SUPPLEMENTARY MATERIALS.

Supplementary methods.

MCAM.

MCA: Selective enrichment of methylated DNA in each sample DNA was conducted by utilizing the MCA methodology. In MCA, the methylated DNA-specific amplification was carried out based upon the serial digestion with a set of isoschizomers, methylation-sensitive SmaI and methylation-insensitive XmaI, followed by XmaI-digested fragment-specific linker PCR. In brief, 5 µg of DNA was digested with *Sma*I and then dephosphorylated using Antarctic phosphatase. DNA was subsequently subjected to digestion with XmaI followed by column-purification with the QIAquick PCR purification kit (Qiagen). The purified DNA was then ligated to a linker by using T4 DNA ligase and column-purified again. The linker was prepared by annealing the following two oligomers: RMCA24 (5'-CCACCGCCATCCGAGCCTTTCTGC-3') and RMCA12 (5'-CCGGGCAGAAAG-3). One hundred ng of linker-ligated DNA was PCR-amplified in a 100µl reaction mix containing 100 pM of RMCA24 as described previously (Estecio, et al. 2007).

Microarray: The 244K Human CpG Island microarray (Agilent Technologies, Santa Clara, CA) was used as the array platform. The hybridization targets were prepared by labeling 5 µg of MCA-processed DNA with Cy-5 or Cy-3 dUTP using the random primer method (BioPrime DNA Labeling System, Invitrogen, Carlsbad, CA). Array hybridization and washing was carried out according to the Agilent CGH microarray protocol. Array raw data acquisition was conducted using an Agilent G2565BA microarray scanner and Feature Extraction Software (Agilent) according to the array-CGH data extraction protocol.

Array data processing: Raw data processing included background subtraction and LOESS normalization using the LIMMA scripts (Wettenhall and Smyth 2004). LOESS normalization was performed based on the probes whose corresponding SmaI-XmaI fragment length were greater than 5kb and thus were not susceptible to PCR-amplification

regardless of the methylation status, as was described previously (Estecio et al. 2007). The normalized log2 intensity ratio to the fully methylated control DNA, CpGenome Universal Methylated DNA (Millipore, Billerica, MA), at each locus was used as the value representing the locus methylation status. When multiple probes corresponded to a SmaI-XmaI fragment, the median of these probes were used as the representative value. The data for probes whose corresponding SmaI-XmaI fragments were 60-2000 nucleotides in length (*i.e.*, optimal size for PCR amplification) were used for the evaluation of loci methylation status (Estecio et al. 2007). Fragments were dropped from analyses when only single corresponding probe presented on the array and the raw signal intensity for this probe was low (i.e., <500 AU) in fully methylated control DNA, as described previously (Estecio et al. 2007). As the results, this MCAM protocol enabled the methylation status assessment of 34,396 SmaI-XmaI restriction fragments that corresponded to 14,213 CGIs (50.4% of all CGIs in the genome). Twenty percent of these SmaI-XmaI fragments located outside of known coding or non-coding genes, and the remaining fragments located proximal to the transcriptional start sites (-2,000 to +500 bases, 63%) or transcribed regions (17%). Annotation of the probes and SmaI-XmaI fragments was based upon the Human Genome Assembly version 18. Array raw data processing was conducted using a LIMMA library-based R script and a sql script.

Legends for Supplementary figure.

Supplementary Figure 1. MCAM data reproducibility and reliability

A. The raw data for two experimental batches of a specimen demonstrated extremely high correlation (R>0.99). Each datapoint represents a probe. The MCAM experiment for these two batches (i.e., DNA processing, array hybridization, and array scanning) was performed on separate days, two weeks apart. **B.** Methylation measurements by MCAM (*X-axis*) and qMSP (*Y-axis*) are plotted for individual specimens at four loci. The results from MCAM and qMSP assays at these loci correlated well (R>0.70, p<.0001) despite of the markedly distinctive basis of methylation measurement for MCAM vs. qMSP (e.g., restriction enzyme digestion vs. bisulfite conversion, single CpG methylation status vs. continuous methylation of all or nearly all CpGs within a region that is 70-120 bases in length).

Supplementary Figure 2. Cluster analysis of the methylation microarray data.

The k-mean Clustergram is shown for the analysis of 18,892 autosomal loci that tended to be differentially methylated between17 CRCs and 8 control NCs (inclusion criteria: t-test p<.1). *Y-axis* represents loci alignment, while *x-axis* represents tissue alignment: o*range, control NCs; pink, CIMP(+) CRCs; blue, CIMP(-) CRCs.* As is expected, control NCs clustered separately from CRCs, and CIMP(-) CRCs formed a cluster separately from most CIMP(-) CRCs. *Gray vertical bars* indicate the clusters of loci whose methylation status in CIMP(+) CRCs differs from that of CIMP(-) CRCs.

Supplementary Figure 3. *ALX3* methylation status for non-neoplastic colonic tissues from CRC cases as well as neoplasia-free control cases.

Median (*bar*), 25-75 percentile range (*box*), and 10-90 percentile range (*whisker*) of all informative specimens are displayed for each tissue category. P-values were calculated by Mann-Whitney test.

Target	Forward primer sequence (5'>3')	Reverse primer sequence (5'>3')	TaqMan probe sequence (5'>3')
ALX3	GTTCGGGTTAGCGTTAATTCGGTTTTC	CTTCCTACTTATCTCTCCCGCTCG	AAGTCGTGGCGAAAGGCGAGAG
CCDC4	TTTTGCGTTTGGAATTGTTGCGGC	CCTAATAAAACTACCGCACGTACGAACG	TTCGTTGTTGTTGATGGAGACGGCG
CHX10	AAAATTTTCGGATTCGGGTTTCGGC	CAAAAAACCTACGAACGCCCCG	ACCGTCCCATCCCTAAACTCAAACTAACC
DOCK8	GGTTTCGGGTAGAGCGCGTC	TAACGCGCAAACAACGACGAACG	AGTATCGGGAGGTTAGTTTCGCGTTGG
GJA7	TTCGGGTAGTTGTCGGTATCGTC	CGCGAAAATATCGAACGACGATCG	AAACGACGCGCACTCGACGAC
GLP1R	AGTTCGGGATTAGTTTTCGTACGC	AACCGAAAACGCCGACCATAACG	TCGTAGGTGGTAGCGATGGTTTAGTTTTGA
HOMER2	GGAAGGGTTACGGGTTTCGC	CGAAAATCTAAAACCACCTAACCCGCG	TTTGTTGGGTGTTCGCGTGTTTCGG
BTG4	ATTCGTTTCGTTTCGCGTTCGTTTC	CGGGAGGGTTTTGAGAGGAGC	AACGACCAACCGTCCTTAAAACCC
miR34b	CGCTCTAAACGACCGAATAACTATAACG	CTCCCAAACCGAAAACCGCG	TACCAAACCTCCCCTTCCCGCAAC
NME4	GTTTCGGGTTGGTATCGTTTTTCGC	CGCCAAAAAAAAACCGCCCATAACG	TTTCGCGTTTATAGCGGTTCGCGG
NPTX1	AGTTTTCGAGCGTTCGTCGTTTGC	TCTAACGCCAAAAAACGACGACCG	TGAGTCGGGGTTGCGTTTTCGTTG
TMEM42	GTAGGGCGTCGTCGTATTTTCGTC	TACCACCCCAACTAACCGCAACG	TTTGTTTCGTCGGGGTTTCGGGTCG
TTLL12	GCGTTTGTGGTTTGGTTGTCGTC	TCCCGACATTCCCCGAAATCG	TGCGTACGCGTATTTGATGGTGGTTGA
ZNF583	GATTTGCGGTCGTTCGGGGTTTTC	AACCCTAAAACAAACCGCAAAAACCG	TTTGCGTCGCGTCGGTTTCGGATTT

Supplementary Table 1. Oligonucleotide sequences.

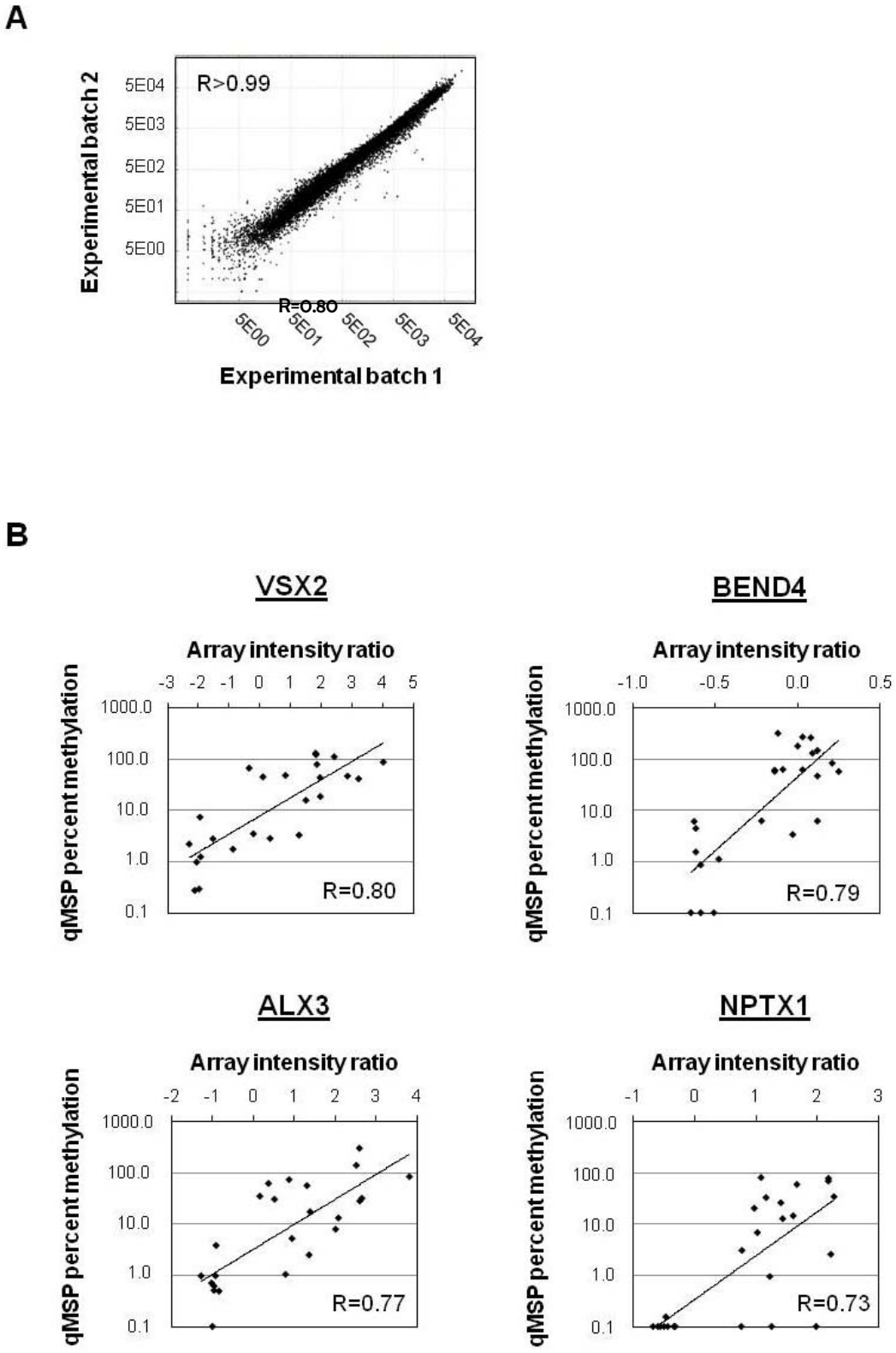
Locus	p-value	CRC mean / NC mean	p-value<0.01 and fold change>0.5	CRC min.> NC max.	Reference
BMP3	2E-02	0.38	no	no	(Zou, et al. 2007)
GATA4	9E-03	0.91	yes	no	(Hellebrekers, et al. 2009)
GATA5	5E-05	1.04	yes	no	(Hellebrekers et al. 2009)
HIC1	9E-06	1.16	yes	no	(Lenhard, et al. 2005)
HPP1	1E-06	2.90	yes	no	(Belshaw, et al. 2004)
ITGA4	1E-05	0.56	yes	no	(Ausch, et al. 2009)
MAL	7E-03	0.93	yes	no	(Mori, et al. 2006)
MGMT	7E-02	0.49	no	no	(Belshaw et al. 2004)
NDRG4	6E-02	0.08	no	no	(Melotte, et al. 2009)
NELL1	1E-04	1.65	yes	no	(Mori et al. 2006)
OSMR	4E-04	1.15	yes	no	(Kim, et al. 2009)
RASSF2	9E-07	1.26	yes	no	(Nagasaka, et al. 2009)
SFRP2	1E-12	4.10	yes	yes	(Muller, et al. 2004)
TFPI2	2E-07	2.98	yes	no	(Glockner, et al. 2009)
VIM	5E-03	0.35	yes	no	(Chen, et al. 2005)
WIF1	1E-01	0.93	no	no	(Lee, et al. 2009)

APC, SEPT9, and ALX4 loci were not informative in the current MCAM analysis.

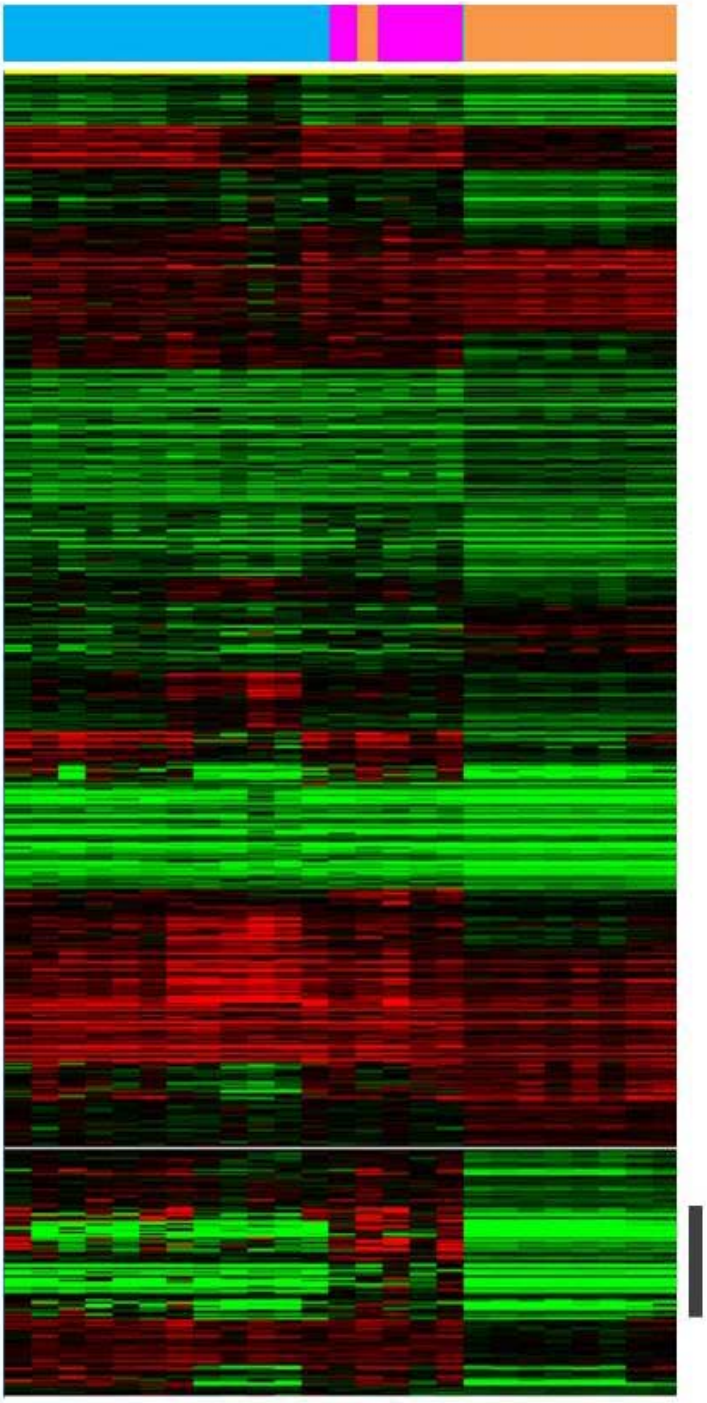
Supplementary Table 2. MCAM-derived methylation profiles of the previously reported CRC methylation markers.

Locus			Microarray (Log2 intensity)				MSP
Nucleotide position (hg18)	Corresponding genes	Location	CRC: mean (SD)	NC: mean (SD)	CRC/NC	t-test p-value	Hypermethylation in CRCs
chr4:154929653-154930045	SFRP2	5'UTR-Exon1	1.37 (1.10)	-2.72 (0.35)	4.10	1E-12	(+)
chr14:73777277-73777554	VSX2	Intron1	1.57 (1.19)	-1.77 (0.45)	3.34	8E-10	(+)
chr6:41714264-41714529	MDFI	Exon1-Intron1	1.89 (0.95)	-1.33 (0.37)	3.23	1E-11	(+)
chr4:154930045-154930197	SFRP2	5'UTR-Exon1	1.82 (1.14)	-1.22 (0.20)	3.04	1E-09	(+)
chr1:110414310-110414409	ALX3	Intron1	1.58 (1.00)	-0.94 (0.12)	2.52	3E-09	(+)
chr17:76066388-76066727	NPTX1	5'UTR	1.48 (0.52)	-0.39 (0.19)	1.87	3E-12	(+)
chr6:39124527-39124750	GLP1R	5'UTR-Intron1	0.79 (0.55)	-0.75 (0.24)	1.54	1E-09	(+)
chr3:44878617-44878768	TMEM42/miR564	Intron1	1.09 (0.18)	-0.16 (0.40)	1.26	6E-12	(+)
chr22:37432155-37432530	GTPBP1	Intron1	0.62 (0.18)	-0.51 (0.22)	1.13	1E-14	(-)
chr11:110888513-110888739	miR34b-BTG4	5'UTR	0.62 (0.32)	-0.42 (0.22)	1.04	3E-10	(+)
chr15:81412032-81412142	HOMER2	Intron1	0.71 (0.37)	-0.28 (0.23)	1.00	8E-09	(+)
chr22:41912965-41913471	TTLL12	5'UTR-Intron1	0.31 (0.21)	-0.65 (0.21)	0.96	3E-12	(+)
chr11:67985025-67985286	SAPS3	Intron1	0.55 (0.29)	-0.28 (0.13)	0.84	6E-10	(-)
chr1:154988383-154990056	HDGF	5'UTR-Intron1	0.45 (0.15)	-0.36 (0.17)	0.82	1E-13	(-)
chr19:3012272-3012428	AES	Intron1	0.30 (0.19)	-0.48 (0.18)	0.78	1E-11	(-)
chr16:387138-387279	NME4	5'UTR-Exon1	0.37 (0.21)	-0.39 (0.10)	0.76	5E-12	(+)
chr9:204673-204810	DOCK8	5'UTR	0.37 (0.23)	-0.36 (0.10)	0.73	8E-11	(+)
chr19:61607426-61607563	ZNF583	5'UTR-Exon1	-0.03 (0.19)	-0.65 (0.14)	0.62	4E-10	(+)
chr17:40262574-40262867	GJC1	Intron1	0.32 (0.19)	-0.29 (0.11)	0.61	1E-10	(+)
chr4:41848650-41849315	BEND4	Intron1-Exon2	0.03 (0.13)	-0.56 (0.09)	0.58	2E-13	(+)

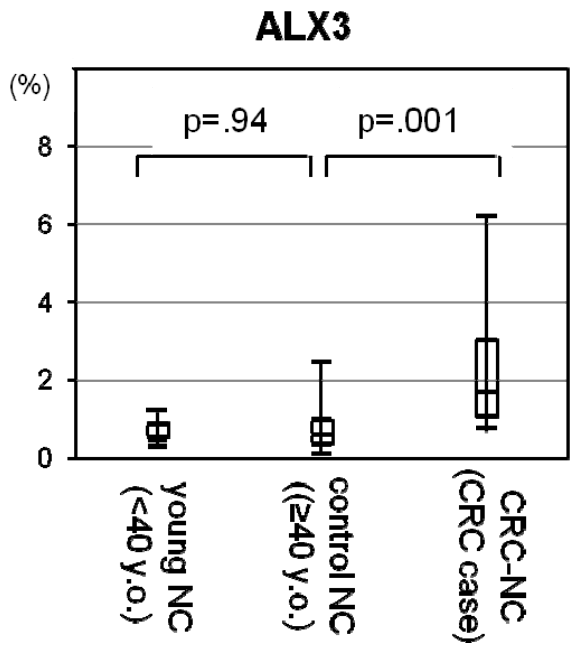
Supplementary Table 3. Preliminary validation results of the twenty candidate loci for cancer-specific hypermethylation.



Supplementary Figure 1. MCAM data reproducibility and reliability



Supplementary Figure 2. Cluster analysis of the methylation microarray data.



Supplementary Figure 3. ***ALX3*** **methylation status for non-neoplastic colonic tissues from CRC cases as well as neoplasia-free control cases.**

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