Epigenomic Analysis of Aberrantly Methylated Genes in Colorectal Cancer Identifies Genes Commonly Affected by Epigenetic Alterations

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ABSTRACT

Background. Determination of the profile of genes that are commonly methylated aberrantly in colorectal cancer (CRC) will have substantial value for diagnostic and therapeutic applications. However, there is limited knowledge of the DNA methylation pattern in CRC.

Materials and Methods. We analyzed the methylation profile of 27,578 CpG sites spanning more than 14,000 genes in CRC and in the adjacent normal mucosa using beadchip array-based technology.

Results. We identified 621 CpG sites located in promoter regions and CpG islands that were significantly hypermethylated in CRC compared to normal mucosa. The genes on chromosome 18 showed promoter hypermethylation most frequently. According to gene ontology analysis, the most common biologically relevant class of genes affected by methylation was the class associated with the cadherin signaling pathway. Compared to the genome-wide expression array, mRNA expression was more likely to be down-regulated in the genes demonstrating promoter hypermethylation, even though this was not statistically significant. We validated 10 CpG sites that were hypermethylated (*ADHFE1, BOLL, SLC6A15, ADAMTS5, TFPI2, EYA4, NPY, TWIST1, LAMA1, GAS7*) and 2 CpG sites showing hypomethylation (*MAEL, SFT2D3*) in CRC compared to the normal mucosa in the array studies using pyrosequencing. The methylation status measured by pyrosequencing was consistent with the methylation array data.

Conclusions. Methylation profiling based on beadchip arrays is an effective method for screening aberrantly methylated genes in CRC. In addition, we identified novel methylated genes that are candidate diagnostic or prognostic markers for CRC.

Colorectal cancer (CRC) is one of the most common cancers in the world. CRC arises as a consequence of the accumulation of genetic alterations and epigenetic alterations that transform colonic epithelial cells into adenocarcinoma cells [1]. The aberrant methylation of CpG islands in the promoter or exon 1 regions of the genes is a recognized epigenetic event that silences the tumor suppressor genes in colorectal cancer [2-3]. These aberrantly methylated genes are promising biomarkers for molecular diagnostics and early detection and are attractive predictive markers for targeted therapies [4].

Colorectal cancer can be prevented through a resection of colorectal adenoma and is treated most effectively when detected at an early stage. A regular colonoscopic examination is recommended, but the high cost and invasiveness of the procedure is an obstacle to its application as a screening test for CRC. Furthermore, although fecal occult blood testing is inexpensive and non-invasive, the sensitivity and specificity of this test are low[5-7]. Therefore, more accurate biomarkers and methods for the early detection of CRC, such as fecal DNA based tests, are needed [8-11]. Fecal DNA tests that employ genetic mutations are complicated, generally expensive, and are inadequately sensitive to adenomas. Recent studies showed that the aberrant DNA methylation of several genes is present in even the earliest steps in the adenoma-carcinoma sequence, such as the aberrant crypt focus[12-15]. Moreover, many genes were silenced by aberrant methylation and might be associated with colorectal tumorigenesis [16-21]. Therefore, the genes with aberrant methylation have the potential to be useful biomarkers for the early detection of colorectal tumors. The ability to detect aberrant DNA methylation from the DNA extracted from a range of samples, including blood, stool and paraffin-embedded formalin-fixed tissue, suggests that these assays are robust with excellent potential to be

used clinically [22-24]. In addition, the DNA methylation patterns can be applied to the molecular classification of neoplasms [25] as well as to the prediction of the therapeutic responsiveness [26-27] and prognosis of CRC [28-29]. Finally, epigenetic therapy, such as 5-azacitidine, has been shown to be effective in treating hematologic malignancies and might be useful for treating CRC [30]. As our understanding of the role of epigenetic alterations in the carcinogenesis of CRC increases, epigenetic therapy for CRC might be realized. In addition, identification of the signaling pathways deregulated by aberrant DNA methylation may provide a means of selecting CRCs that will be particularly sensitive to targeted therapies [31].

A comprehensive assessment of the aberrantly methylated genes in CRCs has the potential to not only improve our understanding of the molecular biology of CRC but also identify the methylated genes that will influence the clinical care of patients with CRC. Therefore, this study analyzed the methylation profile of 27,578 CpG sites spanning more than 14,000 genes in CRC tissue and the adjacent normal tissue using beadchip array-based technology [32-33].

MATERIALS AND METHODS

Subjects

Twenty-two pairs of colorectal cancer and adjacent normal mucosa were collected from patients treated at Samsung Medical Center (Seoul, Korea) for methylation profiling (Table 1). Another 35 pairs and 65 pairs were obtained to validate the candidate genes selected from methylation profiling using pyrosequencing analysis and genome-wide expression array, respectively (Table 1). The protocol of this study was approved by the Institutional Review Board of the institution. None of the patients had clinically apparent polyposis syndrome or Lynch syndrome. The DNA was extracted from snap-frozen sections from these tumors and normal mucosa using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol.

Methylation profiling in CRC and normal mucosa

Human Methylation27 DNA Analysis BeadChip® (Illumina) was used to analyze the methylation profile of the CRCs. This beadchip array can provide methylation information at a single-base resolution for 27,578 CpG sites spanning more than 14,000 genes.

All the samples were bisulfite-converted using an EZ DNA methylation kit (Zymo Research) according to the manufacturer's instructions. After whole-genome amplification with 200 ng of input bisulfite-converted DNA, the product was fragmented,

purified and applied to the BeadChips using Illumia-supplied reagents and conditions. After extension, the array was stained fluorescently, scanned, and the intensities of the unmethylated and methylated bead types were measured.

Thirty five targets of the 27,578 targets with a detection p-value>0.05 were excluded and the remaining 27,543 target CpG sites were used in the final analysis. Each methylation data point is represented by the fluorescent signals from the M (methylated) and U (unmethylated) alleles. The background intensity calculated from a set of negative controls was subtracted from each analytical data point. The ratio of fluorescent signals was then computed from the two alleles ß = (max(M, 0))/(|U| + |M| + 100). The ß-value reflects the methylation level of each CpG site. A ß-value of 0–1.0 indicates the percent methylation from 0% to 100%, respectively, of each CpG site. The difference in the mean ß-value (Δβ) means (mean of ß-value in CRC – mean of ß-value in normal mucosa). Statistical significance of the methylation data was determined using a paired t-test based on the null hypothesis that no difference exists between the means of CRC and normal mucosa in the methylation data. The false discovery rate (FDR) was controlled by adjusting the p value using the Benjamini-Hochberg algorithm.

Hierarchical clustering was performed using complete linkage with a Euclidian metric. Gene ontology analysis for the genes with hypermethylated promoters in CpG islands was performed using the PANTHER Classification System (http://www.pantherdb.org/panther/ontologies.jsp), using the text files containing the Gene ID list and accession number of Illumina probe ID.

Genome-wide expression array in CRC

The total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The RNA samples were labeled according to the chip manufacturer's recommended protocols. Briefly, 0.5 μg of the total RNA from each sample was labeled using the Illumina Total Prep RNA Amplification Kit (Ambion) in a process of cDNA synthesis and *in vitro* transcription. Single-stranded RNA (cRNA) was generated and labeled by incorporating biotin-NTP (Ambion). A total of 1.5 μg of biotin-labeled cRNA was hybridized at 58℃ for 16 hours to the Illumina's Sentrix Human-6 v2 Expression BeadChip (Illumina). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantitated using Illumina's BeadArray Reader Sanner (Illumina) according to the manufacturer's instructions. The array data was processed and analyzed using Illumina BeadStudio version 3.0 software (Illumina). Data normalization was performed using quantile normalization, and the fold changes and statistical significance were determined using the Avadis Prophetic version 3.3 (Strand Genomics).

Validation of methylation status with pyrosequencing analysis

The promoter region of the 12 genes (alcohol dehydrogenase, iron containing, 1 *(ADHFE1),* bol, boule-like (Drosophila) *(BOLL),* solute carrier family 6 (neutral amino acid transporter), member 15 (*SLC6A15),* a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 5 *(ADAMTS5),* tissue factor pathway inhibitor 2 (*TFPI2),* eyes absent homolog 4 (Drosophila) (*EYA4),* neuropeptide Y *(NPY),* twist homolog 1 (Drosophila) *(TWIST1),* laminin, alpha 1 *(LAMA1),* growth

arrest specific 7 *(GAS7)*, SFT2 domain containing 3 *(SFT2D3)*, maelstrom homolog (Drosophila) (*MAEL)*) were amplified using the forward primer and biotinylated reverse primer, which were designed by PSQ Assay Design (Biotage AB). The bisulfite-modified DNA was amplified in a 25-μL reaction with the primer set and f-Taq polymerase (Solgent). The samples were heated to 95^{o}C for 2 min and amplified for 50 cycles of the following: 95^{o}C for 30 seconds, 58~63^{o}C for 30 seconds, and 72^{o}C for 30 seconds, followed by a final extension step at 72^{o}C for 5 minutes. Pyrosequencing reactions were carried out with a sequencing primer on the PSQ HS 96A System (Biotage AB) according to the manufacturer's specifications. Supplementary table 1 lists the primer sequences.

RESULTS

Methylation profiling in CRC

The methylation status of the 27,578 CpG sites in 22 pairs of CRC tissue and adjacent normal mucosa were measured to identify the genes that are commonly methylated aberrantly in CRC. We selected 3,622 CpG sites with an adjusted $P<0.001$ and a minimum $\Delta\beta$ of 0.15. The CpG sites in CpG islands were more likely to be hypermethylated compared to the CpG site outside CpG islands (Supplementary table 2). Six hundred and twenty one (6.3%) of the 9,792 CpG sites located in promoter regions and CpG islands were found to be significantly hypermethylated in CRC compared to the normal mucosa. Table 2 lists the twenty top-ranking genes with hypermethylated or hypomethylated promoters in CpG islands. Hierarchical clustering with the differentially methylated CpG sites showed clear demarcation between CRC and normal mucosa. Genes with hypermethylated or hypomethylated promoters in CpG islands were generally found on all chromosomes (Fig. 1). However, there were differences between chromosomes. Chromosomes 18 and 5 carried hypermethylated genes most frequently and chromosomes 22, 17 and 15 carried the highest frequency of hypomethylated genes.

Validation of methylation status by pyrosequencing analysis

Ten CpG sites showing hypermethylation and 2 CpG sites showing hypomethylation in CRC compared to the normal mucosa were validated by

pyrosequencing to confirm the methylation state of the genes identified to be aberrantly methylated in CRC by the array studies. Among the 10 hypermethylated CpG sites, 4 CpG sites (*ADHFE1, BOLL, SLC6A15, ADAMTS5*) were selected because these genes were the most highly methylated in CRC compared to the normal mucosa, and 6 CpG sites (*TFPI2, EYA4, NPY, TWIST1, LAMA1, GAS7*) were selected based on our previous GoldenGate Methylation Solution (Illumina) results (unpublished) or genome-wide expression array data. The two hypomethylated CpG sites (*MAEL, SFT2D3*) were selected because these genes showed the lowest methylation level in tumor tissue compared to the normal mucosa. The methylation status measured by pyrosequencing showed a good correlation with the methylation status measured by Human Methylation27 DNA Analysis BeadChip® (Fig. 2). This result showed that DNA methylation profiling using beadchip arrays is an accurate method for the genome-wide screening of methylated CpG sites.

Gene ontology categories of hypermethylated or hypomethylated CpG sites

Gene ontology analysis of the hypermethylated or hypomethylated CpG sites located in promoter regions and CpG islands in CRC was performed. The aberrantly methylated CpG sites were distributed across various categories of biological processes, molecular functions or pathways. However, the promoters of the genes related to certain categories appeared to be more likely to be hypermethylated (Table 3). Interestingly, the genes in cadherin signaling pathway were mostly frequently hypermethylated.

Comparison of promoter hypermethylation to "CAN genes"

MLH1 can be inactivated genetically and epigenetically. A germline mutation of *MLH1* causes Lynch syndrome and promoter hypermethylation of *MLH1* causes microsatellite unstable sporadic CRC. Therefore, this study examined whether the promoter of *CAN* genes, described by Sjoblom et al [34], showed hypermethylation. Thirty-seven out of 69 *CAN* genes had promoter regions in the CpG islands and 6 of these 37 genes (cell adhesion molecule with homology to L1CAM (close homolog of L1) *(CHL1),* CUB and Sushi multiple domains 3 *(CSMD3), EYA4,* guanylate cyclase 1, soluble, alpha 2 *(GUCY1A2),* potassium voltage-gated channel, KQT-like subfamily, member 5 *(KCNQ5),* matrix metallopeptidase 2 *(MMP2)*) showed significant promoter hypermethylation (Table 4). These results are consistent with those reported by Schuebel et al [35].

Comparison of promoter hypermethylation to genome-wide expression array data

Finally, genome-wide expression array analysis was performed comparing 6 normal colonic mucosa samples versus 65 CRC tissues to determine the relationship between the gene methylation status and mRNA expression of genes. This approach was used to obtain a preliminary assessment of the proportion of genes that were aberrantly methylated "passenger" genes vs. "driver" genes. The mean fold change was the log ratio of the mRNA expression level for the CRC tissue relative to 6 pooled normal mucosa. There was no statistically significant difference in the mRNA expression level

between promoter hypermethylation group and hypomethylation group (Supplementary table 3). However, mRNA expression was more likely to be down-regulated in the promoter hypermethylation group, even though it was not statistically significant. The genes with promoter hypermethylation whose expression was downregulated more than 2 fold are listed as follows: *ADHFEI,* sodium channel, nonvoltage-gated 1, beta (*SCNN1B),* *C2orf32,* slit homolog 2 (Drosophila) *(SLIT2),* enoyl CoA hydratase domain containing 3 *(ECHDC3),* slit homolog 3 (Drosophila) *(SLIT3),* EGF-containing fibulin-like extracellular matrix protein 1 *(EFEMP1),* somatostatin (*SST),* forkhead box D2 (*FOXD2),* ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (*ST3GAL4),* frizzled-related protein (*FRZB),* transcription elongation factor A (SII)-like 2 (*TCEAL2),* homeobox A5 (*HOXA5),* ubiquitin carboxyl-terminal esterase L1 (*UCHL1),* NDRG family member 2 (*NDRG2),* zinc finger homeobox protein 1b *(ZFHX1B),* *NPY,* zinc finger protein 447 (*ZNF447),* protein phosphatase 1, regulatory (inhibitor) subunit 3C *(PPP1R3C).* The genes with promoter hypomethylation whose expression was upregulated more than 2 fold are as follows: *C19orf33,* interleukin 10 receptor, alpha (*IL10RA),* enoyl CoA hydratase 1, peroxisomal (*ECH1),* myotubularin 1 (*MTM1),* 3-hydroxymethyl-3-methylglutaryl-CoA lyase (*HMGCL).*

DISCUSSION

A genome-wide assessment of the methylation state of CpG's in CRC was assessed using Human Methylation27 DNA Analysis BeadChip® arrays. This array platform was found to be a promising method for identifying the genes with promoter hypermethylation in CRC. These results are consistent with the published genome-wide assessments of aberrantly methylated genes in CRC. Schuebel et al reported that epigenetic unmasking techniques using expression arrays identified the genes affected by promoter CpG island DNA hypermethylation [35]. They confirmed the methylation status of several candidate genes in CRC and normal tissue using nested methylation specific PCR. The results of their validation studies identified the genes that were found in the present study to be methylated in CRCs. For example, *BOLL, EFEMP1,* and junctophilin 3 (*JPH3)* were significantly methylated in both studies. Estécio et al used the Methylated CpG Island Amplification (MCA) method to identify the methylated genes in the RKO colorectal cancer cell line [36]. Sixty-three of the genes that Estécio found to be methylated using the MCA method were represented on the HumanMethylation27 arrays. Among these 63 genes, 6 genes (glial cell derived neurotrophic factor (G*DNF),* GDNF family receptor alpha 1 (*GFRA1),* heart and neural crest derivatives expressed 2 (*HAND2),* orthopedia homeobox (*OTP),* PR domain containing 14 (*PRDM14),* Wilms tumor 1 (*WT1)*) were significantly methylated in our studies. Furthermore, Mori et al employed epigenetic unmasking to identify 54 genes that showed CRC-specific promoter methylation [37]. Among the candidate genes, the promoters of NEL-like 1 (chicken) (*NELL1),* A kinase (PRKA) anchor protein 12 (*AKAP12),* mal, T-cell differentiation

protein (*MAL)*, *SST* and tachykinin, precursor 1 *(TAC1)* were significantly methylated in our results. Finally, methylated DNA immunoprecipitation was used to identify aberrantly methylated genes in the CRC through its application to the colorectal cancer cell line. Among the genes identified as hypermethylated in SW48, we found that two genes, ADAM metallopeptidase domain 12 (*ADAM12)* and zinc finger protein 677 (*ZNF677),* were hypermethylated in the CRC tissue compared to normal tissue [38]. To our knowledge, this study is the first report of methylation profiling using Human Methylation27 DNA Analysis BeadChip® in CRC. A comparison of our results demonstrated modest overlap in the genes found to be commonly methylated in CRCs compared to previously published studies. This may represent differences in the sensitivity of the assays, differences between the cell lines and primary tumors or differences in the epigenome of tumors that occur in Western populations vs. Asian populations.

We obtained a list of 621 genes with the hypermethylated promoter in CpG islands. It is postulated that the number of epigenetically altered genes is higher than genetically altered genes in tumor tissue [35]. However, the expression of all the 621 hypermethylated genes was not down-regulated in the CRC tissue compared to normal mucosa. The correlation between promoter hypermethylation and the mRNA expression level was modest at best, even though mRNA expression tended to be down-regulated in the genes showing promoter hypermethylation. This likely reflects the fact that many epigenetic and genetic alterations in cancers are passenger events that are not important in the pathogenesis of cancer [39]. Moreover, multiple mechanisms regulate gene expression in addition to methylation, and these mechanisms are altered in CRC, which confound our

ability to identify a correlation between methylation and gene expression. Although the expression is not down-regulated, cancer-specific promoter hypermethylation can be valuable as a biomarker.

The following interesting patterns were identified through an analysis of the methylome of CRCs: 1) genes on chromosome 18 were most frequently methylated; 2) *CAN* genes can be affected by mutations and aberrant methylation; and 3) genes involved in cadherin function are often subject to aberrant DNA methylation. A previous study showed that the genes on chromosome 18 were most frequently down-regulated in rectal cancer [40]. In addition, a loss of chromosome 18 occurs at early stages of colorectal carcinogenesis [41]. This suggests that the aberrant methylation of genes appears to cooperate with the genetic alterations to drive the initiation and progression of CRC [42]. In comparison of our result with Sjoblom's *CAN* genes, we could get the methylation level of *37* CAN genes with the promoter in CpG islands and the promoters of 6 genes were hypermethylated. This proportion is meaningful considering that some of 37 genes can have oncogenic effect. Ontology analysis of the genes showed that promoter hypermethylation occurred at various biological processes and molecular functions. Among them, the cadherin signaling pathway attracted attention. The cadherin gene family (E-cadherin, N-cadherin, P-cadherin) encodes the proteins that mediate calcium-ion-dependent adhesion. Cadherin-catenin complex is the central part of this pathway. It has been suggested that they are involved in colorectal carcinogenesis.

This study identified new candidates of methylation markers for CRC. Ten genes with promoter hypermethylation were validated using pyrosequencing analysis. To our knowledge, 7 genes have not been reported to undergo DNA methylation in CRC. *TFPI2*

is a Kunitz-type serine proteinase inhibitor that protects the extracellular matrix of cancer cells from degradation and inhibits in vitro colony formation and proliferation [43]. Promoter hypermethylation of *TFPI2* was observed in various cancers including esophageal cancer, gastric cancer, pancreatic cancer, cervical cancer and malignant melanoma [44-50]. Methylation of *TFPI2* in stool DNA was recently reported to be a potential novel biomarker for the detection of CRC [43]. *EYA4* encodes a protein acing as a transcriptional activator through its protein phosphatase activity, which is important for eye development and for the continued function of the mature organ of Corti[51]. Aberrant methylation was observed in esophageal and colorectal cancer [52-53]. *BOLL* belongs to the DAZ gene family that is required for germ cell development. One report showed that *BOLL* was hypermethylated in colon cancer cell lines [35].

Other genes were reported to be associated with carcinogenesis. For example, *TWIST1* promoter methylation was reported to be significantly more prevalent in malignant breast tissue than in healthy tissue [54]. *NPY* can reduce the invasive potential of colon cancer cells in vitro [55]. Further study will be needed to confirm the usefulness of these promoter hypermethylation as biomarkers and clarify the functional role of these genes in colorectal carcinogenesis. In addition, it is important to validate the methylation status and clarify the functional role of the genes with promoter hypermethylation, in which expression was down-regulated in CRC.

In conclusion, we have shown that methylation profiling based on beadchip arrays is an effective method for screening the genes with promoter hypermethylation. In addition, we identified new potential candidates of methylation markers in CRC.

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TABLE 1 Characteristics of the subjects

	Methylation profiling group (n=22)	Validation group (n=35)	Expression array group (n=65)
Age (years)[a]	63 (42-77)	58 (43-77)	59 (41-77)
Sex			
Male	16	23	41
Female	6	12	24
Location			
Right colon	7	10	7
Left colon	9	18	19
Rectum	6	7	39
Stage			
I	6	3	0
II	9	21	42
III	7	9	23
IV	0	2	0

[a] Median (range)

TABLE 2 Twenty top-ranking genes with hypermethylated or hypomethylated promoters

Hypermethylation[a]		Hypomethylation[b]	
ADHFE1	*FLJ30834*	*SFT2D3*	*FLJ36046*
GPR75	*SLC18A3*	*MAEL*	*LAMB1*
BOLL	*WDR78*	*EDG6*	*SLC6A6*
SLC6A15	*KCNQ5*	*LILRA4*	*GPSM1*
ADAMTS5	*PRKAR1B*	*HIST1H2BO*	*MGC11257*
VGCNL1	*GABBR2*	*GPR109A*	*INT1*
TFPI2	*PCDHGC4*	*CARD14*	*SLC6A18*
CUTL2	*ADCY1*	*FLJ36116*	*FLJ27365*
UNC5C	*FIGN*	*GRAP*	*NUP50*
SPG20	*GALR2*	*NRXN1*	*ABHD7*

[a] twenty top-ranking genes hypermethylated in colorectal cancer tissue compared to normal colorectal mucosa, which were selected based on statistical significance

[b] twenty top-ranking genes hypomethylated in colorectal cancer tissue compared to normal colorectal mucosa, which were selected based on statistical significance

TABLE 3 Biological process, molecular function, and pathway categories with methylated genes[a]

	Total	Hypermethylaton	Hypermethylaton (expected)	p-value
Biological process				
Signal transduction	1560	188	99.8	1.29E-17
Developmental processes	1157	148	74.0	2.62E-15
Neuronal activities	299	64	19.1	2.91E-15
Cell communication	539	91	34.5	7.33E-15
Cell surface receptor mediated signal transduction	686	100	43.9	1.94E-12
Ectoderm development	390	70	25.0	2.61E-12
Neurogenesis	360	64	23.0	7.94E-11
Cell adhesion	257	49	16.4	8.06E-10
mRNA transcription regulation	841	104	53.8	1.41E-08
Cell adhesion-mediated signaling	152	35	9.7	3.36E-08
G-protein mediated signaling	307	52	19.6	7.79E-08
Protein metabolism and modification	1506	55	96.3	1.77E-05
mRNA transcription	1080	112	69.1	2.89E-05
Biological process unclassified	2877	133	184.0	5.50E-05
Synaptic transmission	147	26	9.4	7.18E-04
Cell proliferation and differentiation	586	62	37.5	2.66E-03
Sensory perception	139	21	8.9	1.03E-02
Ion transport	278	36	17.8	1.04E-02
Electron transport	120	0	7.7	1.37E-02
Cation transport	226	31	14.5	1.64E-02
Other neuronal activity	84	16	5.4	2.04E-02
Other metabolism	308	7	19.7	2.54E-02
Mesoderm development	293	36	18.7	2.84E-02
Pre-mRNA processing	166	1	10.6	3.80E-02
Nerve-nerve synaptic transmission	38	10	2.4	4.21E-02
Action potential propagation	9	5	0.6	4.68E-02
Molecular function				
Receptor	553	97	35.4	9.24E-18
G-protein coupled receptor	146	36	9.3	2.33E-09
Cell adhesion molecule	150	34	9.6	1.34E-08
Homeobox transcription factor	142	34	9.1	1.85E-08
Transcription factor	1118	124	71.5	2.03E-08
Extracellular matrix	144	32	9.2	7.00E-08
Cadherin	39	17	2.5	2.04E-07
Ion channel	164	31	10.5	4.44E-06
HMG box transcription factor	18	9	1.2	5.37E-04
Molecular function unclassified	2736	131	175.0	7.78E-04
Ligase	231	2	14.8	1.20E-03
Voltage-gated ion channel	76	16	4.9	7.27E-03
Ligand-gated ion channel	41	11	2.6	1.42E-02
Basic helix-loop-helix transcription factor	71	14	4.5	4.12E-02
Pathway				
Cadherin signaling pathway	88	20	5.6	2.86E-04

[a] Gene ontology analysis was performed using the PANTHER Classification System.

TABLE 4 Assessment of promoter hypermethylation in "*CAN* genes"[a]

CAN gene	p-value	Δβ(mean)	β(mean)_normal	β(mean)_tumor
ABCA1	7.40E-01	-0.001	0.023	0.024
ACSL5	1.37E-07	0.265	0.854	0.589
ADAMTS15	1.45E-01	-0.005	0.031	0.036
ADAMTS18	6.86E-01	0.014	0.252	0.238
APC	1.01E-01	-0.042	0.037	0.080
CD109	3.18E-02	-0.040	0.045	0.085
CHL1	5.74E-08	-0.270	0.169	0.440
CNTN4	4.61E-02	-0.062	0.256	0.318
CSMD3	2.21E-04	-0.156	0.070	0.227
EPHA3	1.25E-01	-0.041	0.188	0.229
EPHB6	1.14E-01	-0.011	0.018	0.029
ERCC6	3.06E-01	-0.003	0.019	0.022
EYA4	1.70E-11	-0.510	0.035	0.545
FBXW7	1.44E-01	0.006	0.084	0.078
GALNS	8.73E-01	0.000	0.029	0.029
GNAS	7.05E-01	-0.012	0.526	0.538
GUCY1A2	4.09E-04	-0.218	0.123	0.341
KCNQ5	8.24E-12	-0.491	0.037	0.528
KRAS	1.83E-01	-0.007	0.021	0.028
LRP2	4.60E-03	-0.098	0.082	0.180
MAP2	1.23E-03	-0.104	0.379	0.483
MLL3	4.09E-02	-0.028	0.073	0.101
MMP2	1.61E-08	-0.286	0.214	0.500
NF1	2.57E-01	0.007	0.079	0.073
PHIP	3.39E-01	-0.002	0.035	0.037
PKNOX1	8.50E-01	0.000	0.048	0.047
PRKD1	2.31E-01	-0.063	0.300	0.364
PTPRU	8.11E-05	0.018	0.051	0.033
RET	1.39E-02	-0.107	0.075	0.182
SCN3B	3.43E-01	-0.031	0.094	0.126
SFRS6	5.15E-02	-0.004	0.028	0.031
SLC29A1	2.49E-01	0.003	0.055	0.052
SMAD4	5.40E-02	-0.003	0.027	0.031
TCF7L2	7.88E-01	-0.001	0.053	0.054
TGFBR2	2.84E-02	-0.012	0.026	0.038
UHRF2	1.46E-01	-0.003	0.036	0.039
UQCRC2	4.59E-02	-0.007	0.031	0.038

[a] "CAN genes" are candidate colorectal cancer genes described by Sjoblom et al[34].

Figure Legend

FIG. 1 Chromosomal distribution of hypermethylated or hypomethylated promoters in CpG islands. The percent means (the number of hypermethylated or hypomethylated CpG sites X 100)/(the number of total CpG sites located in promoter regions and CpG islands on individual chromosome). This shows that genes on chromosome 18 are hypermethylated most frequently.

FIG. 2 Pyrosequencing analysis. Methylation level of 10 hypermethylated genes (*ADHFE1, BOLL, SLC6A15, ADAMTS5, TFPI2, EYA4, NPY, TWIST1, LAMA1, GAS7*) and 2 hypomethylated genes (*MAEL, SFT2D3*) was confirmed by pyrosequencing analysis.