RESEARCH PAPER

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DNA methylation-associated colonic mucosal immune and defense responses in treatment-naïve pediatric ulcerative colitis

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Abbreviations: AUC, area under the curve; CD, Crohn's disease; CpG, cytosine-guanine dinucleotide; DMR: differentially methylated region; FDR, false discovery rate; IBDs, inflammatory bowel diseases; PCA, principal component analysis; RMA, robust multi-array average; SNP, single nucleotide polymorphism; UC, ulcerative colitis.

Inflammatory bowel diseases (IBD) are emerging globally, indicating that environmental factors may be important in their pathogenesis. Colonic mucosal epigenetic changes, such as DNA methylation, can occur in response to the environment and have been implicated in IBD pathology. However, mucosal DNA methylation has not been examined in treatment-naïve patients. We studied DNA methylation in untreated, left sided colonic biopsy specimens using the Infinium HumanMethylation450 BeadChip array. We analyzed 22 control (C) patients, 15 untreated Crohn's disease (CD) patients, and 9 untreated ulcerative colitis (UC) patients from two cohorts. Samples obtained at the time of clinical remission from two of the treatment-naïve UC patients were also included into the analysis. UC-specific gene expression was interrogated in a subset of adjacent samples (5 C and 5 UC) using the Affymetrix GeneChip PrimeView Human Gene Expression Arrays. Only treatment-naïve UC separated from control. One-hundred-and-20 genes with significant expression change in UC (> 2-fold, $P < 0.05$) were associated with differentially methylated regions (DMRs). Epigenetically associated gene expression changes (including gene expression changes in the *IFITM1*, *ITGB2*, *S100A9*, *SLPI*, *SAA1*, and *STAT3* genes) were linked to colonic mucosal immune and defense responses. These findings underscore the relationship between epigenetic changes and inflammation in pediatric treatment-naïve UC and may have potential etiologic, diagnostic, and therapeutic relevance for IBD.

Introduction

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are emerging globally.[1] IBD is associated with high morbidity, leading to a significant healthcare burden.[2] IBD incidence is the highest in young adulthood. Remarkably, IBDs are becoming more common, especially in the pediatric population.[3-5] The increased number of pediatric cases appear to result from an overall amplified incidence rather than a shift toward disease onset at younger age.[6] About 20% of IBD presents in children,[7-9] in whom the diseases usually develop a more aggressive phenotype than in adults.[10]

While the cause or causes of IBD remain to be identified, dietary and environmental changes in the industrialized world have been proposed as possible etiologic factors. Such factors may impose critical alterations in the key physiologic components of IBD pathogenesis during prenatal and/or pediatric development.[11,12] One molecular process that can dynamically respond to nutritional changes in the gut mucosa is DNA methylation.[13] DNA methylation is an epigenetic change occurring at cytosines in CpG dinucleotides and plays an important role in gene expression regulation. Epigenetic maturation relevant for intestinal immune regulation continues beyond infancy in mammals, indicating that even postnatally occurring DNA methylation changes may be important in

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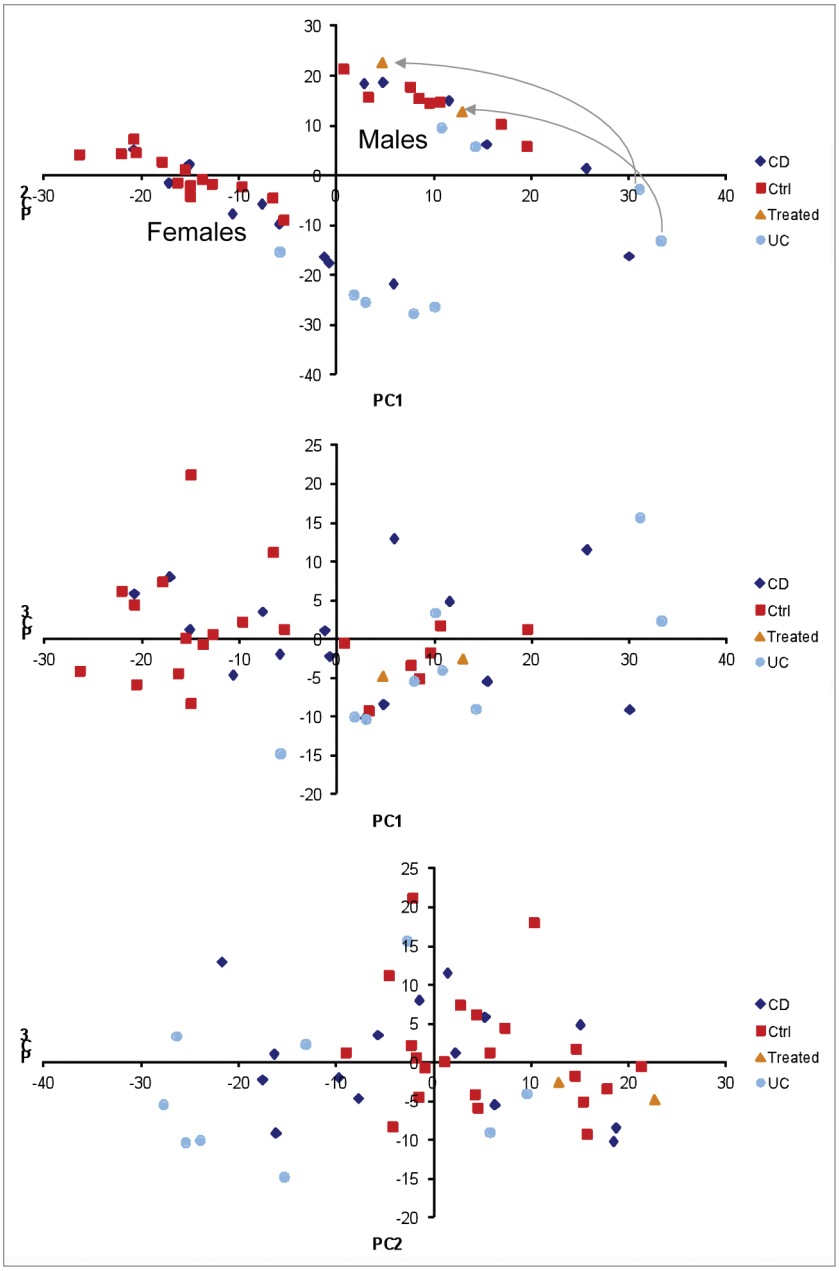

Figure 1. Principal component analysis (PCoA) of DNA methylation. CD: Crohn's disease; Ctrl: Control; UC: ulcerative colitis. The ulcerative colitis DNA methylome separated in one dimension from controls both in males and females (upper panel). Interestingly, males and females separated from each other regardless of disease status in this dimension. The UC-specific separation was lost upon treatment-induced clinical remission in 2 of the male patients studied (gray arrows). There was no obvious disease-specific separation in the other dimensions of the analysis (middle and lower panels).

IBD pathogenesis.[14] Furthermore, maternal micronutrient supplementation can induce colonic mucosal DNA methylation modification relevant for murine colitis susceptibility, implicating this epigenetic process in the developmental origins of IBD.[13,15]

Differentially methylated CpG sites in inflamed intestinal tissues of IBD patients have been detected in limited molecular screens (1,505 methylation sites interrogated),[16] supporting the hypothesis that this epigenetic change is a potential etiologic component in IBD. Metabolite profiling also supported the role of DNA methylation in mammalian mucosal inflammation.[17] A functional methylome map of UC was recently laid out based on colonic mucosal samples from adult discordant monozygotic twin pairs.[18] However, none of the prior studies examined treatment-naïve or pediatric samples. In the meantime, immunotherapy and long standing disease may complicate the identification of pathogenic DNA methylome changes in IBD. Additionally, secondary to its more aggressive course, pediatric IBD may carry unique molecular characteristics compared with adult IBD. Therefore, we set out to explore the association between DNA methylation and gene expression in the colonic mucosa of untreated children with pediatric IBD.

Results

Mucosal DNA Methylation Separates in Treatment-naïve Pediatric Ulcerative Colitis

Overall methylation variation between combined treatment-naïve samples and 2 treated UC samples was examined by correlation based principal component analysis (PCA). There was no consistent separation within the discovery and validation samples when CD cases were compared with controls (**Fig. 1**). On the contrary, all treatment-naïve UC mucosal samples segregated distinctly from controls by DNA methylation-driven PCA in one dimension of the 3 dimensional PCA (**Fig. 1**, upper panel). Interestingly, in the same dimension, there was a gender-based separation between the samples as well. Nevertheless, treatment-naïve UC mucosal DNA methylomes separated from controls both in the female and male groups. Importantly, this separation was lost in the 2 males examined upon treatment with infliximab or 6-mercaptopurine (**Fig. 1**, upper panel, gray arrows), which induced clinical remission in both patients, according to the pediatric ulcerative colitis activity index (PUCAI[19]).

Differentially Methylated Regions in Pediatric Colonic IBD Mucosa

Regional DNA methylation variation may be more important than single CpG site-specific DNA methylation in the regulation of gene expression. Therefore, we performed bump-hunter analysis[20] to identify differentially methylated regions (DMRs) in pediatric IBD colonic mucosa compared with controls. We included the treated samples into the analysis to decrease the inevitable bias induced by inflammation, as shown in the section above. We chose an arbitrary *P* value cut-off of 10^{-4}. By this means, we found 182 CD-associated (**Table S2**) and

3,365 UC-associated DMRs (**Table S3**). These DMRs linked to 108 CD-associated and 2,243 UC-associated genes, respectively. Fifty-eight (53.7%) of the CD-associated genes with DMRs links overlapped with the UC-associated genes with DMRs. This finding indicated that a large portion of the IBD associated mucosal epigenetic changes overlap and that those may arise from non-specific inflammation, which may be more pronounced in face of the confluent colitis characteristic of UC.

Mucosal Epigenetic Changes in UC Associate with Immune and Defense Responses

Gene expression was interrogated by microarrays in a subset of the patients (5 C and 5 UC) from adjacent biopsy samples to examine the direct functional relevance of the UC-specific regional DNA methylation changes. A total of 809 genes showed increased expression (> 2-fold, $P < 0.05$; **Table S4**). Three hundred and 53 showed decreased expression in UC mucosa compared with controls (**Table S5**). Ninety-five (11.7%) of the genes with increased expression showed UC-linked DMR associations (**Table S6**). Twenty-five (7.01%) of the genes with decreased expression showed UC-linked DMR associations (**Table S7**).

There was no significant enrichment of biological processes among the genes with decreased expression that showed DMR associations, compared with the rest of the genome. On the contrary, immune response, defense response, antigen processing and presentation of peptide antigen, antigen processing and presentation, MHC class I peptide loading complex, and MHC protein complex were significantly enriched among the genes with increased expression that had DMR association (*P* corrected < 0.05).

Discussion

The peak incidence of IBD in young adulthood suggests that epigenetic changes occurring during childhood may be important in the etiology of these disorders.[14] Furthermore, the limited length of the disease and the usual absence of co-morbid conditions at the onset of pediatric IBD present a unique opportunity to examine dynamic molecular processes, such as epigenetic changes, relative to the developmental origins of the disease group.[21] Our study includes the most in depth Infinium Methylation Array-based interrogation of intestinal mucosal samples in IBD and is the first to examine pediatric cases.

Our findings indicate that DNA methylation changes in the transverse colonic mucosa are more prominent and, therefore, lead to a more significant separation in pediatric UC than in CD, compared with controls.

The recently developed methylation microarray employed herein has been previously validated in normal adult colonic mucosa and colorectal cancer cell lines.[22] Our validation also supports the reliability of this microarray in human colonic mucosal studies (**Fig. S1**). Although we examined small cohorts, the independent interrogation of discovery and validation sets using comprehensive genomic microarrays (including 482,421 CpGs) and its combined analysis provide substantial strength to our results. Additionally, the treatment-naïve nature of the examined samples is unique compared with any other epigenomic study on mucosal DNA in relationship to IBD thus far. Geographical bias can also complicate multi-center studies on flexible biological systems involved in IBD pathogenesis.[23] Our validation cohort contained samples from a single center eliminating this potential confounding factor.

We observed a remarkable colonic mucosal epigenetic separation of UC compared with CD (**Fig. 1**). This separation may in part be linked to the clinically less diverse nature of UC. Furthermore, the more prominent UC clustering may have resulted from the more intense and confluent inflammation that frequently characterizes the disease, compared with CD. This predicament is supported by the fact that the two biopsy samples from treated patients (in clinical remission) clustered together with controls, as opposed to their treatment-naïve counterparts (**Fig. 1**, gray arrows). One of the most important observation from this work is the loss of colonic mucosal DNA methylome separation upon conventional treatment of UC. This result indicates that the vast majority of identified DNA methylation changes were secondary to the inflammatory process in UC, and are not persistent when the severity of the disease declines.

Over 50% of the CD-associated DMRs were identified in UC mucosa as well. This finding also supports our conclusion that a large portion of the colonic mucosal epigenetic modifications in pediatric IBD arises from non-specific inflammatory changes. Unfortunately, our study does not have sufficient statistical power to determine whether disease-specific DNA methylation differences exist in treatment-naïve colonic mucosa. Whether the DNA methylome can differentiate UC from CD will require the examination of much larger sample groups from each of the two major IBD classes. Although obtaining mucosal biopsies is more invasive than stool collection or phlebotomy, colonoscopy is generally performed to confirm the diagnosis of IBD. Sampling for DNA methylation-based differential diagnostic purposes could take place during this procedure. DNA methylation studies on more readily available cells, such as peripheral blood leukocytes (PBL), did not differentiate between UC and CD in treatment-naïve children in studies using the same arrays used in this work.[24] This may be secondary to the need for cell type-based fractionation of PBLs prior to DNA methylation interrogation,[25,26] since epigenetic patterns are cell- and tissue-specific. Similarly, mucosal cell sub-fractionation from colonic biopsy specimens[27] may further our understanding of IBD-epigenome relationships. However, sub-fractionation required the pooling of biopsy samples to generate sufficient DNA material for high-throughput analyses, limiting its clinical feasibility. Cell sub-fractionation may also result in the potential loss of critical epigenetic information, a possibility that requires further analyses in the future.

The relationship between DNA methylation and gene expression delineated in this study highlight the functional relevance of the identified UC-specific epigenetic changes. In treatment-naïve UC samples, the expression of genes involved in immune and defense responses was significantly increased in association with DNA methylation changes. This result

Table 1. Demographics and disease characteristics of the discovery and validation cohorts.

Cohorts		Age (y)	G	R	Diagnosis			Cohorts		Age (y)	G	R	Diagnosis		
Discovery cohort	C	3.5	M	W	Juvenile polyp			Validation cohort	C	11	F	H	Abdominal pain		
	C	15	F	W	Hematochezia				C	17	M	H	Diarrhea, weight loss		
	C	17	M	W	Perianal fissure				C	15	M	W	Abdominal pain		
	C	17	F	W	Abdominal pain				C	17	M	AA	Abdominal pain		
	C	17	F	W	IBS				C	14	F	W	Abdominal pain		
	C	17.5	F	W	Healthy				C	15	F	W	Abdominal pain		
	C	8	F	W	Hematochezia				C	16	F	W	Diarrhea		
	C	12	M	W	Diarrhea				C	16	F	W	Abdominal pain		
	C	13	M	W	Abdominal pain				C	15	F	W	Abdominal pain		
	C	10	M	W	Abdominal pain				C	16	F	W	Hematochezia		
					Montreal classification				C	15	M	W	Abdominal pain, hematochezia		
					Age	**Location**	**Behavior**		C	9	F	W	Abdominal pain, hematochezia		
	CD	16	M	W	A2	L3	B1						**Montreal classification**		
	CD	17	M	W	A2	L3	B1						**Age**	**Location**	**Behavior**
	CD	15	M	W	A1	L3 L4	B1		CD	6	F	W	A1	L3 L4	B1
	CD	8	F	W	A1	L3 L4	B1		CD	11	M	A	A1	L2	B1
	CD	16.5	F	W	A2	L3 L4	B1		CD	11	M	W	A1	L2 L4	B2
	CD	13	F	W	A1	L3 L4	B1		CD	19	F	AA	A2	L2	B2
	CD	8	F	W	A1	L2 L4	B1		CD	16	F	H	A2	L2	B1
	CD	15.5	F	AA	A2	L3 L4	B1						**Montreal classification of extent of UC**		
	CD	17.5	M	W	A2	L3 L4	B1								
	CD	13	F	na	A1	L2	B2		UC	12	F	W	E3		
					Montreal classification of extent of UC				UC	11	M	AA	E3		
									*UC	16	M	W	E3		
	UC	10	F	W	E3				UC	13	F	AA	E3		
	UC	15	F	W	E3				UC	5	F	H	E3		
	UC	19	M	na	E2										
	*UC	13	M	W	E3										

Note: (y):Age (years); A:Asian; AA:African American; B:Behavior, C:Control; CD:Crohn Disease; E:Extent; F:Female; G:Gender; H:Hispanic; L:Location; M:Male; na:non-applicable; R:Race; UC: Ulcerative Colitis; W:White. * indicates patients whose post-treatment samples were also interrogated.

highlights the importance of epigenetic mechanisms in the regulation of the inflammatory cascade characteristic for UC.

Among the genes where UC-specific increased expression associated with DNA methylation changes, *S100 calcium binding protein A9* (*S100A9*) has previously been described as showing increased expression in both CD and UC colonic mucosa.[28] In agreement with our results, other investigations on *S100A9* performed in samples from outside the GI tract have shown *S100A9* increased expression upon treatment with 5-aza-2'-deoxycitidine, and inhibitor of DNA methyltransferase 1 (DNMT1).[29] Similar to our findings, *secretory leukocyte peptidase inhibitor* (*SLPI*) expression was selectively increased in UC mucosa that appeared to originate from mucosal inflammatory cells (this was not observed in CD).[30] This latter work indicates that some of the gene expression signals detected in our study may have derived from mucosal immune cells rather than epithelial, albeit specific to UC. Another important observation of our study is the increased expression of *STAT3* in the colonic mucosa of treatment-naïve UC patients in association with decreased gene body methylation overlapping 2 exons (**Table S6**). The importance of the STAT3 signaling pathway has been highlighted in studies of pediatric onset UC, in which increased expression of the gene was noted in colonic mucosa as well[31]. In support of our findings suggesting that epigenetic changes may be an important element in the regulation of the STAT3 signaling pathway, an inverse correlation between gene methylation and *STAT3* expression level has been recently demonstrated.[32] Similarly, other publications validate our findings on inflammation-associated increased gene expression of specific genes (e.g., *SAA1*,[33] *IFITM1* and *ITGB2*[34]).

This work includes the first high-throughput DNA methylation and gene expression association study on IBD from

treatment-naïve colonic mucosal samples. The results indicate the potential importance of epigenetic mechanisms in the modulation of the inflammatory changes characteristic of UC. We also conclude that the large majority of DNA methylation and gene expression changes at the time of diagnosis are transient and respond to treatments and/or disease remission. Further work toward deciphering the contribution of different cell types on whole mucosal biopsies is needed. Our findings may promote the implementation of DNA methylation-based differential diagnostic and therapeutic modalities for pediatric UC.

Table 2. Summary of cohort groups including the diagnosis, age and gender of the patients in this study

Cohorts	Diagnosis	Age (years)			Gender
		Mean	Median	Min to Max	(M;F)
Discovery Cohort (n = 24)	C	13.0	14.0	3.5–17.5	5;5
	CD	14	15	8–17.5	4;6
	UC	14	14	10–19	2;2
Validation Cohort (n = 22)	C	14.67	15.0	9–17	4;8
	CD	12.6	11.0	6–19	2;3
	UC	11.4	12.0	5–16	2;3

Note: C:Control; CD:Crohn's Disease; F:Female; M:Male; UC: Ulcerative Colitis.

Patients and Methods

Patients and Samples

Secondary to the dynamic nature of epigenetic changes, the following factors were controlled: age, gender, colonic location,[35,36] and treatment status.[37] Hence, only treatment-naïve patients and controls were examined. Discovery [10 controls (C), 10 Crohn's disease (CD), and 4 ulcerative colitis (UC)] and validation cohorts (12 C, 5 CD, 5 UC) of patients were included (**Table 1 and 2**). Control patients in the discovery cohort were recruited prior to endoscopy through the pediatric gastroenterology tissue bank of the Pediatric Inflammatory Bowel Disease Consortium Registry of the Baylor College of Medicine (BCM), Charles University, Prague, Czech Republic, and Mass General Hospital for Children, which were established in agreement with local and federal regulations. Only patients with grossly and histologically normal mucosa at colonoscopy were designated as controls. Treatment-naïve IBD cases in the discovery cohort were recruited prior to their first diagnostic colonoscopy in the latter two locations and their disease was determined based upon clinical, biochemical and histological characteristics. The validation cohort patients were all enrolled at BCM to eliminate potential center bias. Left sided (from mid transverse to rectal) colonic mucosal samples were snap frozen on dry ice or in liquid nitrogen immediately after biopsy and stored at -80 °C until further analysis. The inflammatory cell infiltration in the adjacent transverse colon biopsies of UC patients was rated between mild to severe on a numeric scale by a blinded pathologist. Treated samples from one patient each from the two cohorts were also included into the analyses to examine and reduce inflammation bias (**Table 1***).

DNA Extraction

After thawing, the colonic mucosal biopsies of the discovery cohort were centrifuged at 14,000 rpm for 30 s and resuspended in 500 µl RLT buffer (Qiagen, Valencia, CA) (with β- mercaptoethanol). Sterile 5 mm steel beads (Qiagen, Valencia, CA) and 500 µl sterile 0.1 mm glass beads (Scientific Industries, Inc., NY, USA) were added for complete bacterial lyses in a Qiagen TissueLyser (Qiagen, Valencia, CA), run at 30Hz for 5min. Samples were centrifuged briefly and 100µl of 100% ethanol added to a 100µl aliquot of the sample supernatant. This mixture was added to a DNA spin column, and DNA recovery protocols were followed as instructed in the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) starting at step 5 of the Tissue Protocol. DNA was eluted from the column with 30µl water and samples were diluted accordingly to a final concentration of 20ng/µl. As for the discovery cohort, genomic DNA was isolated by standard proteinase-k digestion and phenol-chloroform extraction and bisulfite converted as described previously.[38] This approach eliminated potential DNA extraction method based bias. DNA concentrations were determined by a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

RNA Extraction and Processing for Microarrays

Colonic mucosal RNA was isolated by Qiangen-Qiazol - miRNA Isolation Kit. cDNA amplification and labeling was performed with Ovation Pico WTA System V2 and Encore Biotin Module (NuGEN), respectively. Array hybridization was performed according to Affymetrix FS450_0002 Hybridization Protocol for gene expression. The Affymetrix GeneChip® PrimeView™ Human Gene Expression Arrays were scanned with Affymetrix Genechip Scanner 7G.

Infinium Methylation Assay Microarrays

The transverse colonic mucosal DNA sample quality was examined with PicoGreen (http://probes.invitrogen.com/media/pis/mp07581.pdf) before processing toward the microarrays. The samples that passed quality control were processed by Infinium HumanMethylation450 BeadChip Kits (Illumina San Diego, CA, USA; http://www.illumina.com/products/methylation_450_beadchip_kits.ilmn) according to the manufacturer's recommendations through automated processes in the Core Laboratory for Translational Genomics of the Baylor College of Medicine. Arrays were imaged with BeadArray Reader using standard Illumina scanner settings. The R Bioconductor minifi package[39] was used to generate β values normalized to internal control probes. Internal controls determined the array processing to be of good quality. After removal of probes containing SNPs (http://www.rforge.net/IMA/snpsites.txt), 390,433 CpG probes on the array were used for subsequent analysis. The R Linear Models for Microarray Data (Limma) package[40] was used to compare β values and identify differentially methylated probes between controls and CD, or UC affected individuals. Limma fitted a linear model to β values for each probe in the compared samples

and then calculated a t-statistic using an empirical Bayesian model that moderates the standard errors across probes. *P* values were calculated from the moderated t-statistics and multiple testing correction of the p-values was performed using Benjamini and Hochberg's method[41] (false discovery rate: FDR) to identify differentially methylated probes. The R Bioconductor bumphunter package was used for identifying differentially methylated regions. The R prcomp function was used to perform principal component analysis (PCA) based on β values for the samples. The raw data of the microarrays was uploaded to Gene Expression Omnibus (GEO; Series GSE32146) and is accessible at: Discovery cohort: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=frutfkgmamamkvi&acc=GSE32146.

Validation cohort: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pvojjiqcqiuqori&acc=GSE42921.

Bisulfite Pyrosequencing Validation

Colonic mucosal DNA from the discovery cohort was bisulfite converted with EZ DNA Methylation-Gold Kit (D5006, Zymo Research, Orange, CA, USA). DNA was amplified with traditional primer biotinylation following bisulfite conversion. A quantitative bisulfite pyrosequencing protocol was used for all methylation analyses with the utilization of the Pyro Q CpG program (QIAGEN GmbH, QIAGEN Strasse 1. 40724 Hilden, Germany). Infinum array validation was performed at 5 candidate loci with corrected $P < 0.01$ in the discovery cohort: (*SMAD3* associated: chr15:67442893–67442893; *C8orf74* associated: chr8:10555466–10555466; *FLT1* associated: chr13:28975690–28975690; "Chr1": chr1:120289923–120289923 and "Achr1": chr1:150535935–150535935). **Table S1** shows the pyrosequencing primers utilized for the validation.

Bisulfite pyrosequencing at 5 independent loci correlated significantly with the microarrays of the discovery cohort ($r = 0.87$, $P < 0.0001$; **Figure S1**) supporting the reliability of the results. **Table S1** shows the pyrosequencing primers utilized for the validation of the discovery microarrays.

Gene Expression Microarray Analysis

The R Bioconductor affy package[42]_ENREF_43 RMA (robust multi-array average) function was used to compute expression. The CEL files and RMA normalized expression values can be downloaded from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pzyffweqggmsgnk&acc=GSE42911.

Affymetrix GeneChip PrimeView Human Gene Expression Array probe annotations were associated with Infinium HumanMethylation450 v1.1 probe annotations based on gene symbols. The β values of Infinium probes showing differential methylation between UC and control samples were compared with the averaged RMA values for genes with multiple probe sets based on the gene symbol association.

Statistical and Bioinformatic Analysis

Unpaired, two tailed, *t* test; was used in the group comparisons. Statistical significance was declared at $P < 0.05$. DAVID (http://david.abcc.ncifcrf.gov/) was utilized to examine gene ontology enrichment in the select gene lists; significance was declared at P values adjusted for multiple testing $P < 0.05$.

Disclosures

The authors have no conflict of interests to declare.

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Data Access

DNA methylation: Gene Expression Omnibus (GEO; Series GSE32146) and is accessible at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=frutfkgmamamkvi&acc=GSE32146.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pvojjiqcqiuqori&acc=GSE42921

Gene expression: The CEL files and RMA normalized expression values can be downloaded from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pzyffweqggmsgnk&acc=GSE42911.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/29446/

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