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Short Report

Identification of disease-associated DNA methylation in intestinal tissues from patients with inflammatory bowel disease

Lin Z, Hegarty JP, Cappel JA, Yu W, Chen X, Faber P, Wang Y, Kelly AA, Poritz LS, Peterson BZ, Schreiber S, Fan J-B, Koltun WA. Identification of disease-associated DNA methylation in intestinal tissues from patients with inflammatory bowel disease. Clin Genet 2011: 80: 59–67. © John Wiley & Sons A/S, 2010

Overwhelming evidence supports the theory that inflammatory bowel disease (IBD) is caused by a complex interplay between genetic predispositions of multiple genes, combined with an abnormal interaction with environmental factors. It is becoming apparent that epigenetic factors can have a significant contribution in the pathogenesis of disease. Changes in the methylation state of IBD-associated genes could significantly alter levels of gene expression, potentially contributing to disease onset and progression. We have explored the role of DNA methylation in IBD pathogenesis. DNA methylation profiles (1505 CpG sites of 807 genes) of matched diseased ($n = 26$) and non-diseased ($n = 26$) intestinal tissues from 26 patients with IBD [Crohn's disease (CD) $n = 9$, ulcerative colitis (UC) $n = 17$] were profiled using the GoldenGate™ methylation assay. After an initial identification of a panel of 50 differentially methylated CpG sites from a training set (14 non-diseased and 14 diseased tissues) and subsequent validation with a testing set (12 non-diseased and 12 diseased tissues), we identified seven CpG sites that are differentially methylated in intestinal tissues of IBD patients. We have also identified changes in DNA methylation associated with the two major IBD subtypes, CD and UC. This study reports IBD-associated changes in DNA methylation in intestinal tissue, which may be disease subtype-specific.

Conflict of interest

None declared by authors.

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Inflammatory bowel disease (IBD) is a heterogeneous disease with two major subtypes, Crohn's disease (CD) and ulcerative colitis (UC). Currently more than 32 susceptibility loci have been identified for IBD (1–6). However, all these genetic risk factors can only account for approximately 20%

of the genetic risk (7, 8), suggesting that other factors, including possible epigenetic factors, are involved in IBD pathogenesis (9).

Heritable and reversible epigenetic alteration is being recognized as a factor affecting disease pathogenesis (10, 11). DNA methylation and histone modification are the most studied epigenetic events. The role of DNA methylation has been widely studied in various cancers (12, 13) and several other human diseases including IBD. Abnormal DNA methylation has been observed in UC patients in the estrogen receptor (ER), $P14^{ARF}$, and E-cadherin gene (14), and there is an emerging evidence of a role of epigenetic factors in the regulation of gene activity as a factor in the etiopathogenesis of IBD (15).

In the present study, we measured the methylation status of 1505 CpG sites representing 807 genes in intestinal tissues from IBD patients to identify IBD-associated changes in DNA methylation.

Materials and methods

Study samples

Fifty-two intestinal tissues (diseased and paired normal) from 26 patients with IBD were used in this study. These were 9 CD patients (5 females, 4 males) and 17 UC patients (6 females, 11 males). All tissues were collected at the time of surgery and classified by a pathologist (Table 1) following a protocol approved by the Penn State College of Medicine Institutional Review Board.

Bisulfite conversion of DNA and GoldenGate™ methylation assay

Genomic DNA from frozen human intestinal tissues was isolated with the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). One microgram of DNA was used for bisulfite conversion by the Methylation-Gold Kit (Zymo Research, Orange, CA).

Bisulfite-converted genomic DNA was used for the GoldenGate methylation assay (16), which was performed at the Cleveland Clinic.

Bisulfite polymerase chain reaction-based restriction fragment length polymorphism analysis

For microarray data validation, we used polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) to confirm the methylation status of two IBD-associated CpG sites: MAPK10_E26_F and GABRA5_P862_R. The PCR-based RFLP method was described previously (17). Bisulfite-converted DNAs from four samples were used for PCR. PCR products were digested with Dpn I (for MAPK10_E26_F) and BstB I (for GABRA5_P862_R) and separated by 8% polyacrylamide gel electrophoresis (PAGE). The band quantification was performed using ImageJ adjusted for β-value calculation.

Data analysis

DNA methylation assays were performed by Illumina BeadArray Technology (16). A β value of 0–1.0 was reported for each CpG site, signifying

Table 1. Intestinal tissues used in this study

Sample type	Disease status	Tissue location	Training set (*n*)	Testing set (*n*)	Total (*n*)
CD patients	Diseased	Small bowel	1		1
	Diseased	Terminal ileum	2	2	4
	Diseased	Cecum	2	2	4
					[9]
	Matched normal	Small bowel	1		1
	Matched normal	Terminal ileum	2	2	4
	Matched normal	Cecum	2	2	4
					[9]
UC patients	Diseased	Terminal ileum	1	1	2
	Diseased	Cecum	6	7	13
	Diseased	Left colon	2		2
					[17]
	Matched normal	Terminal ileum	8	7	15
	Matched normal	Cecum	1	1	2
					[17]
IBD patients	Diseased		14	12	26
	Matched normal		14	12	26
Non-IBD patient (diverticulitis)	Normal	Small bowel	1	1	2
	Normal	Colon	1	1	2

CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis. Numbers in parentheses equal total patients for disease subtype.

the methylation level. The β values were calculated by subtracting background with use of negative controls on the array and taking the ratio of methylated signal intensity to the sum of both methylated and unmethylated signals. To assess the differences between diseased and non-diseased groups, Wilcoxon-signed rank test was used to compare β values due to small size in this experiment and violation of normality assumption.

Results

To identify IBD-associated DNA methylation, we employed the Illumina GoldenGate methylation assay (1505 CpGs, 807 genes). This array has been validated on different human tissues. The array genes are involved in cell cycle control, differentiation, DNA repair, apoptosis, and immune response (16). IBD is an inflammatory disease with an increased epithelial turnover rate and an increased risk for developing colorectal cancer (CRC). Therefore, using the GoldenGate methylation assay for studying DNA methylation in IBD is intuitively appropriate as an efficient first pass study approach.

IBD-associated DNA methylation was first identified using a training set of matched human intestinal tissue specimens from IBD patients and then validated using a second testing set of specimens (Table 1). In each set, DNA methylation was compared between diseased and macroscopically normal intestinal tissues from the same patient to minimize inter-subject variability.

Identification of IBD-associated methylation with a training set of intestinal tissues from IBD patients

In the training set, we analyzed 14 diseased and 14 matched normal intestinal tissues from 14 IBD (CD 5, UC 9) patients. As listed in Table 2, a total of 50 CpGs were significantly associated ($p < 0.05$) with diseased tissue. The difference in mean methylation level (expressed as β value) between diseased and normal intestinal tissues ranged from 0.004 to 0.177 in the 50 CpG sites (>0.05 in 32 CpGs and >0.10 in 14 CpGs). None of the top 50 loci was significant at false discovery rate (FDR) level of 0.2 after adjusting for multiple comparisons. In this pilot project, rather than looking for solid statistical evidence, our focus was on biological discovery, which will be verified in follow-up experiments with more patients.

Validation of IBD-associated methylation with a testing set of intestinal tissues from IBD patients

Fifty CpG sites ($p < 0.05$) from the training set were validated using an independent testing set that included 12 diseased and 12 matched, macroscopically normal intestinal tissues from 12 IBD (CD 4, UC 8) patients. The results are listed in Table 2. The difference in β value between diseased and normal intestinal tissues ranged from 0.0003 to 0.212 in the 50 CpG sites. The difference in the β value in these 50 CpGs was >0.05 in 25 CpGs and >0.10 in 15 CpGs, respectively.

Analysis of the training and testing set data identified seven CpG sites suggested to have significant IBD-associated changes in DNA methylation. The seven CpG sites had mean β-value differences >0.05 (range: 0.065–0.190) across diseased and normal samples and p values <0.05 (Table 2, **bold** entries). These CpG sites were AATK_P709_R, BGN_P333_R, GABRA5_P862_R, MAPK10_E26_F, SERPINA5_P156_F, STAT5A_P704_R, and TNFRSF1A_P678_F. Figure 1 shows the hierarchical clustering analysis of IBD-associated methylation CpG sites in the intestine training and testing sets of samples. Overall, clustering analysis revealed differences in gene methylation between the normal and disease-affected areas of these tissues. CpG sites of three genes, STAT5A, SERPINA5, and BGN, showed consistently different methylation in diseased tissues *vs* normal, with a β value >0.10. Gene annotations and microarray data for all 1505 CpG sites in the 26 samples are provided as Appendix S1, Supporting Information.

Disease subtype-associated DNA methylation in the intestinal tissues from CD and UC patients

CD and UC specimens from the training and testing groups were combined to study IBD subtype-associated DNA methylation. In defining the list of loci differentially methylated, we applied two constraints: (i) a statistical analysis value of $p \leq 0.04$ and (ii) a mean methylation level difference between normal and disease-affected tissues of $\beta \geq 0.10$. None of the top 50 loci was significant at FDR level of 0.2 after adjusting for multiple comparisons due to the small overall sample size.

For nine matched CD tissues, 25 methylation CpG sites showed significantly differential methylation ($p < 0.02$ and β-value difference >0.1). Figure 2a shows the hierarchical clustering analysis of the top 25 associated CpG sites of 23 genes in CD patients. The heat map depicts the relative methylation difference between diseased and

Table 2. Differentially methylated CpG loci in IBD intestinal tissue

	Training set						Testing set					
Target ID[a]	Diseased mean[b]	Diseased SD	Normal mean[b]	Normal SD	Difference	p Value	Diseased mean[b]	Diseased SD	Normal mean[b]	Normal SD	Difference	p Value
BCL6_P248_R	0.009	0.010	0.041	0.033	0.032	0.001	0.048	0.058	0.045	0.046	0.002	0.931
HIF1A_P488_F	0.079	0.052	0.027	0.034	0.052	0.004	0.033	0.048	0.033	0.035	0.0004	0.707
TJP1_P390_F	0.059	0.037	0.023	0.019	0.036	0.004	0.052	0.077	0.041	0.027	0.010	0.402
APC_P280_R	0.008	0.009	0.023	0.020	0.015	0.009	0.021	0.025	0.020	0.019	0.001	0.977
TNFSF10_P2_R	0.155	0.121	0.075	0.148	0.080	0.009	0.073	0.078	0.072	0.063	0.001	0.795
BGN_P333_R	**0.568**	**0.169**	**0.716**	**0.118**	**0.148**	**0.012**	**0.629**	**0.163**	**0.733**	**0.088**	**0.104**	**0.040**
EYA4_P794_F	0.283	0.159	0.161	0.100	0.122	0.013	0.211	0.114	0.207	0.133	0.004	0.795
GAS1_E22_F	0.009	0.003	0.013	0.004	0.004	0.013	0.011	0.006	0.011	0.005	0.0003	0.931
PTPN6_P282_R	0.506	0.188	0.345	0.149	0.161	0.013	0.332	0.162	0.349	0.126	0.017	0.795
LAT_E46_F	0.647	0.137	0.732	0.215	0.086	0.015	0.632	0.190	0.740	0.101	0.108	0.126
PURA_P928_R	0.004	0.003	0.010	0.011	0.007	0.017	0.009	0.010	0.007	0.011	0.001	0.402
EGF_E339_F	0.979	0.015	0.992	0.008	0.012	0.017	0.975	0.048	0.992	0.009	0.017	0.112
GATA6_P726_F	0.070	0.034	0.041	0.030	0.029	0.019	0.057	0.047	0.060	0.033	0.003	0.544
RIPK4_E166_F	0.057	0.029	0.033	0.019	0.024	0.019	0.051	0.040	0.049	0.022	0.002	0.707
HFE_E273_R	0.002	0.001	0.006	0.007	0.004	0.022	0.011	0.018	0.008	0.017	0.003	0.260
EVI1_E47_R	0.056	0.093	0.136	0.120	0.081	0.022	0.102	0.065	0.091	0.066	0.011	0.665
IFNGR2_P377_R	0.698	0.194	0.828	0.092	0.130	0.022	0.711	0.170	0.839	0.072	0.127	0.078
MAS1_P469_R	0.898	0.098	0.721	0.215	0.177	0.022	0.886	0.153	0.799	0.181	0.087	0.371
SERPINA5_P156_F	**0.343**	**0.154**	**0.493**	**0.180**	**0.150**	**0.022**	**0.396**	**0.167**	**0.562**	**0.122**	**0.166**	**0.010**
FABP3_E113_F	0.655	0.211	0.753	0.224	0.098	0.024	0.682	0.209	0.822	0.088	0.140	0.175
IL18BP_P51_R	0.490	0.113	0.589	0.189	0.099	0.024	0.430	0.246	0.595	0.125	0.165	0.126
LMTK2_P1034_F	0.671	0.152	0.528	0.162	0.144	0.024	0.628	0.192	0.549	0.192	0.079	0.285
MMP3_P16_R	0.860	0.088	0.928	0.046	0.068	0.024	0.886	0.128	0.941	0.032	0.055	0.260
RHOH_P121_F	0.783	0.122	0.837	0.188	0.054	0.024	0.714	0.235	0.876	0.092	0.162	0.061
TNFRSF1A_P678_F	**0.873**	**0.094**	**0.932**	**0.043**	**0.058**	**0.024**	**0.863**	**0.135**	**0.939**	**0.034**	**0.076**	**0.035**
NOTCH1_E452_R	0.017	0.047	0.028	0.032	0.011	0.025	0.002	0.005	0.008	0.016	0.006	0.472
PDE1B_P263_R	0.030	0.020	0.016	0.009	0.014	0.027	0.030	0.032	0.026	0.013	0.004	0.341
PDGFRB_E195_R	0.557	0.208	0.689	0.232	0.131	0.027	0.555	0.268	0.767	0.103	0.212	0.069
FOLR1_E368_R	0.969	0.025	0.979	0.023	0.011	0.031	0.959	0.052	0.983	0.011	0.024	0.341
HTR2A_P853_F	0.092	0.087	0.177	0.113	0.084	0.031	0.113	0.080	0.173	0.097	0.060	0.141
AATK_P709_R	**0.823**	**0.098**	**0.895**	**0.072**	**0.072**	**0.035**	**0.838**	**0.120**	**0.918**	**0.048**	**0.080**	**0.030**
ABCC2_E16_R	0.970	0.046	0.877	0.128	0.092	0.035	0.929	0.093	0.878	0.106	0.051	0.260
EFNB3_P442_R	0.077	0.112	0.029	0.020	0.048	0.035	0.040	0.028	0.048	0.027	0.008	0.470
IL1B_P829_F	0.795	0.106	0.874	0.079	0.079	0.035	0.585	0.178	0.707	0.098	0.122	0.061
FLJ20712_P984_R	0.547	0.188	0.670	0.135	0.123	0.039	0.567	0.223	0.723	0.090	0.156	0.069
GABRA5_P862_R	**0.875**	**0.079**	**0.927**	**0.052**	**0.052**	**0.039**	**0.882**	**0.117**	**0.943**	**0.037**	**0.060**	**0.026**
HOXB2_P99_F	0.725	0.202	0.856	0.105	0.131	0.039	0.744	0.198	0.855	0.145	0.112	0.069
MAPK10_E26_F	**0.802**	**0.115**	**0.873**	**0.108**	**0.071**	**0.039**	**0.820**	**0.155**	**0.903**	**0.051**	**0.083**	**0.035**
MAS1_P657_R	0.939	0.073	0.794	0.180	0.144	0.039	0.886	0.153	0.799	0.181	0.087	0.371

Table 2. Continued

	Training set						Testing set					
Target ID[a]	Diseased mean[b]	Diseased SD	Normal mean[b]	Normal SD	Difference	p Value	Diseased mean[b]	Diseased SD	Normal mean[b]	Normal SD	Difference	p Value
NEU1_P745_F	0.714	0.170	0.810	0.177	0.095	0.039	0.708	0.232	0.852	0.098	0.144	0.126
S100A4_P194_R	0.691	0.224	0.800	0.174	0.109	0.039	0.726	0.191	0.852	0.072	0.127	0.100
GPR116_E328_R	0.956	0.046	0.978	0.019	0.022	0.043	0.953	0.060	0.982	0.020	0.029	0.078
GPX3_E178_F	0.048	0.026	0.075	0.028	0.026	0.043	0.058	0.051	0.062	0.026	0.004	0.341
MAGEL2_P170_R	0.946	0.028	0.965	0.023	0.020	0.043	0.953	0.064	0.973	0.019	0.020	0.544
SLC22A18_P472_R	0.878	0.078	0.927	0.033	0.049	0.043	0.875	0.101	0.925	0.030	0.050	0.088
STAT5A_P704_R	**0.498**	**0.214**	**0.665**	**0.194**	**0.167**	**0.043**	**0.514**	**0.224**	**0.705**	**0.198**	**0.190**	**0.046**
ASCL2_P360_F	0.092	0.131	0.039	0.036	0.053	0.048	0.078	0.081	0.049	0.037	0.029	0.665
ESR2_E66_F	0.012	0.009	0.007	0.004	0.004	0.048	0.010	0.007	0.012	0.009	0.002	0.885
IL16_P93_R	0.566	0.162	0.687	0.117	0.121	0.048	0.585	0.178	0.707	0.098	0.122	0.061
RUNX1T1_E145_R	0.208	0.120	0.144	0.067	0.064	0.048	0.237	0.116	0.204	0.085	0.033	0.470

IBD, inflammatory bowel disease.
Bold entries denote CpG sites with a mean β-value difference >0.05 across diseased and normal samples and a p value <0.05.
[a]Gene symbols are contained within the target ID before the first underscore.
[b]Mean indicates mean β value (fractional methylation from 0 to 1).

normal CD tissue. Overall, the clustering analysis revealed some separation of gene methylation between the normal and disease-affected areas on the tissues from CD patients. The highest β-value difference between normal and disease-affected tissues was 0.25 for genes NOTCH4 and TJP2; a difference of >0.17 was observed in 15 CpG sites. The lowest p value was 0.002 for CpG sites of genes, GNAS and RHOH.

For matched tissues from UC patients, 13 methylation CpG sites showed significant differential methylation between diseased and normal intestinal tissues ($p < 0.02$ and β-value difference >0.1). Figure 2b shows the hierarchical clustering analysis of the top 13 associated CpG sites of 13 genes in UC intestinal tissue. Overall, clustering analysis revealed some separation of gene methylation between the normal and disease-affected areas of the tissues from UC patients. The highest β-value difference between normal and disease-affected tissues was 0.32 in gene FMR1; a difference of >0.17 was observed in two other CpG sites. The lowest p value was 0.01 for gene HLA-DQA2.

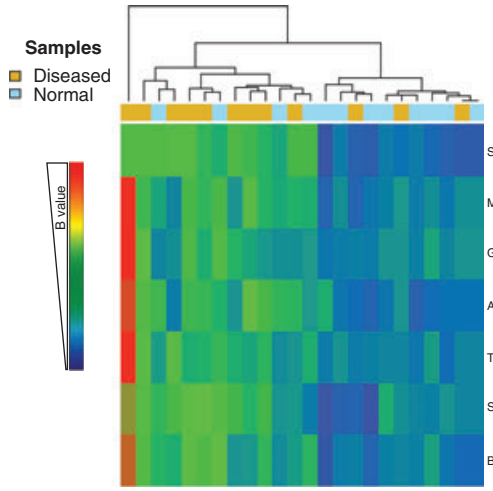
The top disease-associated CpG sites ($p < 0.02$ and the β-value difference >0.1) described above appear to be disease subtype-specific. There were common CpG sites observed for both CD and UC when $0.5 > p \geq 0.02$ and the β-value difference <0.1. Gene annotations are listed in Appendix S2, Supporting Information.

Validation of Bead Array DNA methylation data by bisulfite PCR-based RFLP

To validate our GoldenGate methylation assay data, we used an independent bisulfite PCR-based RFLP method to analyze the methylation status of two IBD-associated CpG sites: MAPK10_E26_F and GABRA5_P862_R. The results for MAPK10_E26_F are shown in Fig. 3a and for GABRA5_P862_R in Fig. 3b. For each CpG site, we analyzed four samples with different β values. The trend of β values for both MAPK10_E26_F and GABRA5_P862_R was the same among the four samples analyzed and between these two assays. Thus, the results from the Illumina Methylation assay were validated using this PCR-based RFLP technique.

Discussion

While a patient's genetic makeup is important in defining disease susceptibility, clinical presentation, and clinical course of IBD, it is also clear that other factors play a significant role in the disease



	Training set						Testing set					
	D_mean	D_sd	N_mean	N_sd	Differ	p	D_mean	D_sd	N_mean	N_sd	Differ	p
STAT5A_P704_R	0.498	0.214	0.665	0.194	0.167	0.043	0.514	0.224	0.705	0.198	0.190	0.046
MAPK10_E26_F	0.802	0.115	0.873	0.108	0.071	0.039	0.820	0.155	0.903	0.051	0.083	0.035
GABRA5_P862_R	0.875	0.079	0.927	0.052	0.052	0.039	0.882	0.117	0.943	0.037	0.060	0.026
AATK_P709_R	0.823	0.098	0.895	0.072	0.072	0.035	0.838	0.120	0.918	0.048	0.080	0.030
TNFRSF1A_P678_F	0.873	0.094	0.932	0.043	0.058	0.024	0.863	0.135	0.939	0.034	0.076	0.035
SERPINA5_P156_F	0.343	0.154	0.493	0.180	0.150	0.022	0.396	0.167	0.562	0.122	0.166	0.010
BGN_P333_R	0.568	0.169	0.716	0.118	0.148	0.012	0.629	0.163	0.733	0.088	0.104	0.040

Fig. 1. Cluster analysis of differentially methylated CpG sites in inflammatory bowel disease (IBD) intestinal tissues. (**Left**) Hierarchical clustering of methylation data from a training set of matched normal and disease-affected intestinal tissues from IBD (Crohn's disease and ulcerative colitis) patients. Methylation profiles of the seven loci from the training and testing sets ($p < 0.05$) were clustered using un-centered correlation and pairwise average linkage. Columns represent samples: yellow is diseased and blue is normal; rows correspond to CpG sites. In the heat map, red indicates increased methylation while blue indicates decreased methylation. (**Right**) Data analysis of the seven loci showing significant associated with IBD in the training and testing sets.

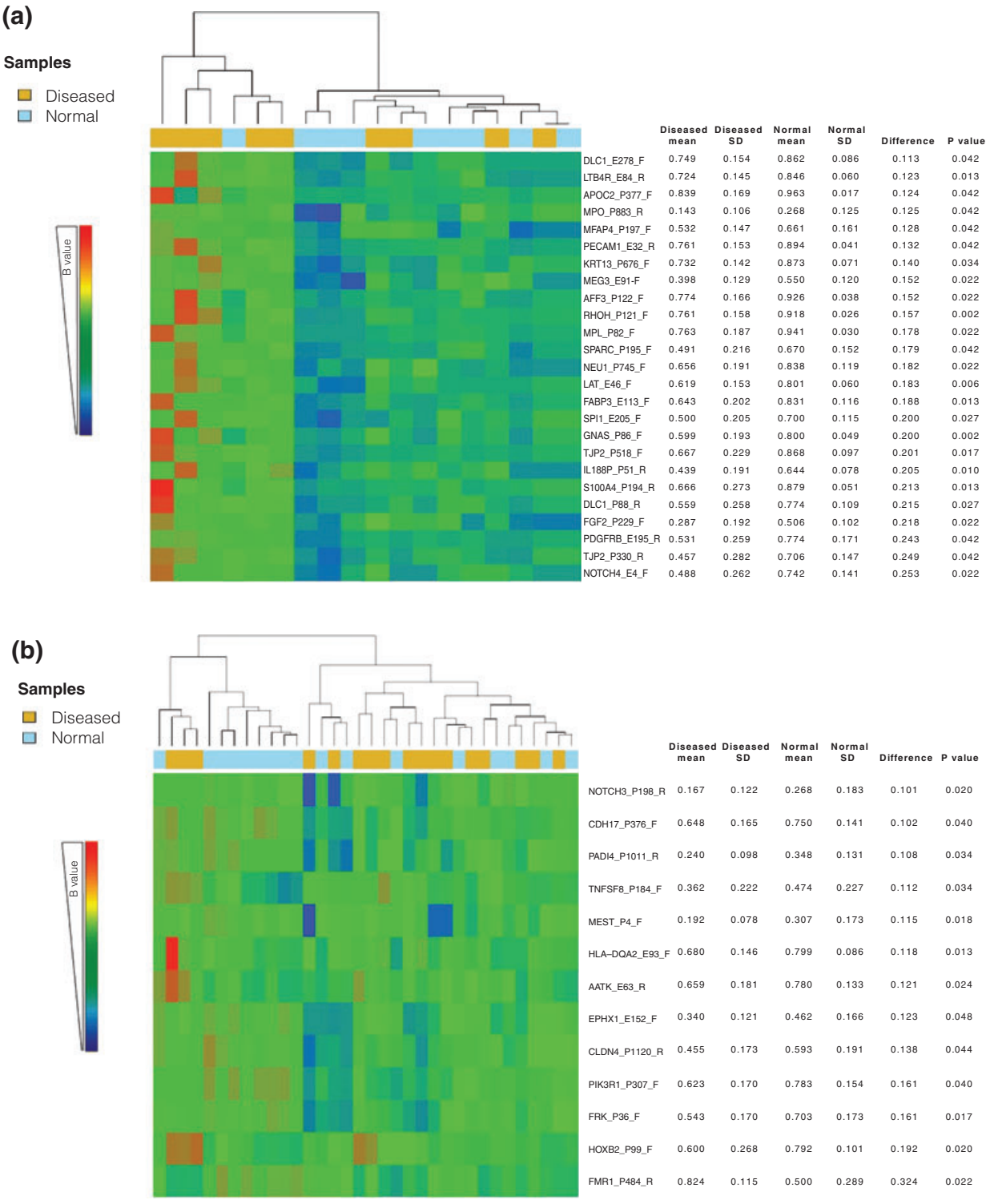
process. This is underscored by the observation of discordant disease between monozygotic twins and the lack of clinical disease in spite of homozygous mutations known to predispose to IBD. Environmental factors, such as diet and commensal flora, can impact an individual's epigenetic profile, thereby affecting disease expression.

DNA methylation in CRC has been well studied and several tumor-associated DNA methylation patterns have been identified (18, 19). Previous IBD-related DNA methylation studies have focused on the development of CRC in UC patients. Initial studies reported that four genes were highly methylated in high-grade dysplastic epithelium from patients with UC (20). Subsequent observations of disease-related methylation status were made for the ER, HPP1, MLH1, RAB32, MGMT, and P14ARF (21–23) and E-cadherin genes from UC patients (14, 24, 25).

Similarly, intrinsic changes in DNA methylation may play a key role in IBD susceptibility. An increased frequency and level of methylation of the MDR1 gene promoter was found to occur in inflammatory rectal mucosa from UC patients, especially in patients with shorter duration and younger onset of disease (15). Recently, expression of the MD-2, a critical TLR4 coreceptor upregulated in IBD but not in normal or diverticulitis patients, has been recently shown to be upregulated by demethylation of the MD-2 promoter region (26).

Multiple genetic risk loci have been found to be common in both CD and UC, indicating a significant shared genetic component in the etiopathophysiology of these diseases (27, 28). In this study, we analyzed methylation profiles of IBD patients using a high throughput, multiplexing microarray-based platform with single-site CpG resolution spanning 1505 CpG loci from 807 genes. The study samples included matched diseased and normal intestinal tissues from each IBD patient. After a training set identification and testing set validation, seven loci of IBD-associated methylation loci were identified. We identified seven differentially methylated CpG sites from the intestinal tissues of IBD patients. As the tissue and disease composition of the training set was different from the testing set, making absolute matched comparison is difficult due to tissue-specific DNA methylation patterns. We partially addressed this issue by focusing on the common changes in DNA methylation in the diseased intestinal tissue. The observation of significant DNA methylation changes among the IBD patients in this study probably represents a disease-associated phenomenon, rather than being an artifact of tissue type or location.

We also observed that DNA methylation varied according to IBD subtype, the identified loci containing these IBD-associated methylation. While these changes are of significant interest, the findings need to be replicated in a larger number of patients, ideally using a single cell type. It should be noted the Illumina



(a)

	Diseased mean	Diseased SD	Normal mean	Normal SD	Difference	P value
DLC1_E278_F	0.749	0.154	0.862	0.086	0.113	0.042
LTB4R_E84_R	0.724	0.145	0.846	0.060	0.123	0.013
APOC2_P377_F	0.839	0.169	0.963	0.017	0.124	0.042
MPO_P883_R	0.143	0.106	0.268	0.125	0.125	0.042
MFAP4_P197_F	0.532	0.147	0.661	0.161	0.128	0.042
PECAM1_E32_R	0.761	0.153	0.894	0.041	0.132	0.042
KRT13_P676_F	0.732	0.142	0.873	0.071	0.140	0.034
MEG3_E91-F	0.398	0.129	0.550	0.120	0.152	0.022
AFF3_P122_F	0.774	0.166	0.926	0.038	0.152	0.022
RHOH_P121_F	0.761	0.158	0.918	0.026	0.157	0.002
MPL_P82_F	0.763	0.187	0.941	0.030	0.178	0.022
SPARC_P195_F	0.491	0.216	0.670	0.152	0.179	0.042
NEU1_P745_F	0.656	0.191	0.838	0.119	0.182	0.022
LAT_E46_F	0.619	0.153	0.801	0.060	0.183	0.006
FABP3_E113_F	0.643	0.202	0.831	0.116	0.188	0.013
SPI1_E205_F	0.500	0.205	0.700	0.115	0.200	0.027
GNAS_P86_F	0.599	0.193	0.800	0.049	0.200	0.002
TJP2_P518_F	0.667	0.229	0.868	0.097	0.201	0.017
IL18BP_P51_R	0.439	0.191	0.644	0.078	0.205	0.010
S100A4_P194_R	0.666	0.273	0.879	0.051	0.213	0.013
DLC1_P88_R	0.559	0.258	0.774	0.109	0.215	0.027
FGF2_P229_F	0.287	0.192	0.506	0.102	0.218	0.022
PDGFRB_E195_R	0.531	0.259	0.774	0.171	0.243	0.042
TJP2_P330_R	0.457	0.282	0.706	0.147	0.249	0.042
NOTCH4_E4_F	0.488	0.262	0.742	0.141	0.253	0.022

(b)

	Diseased mean	Diseased SD	Normal mean	Normal SD	Difference	P value
NOTCH3_P198_R	0.167	0.122	0.268	0.183	0.101	0.020
CDH17_P376_F	0.648	0.165	0.750	0.141	0.102	0.040
PADI4_P1011_R	0.240	0.098	0.348	0.131	0.108	0.034
TNFSF8_P184_F	0.362	0.222	0.474	0.227	0.112	0.034
MEST_P4_F	0.192	0.078	0.307	0.173	0.115	0.018
HLA–DQA2_E93_F	0.680	0.146	0.799	0.086	0.118	0.013
AATK_E63_R	0.659	0.181	0.780	0.133	0.121	0.024
EPHX1_E152_F	0.340	0.121	0.462	0.166	0.123	0.048
CLDN4_P1120_R	0.455	0.173	0.593	0.191	0.138	0.044
PIK3R1_P307_F	0.623	0.170	0.783	0.154	0.161	0.040
FRK_P36_F	0.543	0.170	0.703	0.173	0.161	0.017
HOXB2_P99_F	0.600	0.268	0.792	0.101	0.192	0.020
FMR1_P484_R	0.824	0.115	0.500	0.289	0.324	0.022

Fig. 2. Genes showing significant differential methylation in intestinal tissues from inflammatory bowel disease patients. (**a**) (**Left**) Hierarchical clustering of methylation data from matched normal and disease-affected intestinal tissues from Crohn's disease (CD) patients. Methylation profiles of the 25 CpG sites were clustered using un-centered correlation and pairwise average linkage. (**Right**) A list of loci showing significant associated with CD ($p < 0.04$, $\beta > 0.1$). (**b**) (**Left**) Hierarchical clustering of methylation data from matched normal and disease-affected intestinal tissues from ulcerative colitis (UC) patients. Methylation profiles of the 13 CpG sites were clustered using un-centered correlation and pairwise average linkage. (**Right**) A list of loci showing significant associated with UC ($p < 0.04$, $\beta > 0.1$). Columns in (**a**) and (**b**) represent samples: yellow is diseased and blue is normal; rows correspond to CpG sites. In the heat map, red indicates increased methylation; blue indicates decreased methylation.

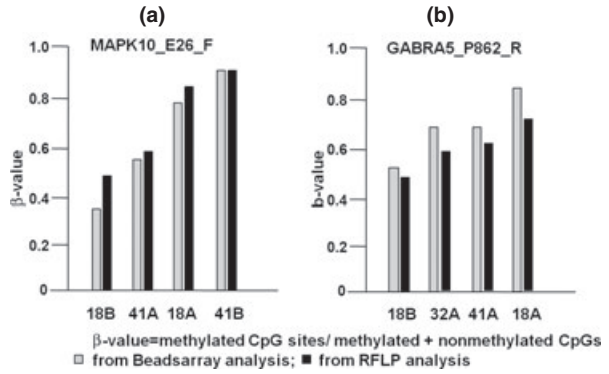


Fig. 3. Validation of microarray results by polymerase chain reaction (PCR)-based bisulfite restriction fragment length polymorphism (RFLP). (**a**) Microarray results and corresponding RFLP-PCR for MAPK10_E26_F in four intestinal tissues. After PCR reaction, the PCR products were treated with Dpn I at 37°C for 6 h, and were separated by 8% polyacrylamide gel electrophoresis (PAGE). (**b**) Microarray results and corresponding RFLP-PCR for GABRA5 in four intestinal tissues. After PCR amplification, the PCR products were treated with BstBI at 37°C for 6 h, and were separated by 8% PAGE. Band quantification was performed using ImageJ software. The β value was calculated as methylated CpGs/methylated+nonmethylated CpGs.

GoldenGate methylation assay is limited by single nucleotide polymorphisms (SNPs) or repetitive elements within or overlapping many of its probes, which may interfere with DNA methylation analyses (29). Nine of the top disease-associated CpG sites reported in this paper contained SNPs (APOC2_P377_F, PECAM1_E32_R, MEG3_ E9 1_F, RHOH_P 121_F, IL18_P51_r, TJP2_P330_R, NOTCH4_E4_F, MEST_P4_F, FMR1_P484_R, while an additional 10 disease-associated CpG sites contained repetitive elements (STAT5A_P7 04_R, GABRA5_P862_R, TNFRSF1A_P678_F, MAPK10_E26_F, LTB4R_E64_R, SPARC_P1 95_F, EPHX1_E152_F, CLDN4_P1120_R, FMR 1_P484_R). Two of these loci were re-evaluated using an independent PCR-based bisulfite RFLP method (Table 3) and found to be consistent with the GoldenGate array results.

Overall, the results suggest that epigenetic modifications may be a factor in the pathogenesis and clinical expression of IBD. As changes in DNA methylation already show promise as biomarkers for cancer diagnosis (30), further study of IBD-associated DNA methylation may lead to its clinical application in the diagnosis and treatment of IBD.

Supporting Information

The following Supporting information is available for this article: Appendix S1. GoldenGate array data.

Table 3. Annotations on genes with IBD-associated CpG methylation genes with IBD-associated changes in CpG methylation as in Figure 1

Genes	Annotation
Genes with IBD-associated changes in CpG methylation shown in Figure 1	
STAT5A	Signal transducer and activator of transcription 5A
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
SERPINA5	Plasminogen activator inhibitor III (PAI-3)
MAPK10	Mitogen-activated protein kinase 10
AATK	Apoptosis-associated tyrosine kinase; LMTK1
GABRA5	Gamma-minobutyric acid A receptor, alpha 5
BGN	Bone/cartilage proteoglycan I (Biglycan)
Genes with CD-associated changes in CpG methylation shown in Figure 2	
IL18BP	Signal transducer and activator of transcription 5A
LTB4R	Leukotriene B4 receptor
PDGFRB	Platelet-derived growth factor beta
PECAM1	Platelet-endothelial cell adhesion molecule-1; CD31
FABP3	Intestinal fatty acid binding protein 3
FGF2	Basic fibroblast growth factor
S100A4	S100 calcium binding protein A4
SPARC	Osteonectin
LAT	Linker for activation of T cells
DLC1	Deleted in liver cancer 1
AFF3	AF4/FMR2 family, member 3 nuclear transcriptional activator.
NEU1	Sialidase 1 – lysosomal neuraminidase
TJP2	Zona occluden 2 (ZO-2) tight junction protein
MFAP4	Microfibrillar-associated protein 4
GNAS	Guanine nucleotide adenylate cyclase-stimulating G alpha protein
RHOH	Ras homolog gene family, member H
MEG3	Maternally expressed 3
KRT13	Keratin 13
APOC2	Apolipoprotein C-II
NOTCH4	Notch homolog 4
MPL	Myeloproliferative leukemia virus oncogene; CD110
MPO	Myeloperoxidase
SPI1	PU.1 transcription factor
Genes with UC-associated changes in CpG methylation shown in Figure 3	
TNFSF8	Tumor necrosis factor ligand superfamily, member 8; CD30L
PIK3R1	p85 regulatory subunit alpha of PI3-kinase
PADI4	Peptidyl arginine deiminase 4
CLDN4	Claudin 4 tight junction protein
CDH17	Liver–intestine cadherin
HLA-DQA2	Major histocompatibility complex, Class II, DQ Alpha 2
HOXB2	Homeobox B2 transcription factor
FRK	Fyn-related tyrosine kinase
AATK	Apoptosis-associated tyrosine kinase; LMTK1
MEST	Mesoderm-specific transcript homolog; PEG1
EPHX1	Microsomal epoxide hydrolase
NOTCH3	Notch homolog 3
FMR1	Fragile X mental retardation protein

Appendix S2. Annotations on genes with IBD-associated CpG methylation.

Additional Supporting information may be found in the online version of this article.

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