and *CDH13* (H-cadherin), are located on the long arm of chromosome 16 [16q (2)], where loss of heterozygosity was reported in several human cancers including colorectal cancer (3–5). Loss of expression of cadherins has been described in many epithelial cancers, and it may play a role in tumor cell invasion and metastasis (1, 6, 7).

Inactivation of tumor suppressor genes may occur via point mutations, loss of heterozygosity, homozygous deletions, or aberrant methylation (8–10). Aberrant methylation of 5′ gene promoter regions associated with gene silencing is an epigenetic phenomenon observed in many cancer types (11), and the number of methylated genes in individual cancers is estimated to be very high (12). We and others have reported that *CDH13* expression is frequently silenced by aberrant methylation of the 5′ region of the *CDH13* gene in breast and lung cancers (9, 13). In ovarian tumors, the combination of deletion and aberrant methylation has been reported to inactivate *CDH13* (14). However, because aberrant methylation is not limited to CpG dinucleotides of the promoter region (15), one of the most important criteria for functional significance of the target region of *CDH13* methylation in colorectal cancer is good concordance between gene expression and aberrant methylation of this region. Thus, it is critical to confirm the promoter activity for the putative promoter region. In this study, (*a*) we performed a luciferase assay to examine the promoter activity of the putative promoter region; (*b*) we examined the relationship between *CDH13* expression and methylation in colorectal cell lines, and furthermore, we treated *CDH13*-methylated cell lines with 5-Aza-CdR[3] to restore *CDH13* expression; (*c*) we also examined *CDH13* methylation status in primary colorectal cancer and corresponding nonmalignant tissue and adenomas.

Materials and Methods

Reporter Gene Plasmid Constructs and Luciferase Activity Assays. The 5′-flanking region of *CDH13* [GenBank accession number AB001090; nucleotides 1042–1645, named RLUC (region for luciferase assay)] was tested for the ability to drive the expression of the firefly luciferase reporter gene in the promoterless plasmid pGL3-Basic (Promega, Madison, WI). The target region was amplified by the following primers: forward primer, 5′-GCAAGCTCGAATTGATCTGTCAT-3′; and reverse primer, 5′-CGGCGTTTTCATTCATGCA-3′. The sense and antisense sequence fragments of RLUC were inserted upstream of the firefly luciferase reporter gene in pGL3-Basic (CDH13S-pGL3 and CDH13AS-pGL3). The antisense sequence construct (CDH13AS-pGL3) and promoterless pGL3-basic were used for control of the luciferase activity. The plasmid DNA purification was carried out using the Qiagen Midi-prep kit (Qiagen, Inc., Valencia, CA). Orientation and sequence of the insert were verified by sequencing.

HEK293T cells (5.0×10^4) were plated in 24-well dishes. After a 24-h incubation, HEK293T cells were cotransfected with a specific vector plasmid (CDH13S-pGL3, CDH13AS-pGL3, and promoterless pGL3-basic) plus Renilla luciferase plasmid pRL-TK (as a control for transfection efficiency; Promega) complexed with 5 μl of LipofectAMINE PLUS (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The cell extracts were prepared 24 h after transfection, using 100 μl of Passive Lysis Buffer (Promega). The dual luciferase reporter assays were carried out on each lysate (20 μl), as recommended by the manufacturer (Promega). Firefly and Renilla luciferase activities were measured sequentially in a lumicounter. The firefly luciferase activity of each construct was normalized to the Renilla luciferase internal control. All transfections were carried out in duplicate and repeated at least twice.

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[3] The abbreviations used are: 5-Aza-CdR, 5-aza-2′-deoxycytidine; MSP, methylation-specific PCR; RT, reverse transcription.

Cell Lines. Thirteen colorectal cancer cell lines (COLO320DM, COLO201, SW480, LoVo, HCT116, HT-29, RKOAU, SNUC1, SCC10, SW1417, NCI-H630, LS174T, and UMC11) were obtained from American Type Culture Collection (Manassas, VA) or established by us (16). They were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO_2 at 37°C.

Expression of the *CDH13* Gene. Expression of the *CDH13* gene was analyzed by RT-PCR. Total RNA was extracted from the cell lines with Trizol (Life Technologies, Inc.) following the manufacturer's instructions. The RT reaction was performed on 2 μg of total RNA with SuperScript II First-Strand Synthesis using the oligo(dT) primer system (Life Technologies, Inc.). Primer sequences and conditions for RT-PCR product were as described previously (9, 13) and confirmed that genomic DNA was not amplified with these primers. The housekeeping gene *GAPDH* was used as an internal control to confirm the success of the RT reaction (forward primer, 5′-GACCACAGTCCATGCCATCACT-3′; reverse primer, 5′-GCTTCACCACCTTCTTGATGTCA-3′). PCR products were analyzed on 2% agarose gels.

5-Aza-CdR Treatment. Four tumor cell lines with *CDH13* promoter methylation and absent gene expression were incubated in culture medium with 5-Aza-CdR at a concentration of 4 μM for 6 days, with medium changes on days 1, 3, and 5.

Clinical Samples. Surgically resected specimens from 35 primary colorectal tumors, 33 corresponding nonmalignant tissues from these patients, and 19 samples of colorectal adenomas were obtained from Dallas, Texas and Baltimore, Maryland (Table 1). For the *CDH13* expression study, two cases of nonmalignant colorectal epithelium were obtained from nonmalignant epithelium of the resected specimen. Epithelial cells from buccal swabs of eight healthy nonsmoking volunteers and peripheral blood lymphocytes from 12 healthy volunteers were obtained. Appropriate institutional review board permission was obtained from both participating centers, and written informed consent was obtained from all patients.

Collection of Colorectal Epithelial Cells. To reduce the contamination of nonepithelial cells in surgically obtained specimens used for sequencing the promoter region of *CDH13*, we developed a simple, rapid method for enriching epithelial cells using 6-inch, sterile, disposable, nylon bristle brushes (CytoSoft Cytology Brushes; CYB-1, Medical Packaging Corporation, Camarillo, CA). Briefly, colorectal epithelial cells from the mucosal surface of the tumor from resected surgical specimens were obtained by gentle side to side scraping with the brush. In addition, three widely separated areas of normal-appearing colorectal mucosa were sampled per case. The specimens were fixed in the PreservCyt solution (Cytyc Corp., Boxborough, MA), and ThinPrep (Cytyc Corp.) smears were prepared and stained. Papanicolau-stained smears were evaluated for the purity of the cells collected. Those specimens with considerable stromal cell and peripheral blood cell contamination were excluded from the study. Solid pieces of corresponding tumor and mucosal tissues were also obtained from all cases and analyzed separately.

DNA Extraction and MSP. Genomic DNA was obtained from cell lines, primary tumors, and nonmalignant cells by digestion with proteinase K (Life Technologies, Inc.), followed by phenol/chloroform (1:1) extraction (17). Aberrant promoter methylation of *CDH13* was determined by MSP as reported by Herman *et al.* (18) using primers for *CDH13*-methylated and unmethylated sequences (9, 13). Briefly, 1 μg of genomic DNA was denatured by NaOH and modified by bisulfite. The modified DNA was purified using the Wizard DNA purification kit (Promega), treated with NaOH to desulfonate it, precipitated with ethanol, and resuspended in water. PCR amplification was done with bisulfite-treated DNA as a template, using specific primer sequences for the methylated and unmethylated forms of the gene. DNAs from peripheral blood lymphocytes ($n = 12$) and buccal mucosa ($n = 8$) from healthy nonsmokers were used as negative controls for methylation-specific assays. DNA from the lymphocytes of a healthy volunteer treated with *Sss*1 methyltransferase (New England BioLabs, Bevely, MA) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. Water blanks were included with each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP for all samples.

Table 1 *Aberrant promoter methylation of CDH13 in samples*

Sample	No. of *CDH13*-methylated samples (%)
Colorectal cancers	
Cell lines ($n = 13$)	7 (54%)
Tumors ($n = 35$)	17 (49%)
Adenomas ($n = 19$)	8 (42%)
Adenomatous polyps ($n = 9$)	5 (56%)
Villous or tubulovillus adenomas ($n = 7$)	2 (29%)
Adenomas with high-grade dysplasia or cancers ($n = 3$)	1 (33%)
Nonmalignant specimens	
Nonmalignant colorectal mucosa ($n = 33$)	2 (6%)[a]
Peripheral blood lymphocytes ($n = 12$)	0
Buccal mucosa ($n = 8$)	0

[a] Specimens resected for colorectal cancer were also methylated for the *CDH13* gene.

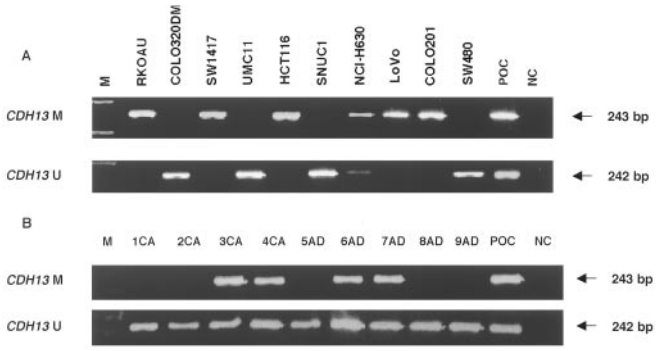

Fig. 1. Representative examples of MSP in colorectal tumors. *A*, cell line samples; *B*, colorectal cancers (*CA*) and adenomas (*AD*). In tumor samples, most of which consist of mixtures of tumor cells and nonmalignant cells, either the unmethylated band only or both the methylated and unmethylated bands were present. *CDH13 M*, *CDH13*-methylated form; *CDH13 U*, *CDH13*-unmethylated form; *POC*, positive control; *NC*, negative control (water blank).

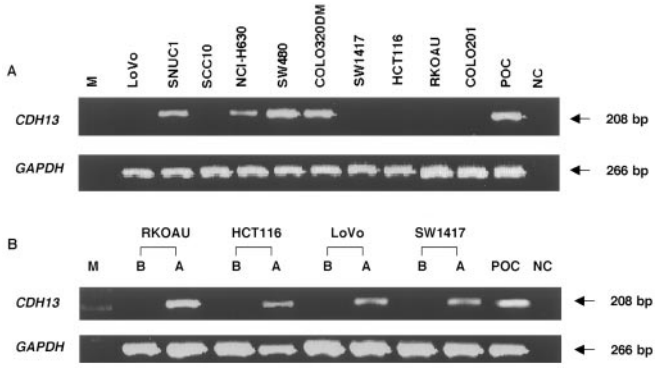

Fig. 2. RT-PCR for *CDH13* expression in colorectal cancer cell lines. *A*, representative examples of RT-PCR for *CDH13* expression in colorectal cancer cell lines. *B*, the effect of 5-Aza-CdR treatment on *CDH13*-negative cell lines with *CDH13* methylation. Treatment with 5-Aza-CdR restored expression of *CDH13* in all four methylated cell lines tested. Expression of the housekeeping gene *GAPDH* was run as a control for RNA integrity. *POC*, positive control; *NC*, negative control (genomic DNA). Before (*B*) and after (*A*) treatment with 5-Aza-CdR.

Map of 5′-flanking Region of *CDH13* and Bisulfite DNA Sequencing. The location of the CpG dinucleotides and the regions for luciferase assay (RLUC), MSP amplicon (RMSP), and bisulfite DNA sequencing (RBSSQ) in the *CDH13* 5′-flanking region are shown in Fig. 3*A*. Bisulfite-treated DNA of RBSSQ was amplified by PCR using primers as described previously (9). These primers were designed to exclude binding to any CpG dinucleotide to ensure amplification of both methylated and unmethylated sequences. PCR products were cloned into plasmid vectors using the Topo TA cloning kit (Invitrogen), following the manufacturer's instructions. Plasmid DNAs were purified using the Wizard Plus miniprep kit (Promega) and then sequenced by the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA). This region included the MSP primer sites and amplicon and encompassed 24 CpG dinucleotides.

Results

Luciferase Activity Assays. The luciferase assay was performed to examine the promoter activity of the putative promoter region of

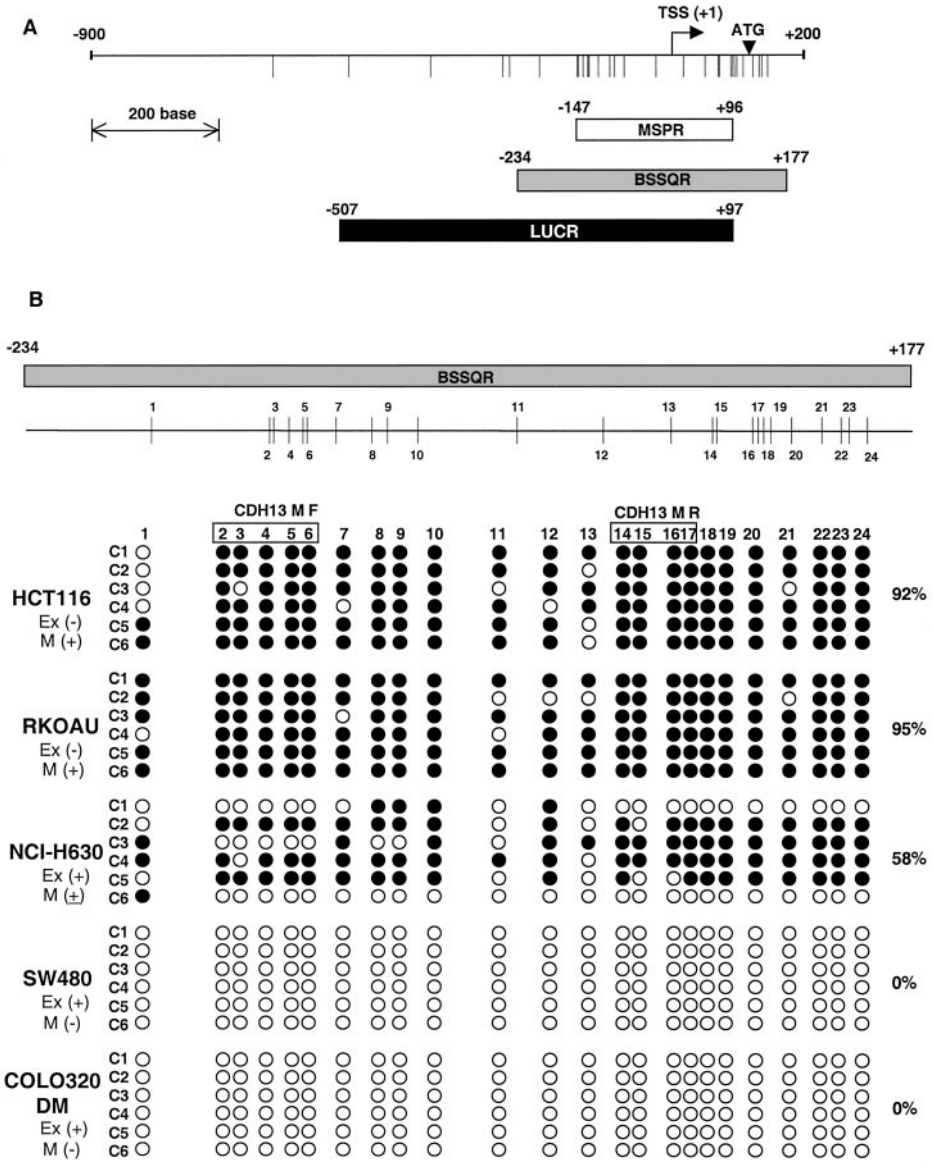

Fig. 3. The 5′ region of the *CDH13* gene and the bisulfite genomic DNA sequence. *A*, positions of CpG dinucleotides in the genomic sequence (GenBank accession number AB001090) are indicated by *thin vertical lines*. The *bent arrow* indicates the transcription start site (*TSS*) (+1), and the *arrowhead* indicates the translation start site (*ATG*). The *horizontal open bar* indicates the region for the amplicon of MSP (RMSP). The *horizontal gray bar* indicates the region for the bisulfite genomic sequence (RBSSQ). The *horizontal black bar* indicates the region of the insert for luciferase assay (RLUC). *B*, location and methylation status of methylated CpG dinucleotides in RBSSQ. Methylation status of individual cloned DNA fragments of five colorectal cell lines and four pairs of enriched primary tumors and corresponding epithelial tissues are shown. Each *row* represents one sequenced allele. Each *circle* represents a CpG dinucleotide. *Filled circle*, methylation; *open circle*, no methylation. Clonal numbers are indicated by the prefix *C* to the *left*. The *numbers* at the *top* indicate the CpG dinucleotide in the amplicon (5′ to 3′). The percentage to the *right* indicates the rate of methylated CpG dinucleotides in each sample. The positions of CpG dinucleotides included in MSP primers (CDH13MF and CDH13MR) are indicated by *boxes*. *M (+)*, positive for the *CDH13*-methylated form by MSP; *M (−)*, negative for *CDH13*-methylated form by MSP; *M (±)*, positive for both the *CDH13*-methylated and unmethylated forms by MSP; *Ex (+)*, *CDH13*-positive by RT-PCR; *Ex (−)*, *CDH13*-negative by RT-PCR.

the *CDH13* 5′-flanking region (RLUC). RLUC included the regions for MSP (RMSP) and bisulfite genomic DNA sequence (RBSSQ) (Fig. 3*A*). The sense sequence of RLUC showed 6.9-fold higher luciferase activity than the antisense sequence and 4.5-fold higher luciferase activity than the vector control.

Aberrant Methylation and Expression of *CDH13* in Cell Lines. We determined the sensitivity of our MSP assay for *CDH13* by performing serial dilutions of known methylated and unmethylated cell lines. The assay could detect 1 methylated allele in the presence of 1000–2000 unmethylated alleles. Aberrant methylation was absent in DNA from peripheral blood lymphocytes and buccal swabs from volunteers (Table 1). We performed MSP for *CDH13* in colorectal cell lines, and representative examples are illustrated in Fig. 1*A*. Aberrant methylation was found in 7 of 13 (54%) cell lines. Only the methylated or unmethylated forms of the gene were present in 12 of 13 (92%) cell lines, and both forms were present in 1 of 13 cell lines. Expression of *CDH13* was examined by RT-PCR, and representative examples are shown in Fig. 2*A*. *CDH13* expression was present in nonmalignant colorectal epithelial cells from two individual samples. However, loss of *CDH13* expression was observed in 6 of 13 (46%) colorectal cell lines, and aberrant methylation was detected in all 6 of these cell lines. *CDH13* expression was present in all six unmethylated cell lines and in one cell line (NCI-H630) that showed both methylated and unmethylated forms by MSP. The concordance between loss of gene expression and aberrant methylation of *CDH13* was 88%.

5-Aza-CdR Treatment. Four cell lines (RKOAU, HCT116, LoVo, and SW1417) that showed loss of expression and methylation by MSP were cultured with 5-Aza-CdR (4 μM, 6 days). *CDH13* expression was restored after treatment in all four methylated cell lines tested (Fig. 2*B*).

Aberrant Methylation of *CDH13* in Primary Colorectal Cancers, Adenomas, and Mucosa. We also examined *CDH13* methylation status for primary colorectal cancers and corresponding nonmalignant colorectal tissues and adenomas by MSP (Table 1; Fig. 1*B*). Aberrant methylation was found in 17 of 35 (49%) cases of primary colorectal cancer, 2 of 33 (6%) corresponding mucosal tissues, and 8 of 19 (42%) adenomas. In both cases where the nonmalignant tissues were methylated, the corresponding tumor samples were also methylated. The histology of the adenomas is shown in Table 1. There was no significant difference in the rates of methylation in colorectal cancers and adenomas. Samples of mucosa and tumors enriched for epithelial cells gave methylation results identical to the corresponding nonenriched samples.

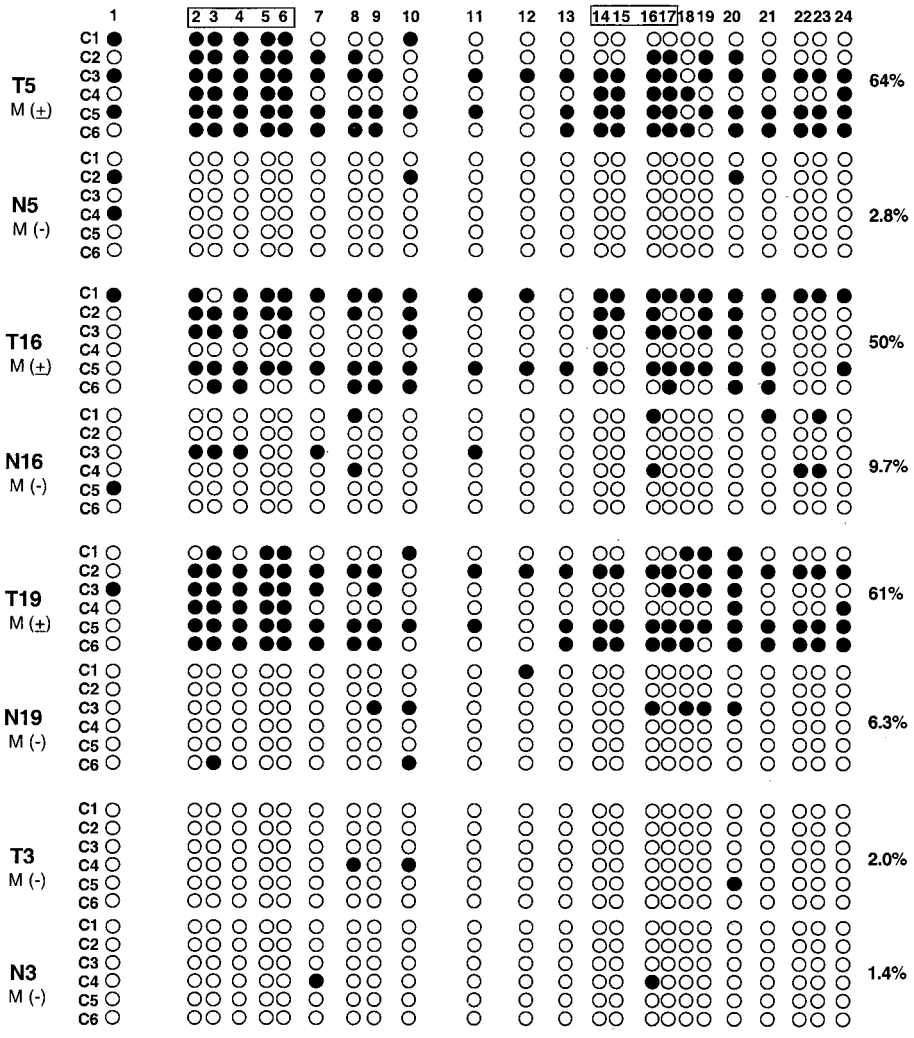

Fig. 3. *Continued.*

In colorectal cancers, there were no differences between methylation-positive and -negative groups with respect to gender, age, stage, tumor location, and tumor size.

Bisulfite Genomic DNA Sequencing. Randomly selected *CDH13* 5′-flanking region (RBSSQ) amplified by PCR was examined for the methylation status of 24 CpG dinucleotides within the RBSSQ region. Using methylation-independent primers, PCR amplification was performed to create a RBSSQ fragment (411 bp) from five cell lines (HCT116, RKOAU, NCI-H630, COLO320DM, and SW480) and from four pairs of primary tumors and corresponding nonmalignant epithelial tissues. PCR amplicons were cloned to sequence the RBSSQ region, which included the MSP primer attachment sites and their amplicon. The results are shown in Fig. 3*B*. In three cell lines that showed only the methylated band by MSP, >92% of all CpG dinucleotides were methylated (HCT116, 92%; RKOAU, 95%). The percentage was obtained by using the following formula: (number of methylated CpG dinucleotides/total number of CpG dinucleotides in six clones × 100). One cell line (NCI-H630) that showed both methylated and unmethylated bands by MSP revealed moderate methylation (58%). Two cell lines (SW480 and COLO320DM) lacking methylated bands by MSP were completely unmethylated at all 24 CpG dinucleotides in six cloned alleles.

Although there was considerable heterogeneity in enriched cancer and corresponding nonmalignant cells from resected specimens (Fig. 3*B*), primary cancer cells were highly methylated (50–64%) compared with the corresponding nonmalignant cells (6–10%) in the samples that showed methylation in cancers by MSP.

Discussion

One of major mechanisms for suppression of tumor suppressor gene function is aberrant methylation of the promoter region, resulting in down-regulation of gene expression (10). In lung, breast, ovarian, bladder, and prostate cancers, aberrant methylation of the *CDH13* 5′-flanking region has been reported (9, 13, 14, 19–21). However, colorectal carcinomas have not been studied previously. It is important to confirm that the target region studied for methylation actually has promoter activity because methylation may occur in non-promoter regions not related to gene silencing in nonmalignant cells (15). In this study, we performed the luciferase assay for the *CDH13* 5′-flanking region (RLUC), which included the MSP primer sites and sequenced region. We found promoter activity in RLUC, confirming that the selected region is appropriate for methylation study (MSP and CpG dinucleotide sequencing).

We examined the expression and methylation status of *CDH13* in colorectal cancer cell lines. Treatment with 5-Aza-CdR restored *CDH13* expression in methylated cell lines, confirming that methylation was responsible for silencing *CDH13* expression in these cell lines. We also performed MSP on colorectal samples. Only occasional methylation was present in nonmalignant tissues adjacent to cancers, whereas a relatively high percentage of colorectal

cancers (49%) and adenomas (42%) were positive for *CDH13* methylation. Of interest, *CDH13* methylation was frequently observed in colorectal adenomas, and the differences in the rates of methylation in adenomas and carcinomas were not statistically significant, suggesting that methylation precedes adenoma formation. Frequent aberrant methylation of the *APC* and *HPP1* genes was reported in colorectal adenomas (22, 23). These findings support the concept that the adenoma is the principal precancerous lesion in the colorectum and that tumorigenesis is thought to be a multistep process in which genetic alterations accumulate, ultimately producing the neoplastic phenotype (24).

Whereas heterogeneity of methylation was noted in the promoter region of *CDH13*, the fact that the MSP primer attachment sites were consistently methylated in two MSP-positive cell lines and that MSP results showed excellent correlation with expression suggested the importance of these CpG dinucleotides for gene silencing. A moderate degree of heterogeneity of methylation was observed in NCI-H630, which showed both methylated and unmethylated alleles by MSP with positive *CDH13* expression. Analysis of cloned alleles indicates heterogeneity of methylation in the promoter region, with some alleles partially methylated or completely unmethylated. These alleles would not be silenced, resulting in mRNA transcription.

Previous work has indicated that in epithelial cells, CpG island methylation actually increases with age and that CpG dinucleotide methylation may be present in some nonmalignant cells (25–27). To confirm the tumor specificity of *CDH13* methylation, we also sequenced enriched primary tumor cells and corresponding epithelial cells in which there was rare contamination of nonepithelial cells. Although our sequence data represented only a heterogeneous population, it was obvious that the number of methylated CpG dinucleotides was much larger in cancer cells than in the corresponding epithelial cells, indicating that the aberrant methylation of *CDH13* was specific for cancer cells. Heterogeneity of methylation in some tumors and cell lines may result in considerable tumor cell phenotypic variation, as has been described for *CDH1* (28).

Our results strongly suggest that the silencing of *CDH13* expression by aberrant promoter methylation plays a role in the pathogenesis of colorectal cancers. Furthermore, methylation of *CDH13* occurs at an early stage in the multistage process of oncogenesis. Because the MSP for *CDH13* is sensitive, detection of *CDH13* methylation in stool or other clinical samples (29) may be a useful test for early detection of colorectal cancers for the identification of individuals at increased risk.

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