Genes with Aberrant Expression in Murine Preneoplastic Intestine Show Epigenetic and Expression Changes in Normal Mucosa of Colon Cancer Patients

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Abstract
An understanding of early genetic/epigenetic changes in colorectal cancer would aid in diagnosis and prognosis. To identify these changes in human preneoplastic tissue, we first studied our mouse model in which Mthfr+/− BALB/c mice fed folate-deficient diets develop intestinal tumors in contrast to Mthfr+/+ BALB/c mice fed control diets. Transcriptome profiling was performed in normal intestine from mice with low or high tumor susceptibility. We identified 12 upregulated and 51 downregulated genes in tumor-prone mice. Affected pathways included retinoid acid synthesis, lipid and glucose metabolism, apoptosis and inflammation. We compared murine candidates from this microarray analysis, and murine candidates from an earlier strain-based comparison, with a set of human genes that we had identified in previous methylome profiling of normal human colonic mucosa, from colorectal cancer patients and controls. From the extensive list of human methylome candidates, our approach uncovered five orthologous genes that had shown changes in murine expression profiles (PDK4, SPRR1A, SPRR2A, NR1H4, and PYCARD). The human orthologs were assayed by bisulfite-pyrosequencing for methylation at 14 CpGs. All CpGs exhibited significant methylation differences in normal mucosa between colorectal cancer patients and controls; expression differences for these genes were also observed. PYCARD and NR1H4 methylation differences showed promise as markers for presence of polyps in controls. We conclude that common pathways are disturbed in preneoplastic intestine in our animal model and morphologically normal mucosa of patients with colorectal cancer, and present an initial version of a DNA methylation-based signature for human preneoplastic colon.

Cancer Prev Res; 6(11); 1171–81. ©2013 AACR.

Introduction
Nearly one million people worldwide develop colorectal cancer every year (1). Colorectal cancer results from a combination of environmental and genetic factors that convert normal epithelium into a malignant tumor through multiple stages. An understanding of early events in tumorgenesis will lead to timely diagnoses and improved outcomes.

Epigenetic changes are early events in colorectal cancer and other neoplasias. There are numerous genes with methylation differences between colorectal tumors and adjacent tissues (2–4). However, there are limited data on differential methylation in normal colonic mucosa between controls and patients with colorectal cancer (5, 6 and our own recent report (7)). To identify protumorigenic changes in normal human colonic mucosa, we began with the identification of candidate genes in our mouse model that had previously been shown to develop intestinal tumors after administration of low folate diets (8). Since folates generate methyl groups for DNA methylation, we predicted that there would be genetic/epigenetic changes in preneoplastic intestine in our mouse model and that some of these changes might be similar to those seen in human colonic mucosa. We therefore compared murine candidates to the unrestricted list of preliminary human genes that had shown changes following methylation profiling of normal colonic mucosa in colorectal cancer patients and controls (7 and unpublished data).

Our mouse model reflects some of the genetic and nutritional factors that affect risk for human colorectal cancer. Individuals with low folate intake are more susceptible to colorectal cancer than individuals with adequate intake (9). A polymorphism in methylenetetrahydrofolate reductase...
arrays to compare BALB/c early events in intestinal neoplasia. In this report, to identify ity would increase oxidative stress/damage and lead to tent with the hypothesis that enhanced RXR/PPAR activ-

rectal cancer. Furthermore, these methylation differences state, are shared by our mouse model and human colo-
tumorigenic mechanisms, reflecting an altered metabolic cancer and controls. Our results suggest that common human colonic mucosa between patients with colorectal as well as significant expression differences, in normal human genes that showed significant pyrosequencing-
our inter-species comparison led to the identification of 5 human genes that showed significant pyrosequencing-
tumor-susceptible BALB/c and tumor-resistant C57BL/6 mice were fed folate-deficient (FD) or control diets (CD) for one year. Tumors were only observed in BALB/c mice fed FD (8, 12); a single functional copy of the Mthfr gene increased the number of FD mice with tumors (8). Several tumor-predisposing candidate genes involved in cell-cycle control, cell survival, and DNA repair were identified by comparing expression profiles of tumor tissue with normal tissue (13). These observations, in combination with increased DNA damage (14, 15), decreased expression of tumor suppressors and increased retinoid/PPARA pathway activity in BALB/c normal pre-neoplastic intestine (15), could explain the susceptibility of these mice to intestinal tumorigenesis. We observed differential expression of Bcmo1, Aldh1a1, and Spry2a when comparing CD- and FD-fed BALB/c mice. Expression differences were also seen for Bcmo1 between Mthfr+/− and Mthfr+/+ mice. These changes were consistent with the hypothesis that enhanced RXR/PPAR activity would increase oxidative stress/damage and lead to neoplasia (15).

Our unique mouse model, without germline mutation or carcinogen induction, provides an opportunity to study early events in intestinal neoplasia. In this report, to identify specific candidate genes in tumorigenesis, we used microarrays to compare BALB/c Mthfr+/− CD mice and BALB/c Mthfr−/− FD mice, which have relatively lower and higher intestinal tumor susceptibility, respectively (15). We found significant differences in retinoid/PPARA pathway genes between BALB/c mice fed FD and CD. The activation of this pathway is consistent with the findings from our previous report on gene expression profiling between tumor-susceptible BALB/c and tumor-resistant C57BL/6 mice (15).

We compared our murine candidates, obtained from both of the aforementioned expression microarrays, to the extensive list of candidate human genes identified through methylation profiling (7 and unpublished data). Our inter-species comparison led to the identification of 5 human genes that showed significant pyrosequencing-based methylation differences, in 14 CpG dinucleotides, as well as significant expression differences, in normal human colonic mucosa between patients with colorectal cancer and controls. Our results suggest that common tumorigenic mechanisms, reflecting an altered metabolic state, are shared by our mouse model and human colorectal cancer. Furthermore, these methylation differences contribute to an epigenetics signature for diagnosis of colonic neoplasia.

Materials and Methods

Mice and diets

Animal experimentation was approved by the Montreal Children’s Hospital Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines. After weaning, BALB/c Mthfr+/− and Mthfr−/− mice were fed CD (2 mg folate/kg diet) or FD diets (0.3 mg folate/kg diet) for one year. Incidence of neoplasia was consistent with our previous reports (8, 15).

Control subjects

Research was approved by the Temple University Office for Human Subjects Protections Institutional Review Board, protocol 11910. We collected biologic specimens from subjects undergoing routine screening colonoscopy at Temple University Medical Center to serve as the control arm of the study (Supplementary Table S1). We excluded subjects with a personal or first-degree family history of any cancer and subjects with a previous colonoscopic finding of polyps. Subjects who were not excluded underwent complete colonoscopic evaluation by a board certified gastroenterologist. If the colonoscope could not be passed to the appendiceal orifice, the subject was excluded. If the complete colon was visualized, two cold forceps biopsies of normal colonic mucosa from the ascending colon (proximal to the hepatic flexure) were pooled.

Cancer patients

We collected biologic specimens from patients undergoing colon resection for presumed or biopsy-proven colon cancers. Patients were considered eligible if they had no personal or family history of colon cancer before this encounter. Patients with known or clinical features of hereditary cancer syndromes (specifically, hereditary non-polyposis colorectal cancer, or familial adenomatous polyposis syndrome) were excluded. Patients with any personal history of chemotherapy or radiotherapy were also excluded. Patients who remained eligible (Supplementary Table S2) underwent colon resection at a single National Cancer Institute designated Comprehensive Cancer Center (Fox Chase Cancer Center/Temple University, Philadelphia, PA). Specimens, determined by a board certified pathologist to be normal appearing colon mucosa, were obtained well away from the lesion (~10 cm).

DNA and RNA isolation from human normal tissue

Morphologically normal colon mucosa specimens were obtained from colorectal cancer patients or from controls undergoing screening colonoscopy (7). Samples were trea-

RNA extraction from murine normal preneoplastic intestine

RNA was extracted as described (15). Eight samples for microarrays were prepared from 4 BALB/c Mthfr+/− mice fed FD and 4 BALB/c Mthfr−/− mice fed CD. High quality of RNA was verified (Supplementary Fig. S1). In addition, 16 RNA samples were extracted from BALB/c Mthfr−/− and
BALB/c Mthfr+/− mice fed CD and FD (4 mice per group). These biologic replicates were used to confirm microarray results by quantitative real-time RT-PCR (qRT-PCR) and verify effects of genotype and diet on expression.

Microarray analysis and quantitative real-time RT-PCR

Microarray experiments were performed using Affymetrix Mouse Gene 1.0 ST Array Chips, as previously described (15). We considered BALB/c Mthfr+/− mice fed CD as the group with higher tumor resistance and BALB/c Mthfr+/− FD group as the tumor-susceptible group. Genes with expression fold changes more than 1.4 and a P value less than 0.01 after false discovery rate correction were considered significant. Ingenuity Pathways Analysis (IPA) was used to assess biologic processes with the most significant changes.

qRT-PCR was performed as described to confirm microarray data (15). Primers were designed (Supplementary Table S3) and amplified fragments of expected sizes (data not shown). We used 16 mice in 4 groups (4 mice per group); the groups were Mthfr+/+, CD, Mthfr+/−, FD, Mthfr+/+, CD, and Mthfr−/−, FD.

RNA extraction, cDNA synthesis, and gene-specific TaqMan probes (Applied Biosystems) were done as described (7), to measure steady-state levels of PDK4, SPRR1A, SPRR2A, NR1H4, and PYCARD in normal colon mucosa from patients with cancer and controls. Primers and probes are described in Supplementary Table S4.

Quantitative CpG methylation analysis by pyrosequencing

We used bisulfite pyrosequencing to measure methylation of specific CpGs in the 5′ region of human PDK4, SPRR2A, NR1H4, and PYCARD. CpGs in mouse orthologous regions were also assessed, as described (15). Genes and relevant oligonucleotides are presented in Supplementary Table S5. Representative pyrograms are shown in Supplementary Fig. S2A–S2F.

Statistical analysis

Quantitative data are presented as average value of replicates ± SEM. Levene test was performed to assess equality of variance. Two-factor ANOVA was used to evaluate effects of diet and genotype on gene expression; strain and diet were also compared in some cases. Student t test for independent samples was performed for specific comparisons where indicated. Analyses were done using SPSS for WINDOWS software, version 11.0. P values < 0.05 were considered significant.

Results

Differences between BALB/c Mthfr+/− FD and Mthfr+/− CD gene expression profiles

Our microarray results have been deposited in the Gene Expression Omnibus database (GEO, ref. 16, GEO accession no. GSE44011). There were 63 genes with significant expression changes (51 increased and 12 decreased; Supplementary Fig. S3A) in FD Mthfr+/− BALB/c mice compared with CD Mthfr+/− BALB/c mice (Supplementary Table S6).

These 63 genes were grouped based on functions using IPA (Supplementary Fig. S3B). The top 3 categories were lipid metabolism, small-molecule biochemistry, and nucleic acid metabolism. Fatty acid metabolism, LPS/IL-1–mediated inhibition of RXR function, and PXR/RXR activation were identified as pathways with the most significant changes (data not shown).

Evidence for involvement of PPARA in tumorigenesis

Our previous study compared gene expression between the C57BL/6 and BALB/c mouse strains with different sensitivity to intestinal tumorigenesis. In that report, we showed that the PPARA-oxidation pathway plays a critical role (15). The present work, based on diet and genotype comparisons in BALB/c, confirms the involvement of PPARA. Our gene expression profiling identified several genes related to PPARA activation and oxidative stress that are affected by diet and/or Mthfr genotype (Supplementary Table S7), as well as PPARA-responsive genes (Supplementary Table S8). Responsiveness to PPARG was also reported for some of these genes (references listed in Supplementary Table S8).

Confirmed expression changes for eight genes that may promote tumorigenesis in Mthfr−/− FD mice

Expression of eight genes that may influence tumorigenesis was confirmed by qRT-PCR (Supplementary Fig. S4). These genes are involved in regulation of proliferation (Aif5; ref. 17), apoptosis (Plscr1 and Plscr2; ref. 18), cell survival (Ppme; ref. 19–21), recognition of aberrant unmethylated DNA (Pcktrem; ref. 22), overexpression or chromosomal rearrangement related to cancer (Lhfl2; refs. 23, 24), or reduction of retinaldehyde levels (Rdh18 and Akr1c13; ref. 25).

Five additional murine genes and their human orthologs show changes in expression or methylation in mice and in normal intestinal mucosa of colorectal cancer patients

We hypothesized that some protumorigenic mechanisms in our mice might be shared by human preneoplastic colon. To pinpoint the involved genes, we first looked at genes identified in the aforementioned murine microarray (affected by diet or Mthfr genotype, Supplementary Table S6), that would match human orthologs with demonstrated methylation changes in our recent genome-wide profiling of DNA methylation of normal colonic mucosa in patients with colorectal cancer and controls (ref. 7 and C. Sapienza, 2012, unpublished data). We limited our selection to human genes with methylation changes more than 2% and for which increased/decreased methylation could correspond to increased/decreased expression in murine mucosa. We also focused on genes that were related to the PPAR/oxidative stress pathway. Only three genes passed this stringent screen: PDK4, SPRR2A, and NR1H4. We applied the same filters...
to the list of mouse genes deduced from our previously published strain comparison (15); this scan generated only two additional candidates: SPRR1A and PYCARD. Microarray-based methylation changes in PYCARD had been published (7); the other four genes had not been reported.

Confirmation of microarray data for these five murine genes was undertaken. Pdk4, a target of PPARα, is a positive regulator of glycolysis (26). It is upregulated by FD in mice with both Mthfr genotypes (Fig. 1A). Expression of Pdk4 is also higher for Mthfr+/− mice than Mthfr−/− mice, for both diets. We previously reported that Spr2a is downregulated in BALB/c compared with C57BL/6 (15). This ROS scavenger plays a role in tumorigenic events related to oxidative stress. FD lowered Spr2a in both genotypes (Fig. 1B). Nr1h4, also known as Fxr, upregulates cell growth and induces PPARα (27). FD Mthfr+/−/C0 mice demonstrated higher expression of Fxr than wild-type mice. Folate deficiency also stimulated its expression in Mthfr+/−/C0 mice (Fig. 1C). Spr1a has a similar role to Spr2a and showed decreased levels in BALB/c mice compared with C57BL/6 (Fig. 1D). Surprisingly, a marked elevation by FD was seen in C57BL/6 (Fig. 1D), whereas this was not observed for Spr2a (Fig. 1B). PYCARD downregulation is well documented in colorectal cancer (28). Pycard showed lower expression in BALB/c compared with C57BL/6, in both diets (Fig. 1E).

The human orthologs of the five mouse genes presented in Fig. 1 showed altered methylation levels in our genome-wide methylation array (7). We confirmed these changes by bisulfite pyrosequencing-based assays for 6 CpGs in the 5 genes in 6 controls and 36 patients with colorectal cancer. There was excellent correlation between the two methods when β-values from microarrays were compared with %methylation from pyrosequencing data for the 12 individuals: Spearman $r = 0.94$, a total of 72 points; linear regression $r^2 = 0.93, P < 0.001$ (Supplementary Fig. S5). We then examined a new cohort of 29 controls and 29 patients with colorectal cancer, who had not been tested in the original arrays, by pyrosequencing (Supplementary Fig. S6, left panel for each marker). They were compared with 12 controls and 24 patients with colorectal cancer that had been tested for these same 6 CpGs by arrays only (Supplementary Fig. S6, right panel for each marker). There are significant differences between controls and patients, for these independent cohorts of 58 and 36 individuals tested by pyrosequencing and microarrays, respectively.

We then expanded our pyrosequencing assessment to a total of 14 CpGs for these 5 genes (for 35 controls and 35 patients) as seen in Fig. 2. The additional dinucleotides were in the vicinity of the originally interrogated CpGs. All 14 tested CpGs showed significant differences in methylation in normal mucosa between patients with colorectal cancer and controls (Fig. 2A–E). CpGs showed significantly
decreased methylation in normal colon of patients with colorectal cancer for PDK4 (Fig. 2A) and NR1H4 (Fig. 2C). Significantly higher methylation was observed for SPRR2A (Fig. 2B), SPRR1A (Fig. 2D), and PYCARD (Fig. 2E). We then questioned whether these differences could be used as methylation-based biomarkers for presence or absence of polyps in controls. Approximately half of the controls had polyps. The average methylation of NR1H4 was lower for subjects with polyps, although the difference did not reach statistical significance (Fig. 3A). However, we observed an increased average methylation for the five PYCARD CpGs, with significance for two CpGs (16:31121937 and 16:31121918), and borderline significance for three CpGs (16:31121929, 16:31121927 and 16:31121902; Fig. 3B).

We compared transcript levels of these five genes in normal mucosa of colorectal cancer patients and controls, and observed significantly increased expression for the five markers in patients with colorectal cancer (Fig. 4A–E). No expression differences were seen between controls with polyps and those without polyps.

Although average methylation of our five candidate genes was significantly different between patients with colorectal cancer and controls (Fig. 2A–E), there is some overlap between the two groups, for each of the five markers. We hypothesized that the methylation pattern for a collection of markers could have significant diagnostic power to distinguish normal mucosa between colorectal cancer patients and controls. Indeed, hierarchical clustering for classification of 70 individuals led to the identification of 2 major clusters: one cluster comprised predominantly of colorectal cancer patients and a second cluster composed exclusively of controls (Fig. 5A).

We applied the same principle to evaluate the discriminatory ability for classification of 35 controls with (n = 17)
or without \((n = 18)\) polyps. Empiric combinatory comparison using different sets of these five biomarkers (data not shown) revealed that \(NR1H4\) and \(PYCARD\) aggregated data provide the best combination for a potential methylation signature (Fig. 5B). Hierarchical clustering yielded one group almost exclusively composed of controls with polyps (8/9 subjects) and a second group enriched in controls without polyps (17/26 subjects).

**Discussion**

DNA-based biomarkers in normal colonic mucosa would be extremely useful because they have the potential to be diagnostic of colon cancer in the near term or, upon further development, may become prognostic indicators of colon cancer risk. Such biomarkers would provide discriminatory and quantitative biochemical measures to supplement the current endoscopic screening test that is both invasive and subjective.

Environmental factors, such as diet, may be the most important influences on colorectal cancer risk. Low dietary folate is one such risk factor. The polymorphism in \(MTHFR\) (677C→T) can also increase cancer risk when folate status is inadequate. Our mouse model, which develops intestinal neoplasia after low dietary folate, is a relevant model for human sporadic colorectal cancer because the mice do not have germline mutations and develop tumors over an extended period of time, without carcinogen induction. The use of \(Mthfr\)-deficient mice allows us to examine gene-nutrient interactions that have also been observed in human colorectal cancer. DNA methylation is altered by \(MTHFR\) 677C→T genotype and folate levels, with folate-deficient TT individuals showing the lowest global DNA methylation and the highest prevalence of cancer history (29).

Another dietary risk factor for colorectal cancer is high fat (30). Expression profiling in mice revealed significant differences for genes downstream of \(PPAR\), a major regulator of lipid and glucose metabolism. We hypothesize that a disturbance in folate metabolism can result in activation of the RXR/PPAR pathway that increases fatty acid oxidation, generates oxidative stress/damage, and enhances glycolysis, setting the stage for tumorigenesis. Tumors have altered energy metabolism, with a preference for aerobic glycolysis (Warburg effect), instead of the tricarboxylic acid cycle (31). Because of the strong link between high fat diets and development of colon cancer, we had previously argued that the epigenetic reprogramming of lipid and carbohydrate metabolism probably preceded tumor development and was not programmed by the tumor at distant sites (7). Interestingly, only 25% of BALB/c \(Mthfr^{+/−}\) mice developed tumors (15), and the BALB/c \(Mthfr^{+/−}\) mice used for confirming mouse expression microarray data by qRT-PCR did not have tumors. These findings directly support the hypothesis that gene expression changes and reprogramming occur before tumor formation.

The retinoid pathway and metabolism of lipids and carbohydrates were predominant in murine expression profiling (Supplementary Fig. S3B) and human genome-wide methylation assessment (7). Correlation between vitamin A deficiency and tumor initiation has been confirmed in several studies (32 and references therein). Retinoic acid regulates gene expression through the retinoic acid receptor and retinoid X receptor (RAR/RXR) heterodimer. In addition, RXR interacts with other nuclear receptors such as PPARs. Retinoids cannot be synthesized in humans; they are converted from dietary carotenoids (33). Beta-carotene is the major provitamin A carotenoid. Retinaldehyde, the product of BCDO1, prevents formation of the RXR/PPAR heterodimer. Downregulation of \(Bcno1\), as observed in \(Mthfr^{+/−}\) and FD mice, would result in lower retinaldehyde levels and increased PPARA activity. Retinaldehyde can be converted to retinoic acid or retinol by aldehyde dehydrogenases or aldo-keto reductases, respectively. We observed higher \(Aldh1al\) expression in FD-fed BALB/c mice (Supplementary Table S7 and Fig. 4B of ref. 15) and higher expression of \(Akrlc13\) in BALB/c \(Mthfr^{+/−}\) FD mice (Supplementary Fig. S4); these changes would also contribute to lowering retinaldehyde. There was increased expression of \(Rdh18\) in BALB/c \(Mthfr^{+/−}\) FD mice; retinol dehydrogenases can also metabolize retinaldehyde, although it is unclear.
whether the Rdh18 transcript encodes a functional protein (GenBank accession# AY053573). We have recently shown that inhibiting BCMO1 expression increases invasion and migration in human colorectal cancer cells, and that β-carotene, the BCMO1 substrate, upregulates the gene and reverses these effects (34).

To identify early human colorectal cancer events, we compared our list of murine candidate genes (Supplementary Table S6 in the present report and Supplementary Table S6 in ref. 15) with genes identified in methylation profiling of normal human colon (7 and C. Sapienza, 2012, Unpublished data) because disturbances in folate metabolism perturb methylation in both species (12, 35). This approach identified five mouse and human orthologous genes that showed, respectively, changes in expression in murine microarrays and changes in human methylation profiling. Bisulfite-pyrosequencing of human DNA in independent samples of normal mucosa of patients with cancer and controls confirmed significant methylation differences in a total of 14 CpGs (Fig. 2). These genes were: NR1H4 which can activate PPARα (27); PDK4, a target of PPARα that enhances glycolysis (26); PYCARD, a proapoptotic gene (28); and two different members of the SPRR family, involved in protection against oxidative damage. Although different methods and sites of biopsy could result in samples with different cell types, our analysis of methylation at more than 27,000 CpGs revealed differences for only 909 (3.3%) between cancer and control specimens at our least stringent statistical threshold (7). To address the issue of tumor sidedness, we examined normal colon from colorectal cancer patients with left- or right-sided tumors (n = 15 in each group). When we compared the 14 CpGs between groups, there was only one marker (one of the three CpGs in SPRR2A) that showed a significant difference in methylation (data not shown). Aging can also influence DNA methylation and modify colorectal cancer risk (36). However, since the age ranges in controls and patients were similar and there was no difference between the mean ages of these groups in our original study (7), from which the five candidates were selected for validation in this study, it is unlikely that age was a confounder.

Some of the controls had polyps, and one of the above genes, PYCARD, showed significant methylation differences between controls without polyps and controls with polyps (Fig. 3). Interestingly, controls included two cases of HPP (hyperplastic polyps), thought not to give rise to colon tumors, and they are at the very low end of the normal PYCARD methylation distribution while other controls
with polyps are all in the upper part of the normal \textit{PYCARD} distribution (Supplementary Table S1). Furthermore, in the heatmap in Fig. 5B, these 2 subjects cluster with the controls without polyps.

A major outcome of this study is the potential for using the methylation differences \textit{per se} as molecular biomarkers for diagnosis. Although some methylation differences were relatively small, they were observed in two different cohorts, using two different methodologies (Supplementary Figs. S5 and S6), and are therefore reliable. These five genes also exhibited significant expression differences in normal human mucosa between controls and patients with colorectal cancer, with increased expression in patients (Fig. 4). Increased expression was associated with both decreased and increased methylation. While it is true that there is a general (but not absolute) inverse correlation between DNA methylation in the promoter region and transcript levels, our finding is not unusual since methylation within the body of genes and distal to genes is often positively correlated with transcript levels (37, 38). Specific mechanisms involving binding efficiency of transcriptional repressor(s) to methylated regions for example, may also result in changes in expression (39). This mechanism may be relevant here, since the assessed CpGs are all in the 5' region of genes.

The increased expression of \textit{PDK4} and \textit{NR1H4} in patients with colorectal cancer (Fig. 4A and C) is consistent with the higher expression of their orthologs in BALB/c FD\textsuperscript{+/−} mice (Fig. 1A and C). These results suggest that similar molecular changes exist in human and murine preneoplastic intestine. Higher expression of \textit{SPRR1A}, \textit{SPRR2A}, and \textit{PYCARD} in normal tissue of patients with colorectal cancer may represent compensatory effects in response to the tumorigenic environment. Only 20% of mice show tumors at one year and they are quite small (1–2 mm); patients with colorectal cancer have well-developed tumors and their normal mucosa may have had sufficient time for compensatory expression changes. Mice lacking Pycard demonstrate polyp formation (40); PYCARD is an inflammasome-associated molecule with influences on diabetes, obesity, and cancer (41).
SPRR genes are often induced in response to stress and extensively upregulated in various types of cancer (42). SPRR2A is overexpressed in early stages of prostate tumorigenesis (43), and is modulated by 5-aza-2'-deoxycytidine despite the absence of CpG islands (44).

Increased expression of PDK4 and NR1H4 in normal mucosa of colorectal cancer patients and in Mthfr<sup>+/−</sup> mice fed FD may be highly tumorigenic. As mentioned, the shift away from mitochondrial respiration is a hallmark of tumor metabolism (31, 45). Inhibition of pyruvate dehydrogenase (PDH) through phosphorylation, by pyruvate dehydrogenase kinases (PDK), results in decreased respiration in tumors (45). PDKs are a family of four kinases in humans (26); siRNA-based knockdown of PDK1 reverses PDH inhibition and the Warburg effect, and can inhibit tumor growth (45). PDK4 expression is increased by PPARα, by consumption of high fat diets and in diabetic states (26); its role in transformation has not been well studied. Decreased methylation of PDK4 in colorectal cancer mucosa is consistent with increased expression. We also observed significantly decreased methylation in the <sup>5</sup>′ region of Pdk4 in Mthfr-deficient mice, for both diets (data not shown).

NR1H4 encodes the farnesoid-X-receptor (FXR). Bile acids, natural ligands for this receptor, can induce PPARα through a FXR response element in the human PPARα promoter (27). NR1H4 activation increases PDK4 expression (46). Decreased methylation of NR1H4 in human colorectal cancer mucosa is consistent with increased expression. In mice, we observed increased Nr1h4 methylation for Mthfr<sup>+/−</sup>- FD mice compared with Mthfr<sup>+/+</sup> FD mice, and a trend for increased methylation at two CpGs in the <sup>5</sup>′ region, in Mthfr<sup>+/−</sup>- FD mice compared with Mthfr<sup>+/+</sup>- CD mice (data not shown).

Because tumorigenesis is a complex process, there are certainly other genes identified through microarrays in mice or humans that could contribute. For example, in Supplementary Fig. S4, we show 6 genes, in addition to Rdh18 and Ahr1c13 (already mentioned), with confirmed expression changes due to folate or Mthfr deficiency.

Our cluster analysis of bisulfite pyrosequencing-based DNA methylation data provides an initial epigenetic signature for distinguishing normal mucosa from controls versus normal mucosa in patients with colorectal cancer (Fig. 5A). Additional methylation markers could improve the power of this diagnostic assay, and some of the genes discussed above, as well as other genes in our microarrays, could potentially improve the discriminatory power. However, we cannot exclude the possibility that some misclassification in Fig. 5 may have been due to a false negative finding during colonoscopy. Clustering of controls without polyps versus those with polyps (Fig. 5B) is also of interest, but requires additional markers. We used a systematic human genome-wide methylation marker discovery study with patients that were not screened for a specific cause of colorectal cancer, although none of the subjects had a history of familial cancer, colon polyps, or inflammatory bowel disease (7). Candidate genes were intersected with expression profiling data from our mouse model. This original, two-filter approach, resulting in gene identification within common pathways, provides a solid basis for an epigenetics signature for normal intestine in colon cancer. As colorectal cancer development proceeds through multiple stages, it would be useful to develop diagnostic tests for early intervention. Although many studies have reported methylation differences between normal colon and tumors, there are very few genes that have been confirmed by quantitative methods to exhibit methylation differences between controls and patients with colorectal cancer in normal mucosa (5, 7). Our approach has identified 5 genes, at 14 CpG sites, using the highly quantitative pyrosequencing method which could be adapted into a clinical setting (47). Methylation changes accumulate over years and could serve as sentinel markers before the appearance of polyps. Shedding of colon-derived DNA into stool could allow noninvasive testing (48). If differences are systemic, measurements of methylation changes in peripheral blood or saliva would also be extremely useful. Additional studies using our two-pronged approach may lead to identification of other biomarkers for the establishment of a biochemical measure of cancer risk that may be more objective than routine endoscopy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Drs. Elin Sigurdson and Andrew Godwin (Chase Cancer Center) for assistance with colon biopsies and tissue acquisition, and Nuala O’Leary (NCBI) for discussions on the Rdh18 gene.

Grant Support
This research was supported by the Canadian Institutes of Health Research (grant no. MOP 77596 to R. Rozen) and NIH (T32 CA 103652-05) and the Fels Institute for Cancer Research (to C. Sapienza). This work was also supported by a McGill University Health Centre Research Institute Fellowship (to N. Lévesque). The Research Institute is supported by a Centres grant from the Fonds de Recherche du Quebec - Santé.

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Received May 30, 2013; revised August 23, 2013; accepted September 3, 2013; published OnlineFirst October 29, 2013.
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Cancer Prevention Research

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