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Aberrant DNA methylation occurs in colon neoplasms arising in the azoxymethane colon cancer model

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Abstract

Mouse models of intestinal tumors have advanced our understanding of the role of gene mutations in colorectal malignancy. However, the utility of these systems for studying the role of epigenetic alterations in intestinal neoplasms remains to be defined. Consequently, we assessed the role of aberrant DNA methylation in the azoxymethane (AOM) rodent model of colon cancer. AOM induced tumors display global DNA hypomethylation, which is similar to human colorectal cancer. We next assessed the methylation status of a panel of candidate genes previously shown to be aberrantly methylated in human cancer or in mouse models of malignant neoplasms. This analysis revealed different patterns of DNA methylation that were gene specific. *Zik1* and *Gja9* demonstrated cancer-specific aberrant DNA methylation, whereas, *Cdkn2a/p16*, *Igfbp3*, *Mgmt*, *Id4*, and *Cxcr4* were methylated in both the AOM tumors and normal colon mucosa. No aberrant methylation of *Dapk1* or *Mlt1* was detected in the neoplasms, but normal colon mucosa samples displayed methylation of these genes. Finally, *p19^{Arf}*, *Tslc1*, *Hltf*, and *Mlh1* were unmethylated in both the AOM tumors and normal colon mucosa. Thus, aberrant DNA methylation does occur in AOM tumors, although the frequency of aberrantly methylated genes appears to be less common than in human colorectal cancer. Additional studies are necessary to further characterize the patterns of aberrantly methylated genes in AOM tumors.

Keywords

DNA methylation; azoxymethane; colorectal cancer; epigenetics

Introduction

Colorectal cancer affects ~148,000 people/year in the United States and is the third most common cancer in men and women in this country [1]. Most colorectal cancers arise from

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adenomatous polyps, and the risk of developing colon polyps and cancer appears to be a result of the effect of genetic and environmental factors that promote the formation of adenomas and/or the progression of these adenomas to cancer [2]. These environmental and genetic factors contribute to colorectal cancer formation by promoting the accumulation of gene mutations and epigenetic alterations in colon epithelial cells that drive the polyp→cancer formation process.

Many of the genetic events that occur in colon cancer have been identified, and their effects on cancer initiation and progression have been determined through the use of mouse models and human genetics. For instance, the role of *APC* as an initiating event in the adenoma-cancer progression sequence was determined through studies of the cancer family syndrome, Familial Adenomatous Polyposis, which is caused by germline mutations in *APC*, and through studies of the *Apc^{Min}* mouse, which develops intestinal adenomas. Mouse models of cancer have further revealed how gene mutations cooperate in the carcinogenesis process to promote the progression of adenomas initiated by *APC* mutations [3,4].

Although aberrant DNA methylation has been recently shown to occur commonly in colorectal cancer, the causal role of these epigenetic changes in the process of cancer initiation and promotion is poorly understood at this time. It has been established that the aberrant hypermethylation of tumor suppressor genes can result in their transcriptional silencing, which is the mechanism through which DNA methylation is believed to promote cancer formation. DNA methylation appears to cooperate with concurrent alterations in chromatin structure to repress transcription [5–7]. However, little is known regarding the precise timing of these epigenetic alterations in the transition of normal colon epithelial cells to cancer cells through the polyp→cancer progression sequence. Furthermore, the biological role that these aberrantly methylated genes have on driving the formation of colorectal cancer is also poorly understood [8].

A well-established mouse model of colorectal cancer that has the potential to provide insight into the role of aberrant DNA methylation in the molecular pathogenesis of the polyp→cancer progression sequence is the azoxymethane (AOM) rodent colon cancer model. This model employs the carcinogen AOM to induce neoplasms that recapitulate the adenoma-carcinoma sequence in the mouse colon [9,10]. The AOM model also displays some of the common molecular events seen in human colorectal cancer, including the accumulation of *Kras* mutations and increased COX2 expression [11–13]

Mouse models have already proven useful in studying the role of DNA methylation in the mouse skin multistage carcinogenesis [14]. Fraga *et al* assessed the role of DNA methylation in this well-characterized cancer model and found that specific epigenetic events correlated with both initiation steps and with progression steps. They identified several novel genes that were methylated in the mouse model and verified that they are also methylated in primary human cancers [14]. Aberrantly methylated genes have also been identified in mouse models of malignant fibrous histiocytomas, lung cancer, bladder cancer, and leukemia, demonstrating the potential to use mouse models to study the role of epigenetic alterations in cancer initiation and progression [15–19].

Furthermore, with regards to mouse models of intestinal cancer and epigenetic alterations, recently, Hahn *et al* investigated the glutathione peroxidase *Gpx1* and *Gpx2* double knockout mouse using the genome wide methylation analytical technique MIRA (Methylated CpG island recovery assay) and identified a group of genes hypermethylated in chronically inflamed, aged, or neoplastic tissue, suggesting that mouse models of intestinal cancer likely display aberrant DNA hypermethylation [20]. It has also already been shown in both the AOM model and mutant *Apc* mouse models of intestinal cancer that the global

DNA hypomethylation observed in human colorectal malignancy is present in tumors arising in these mice suggesting the epigenetic alterations related to DNA methylation will be similar in these models to human colorectal cancer [21–23]. Furthermore, Linhart et al observed in *Apc^{min/+}* mice over expressing DNA methyltransferase 3b1 (*Dnmt3b1*) that the mice develop larger, more frequent intestinal tumors compared to mice that express normal levels of *Dnmt3b1* [24]. In aggregate, these studies suggest that alterations in DNA methylation can contribute to tumor pathogenesis in mouse models. Consequently, we assessed the methylation state of tumor suppressor genes in the AOM colon cancer model to determine the role of epigenetic alterations in cancer initiation and progression.

In this study, we have found that AOM induced tumors are globally hypomethylated compared to normal colonic mucosa, an observation frequently seen in human neoplasms [25]. Using methylation specific PCR (MSP), we investigated the methylation status of eleven candidate genes known to be aberrantly methylated in human cancer or mouse tumor models and demonstrate that these genes show gene specific patterns of methylation in the tumors and normal tissues. Of note, two of these genes, *Zik1* and *Gja9*, show aberrant DNA methylation in the tumors that is not present or is present at a low frequency in the normal colon. These results suggest that epigenetic events that recapitulate human colorectal cancer occur in the AOM model.

Materials and Methods

Generation of AOM induced tumors

The AOM induced colon neoplasms were generated as described previously [26]. At 6 weeks of age, the mice were treated with AOM subcutaneously twice a week for six weeks, and sacrificed at age 22 to 29 weeks.

Tissue Harvesting

Tissue was harvested as described [26] from mice aged 22 to 29 weeks. After the mice were sacrificed, the intestine was flushed with saline, cut longitudinally with scissors and inspected for tumors. Tumors were dissected from normal mucosa with a razor blade and frozen in liquid nitrogen. A sample of each tumor was sectioned and analyzed by standard histological preparation with hematoxylin/eosin staining. Tumor grade was determined by an experienced pathologist (M. Kay Washington) using criteria defined by the Consensus Report of the Pathology of Mouse Models of Intestinal Cancer [9].

To obtain normal tissue for MSP analysis, wild type FVB mice were sacrificed at age 12–24 weeks and normal colonic epithelium scrapings were collected by cutting open the colon longitudinally and scraping the mucosal surface with a glass slide. Scrapings were collected into microcentrifuge tubes and snap frozen in liquid nitrogen. Colonic crypt epithelial cells, used for global methylation analysis, were isolated by first dissecting colonic epithelium (from cecum to rectum) from 12–24 week old mice. After sacrifice, the colon was cut open longitudinally with scissors and soaked in Hanks' Balanced Salt Solution without Calcium or Magnesium (HBSS, Gibco, Carlsbad, CA) for 5 minutes on ice, then incubated for 5 minutes in HBSS + 25% HEPES + 1% Fetal Bovine Serum. The mucosa was then transferred to a 15 ml conical tube with 10 ml HBSS + 10 mM EDTA and vigorously shaken by hand for five minutes, followed by rotation for 20 minutes at 4°C. This process of shaking/rotating was repeated three times. The colon was then removed from the 15 ml conical tube using forceps and the remaining epithelial cell suspension was centrifuged at 2000 rpm for 5 minutes at 4°C. The supernatant was removed and the cell pellet was frozen in liquid nitrogen for future genomic DNA extraction.

DNA extraction and sodium bisulfite treatment

Genomic DNA was isolated either from frozen tissue samples using the Puregene kit (Qiagen, Valencia, CA) or from formalin fixed, paraffin embedded tissue sections using InstaGene Matrix (Bio-Rad, Hercules, CA) as previously published [27]. Enzymatically methylated DNA was generated using *SssI* methylase (New England Biolabs, Beverly, MA) and used as a positive methylated DNA control for MSP assays. Whole genome amplified (WGA) genomic DNA, which is completely unmethylated, was created using the Repli-g kit (Qiagen, Valencia, CA) following the manufacturer's protocol and was used as a negative control for MSP assays. The resulting DNA was sodium bisulfite modified as previously described [28].

Global methylation analysis

Global DNA methylation was determined using a slot-blot and anti-methyl cytosine antibody method adapted from Tao et al and Vertosick et al [29,30]. Genomic DNA from colonic crypt epithelial cells was isolated from mock treated mice and purified as described. DNA was quantified using Picogreen according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The DNA (40 ng) was diluted into 50 μ l Tris Buffered Saline (TBS) and denatured at 95° C for 10 minutes. Each sample was then slot blotted three times onto an Optitran BA-s Nitrocellulose Transfer membrane (Whatman, Schleicher & Schuell, Germany) and bound to the membrane using a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA) set to 150 mJoules. The cross-linked blot was blocked in 5% fat free milk in TBS for 1 hour at room temperature, then hybridized overnight at 4°C with an anti-5'-methyl cytosine antibody (Eurogentec, San Diego, CA) in 5% milk + TBS at a dilution of 1:500. The blot was washed 3 times in TBS then incubated with an anti-mouse secondary antibody conjugated to an infrared dye (IRD 800) in 5% milk + TBS for 4 hours at room temperature. The blot was washed 3 times with TBS and imaged using a LI-COR infrared scanner (LICOR, Lincoln, NE). Supplementary figure 2 demonstrates linearity of the assay using control calf thymus DNA.

Methylation Specific PCR (MSP)

MSP analysis of bisulfite treated DNA was performed using the methylated and unmethylated primer sets listed in Supplementary Table S1. MSP reactions were performed as previously described [31]. SSSI treated DNA and WGA amplified DNA subjected to bisulfite modification was used as methylated and unmethylated controls for the MSP assays.

Bisulfite Sequencing

Bisulfite treated DNA was PCR amplified with bisulfite sequencing primers generated using MethPrimer, listed in Supplementary Table S1 [32]. PCR conditions were as follows: 95° \times 15"; (94° \times 30"; 60° \times 30"; 72° \times 30"; 72° \times 10') \times 35 cycles. PCR amplicons were cloned into a TA vector (Invitrogen, CA), transformed, subjected to DNA extraction (plasmid mini-prep kit, Qiagen) and sequenced on an ABI 3730x1 DNA Analyzer in the FHCRC Genomics Shared Resource Core following the manufacturer's suggested protocol.

Quantitative RT-PCR

RNA was isolated from EDTA isolated crypt cells or AOM induced tumors using Trizol (Invitrogen, CA) followed by RNeasy purification (Qiagen, CA) following the manufacturer's instructions. cDNA was synthesized using SSII reverse transcriptase (Invitrogen, CA) per reagent protocol. TaqMan assays (Applied Biosystems, CA) for *Zik1* (Assay # Mm00494365) and *Gja9* (Assay # Mm00439121) were performed on an Opticon 2 thermocycler (Bio-Rad, CA) in triplicate and normalized using *Gusb* expression (Assay #

Mm01197698). Each assay was repeated three times and error bars were generated by calculation of the standard error of the mean.

Results

AOM-induced tumors display global DNA hypomethylation compared to normal colonic mucosa

We initially assessed the global DNA methylation status of genomic DNA isolated from AOM induced tumors and compared this to DNA from the colon mucosa. To quantify the amount of total DNA methylation of AOM induced neoplasms, we used a novel anti-methyl cytosine slot-blot method. Single stranded DNA from each normal and tumor sample was spotted in triplicate on a nitrocellulose blot and probed with an antibody that recognizes methylated cytosines. The signals from each spot were quantified using a LI-COR infrared scanner, and the replicates averaged to determine relative methylation levels for each sample. Global methylation levels of five AOM induced colon adenocarcinomas were compared to normal colon crypt epithelial cells by calculating the ratio of methylation signal to total DNA for each sample (Figure 1A). The ratio of signal from the tumor over the normal average represents the fraction of total methylation gained or lost in each sample (Figure 1B). Four out of five tumors were observed to have globally hypomethylated DNA compared to the normal colon epithelium (average decrease of 16.5%, range 4–25%), suggesting that these tumors display decreased DNA methylation across the entire genome, a phenomena observed in human cancer [25]. It is interesting to note that the degree of hypomethylation is specific to an individual tumor as both the T2 and T3 tumors are synchronous tumors from the same mouse. This finding could be due to differing degrees of contamination with normal stromal tissue or may reflect heterogeneity in the underlying molecular pathology of the tumors even in the same individual.

The aberrant methylation of tumor suppressor genes occurs in AOM induced colon cancer

To determine if aberrant DNA methylation occurs in tumors in the AOM model, we isolated genomic DNA from both AOM induced colonic adenocarcinomas and age matched normal mouse colon epithelium not exposed to AOM. We identified candidate genes for methylation analysis by surveying the published literature for genes shown to be methylated in mouse models of cancer and/or to be methylated in human cancer. We selected a total of thirteen genes that met these criteria for further analysis and assessed the methylation status of these genes in the AOM induced tumors (Table 1, Figure 2, and Table 2). We identified four patterns of gene methylation which were as follows: 1) genes methylated more frequently in tumor tissue than in normal colon mucosa; 2) genes methylated in both tumor tissue and normal colon mucosa; 3) genes methylated in colon mucosa but not in the AOM induced tumors; 4) no gene methylation in either colon mucosa or tumor tissue. An example of a gene in group 2 is *Igfbp3*, which is methylated in the majority of tumor samples (70%; N=10), but interestingly, also in a large number of normal epithelium samples (63%; N=8; t=1.0). Methylation was observed in both normal tissue and AOM tumors for *Mgmt*, *Id4*, *Cdkn2a/p16^{Ink4a}*, and *Cxcr4*. Aberrant methylation of *Mgmt* was present in 38% of the normal colon samples (N=8) compared to 25% of the tumors (N=12; p=0.64). We observed similar patterns for *Id4* (18% methylation in tumors, N=11, versus 43%, N=7, in normal mucosa; p=0.33), *Cdkn2a/p16^{Ink4a}* (25% of tumor samples, N=12, versus 44%, N=9, in normal colon mucosa; p=0.26), and *Cxcr4* (8% of the tumors, N=12, versus 25%, N=8, in normal colon samples; p=0.54). Surprisingly, methylation of *Dapk1* and *Mlt1* was only detected in normal mucosa suggesting these genes may be hypomethylated in the AOM tumors. Finally, no promoter methylation was identified in *p19^{Arf}*, *Tslc1*, *Hltf*, or *Mlh1* in either the AOM induced tumors or normal colon mucosa.

Finally, we identified two genes, *Gja9* and *Zik1*, that were aberrantly methylated more commonly in the AOM tumors than in the normal colon mucosa. (Figure 3 and Table 2). *Gja9* was methylated in 93% of AOM tumors (N=15) compared to 50% of normal colon (N=6, p=0.06) and *Zik1* was methylated in 13% of tumors (N=15) and 0% of normal colonic mucosa (N=6, p=0.37). Furthermore, bisulfite sequencing of 10 clones from an AOM tumor that demonstrated methylation of *Gja9* and *Zik1* by MSP confirmed the results of the MSP assays. As shown in Figure 3, *Gja9* demonstrated dense methylation in several clones, although several were completely unmethylated. *Zik1* demonstrated significantly less methylation (present in only 3 of 10 clones), which correlates with the low level of methylation demonstrated by the MSP assay (Figure 2). We propose that the majority of the clones demonstrating no methylation are likely from normal cells that are intermixed in the tumors.

Expression of *Zik1* and *Gja9* was determined using quantitative RT-PCR. TaqMan assays were performed on AOM tumors and normal colon epithelium. *Zik1* demonstrated decreased expression in AOM tumors that carried methylated *Zik1* as well as in tumors that carried unmethylated *Zik1* when compared to normal colonic epithelium (Figure 4). Interestingly, *Gja9* was not expressed in either AOM tumors or normal colon (data not shown).

We further assessed the methylation status of these genes in the AOM tumors by analyzing the methylation status of all of the genes in each individual tumor to determine if a subset of the tumors displayed an excessive proportion of methylated genes similar to the CpG Island Methylator Phenotype (CIMP) observed in human colon cancer[33,34]. To determine if the tumors could be stratified based on the proportion of aberrantly methylated genes, we compared the tumors based on the number of methylated genes from each individual tumor. For tumors that were analyzed by MSP for greater than 2 genes (N=12), an average 1.4 genes were methylated per tumor (range 0–3). A similar pattern was found in normal colonic epithelium, with a mean of 2.1 genes methylated per sample (range 0–5, p=0.27, student's t-test). Thus, we did not identify a subset of tumors that displayed a high proportion of methylated genes.

Discussion

The discovery of aberrantly methylated genes in cancer has led to an intense interest in determining the role of these epigenetic events in the molecular pathogenesis of cancer[34–41]. Mouse models of cancer have the potential for determining the causal factors that lead to the hypermethylation of tumor suppressor genes. Consequently, studies of aberrant gene methylation have been carried out in mouse models of lung cancer, skin cancer, leukemia, and prostate cancer[14,19,24,42,43]. These tumor models have included both genetic and carcinogen-induced models and have revealed that aberrant DNA methylation does occur in the tumors arising in these models. For instance, Patel et al have reported that methylene chloride exposure leads to methylation of the *p16^{Ink4a}* tumor suppressor gene, which is an epigenetic alteration that likely contributes to tumorigenesis in primary lung cancer[44]. Vuilleminot *et al* showed that lung tumors generated by cigarette smoke or other carcinogens display promoter methylation of the H-cadherin and the progesterone receptor genes [43]. Furthermore, Hinoi et al reported tumor specific hypomethylation in the intestinal neoplasms arising in *Apc^{min}* mouse by 5-methylcytosine immunohistochemistry [45]. In the TRAMP prostate cancer model and in the *Il15* transgenic mouse model of acute lymphocytic leukemia, restriction landmark genome scanning has been used to identify genes that are aberrantly methylated in the neoplasms in these mice[19,42].

We have now assessed the role of aberrant gene methylation in a model of colon cancer, the AOM induced colon adenocarcinoma model. We first analyzed global DNA methylation

using a 5-methyl cytosine antibody method and found that AOM tumors are generally hypomethylated compared to normal mouse colonic epithelium. These results are consistent with previously published studies and confirm the use of our model system as being generalizable to other studies of AOM induced colon cancer [23]. This validation is relevant in light of the considerable variability in susceptibility to AOM induced tumors observed between mouse strains [46–48]

Because one of the most common epigenetic alterations observed in human colorectal cancer is hypomethylation of the tumor genome with selective hypermethylation of the promoter regions of tumor suppressor genes, after identifying global DNA hypomethylation in the AOM tumors, we examined the methylation status of a series of candidate tumor suppressor genes [49–54]. The genes were selected primarily based on published studies showing them to be methylated in cancers occurring in mouse models or in human cancer. We identified different patterns of methylation of the genes when the methylation status of the genes in the tumors was compared to normal colon mucosa. A subset of tumors carried methylated *Mgmt*, *Id4*, *Cdkn2a* (*p16^{Ink4a}*), and *Cxcr4*. *Mgmt*, *Id4*, and *Cxcr4* were methylated in <25% of the tumor samples, with similar frequencies detected in normal colon mucosa. Several of the genes analyzed (*Dapk*, *Mlt1*, *Tslc1*, *Hltf*, *Mlh1*, and *p19^{Arf}*) were not methylated in the AOM induced tumors or in the normal colon mucosa. Two additional genes of note are *Dapk* and *Mlt1*, which displayed low levels of methylation in normal colonic mucosa and no methylation in the tumors, suggesting these genes may be hypomethylated in a subset of tumors. These patterns of methylation in normal colon and colon cancer are also observed in human colon and highlight the importance of the reference tissue used to establish the normal methylation state of the gene [55,56].

Finally, we observed aberrant methylation of two genes, *Gja9* and *Zik1*, which represent 15% of the candidate genes assessed. *Gja9* (Gap Junction protein alpha-9), also known as *Cx36*, encodes for a 59 kDa protein involved in gap junction formation and function. It is the main connexin found in the central nervous system that plays an important role in gap junctions responsible for synaptic transmission [57]. *Gja9* is also involved in pancreatic beta islet cell function and mice lacking *Gja9* demonstrate decreased glucose-induced insulin production [54]. The aberrant DNA methylation of *Gja9* has not been reported previously and was identified as a candidate gene based on preliminary results from genome-wide methylation studies that are in progress (Conerly, personal communication). *Zik1* (Zinc finger protein-Interacting with K protein 1) was first identified as a transcriptional repressor that binds to the nuclear ribonucleoprotein particle K protein [58]. *Zik1* has also been identified by Methylation Sensitive Representational Difference Analysis (MS-RDA) as being hypermethylated in intestinal metaplasia (IM) of the stomach. Mihara *et al* reported that *Zik1* is hypermethylated in 100% of human gastric IM samples (N=16), 80% of gastric cancer cell lines (N=10), and 73% of primary gastric cancer tumor samples (N=15), suggesting that epigenetic silencing of *Zik1* may contribute to the pathogenesis of gastric neoplasia [52]. Our discovery that this gene is also hypermethylated in mouse AOM induced tumors suggests that *Zik1* may play an important role in the transformation of gastrointestinal mucosa from normal to cancer. We have also demonstrated that *Zik1* shows decreased expression in AOM tumors regardless of methylation status, further supporting this hypothesis.

Some of our results differ from those of previously published studies which may reflect differences between the normal methylation states of the primary tissues from which the tumors were derived or differences in the assays used to assess the methylation state in the CpG islands of the genes of interest [14,24,43]. Further studies are needed to reconcile these differences. In addition, we found that the methylation state of *Zik1* correlates with decreased expression but that *Zik1* expression is decreased in AOM tumors regardless of

the tumor's methylation status. This finding suggests that *Zik1* may play an important role in the formation of AOM tumors and may be downregulated by both genetic and epigenetic mechanisms. Future studies to determine the role of *Zik1* in colon cancer carcinogenesis are necessary to better understand these findings. In addition, the identification of *Gja9* being a gene that is not expressed in the colon but that shows increased aberrant methylation in AOM tumors compared to normal colon mucosa demonstrates that there are genes similar to methylated *VIM*, which is observed in human colorectal cancer, in the AOM model. *VIM* is not expressed in human colon mucosa or colorectal cancer but methylated *VIM* is commonly detected in colorectal cancers but not in colon mucosa [59].

Importantly, our studies demonstrate that aberrantly methylated genes do arise in the colon cancers arising in the AOM model. In light of our results using a candidate gene approach, we propose that genome wide methods to investigate the DNA methylation status in AOM tumors are likely to identify novel methylated genes that can be used for studying the role of epigenetically altered genes in this model of colorectal cancer. Restriction enzyme based methods, such as Differential Methylation Hybridization (DMH), Methylated CpG Island Amplification (MCA), Restriction Landmark Genomic Scanning (RLGS), or antibody based approaches such as MeDIP, coupled with microarray technology have been effectively used to identify hypermethylated genes in human and mouse tumors[19,42,56,60–64]. Thus, the use of “methylation arrays” to characterize the methylome in AOM induced tumors has the potential to provide a more comprehensive and unbiased assessment of genes methylated in AOM induced tumors.

In summary, we have demonstrated that similar to human colorectal cancer, AOM induced mouse colonic tumors display global DNA hypomethylation and aberrantly hypermethylated genes. Further studies of the epigenetic alterations using genome wide assays should provide a comprehensive assessment of the methylation state of the DNA in these tumors and will demonstrate the potential of this model to be used to further study the molecular pathology of aberrant DNA methylation in colorectal cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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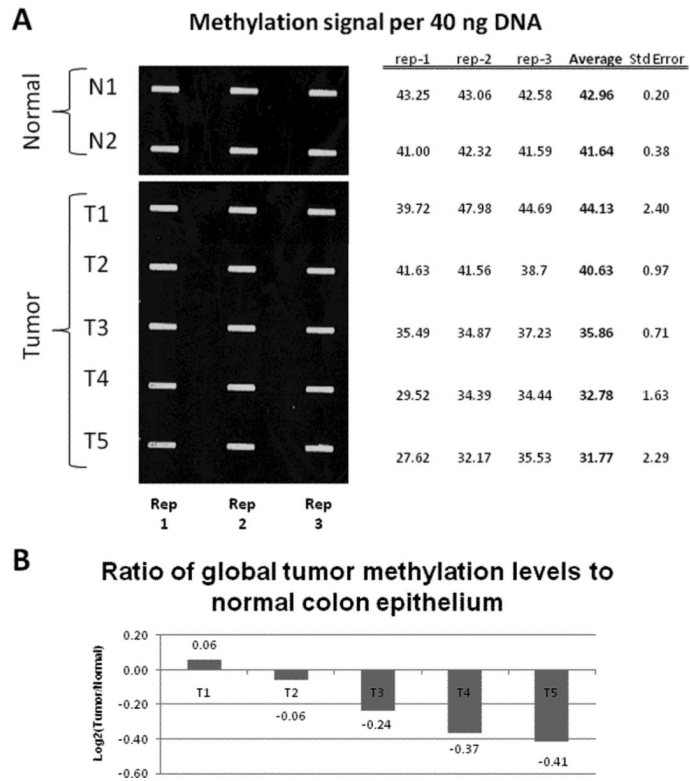


Figure 1. Global methylation analysis of AOM induced tumors

A.) DNA samples from normal colon (N1 and N2) and colon adenocarcinomas (T1–T5) were heat denatured, spotted on a nitrocellulose membrane in triplicate then immobilized with ultraviolet light. The blot was probed first with an antibody against methylcytosine, then with a secondary antibody conjugated to an infrared dye. Three replicates (Rep 1–3) were performed for each sample. The signal for each band was quantified and the average and standard errors were calculated for each sample. B.) The N1 and N2 samples were averaged to set a normal threshold. Each sample was then graphed as the Log₂ ratio of this normal threshold.

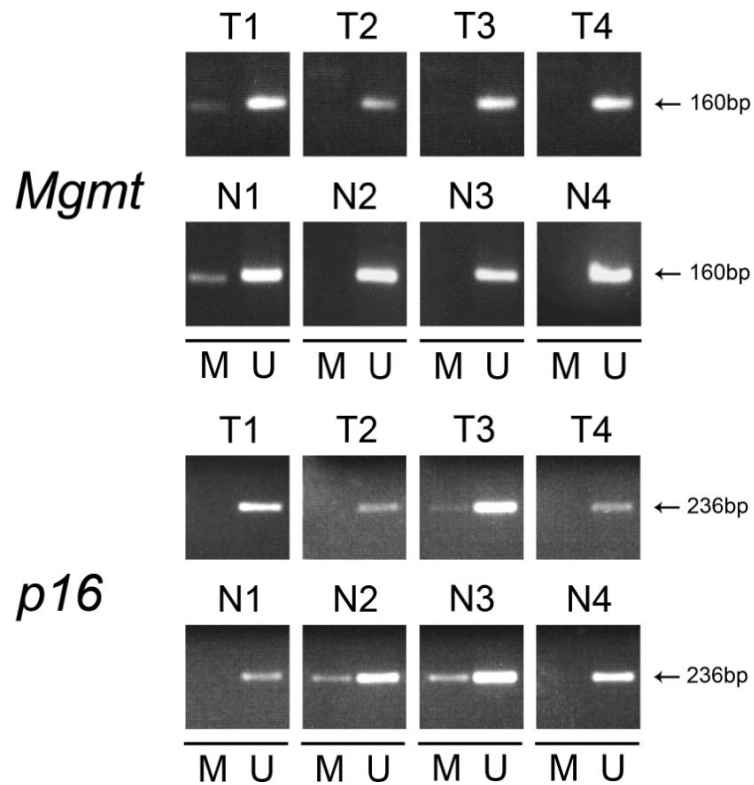


Figure 2. MSP analysis of representative candidate genes in AOM induced colon adenocarcinomas and normal colon mucosa

Representative images from MSP assays for *Mgmt* and *Cdkn2A* (p16^{Ink4a}) from colon neoplasms (T1–T4) and normal colon (N1–N4) are shown. Methylated genes (M) are present in the normal colon mucosa and colon tumor tissues. Unmethylated (U) genes were identified in both normal and tumor cells, which may represent contamination with heterogeneous tissue elements or a clonal mixture of cells. MSP reactions were performed using the primer sets and annealing parameters listed in Supplementary Table 1. Amplicon size (in bp) is shown. *Sssi* (methylation positive control) and WGA (methylation negative control) control DNA samples are not shown.

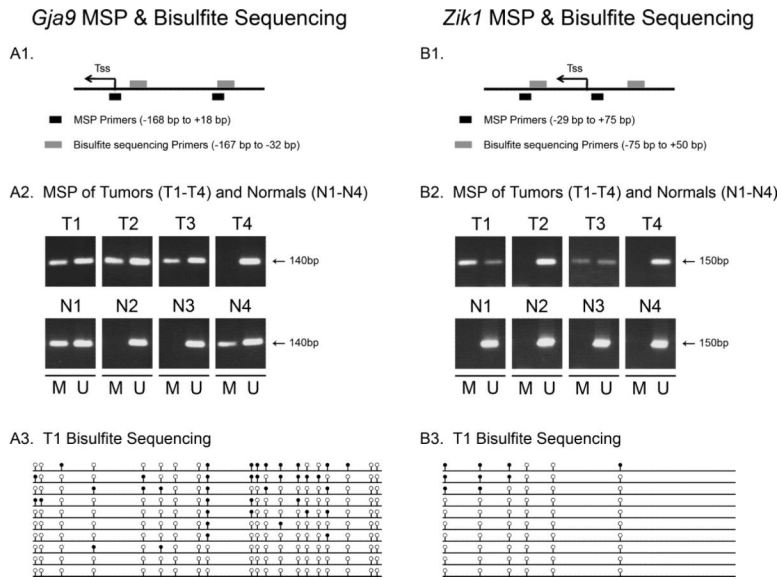


Figure 3. MSP and bisulfite sequencing for *Gja9* and *Zik1*
 A1, B1. Schematic diagrams of the promoter region of *Gja9* and *Zik1*, respectively. Location of the transcription start site (Tss) is shown with an arrow and location of MSP and bisulfite sequencing primers are shown with black and grey boxes, respectively. A2, B2. Results of MSP analysis for *Gja9* and *Zik1* for four AOM tumors (T1–T4) and normal colon samples (N1–N4). Methylated (M) and Unmethylated (U) genes are shown. A3, B3. Results of bisulfite sequencing analysis of the promoter region of *Gja9* and *Zik1*. Ten clones for each gene were isolated and sequenced from a tumor that was shown to carry either methylated *Gja9* or *Zik1* by MSP. Empty and black circles represent unmethylated and methylated CpG dinucleotides, respectively. A subset of the sequenced clones demonstrates methylation for each gene.

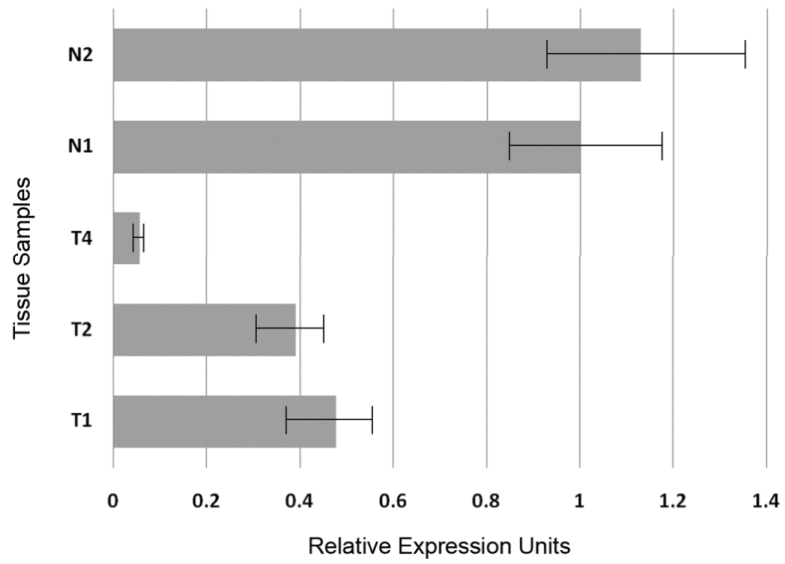


Figure 4. qRT-PCR analysis of *Zik1* expression

Normalized expression of *Zik1* in AOM tumors T1–T4 and normal colonic epithelium N1–N2. The designation of the tumors corresponds to methylation analysis of tumors and normal tissue demonstrated in Figure 3.

Table I

Candidate gene list for investigation of methylation in AOM induced tumors

Gene Name	Symbol	Description	Hypermethylated in human or mouse neoplasms?	Reference
Cyclin dependent kinase inhibitor 2a isoforms p16 ^{Ink4a} and p19 ^{ARF}	<i>Cdkn2a p16^{Ink4a}</i> <i>Cdkn2a p19^{ARF}</i>	Methylated in many human cancers and mouse models of lung, prostate, and pancreatic cancer	Both	[18,65–67].
Death associated protein kinase	<i>Dapk</i>	Hypermethylated in 81% of colorectal carcinomas and mouse lung tumors	Both	[17,68].
O6-methylguanine-DNA methyltransferase	<i>Mgmt</i>	Frequently methylated in colorectal cancer and in skin tumors mouse models of	Both	[14,69]
Inhibitor of DNA Binding 4	<i>Id4</i>	Methylated in both human and mouse leukemia	Both	[19]
Helicase-like transcription factor	<i>Hltf</i>	Hypermethylated in the progression of normal colonic tissue to adenoma to adenocarcinoma	Human	[35,55,70]
MutL homologue 1	<i>Mlh1</i>	Frequently methylated in human colon tumors, contributes to microsatellite instability	Human	[40,71,72]
Methylated in liver tumor 1	<i>Mlt1</i>	Hypermethylated in a SV40 T antigen transgenic mouse model that forms liver tumors.	Mouse	[73]
Tumor suppressor in lung cancer 1	<i>Tslc-1</i>	Hypermethylated in N-nitrosodiethylamine (DEN) induced murine hepatocellular carcinomas	Mouse	[74]
Insulin-like Growth Factor binding protein 3	<i>Igfbp3</i>	Hypermethylated in a mouse carcinogen skin cancer model	Mouse	[14]
Zinc finger protein interacting with K protein 1	<i>Zik1</i>	Hypermethylated in gastric cancer	Human	[52]
Gap junction alpha-10 protein	<i>Gja9</i>	Hypermethylated in mouse tumors	Mouse	(Conerly, personal communication)
C-X-C chemokine receptor type 4	<i>Cxcr4</i>	Methylated in a mouse carcinogen skin cancer model	Mouse	[14]

Table 2

MSP analysis of AOM induced tumors and normal mouse colonic epithelium

Gene	AOM Tumors: Frequency of Methylated Genes (N, %)		Normal Intestinal Epithelium Frequency of Methylated Genes (N,%)	
<i>Cdkn2a/p16^{INK4a}</i>	3/12	(25)	4/9	(44)
<i>Igfbp3</i>	7/10	(70)	5/8	(63)
<i>Mgmt</i>	3/12	(25)	3/8	(38)
<i>Id4</i>	2/11	(18)	3/7	(43)
<i>Cxcr4</i>	1/12	(8)	2/8	(25)
<i>Dapk1</i>	0/8	(0)	3/8	(38)
<i>p19^{Arf}</i>	0/8	(0)	0/7	(0)
<i>Mt1</i>	0/6	(0)	1/8	(13)
<i>Tslc1</i>	0/4	(0)	0/1	(0)
<i>Hlf</i>	0/11	(0)	-	-
<i>Mlh1</i>	0/11	(0)	-	-
<i>Gja9</i>	14/15	(93)	3/6	(50)
<i>Zik1</i>	2/15	(13)	0/6	(0)