

Original Article

Reversibility of Aberrant Global DNA and Estrogen Receptor- α Gene Methylation Distinguishes Colorectal Precancer from Cancer

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Abstract: Alterations in the global methylation of DNA and in specific regulatory genes are two epigenetic alterations found in cancer. However, the significance of epigenetic changes for diagnosis and/or prognosis of colorectal cancer have not been established, although it has been extensively investigated. Recently we have identified a new type of cancer cell called precancerous stem cells (pCSCs) and proposed that cancer may arise from a lengthy development process of tumor initiating cells (TICs) \rightarrow pCSCs \rightarrow cancer stem cells (CSCs) \rightarrow cancer, which is in parallel to histological changes of hyperplasia (TICs) \rightarrow precancer (pCSCs) \rightarrow carcinoma (CSCs/cancer cells), accompanied by clonal evolutionary epigenetic and genetic alterations. In this study, we investigated whether aberrant DNA methylation can be used as a biomarker for the differentiation between premalignant and malignant lesions in the colorectum. The profile of global DNA and estrogen receptor (ER)- α gene methylation during cancer development was determined by analysis of 5-methylcytosine (5-MeC) using immunohistochemical (IHC) staining, dot blot analysis or a quantitative gene methylation assay (QGMA). Herein we show that global DNA hypomethylation and ER- α gene hypermethylation are progressively enhanced from hyperplastic polyps (HPs) \rightarrow adenomatous polyps (APs) \rightarrow adenomatous carcinoma (AdCa). The aberrant methylation can be completely reversed in APs, but not in AdCa by a nonsteroidal anti-inflammatory drug (NSAID) celecoxib, which is a selective inhibitor of cyclooxygenase-2 (Cox-2), suggesting that the epigenetic alterations between colorectal precancer (AP) and cancer (AdCa) are fundamentally different in response to anti-cancer therapy. In normal colorectal mucosa, while global DNA methylation was not affected by aging, ER- α gene methylation was significantly increased with aging. However, this increase did not reach the level observed in colorectal APs. Taken together, reversibility of aberrant global DNA and ER- α gene methylation distinguishes colorectal precancer from cancer.

Key Words: Precancer, DNA methylation, colorectal cancer, estrogen receptor- α , nonsteroidal anti-inflammatory drugs, cancer progression, epigenetic, tumor initiation

Introduction

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. In the United States, it accounts for ~20% of all cancer deaths, with a yearly incidence of about 149,280 new cases and 56,910 deaths [1-3]. About 5-8% of the US population has a lifetime risk of developing colorectal cancer, being most common in people over the age of 50 [1, 2]. Thus, early diagnosis, prevention, and treatment are critical for the cure of colorectal cancer.

Recently we have identified a new type of cancer cells called precancerous stem cells (pCSCs), which have the potential of both benign and malignant differentiation [4, 5]. We have proposed that cancer may arise from a lengthy developing process of tumor initiating cells (TICs) \rightarrow pCSCs \rightarrow cancer stem cells (CSCs) \rightarrow cancer cells, which is in parallel to the histological changes of hyperplasia (TICs) \rightarrow precancer (pCSCs) \rightarrow carcinoma (CSCs/cancer cells), accompanied by clonal evolutionary epigenetic and genetic alterations [4, 6]. Colorectal cancer develops through a

lengthy, complex precancer stage involving progression of histological, epigenetic and genetic alterations [7, 8]. Histological alterations include normal epithelium → dysplastic aberrant crypt foci (ACF) or hyperplastic polyp (HP) → adenomatous polyp (AP) → invasive adenocarcinoma (AdCa) [9]. Colorectal polyps are classified as HPs (metaplasia, non-adenomatous), and APs, which are further classified into tubular adenoma, tubulovillous adenoma, villous and serrated adenoma [10, 11]. The risk of a polyp developing into a carcinoma increases with the increased degree of dysplasia: tubular histology is associated with a 5% risk, tubulovillous histology with a 15-20% risk, and villous histology with up to a 50% risk [12-14]. Both epigenetic and oncogenetic alterations may occur during the development of colorectal cancer from HPs (tumor initiation) → APs (precancer) → AdCa (cancer) [12, 15-17]. While the genetic alterations from precancer to cancer in colorectum have been extensively studied [18], it is not clear whether epigenetic alterations such as DNA methylation can significantly differentiate precancer from cancer. In other word, the significance of epigenetic changes in the diagnosis and prognosis of colorectal cancer has not been well established.

Epigenetic alteration is a mechanism by which gene function is selectively activated or inactivated without changing in the DNA sequence [19, 20]. Alterations of DNA methylation globally and in specific regulatory genes are two epigenetic alterations found in cancer [19, 20]. Most tumors, including colorectal cancer have a 35-60% reduction in 5-MeC in DNA [19, 20]. This global DNA hypomethylation occurs mainly in the “body” (coding regions and introns) of genes and in repetitive DNA sequences, and has been linked to a reduction in the binding of methylated DNA-binding proteins and histone deacetylase (HDAC) resulting in an increased acetylation of histones and opening up of chromatin [21-23]. These events facilitate the transcription of genes including reactivation of transposons of retroviruses [21, 24]. DNA hypomethylation in cancers is accompanied by hypermethylation of regulatory genes, especially tumor suppressor genes [19, 21, 23]. Hypermethylation of CpG islands in the regulatory regions of genes is usually associated with transcriptional silencing of

genes [25]. However, the significance of epigenetic alterations in predicting progression of neoplasms has not been clearly defined.

Estrogen has been shown to be associated with a significant decrease in the risk of colorectal cancer [26]. Consistently, frequent hypermethylation of the ER- α gene has been reported in colorectal cancers [17, 27], suggesting that ER- α gene hypermethylation might be a predictor for colorectal cancer progression. ER- α is a transcription factor that, upon binding to estrogen, translocates to the nucleus where it activates various genes including those involved in the inhibition of cell division [28]. Introduction of ER- α gene into ER-negative colon carcinoma cells was found to suppress cell growth [29]. Recovery of an epigenetically inactivated ER gene resulted in growth suppression of colon cancer cells *in vitro* and *in vivo* [30]. Preclinical studies have demonstrated that ER- α gene is also hypermethylated in azoxymethane (AOM)-induced rat colon cancer cells, suggesting a common molecular alteration between rat and human [31].

Epidemiological studies showed that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) including the cyclooxygenase-2 (Cox-2) selective inhibitor celecoxib, and the nonselective inhibitor aspirin, is associated with an up to 50% risk reduction for colorectal cancer [32-34]. Two recent intervention trials, one in patients with previous colorectal cancer and one in patients with previous adenomas, have offered strong evidence supporting the use of celecoxib to prevent progression of colorectal neoplasia [34-38]. It has been shown in AOM-induced rat colon tumors that short-term (7 to 28 days) treatment with celecoxib reversed both DNA hypomethylation (*i.e.* increased methylation of DNA) and hypermethylation of the ER- α gene (*i.e.* decreased methylation of the gene) [31]. Thus, we hypothesized that global hypomethylation of genes and hypermethylation of the ER- α gene may be a predictor for colorectal cancer progression.

We report here that the level of DNA hypomethylation and the extent to which the ER- α gene is methylated correlate with the stage of progression from normal-appearing epithelium to AdCa. Both alterations were reversed by celecoxib, further supporting the

Table 1 Patient characteristics

Age (years)	Gender	Diagnosis		
		Hyperplastic polyp	Adenomatous polyp	Adenocarcinoma
≤ 50	Female	3	2	4
	Male	5	5	5
>50	Female	3	3	6
	Male	4	3	6
Total		15	13	21

usefulness of global DNA hypomethylation and hypermethylation of ER- α gene as biomarkers for chemoprevention.

Experimental Design and Methods

Patients and Tissues

Frozen or RNAlater (Ambion, Inc., Austin, TX) preserved and paraffin embedded samples of colorectal adenocarcinoma, adenomatous polyp, hyperplastic polyp, and normal mucosa either near (<2.0 cm) or distal (>2.0 cm) to the lesion were retrieved from the Department of Pathology, Ohio State University Medical Center. The age and gender of the study population are listed in **Table 1**. To determine the effect of celecoxib on the methylation of DNA and ER- α gene, biopsies of four colorectal lesions (one hyperplastic polyp, two adenomatous polyps and one adenocarcinoma) were obtained from patients treated with 200 mg/day of celecoxib for 30 days at the Xiangya Medical University Hospital, Hunan Province, China.

Immunohistochemical Study for 5-MeC

Serial sections (5 micron) of paraffin embedded samples were stained with hematoxylin and eosin (H&E) for histopathological diagnosis and were immunohistochemically stained for 5-MeC. After antigen retrieval, the sections to be stained immunohistochemically were rinsed with PBS and treated with 3.0% hydrogen peroxide to quench endogenous peroxidase activity. The sections were covered with 100 μ L of mouse monoclonal primary antibody (5 μ g/mL) specific for 5-MeC (Serotec Inc., Raleigh, NC) and incubated for one hour at 37°C. They were then incubated with biotinylated goat antimouse secondary antibody (Dako, Glostrup, Denmark), reacted with streptavidin-peroxidase (Dako) and treated with diaminobenzidine (Dako). Two

observers (Drs. Rulong Shen and Yiqing Xu) independently scored the staining intensity on a scale of 0 (no staining) to 4+ (intense staining). The weighted immunostaining score was obtained by multiplying the intensity score by the percentages of cells with the score. The results of the two observers were averaged. The lesions were compared with normal tissue from the same patient.

DNA Isolation

DNA was isolated from frozen and RNAlater samples by digestion with 400 μ g/mL RNase A and 200 μ g/mL proteinase K followed by extraction with phenol, chloroform, and isoamyl alcohol, as described previously [31, 39]. For paraffin-embedded samples, the lesion was dissected from 8 to 12 serial sections and pooled. DNA was extracted from the pooled samples according to the protocol of the Pico Pure DNA Extraction Kit (Arcturus System for Microgenomics, Mountainview, CA), and then purified with a Microcon YM-100 Centrifugal Filter Unit (Millipore, Billerica, MA). Approximately 20 ng of DNA was obtained from each paraffin-embedded sample.

DNA Methylation by Dot-Blot Analysis

Purified DNA was denatured and dotted onto the Hybond™ nitrocellulose membrane using a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). The membrane was probed with mouse monoclonal antibody to 5-MeC (Serotec Inc., Raleigh, NC), washed with Tris-buffered saline plus Tween 20, pH7.6, and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary anti-mouse-IgG antibody. The membrane was then treated with enhanced-chemiluminescence western blotting detection reagents and exposed to Kodak autoradiograph films. Optical density (OD) of the dots was determined using a Scion Image Analysis System (Scion Corp., Frederick,

MD). The membrane was then stained with 0.02% methylene blue to determine the relative amount of DNA loaded for each sample.

Quantitation of ER- α Gene Methylation Levels

Quantitative gene methylation assay (QGMA) was performed using the Enzymatic Regional Methylation Assay (ERMA) with modification [40, 41]. Briefly, DNA was denatured in 22 μ L of 0.3M NaOH for 15 min at 37°C followed by addition of 208 μ L of freshly prepared 2.2M sodium metabisulfite containing 0.5M hydroquinone (pH 5.0) and incubation under mineral oil at 50°C for 8 h. The bisulfite modified DNA was recovered with a Microcon YM-100 Centrifugal Filter Unit, and desulfonated with 0.3M NaOH at 37°C for 20 min. After neutralization with 30 μ L of 10M ammonium acetate, the DNA was precipitated with ethanol and dissolved in 20 μ L of nuclease-free water. The bisulfite-modified DNA was globally amplified 500 to 1,000-fold with the GenomePlex Complete Whole Genome Amplification Kit (Sigma-Aldrich Corp., St. Louis, MO). The whole genome amplification (WGA) product (5-12 μ g) was purified with the Microcon YM-100 Centrifugal Filter Unit. Aliquots of the WGA products were PCR amplified for ER- α gene (GenBank accession number: AY425004) using primers designed for bisulfite modified DNA with forward primer, 5'-GATGTTTAAGTTAATGTTAGGGT AAG-3' (nt 1994-2019) and reverse primer, 5'-AACTACTACTATCCAAATACA CCT-3' (nt 2461-2485). The amplified target region of the gene was purified with the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) and quantified fluorometrically with the PicoGreen ds DNA Quantification Kit (Molecular Probes, Inc., Eugene, OR). The PCR product (50 ng) was incubated dually with 1.1 μ Ci and 0.0156 nM S-adenosyl-L-[methyl- 3 H] methionine (Amersham, Piscataway, NJ) and 8 u Sss I methylase to methylate only CpG sites in the gene PCR product. The labeled gene products were spotted onto DE81 filters (Whatman, Maidstone, England) followed by washing 1x with 10% trichloroacetic acid for 20 min, 2x with 5% trichloroacetic acid for 10 min, 1x with 95% ethanol for 10 min, and 1x with 100% acetone for 10 min prior to scintillation counting. The incorporation of 3 H-methyl groups into the gene PCR product was directly

proportional to the number of methylated CpG sites originally present in the target gene region.

Sequencing of Bisulfite-modified DNA

The purified PCR product of ER- α gene was ligated into the TA cloning vector, pCR 2.1 vector and transformed into One Shot TOP10F' chemically competent *E. Coli* using standard protocols (Invitrogen, Carlsbad, CA). Plasmid colonies were grown overnight in LB broth containing 50 μ g/mL kanamycin. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and analyzed by restriction mapping with *EcoR I*, *Hind III* and *Xba I* to confirm the insertion of the PCR-amplified fragments. The clones were automatically sequenced with an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) using PO primers: forward, 5'-ATT GGG CCC TCT AGA TGC-3' and reverse, 5'-TTG GTA CCG AGC TCG GAT-3'.

ER- α mRNA Expression by Real Time RT-PCR

Total RNA was isolated from AdCa or normal colorectal mucosa frozen in liquid nitrogen using the TriZol Reagent [4, 42]. The RNA was subsequently treated with DNase I at 37°C for 30 min. The quality of the RNA samples was determined by capillary electrophoresis through agarose gels and staining with ethidium bromide, the 18S and 28S RNA bands being visualized under UV light. cDNA was synthesized by reverse transcription with Oligo(dT)₁₅ primer and the High Capacity cDNA Achieve kit (Applied Biosystems, Foster City, CA). The cDNA was PCR-amplified with primers specific for the ER- α gene and for the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems Assays-on-Demand Gene Expression Products for the primer and probe sets), using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR amplification was dually performed in a 30 μ L reaction mixture consisting of 600 nM of each primer; 200 nM probe; 5 units of Ampli-Taq Gold; 200 μ M each of dATP, dCTP, and dGTP; 400 μ M dUTP; 5.5 mM MgCl₂; 1 unit of AmpErase uracil N-glycosylase; and 1 x TaqMan buffer A containing a reference dye. The incubations were at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The mRNA

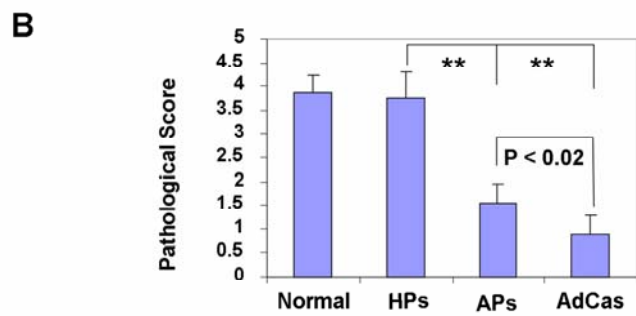
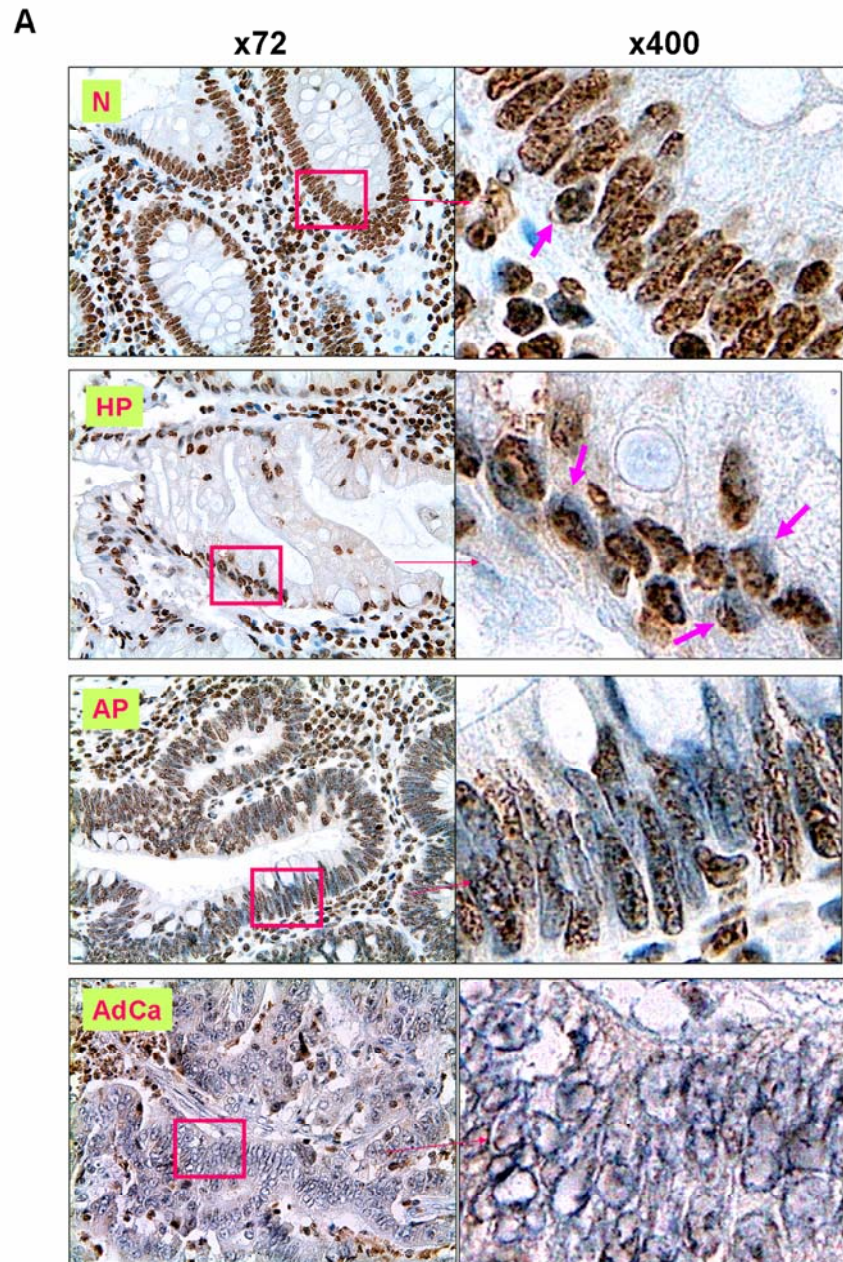


Figure 1 Hypomethylation of global DNA in adenomatous carcinomas. The 5-MeC in DNA of various stages of colorectal lesions was detected by IHC staining. Sections of HPs, APs, AdCas and histologically “normal” mucosa around colorectal cancers were stained with mAb to 5-MeC and counterstained with hematoxylin. The intensity of the staining was scored as described in Materials and Methods. **A.** Micrographs of representative staining for 5-MeC with different scores at low (x72) and high (x400) magnification: N (> 3 ~ 4+), the section of “normal” mucosa distal to or near cancer were stained uniformly and intensely except for a few cells that were not stained uniformly or intensively (arrow); HP (3+), the hyperplastic polyps were stained as intensively but less uniformly (arrows) as “normal mucosa”; AP (2+), the adenomatous polyps were stained weakly or moderately with speckles; and AdCa (< 1+), the adenocarcinomas were essentially not stained. Note that the infiltrated mononuclear cells in the AdCa were positive for 5-MeC. The boxes in the micrographs of x72 magnification are shown as the micrographs of x400 magnification. **B.** Summary of all specimens: Normal, near: n = 49; HPs: n = 15; APs, n = 13, and AdCas: n = 21. **, p < 0.01, as compared to HPs.

expression level was determined by dividing the threshold cycle for the ER- α gene by the threshold for the reference gene GAPDH. The relative gene expression was then used to calculate the ER- α mRNA expression level relative to its expression in normal colorectal mucosa tissue.

Statistical Analysis

The results were analyzed for statistical significance by One Way Analysis of Variance followed by the Bonferroni t-test with a p value of less than 0.05 as significant. Data are presented as mean \pm SE.

Results

Global DNA Methylation is Progressively Decreased with Progression of Colorectal Lesions

To determine the kinetics of epigenetic changes in colorectal precancer and cancer, we investigated the global DNA methylation in histologically “normal” mucosa, HP, AP, and AdCa using immunohistochemical (IHC) staining for 5-MeC in the nuclei of epithelial cells (**Figure 1**). The staining was scored under low magnification (**Figure 1A**). In normal mucosa, the nuclei of superficial matured epithelial cells were uniformly and intensely stained for 5-MeC (~ 4.0+). The intensity of staining was slightly, but seemingly not significantly reduced in the nuclei of HPs (3.0 ~ 4.0+). However, the nuclei of APs demonstrated significantly weaker staining (1.5 ~ 2.0+), and those of AdCa were barely stained (<0.5 ~ 1.0+). Statistical analysis show that global DNA methylation was significantly reduced in APs and AdCas, but not in HPs, compared to “normal” mucosa (**Figure 1B**). It should be noted that under high power, the global DNA methylation of HPs appeared to be

reduced, because the nuclei of epithelial cells were obviously less homogenously stained compared to those of “normal” mucosa (**Figure 1A**, right panel). The results suggested that methylation of global DNA was decreased starting from the stage of HPs, and progressively reduced when HPs progressed to AdCa.

To verify the findings of IHC staining, global DNA methylation in colorectal lesions was further determined by Dot-blot analysis. As shown in **Figure 2**, there were no significant alterations in global DNA methylation between HPs and “normal” mucosa either distal or proximal to the lesions (**Figure 2**). However, the methylation in the DNA of APs was significantly decreased by 27% and further decreased by 40% in AdCa, compared to distal “normal” mucosa. These results indicate that global DNA methylation was progressively decreased from HPs \rightarrow APs \rightarrow AdCa.

ER- α Gene Methylation is Progressively Enhanced with Progression of Colorectal Lesions

Since ER- α gene was hypermethylated in colorectal cancer [17, 27], we examined the kinetics of ER- α gene methylation during the development of colorectal cancer by QGMA. First, the accuracy of QGMA was determined by its correlation with bisulfite sequencing. The ER- α gene clones containing 0, 2, 3 or 5 methylated CpG sites were subjected to QGMA assay and bisulfite sequencing. As shown in **Figure 3A**, the density of Me-CpG sites in each clone determined by QGMA correlated well with the number of Me-CpG sites determined by bisulfite sequencing, suggesting that the QGMA was highly specific and sensitive for quantifying ER- α gene methylation (**Figure 3A**), and that QGMA was, therefore, reliable for evaluation of Me-CpG sites in ER- α gene.

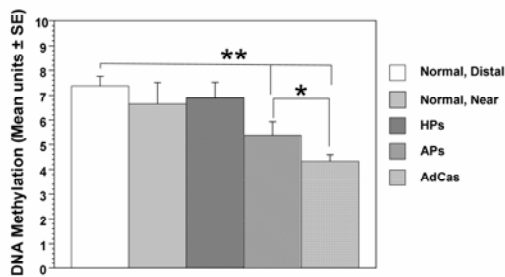


Figure 2 Dot-blot analysis of global DNA methylation in various types of colorectal lesions. DNA was isolated from “normal” mucosa distal to or near cancer, HPs, APs, and AdCas, and blotted onto membranes that were probed with the monoclonal primary antibody specific for 5-MeC. The intensity of the blots was normalized by the intensity of the methylene blue staining of DNA, and the results are expressed as arbitrary units (means ± SE). Normal, distal: n = 49; Normal, near: n = 49; HPs: n = 15; APs, n = 13, and AdCas: n = 21. **, p < 0.05, as APs or AdCas compared to normal, distal; * < 0.05, as compared between APs and AdCas.

Next, we examined the number of methylated CpG (Me-CpG) in the ER- α gene in normal mucosa, HPs, APs, and AdCas using both QGMA assay and bisulfite sequencing (**Figure 3B**). Analysis of Me-CpG sites in ER- α gene from “normal” mucosa, HPs, APs, and AdCas revealed that the number of Me-CpG sites was not significantly different between the “normal” mucosa (either distal to or near the lesions) and HPs (5.3 ~ 6.3 sites), but significantly increased by ~ 3 times in APs (15.4 ± 1.7 Me-CpG sites) compared to “normal mucosa” and HPs (p < 0.01; **Figure 3B**). The level of ER- α gene methylation further increased with progression of APs to AdCas (24.0 ± 3.0 Me-CpG sites; p < 0.01). Thus, methylation of the ER- α gene, in contrast to that of global DNA, was progressively increased with the progression of HP → AP → AdCa. Thus, quantitation of Me-CpG in ER- α gene may be useful for discriminating between colorectal hyperplastic polyp, precancer and cancer.

Effect of Aging on Global DNA Hypomethylation and ER- α Gene Hypermethylation in Normal Colorectal Mucosa

Since the incidence of colorectal cancer increases with age and the population above 50 years old have an especially high risk for

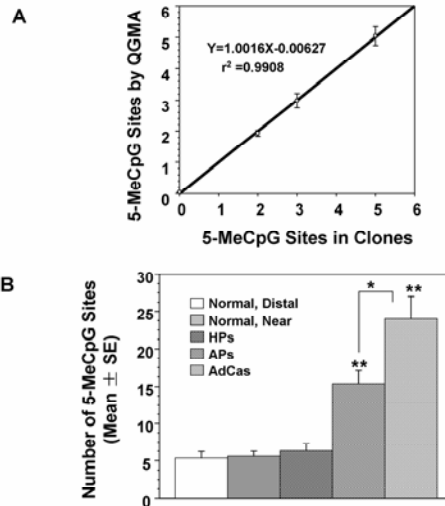


Figure 3 Hypermethylation of ER- α gene in adenomatous carcinomas. **A**. The accuracy of the Quantitative Gene Methylation Assay (QGMA) for ER- α gene. Clones containing 0, 2, 3 and 5 methylated CpG were subjected to the QGMA assay and sequencing. The number of CpG sites determined by the QGMA assay is plotted versus the number determined by sequencing. Results are means ± SE for six determinations. For the regression line, $r^2 = 9908$. **B**. ER- α gene methylation during colorectal neoplastic progression. DNA was isolated from paraffin-embedded tissue sections of normal mucosa, either distal or adjacent to the lesions, HPs, APs and AdCas. After bisulfite-treatment, the DNA was amplified by PCR for the ER- α gene and assayed by QGMA. Results are expressed as the mean number of methylated CpG sites ± SE. The number of specimens used for each group is the same as in **Figure 2**. **, p < 0.01, as compared to normal, distal, normal, near, or HPs; *, p < 0.01, as compared between APs and AdCas.

colorectal cancer [43], we examined whether aging affects global DNA methylation and hypermethylation of the ER- α gene in normal mucosa. Samples of normal colorectal mucosa, both distal to and near the lesions, were divided into two age groups, i.e., younger and older than 50 years of age. While global DNA methylation in both distal and near normal mucosa from patients younger than 50 years of age was not significantly different from that in patients older than 50 years (data not shown), the ER- α gene was significantly hypermethylated in patients older than 50 years (**Figure 4**). The Me-CpG sites in the ER- α gene in the mucosa distal to or near the lesions were increased by ~ 2 fold in patients older than 50 years compared to the younger

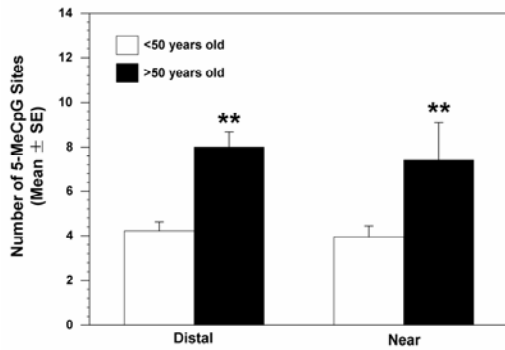


Figure 4 The effect of aging on ER- α gene methylation in normal-appearing colorectal mucosa. DNA samples of normal colorectal mucosa distal to and near the colorectal carcinomas were further divided into two age groups, i.e., younger ($n = 24$) and older ($n = 25$) than 50 years of age. After bisulfite-treatment, the DNA was amplified by PCR for the ER- α gene and assayed by QGMA. Results are expressed as means \pm SE. The asterisks indicate significant difference between the two age groups with p -value < 0.01 .

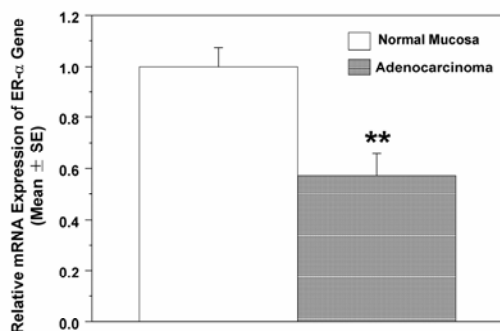


Figure 5 Hypermethylation of ER- α gene inhibits transcription of ER- α gene in colorectal adenocarcinomas. Total RNA was isolated from 8 normal mucosa and 6 adenocarcinomas, and subjected to real-time PCR analysis for ER- α gene expression as described in Materials and Methods. Results are expressed as means of relative ER- α mRNA expression units \pm SE. The asterisk indicates significant difference between normal mucosa and adenocarcinomas, p -value < 0.01 .

patients. The number of methylated sites in ER- α gene was 4.2 ± 0.4 and 3.9 ± 0.5 , respectively, in the normal mucosa distal to and near the lesions from patients younger than 50 years, and 8.0 ± 0.7 and 7.4 ± 1.6 , respectively, from patients older than 50 years ($p < 0.01$). These results indicate that in normal colorectal mucosa the methylation of ER- α gene was increased with aging. However, this

increase did not reach the level observed in colorectal APs (compare **Figures 3B** and **4**).

Reduction of ER- α Gene mRNA Expression in Colorectal Cancer

The biological consequence of ER- α gene hypermethylation was hypothesized to be suppression of ER- α gene transcription. To verify this hypothesis, we examined the level of ER- α gene transcript in adenocarcinomas by real-time PCR. As shown in **Figure 5**, the mRNA level of ER- α gene in adenocarcinoma was significantly decreased by 43%, relative to normal colorectal mucosa. This result is consistent with the increased methylation in ER- α gene in colorectal adenocarcinoma, suggesting that hypermethylation of ER- α gene results in suppression of ER- α gene transcription.

Non-Steroidal Anti-inflammatory Drug Reverses Aberrant Methylation in Colorectal Precancer but not in Cancer

To explore the effect of tumorigenic environment on global DNA and ER- α gene methylation, we investigated the role of inflammation in the hypomethylation of global DNA and the hypermethylation of ER- α gene. Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) used to reduce numbers of colon and rectum polyps in patients with familial adenomatous polyposis [35], and has been shown to modulate global DNA methylation and the methylation of ER- α gene in rat colon tumors [31]. We further investigated whether celecoxib could reverse abnormal methylation in human colorectal cancer. Biopsies were obtained from four colorectal lesions (one hyperplastic polyp, two adenomatous polyps and one adenocarcinoma) of patients before and after treatment with 200 mg celecoxib per day for 30 days, and analyzed for global DNA methylation and methylation in ER- α gene by QGMA. As expected, global DNA methylation was decreased in APs and AdCas before treatment (**Figure 6A**), whereas methylation of the ER- α gene was increased (**Figure 6B**). Interestingly, after a 30-day treatment with celecoxib, global DNA methylation was increased and hypermethylation of the ER- α gene was decreased in APs, but not in AdCas, indicating that the drug reversed global DNA hypomethylation and hypermethylation of ER- α gene in APs, but not in AdCas. This is an interesting finding because the reversibility of

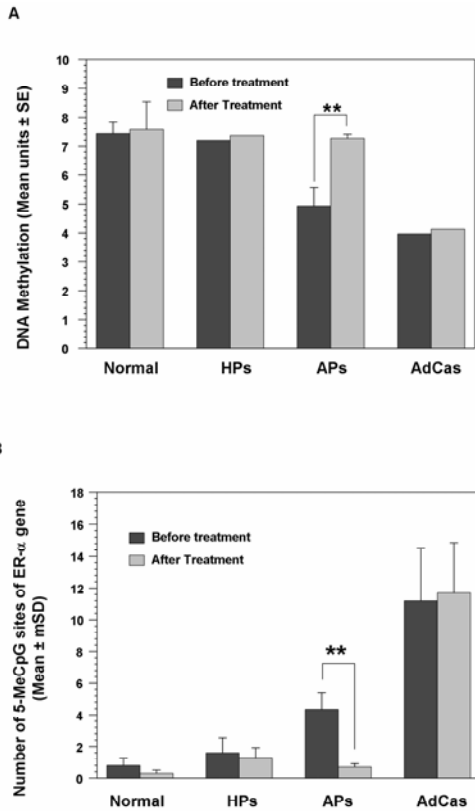


Figure 6 Effect of celecoxib on the methylation of global DNA and ER- α gene in colorectal tumors. Colorectal biopsies were obtained from four colorectal lesions (one hyperplastic polyp, two adenomatous polyps and one adenocarcinoma) before and after treatment of patients with 200 mg celecoxib per day for 30 days. The DNA was extracted and subjected to Dot-blot analysis and QGMA. **A.** Global DNA methylation was analyzed by Dot plot analysis with mAb to 5-MeC. The 5-Mec signal of DNA methylation was normalized by the equal intensity of 0.02% methylene blue staining. Results are expressed as means arbitrary units \pm SE. The asterisk indicates a significant difference before and after treatment with celecoxib, p-value <0.01 . **B.** ER- α gene methylation was analyzed by QGMA, as described in **Figure 3**. 10 colonies of each sample were examined for methylated CpG sites of ER- α gene, and the results are expressed as means \pm SE of 5-Mec-CPG sites per ER- α gene. **, $p < 0.01$, as compared before and after treatment with celecoxib.

global DNA hypomethylation combined with that of ER- α gene hypermethylation may be used as a reliable biomarker for chemoprevention.

Discussion

Colorectal cancer, as a genetic model of cancer, has been extensively studied at epigenetic and genetic levels [7, 18]. While epigenetic alteration has been reported at various developing stages of colorectal cancers [22, 44-46], its significance in diagnosis and prognosis of the diseases has not been well defined. Like other cancer, colorectal cancer may undergo the process of initial lesion (HPs) \rightarrow precancerous lesion (APs) \rightarrow cancerous lesions (AdCas) [6, 9, 47]. Thus, understanding of the epigenetic alteration in the process of colorectal cancer development may be important for early detection, prevention and treatment of the cancer. In this study, we investigated kinetically the alterations of global DNA and ER- α gene methylation from histologically "normal" mucosa \rightarrow HPs \rightarrow APs \rightarrow AdCas, and demonstrated that global DNA methylation was progressively decreased, while ER- α gene methylation was progressively increased from colorectal initial mucosal lesions to APs and AdCas. More importantly, the aberrant methylations in APs but not in AdCas can be reversed by NSAID celecoxib, suggesting a qualitative turning point for colorectal cancer development. These findings have the following important implications for colorectal cancer: a) the epigenetic changes can be used as a biomarker for predicting progression of colorectal neoplasm; b) the reversibility of the aberrant methylation may be used to distinguish the precancerous lesions likely to regress or not progress from those likely to progress to carcinoma in colorectum; c) Precancerous lesions but not cancerous lesions of colorectum can be treated with NSAIDs; and d) the status of global DNA and ER- α gene methylation may be a reliable marker for evaluating the effectiveness of primary and secondary chemoprevention of colorectal neoplasm.

Global DNA hypomethylation in colon adenomas and adenocarcinomas has been reported in colorectal cancer, using high pressure liquid chromatography (HPLC) analysis [48]. Due to the limited supply of tissue obtained from biopsies, we could not use HPLC, but had to develop other methods requiring much less tissue. By IHC and dot-blot analysis, we were able to quantitatively detect 5-MeC-CpG in DNA with a distinct pattern between HPs, APs and AdCas. Especially, IHC staining clearly demonstrated individual cells involved in aberrant methylation. Although the

scoring system currently used may be subjective, the IHC assay for global DNA methylation can be more precisely quantified by combination with digital pathology, by which the level of global DNA methylation in individual cells could be precisely detected and quantified at the initiation stage of cancer development.

Hypermethylation of ER- α gene has been reported in human colon cancers to be as high as 80% or more [17, 27], and increases with patient age, especially in menopausal females more than 50 years old [29, 30, 44, 49]. To quantitatively determine the methylation sites of the gene in paraffin-embedded section, we employed a highly specific and sensitive assay that requires only nanograms of DNA to quantitatively detect the methylated CpG in the promoter of ER- α gene. The QGMA involves bisulfite conversion of unmethylated cytosine to uracil, whole genome amplification, PCR amplification and radiolabelling of the remaining CpG sites with tritium-labeled methyl groups. Our results indicate that the method is highly sensitive without loss of specificity.

In this study, the methylation of both global DNA and the ER- α gene in HPs was not significantly different from that in "normal" mucosa. This, however, does not exclude epigenetic alteration occurring in some particular genes at this stage, such as *BRAF* and *KRAS* genes [45], known as "field effect" [46, 50]. Our study suggests that in HPs, only few or limited genes were involved in genetic alterations, and thus global DNA methylation was not significantly altered compared to "normal" mucosa. Since more and more genes were hypomethylated as HPs progressed to APs and AdCas, the alteration of global DNA methylation was significantly detected in APs and AdCas. Interestingly the kinetics of global DNA hypomethylation is consistent with that of ER- α gene hypermethylation. The significant alterations of global DNA and ER- α gene occurred during the transition from HPs to APs. This suggests that DNA hypomethylation and ER- α gene hypermethylation are two important parameters for predicting the progression of colorectal neoplasms.

One consequence of DNA methylation appears to result in recruitment of histone deacetylase (HDAC) that decreases the acetylation of DNA, leading to heterochromatin and decreased

transcription of genes [23, 51, 52]. The methylation of CpG islands that are included in the heterochromatin can be blocked. Hypomethylation decreases the binding of HDAC and opens up the chromatin, leading to unmasking of the CpG islands and enhanced transcription of genes. It is possible that carcinogen-induced global DNA hypomethylation might subvert the genetic stability, leading to aberrant activation of numerous silenced genes including embryonic stem (ES) cell genes. The activation of ES cell genes may exhibit as "atavism" [6]. The consequence of the "atavism" is supposed to be dependent on environmental niches [4, 6]. It is interesting that while global DNA methylation was progressively decreased during the development of colorectal cancer, ER- α gene methylation was simultaneously increased. The mechanisms underlying this paradoxical phenomenon might be a key to the realm of cancer.

In the clinic, precancerous lesions are reversible, as they may regress or progress to malignant lesions, probably depending on the tumorigenic environment in a given individual [4, 6]. Since the development of colorectal cancer is closely associated with intestinal inflammation [53], which can be prevented by anti-inflammatory drug celecoxib in humans [34-36, 54, 55] and animals [55-58], we explored whether the aberrant DNA methylation in APs and AdCas could be reversed by celecoxib in human patients [56-58]. The patients with APs but not AdCas treated with 200 mg celecoxib per day for 30 days demonstrated that hypomethylation in global DNA and hypermethylation in ER- α gene were synchronically decreased to the level of normal mucosa, suggesting that the aberrant DNA methylation is reversible in APs but not in AdCas. This finding is consistent with previous observation that administering celecoxib to rats with colon tumors reversed both global DNA hypomethylation and hypermethylation of ER- α gene in the tumors [31, 48]. Thus, global DNA and ER- α gene methylation status can potentially be used as a biomarker for distinguishing precancer from cancer in colorectum, or even other cancers. In addition, our finding also supports the notion that celecoxib can be used for secondary chemoprevention from recurrence of colorectal cancer after surgical therapy. Despite the limited number of cases in this study, two recent intervention trials, one in

patients with previous colorectal cancer, and another in patients with previous adenomas have offered strong evidence supporting the use of celecoxib for secondary prevention of colorectal neoplasia [35-38]. Thus, the reversibility of global DNA and ER- α gene methylation after short-term treatment of patients with celecoxib appears to be a reliable parameter for distinguishing APs from AdCas.

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