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Genome-Wide Identification and Validation of a Novel Methylation Biomarker, *SDC2*, for Blood-Based Detection of Colorectal Cancer

TaeJeong Oh,* Nayoung Kim,* Youngho Moon,* Myung Soon Kim,* Benjamin D. Hoehn,* Chan Hee Park,† Tae Soo Kim,‡ Nam Kyu Kim,§ Hyun Cheol Chung,†‡¶ and Sungwhan An*

From Genomictree, Inc., Daejeon, South Korea; and the National Biochip Research Center,† the Cancer Metastasis Research Center,‡ and the Departments of Surgery§ and Internal Medicine,¶ Yonsei University College of Medicine, Seoul, South Korea*

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Address correspondence to Sungwhan An, Ph.D., 829 Tamnip-dong, Yuseong-gu, Daejeon, 305-510, South Korea. E-mail: genomictree1@korea.com.

Aberrant DNA methylation has shown promise as a biomarker for the early detection of cancer. To discover novel genes frequently methylated at an early stage in colorectal cancer (CRC), DNA microarray analysis coupled with enriched methylated DNA was performed in primary tumors and compared with adjacent nontumor tissues of 12 patients with CRC at stages I to IV. Stepwise filtering for candidate selection in microarray data analysis yielded a set of genes that are highly methylated across all CRC tumors and that can be used as a composite biomarker for CRC detection. Verification assay identified the *SDC2* gene as a potential methylation biomarker for early CRC detection. In clinical validation in tissues from 139 CRC patients, a much higher level of aberrant *SDC2* methylation was measured in most primary tumors (97.8%), compared with corresponding nontumor tissue of CRC patients, irrespective of clinical stage. Clinical validation of *SDC2* methylation in serum DNA from CRC patients ($n = 131$) at stages I to IV and from healthy individuals ($n = 125$) by quantitative methylation-specific PCR demonstrated a high sensitivity of 87.0% (95% CI, 80.0% to 92.3%) in detecting cancers, with a specificity of 95.2% (95% CI, 89.8% to 98.2%). Importantly, sensitivity at stage I was 92.3%, indicating the potential of *SDC2* methylation as a blood-based DNA test for early detection of CRC. *(J Mol Diagn 2013, 15: 498–507; http://dx.doi.org/10.1016/j.jmoldx.2013.03.004)*

Colorectal cancer (CRC) is one of the most common types of malignancies worldwide and is a known major cause of cancer morbidity and mortality.[1,2] The mean 5-year survival rate for CRC is estimated to be less than 10% if metastasis occurs, but can be as high as 90% if the cancer is detected at an early stage.[3] Several screening options are currently available to facilitate early detection of CRC, including fecal occult blood testing, fecal immunochemical testing, and colonoscopy. Over the last 10 years, colonoscopy has been the preferred screening test to detect CRC.[4–6] Within the general public, however, the acceptance of screening colonoscopy remains surprisingly low.[7,8] This reluctance may be due to the need for extensive bowel preparation, which many patients may find prohibitive. Thus, a noninvasive test using blood or stool might be an attractive alternative for screening of people for colonoscopy referral.

Aberrant DNA hypermethylation is known to be a major mechanism for inactivation of cancer-associated genes, including tumor suppressor genes, in CRC and in other human cancers.[9,10] Methylated DNA is chemically and biologically stable, is less subject to transient alterations, and is readily detectable in many types of biological samples, including blood and stool; it is therefore well suited for molecular methods of noninvasive or minimally invasive cancer

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detection.[11–13] Cancer-specific aberrant DNA methylation can be detected in plasma or serum from patients with various solid tumors, including CRC.[14,15] Several blood-based markers have been described as potential markers for CRC, including the genes *ALX4*, *SEPT9*, *NEUROG1*, *THBD*, *C9orf50*, and *VIM* (vimentin).[16–20]

In the present study, we performed CpG microarray analysis coupled with methylated DNA isolation assay (MeDIA)[21] to investigate a specific subset of genes aberrantly methylated in primary tumor of CRC. The methylation profiles of these genes were indirectly compared with those of paired, adjacent nontumor tissues through a common reference DNA. Through stepwise filtering processes, we identified the *SDC2* gene as a novel, highly promising methylation marker for detection of CRC. We then conducted validation studies in match-paired CRC tissue samples representing various stages, followed by feasibility testing in cell-free serum DNA derived from CRC patients and from healthy individuals. We demonstrated that *SDC2* has high sensitivity and specificity in both tissues and serum-based tests for early detection of CRC.

Materials and Methods

Reagents

All chemical reagents used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Oligonucleotides were synthesized by Bioneer (Daejeon, South Korea).

Cells and Clinical Specimens

The human colon cancer cell lines Caco-2 (catalog no. 30037.1) and HCT116 (catalog no. 10247) were purchased from the Korean Cell Line Bank (Seoul National University, Seoul, Korea) and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/mL of penicillin, and 100 μg/mL of streptomycin (all from JBI, Seoul, Korea) in an incubator with a humidified, 5% CO_2–enriched atmosphere at 37°C.

Fresh-frozen primary tumors ($n = 139$) and paired, adjacent nontumor tissues ($n = 139$) from CRC patients were collected at the time of surgery at the Yonsei University College of Medicine Cancer Center. We randomly divided these tissue samples into three groups for microarray analysis (12 pairs), confirmation assay (32 pairs), and clinical validation (95 pairs); each group included all four stages of CRC. Each tumor specimen and paired, adjacent nontumor section was histologically verified by a board-certified pathologist and was archived for further DNA studies. For healthy normal control, genomic DNA from two individuals (a 24-year-old male and a 82-year-old female) without any history of malignancy for CRC was purchased from BioChain Institute (Hayward, CA). All normal serum specimens ($n = 125$) derived from healthy individuals were obtained from Innovative Research (Novi, MI), which is a commercial donor center licensed by the U.S. Food and Drug Administration; none of these individuals had any apparent history of malignancy, although they had not undergone colonoscopy screening at the time of blood drawing. Of the 131 serum samples from cancer patients histologically confirmed by a pathologist, 87 samples representing all four stages (stage I, $n = 11$; stage II, $n = 28$; stage III, $n = 36$; and stage IV, $n = 12$) were obtained from CRC patients before surgery at the Yonsei University College of Medicine Cancer Center; the remaining 44 samples, from CRC patients of European continental origin with stage I ($n = 15$) or stage II ($n = 29$) disease, were purchased from BioServe (Beltsville, MD). Blood was drawn, stored at 4°C for 30 minutes, and the serum fraction was separated by centrifugation at 600 × *g* for 15 minutes; serum samples were then immediately frozen in 1-mL aliquots and stored at −80°C until DNA extraction was performed. The characteristics of enrolled patients are described in Table 1. The study was approved by the Institutional Review Board of the Yonsei University College of Medicine Cancer Center. Informed consent was obtained from all participating patients and healthy control subjects.

DNA Isolation

Genomic DNA was isolated from tissue specimens and cell lines using a DNA mini kit (Qiagen, Hilden, Germany;

Table 1 Clinicopathological Features of Colorectal Cancer Patients and Healthy Control Subjects

Characteristics	Colorectal tissue		Serum
Healthy control			$n = 125$*
Sex [no. (%)]			
Male			64 (51.2)
Female			61 (48.8)
Age, mean years (range)			51.0 (40–61)
CRC	$n = 139$	$n = 87$†	$n = 44$‡
Sex [no. (%)]			
Male	86 (61.9)	51 (58.6)	18 (40.9)
Female	53 (38.1)	36 (41.3)	26 (59.1)
Age, mean years (range)	60.7 (35–84)	58.4 (35–84)	58.4 (33–78)
Stage [no. (%)]			
I	31 (22.3)	11 (12.6)	15 (34.1)
II	34 (24.5)	28 (32.2)	29 (65.9)
III	55 (39.6)	36 (41.3)	
IV	19 (13.7)	12 (13.8)	
Differentiation [no. (%)]			
Moderate	108 (77.7)	66 (75.9)	28 (63.6)
Poor	7 (5.0)	4 (4.6)	1 (2.3)
Well	18 (12.9)	12 (13.8)	5 (11.4)
Not determined	6 (4.3)	5 (5.7)	10 (22.7)

*Sera purchased from Innovative Research (Novi, MI).
†Sera obtained from the Yonsei University College of Medicine Cancer Center (Seoul, South Korea).
‡Sera purchased from BioServe (Beltsville, MD).

Valencia, CA) according to the manufacturer's instructions. Serum DNA was extracted using a Dynabeads silane viral NA kit (Life Technologies—Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Genomic DNA was extracted from 800 μL of serum samples. Serum samples were separated into two portions of 400 μL each, and serum was mixed with 100 μL of 20 mg/mL proteinase K; 600 μL of lysis/binding buffer was added, and the samples were mixed by inverting the tubes four to six times. This mixture was then incubated at room temperature for 5 minutes, followed by addition of 300 μL of isopropanol. Dynabeads MyOne silane (100 μL; Life Technologies—Invitrogen) was added, and the mixture was incubated on a rocking platform at room temperature for 10 minutes. The tube was then placed on a bead-isolation magnet (Genomictree, Daejeon, South Korea) for 2 minutes, to let the magnetic beads collect at the magnet. The supernatant was removed, and 850 μL of washing buffer 1 was added. The washing step was repeated two times, and the bead pellet was dried at room temperature for 10 to 15 minutes. Finally, 30 μL of elution buffer was added to the dried pellet, and the mixture was then incubated at 70°C for 3 minutes to elute DNA. The eluted DNA was combined and stored at −20°C until use.

CpG DNA Microarray Analysis

CpG microarray analysis in conjunction with the enrichment of methylated DNA by MeDIA using recombinant protein domain (MBD2bt) which is optimally truncated form of methyl-CpG-binding protein isoform, MBD2b was performed as described previously,[21] with slight modification.

In brief, genomic DNA was isolated from primary tumors and paired, adjacent nontumor tissues from 12 CRC patients across all the stages (stage I, $n = 3$; stage II, $n = 2$; stage III, $n = 5$; and stage IV, $n = 2$) and sonically fragmented. Each fragmented genomic DNA (0.5 μg) was incubated with 2 μg of recombinant MBD2bt protein for 4 hours at 4°C on a rocking platform for enrichment of methylated DNA. The enriched methylated DNA was amplified using a whole-genome amplification kit (Sigma-Aldrich) and labeled with Cy5 dye as recommended by the manufacturer. The common reference DNA was prepared by amplifying pooled genomic DNA from 12 paired, adjacent nontumor tissue samples without methylation enrichment and labeled with Cy3. The labeled DNA samples were purified using a PCR purification kit (Qiagen) and then were cohybridized onto human CpG island microarrays containing 237,000 oligonucleotide probes covering 27,800 annotated CpG islands (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions.

The raw DNA microarray data have been submitted to the Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE36380).

The hybridized images were analyzed using an Agilent DNA microarray scanner, data quantification was performed using the associated Feature Extraction software version 9.3.2.1, and preprocessing of raw data and normalization steps were performed using GeneSpring software version 7.3.1 (Agilent Technologies). To identify differentially methylated targets between primary colorectal tumors and paired, adjacent nontumor tissue samples, a group comparison was performed using a parametric analysis of variance test with Benjamini—Hochberg multiple testing correction ($P < 0.01$). The multiple-probe enriched genes were selected as methylation target genes if their probes yielded a positive or negative call for methylation in tumor, compared with paired, adjacent nontumor tissue, in at least two adjacent probes, allowing for only a one-probe gap within the CpG islands.

Bisulfite Treatment and DNA Purification

Genomic DNA was chemically modified using sodium bisulfite, which converts all unmethylated cytosine to uracil but leaves methylated cytosine unmodified. To this end, we used an EZ DNA methylation kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. In brief, genomic DNA was treated with sodium bisulfite for 2.5 hours at 65°C and desulfonation was performed for 20 minutes at room temperature. Bisulfite-converted DNA was purified using a Zymo-Spin IC column (Zymo Research) and eluted with 10 μL of distilled water. The eluted DNA was either used immediately for methylation analysis or was stored at −20°C until further use.

Quantitative Bisulfite Pyrosequencing Analysis

To quantify methylation levels of target genes in cell lines and tissues, we performed quantitative bisulfite pyrosequencing.[22] Bisulfite PCR and pyrosequencing primers were designed to amplify two to five CpG dinucleotides sites in target sequences, using PyroMark Assay Design software version 2.0 (Qiagen). Primer sequences are listed in Table 2. Briefly, 20 ng of bisulfite-modified DNA was amplified in a 25-μL reaction volume with gene-specific primers and Taq polymerase (Solgent, Daejeon, South Korea). Samples were heated to 95°C for 10 minutes and then were amplified for 45 cycles of 95°C for 45 seconds, optimal annealing temperature for 45 seconds, and 72°C for 60 seconds. All reactions were then incubated at 72°C for 10 minutes for final extension. Pyrosequencing was performed using a PyroGold kit and a PyroMark Q96 ID instrument (Qiagen) according to the manufacturer's instructions.

The methylation index (MtI) for each region of interest and for each sample was calculated as the mean percentage of methylated cytosine for all examined CpGs tested. Methylated non-CpG cytosines were used for internal controls, and to check the fidelity of bisulfite conversion. If the MtI value of the primary tumor was greater than that of the paired,

Table 2 Primers for Bisulfite PCR and Pyrosequencing Assay

Gene		Sequence*	CpG sites†	Size (bp)
CHST11	Forward	Biotin-5′-GAGATTATTTTGGTTAATATGG-3′	+361, +368, +385, +391, +393	207
	Reverse	5′-TTTAAAACRAAATCTCACT-3′		
	Sequencing	5′-TAGGAGAATGGTGTGAAT-3′		
KCNA1	Forward	5′-GGGTGGGTTTYGTAGAGAGTAAG-3′	−420, −410, −398, −394	114
	Reverse	Biotin-5′-CCTCCRACRAATTTACTTTT-3′		
	Sequencing	5′- TTTTTTGGGGGAGGA-3′		
IRX5	Forward	5′-GGGTTYGGGTTAGGTTTTATAA-3′	+2558, +2568, +2572, +2576	113
	Reverse	Biotin-5′-TAACTCCRCAACATTTTC-3′		
	Sequencing	5′-ATTTTAATGGATTAAATTAG-3′		
SIM1	Forward	5′-GGTTTTTAATTAGGAATAATAGTG-3′	−1024, −1021, −1015, −1003	244
	Reverse	Biotin-5′-AACRCCCATCTCTTAACT-3′		
	Sequencing	5′-CATCTCTTAACTATTCTCATACCT-3′		
SDC2 (R1)	Forward	5′-AAAGATAAAGGGGAAGAAAAGAGTATAGAGG-3′	−134, −153	204
	Reverse	Biotin-5′-CCCAAATAAACCCCAAAAAAATTCCTACAAAA-3′		
	Sequencing	5′-AAGGAAGAAAAGGATTGA-3′		
SDC2 (R2)	Forward	5′-GGAGGAGTAAAATTATAGTAGAGTAAGAA-3′	+351, +365, +370	100
	Reverse	Biotin-5′-CCCTCTCACTTATTAATTTCTACACT-3′		
	Sequencing	5′-GTAAGAAGAGTTTTAGAGAGTAGTTT-3′		
SDC2 (R3)	Forward	5′-GGGAGTGTAGAAATTAATAAGTG-3′	+456, +460, +466, +473	228
	Reverse	Biotin-5′-TCCCAACCRCTACTTACAA-3′		
	Sequencing	5′-AGGYGTAGGAGGAGGAA-3′		
SORCS3	Forward	5′-GGGTTTTTTTGGATAAGG-3′	+1741, +1751, +1754, +1763	101
	Reverse	Biotin-5′-CAAACRCRATACTCAATC-3′		
	Sequencing	5′-TTTTTTTGGATAAGGATG-3′		

*R indicates G or A. Y indicates C or T. Biotin indicates 5′ biotinylated sequences.
†The CpG sites, indicated by nucleotide position from transcription start site (+1), determined the methylation status by bisulfite-pyrosequencing.

adjacent nontumor tissue, the result was considered methylation positive.

qMSP

SDC2 methylation was determined by fluorescence-based quantitative methylation-specific PCR (qMSP) in serum DNA using a protocol modified from that of Eads et al.[23] For this assay, two methylation-specific primers and probes were specifically designed to bind to bisulfite-converted methylated DNA for the 5′ untranslated region (5′ UTR, 121 bp; +378 to +498 nt) of the *SDC2* gene. The forward and reverse primers used were 5′-TAGAAATTAA-TAAGTGAGAGGGCGT-3′ and 5′-GACTCAAACTC-GAAAACTCGAA-3′, respectively. The probe was 5′-FAM-AGTAGG-CGTAGGAGGAGGAAGCGA-Iowa Black-3′. To confirm the quality and quantity of bisulfite-modified serum DNA, β-actin (*ACTB*; 133 bp) was used as an internal control to normalize the DNA input. The forward and reverse primers used were 5′-TGGTGATGGAG-GAGGTTTAGTAAGT-3′ and 5′-AACCAATAAAACCT-ACTCCTCCCTTAA-3′, respectively. The probe was 5′-TET-ACCACCACCCAACACACAATAACAAACACA-Iowa Black-3′.

The qMSP analysis was performed on a Rotor-Gene Q real-time PCR system (Qiagen). Thermal cycling conditions were as follows: for *SDC2*, 10 minutes at 95°C and then 50 cycles of 10 seconds at 95°C, 15 seconds at 62°C, 20 seconds at 72°C; for *ACTB*, 10 minutes at 95°C and then 50 cycles of 10 seconds at 95°C, 60 seconds at 58°C. Heating and cooling rates were 20°C per second and 15°C per second, respectively. qMSP was performed using a Rotor-Gene probe PCR kit (Qiagen) at a 1× final concentration in a 20-μL reaction volume for all assays. The reaction mixture contained a 1/3 volume of bisulfite-converted serum DNA from 0.8 mL of serum, 100 nmol/L of each of the *SDC2* or *ACTB* primers, and 80 nmol/L of each of the *SDC2* or *ACTB* oligonucleotide probes with 5′-fluorescent reporter dyes. The percentage of methylated reference (PMR) was defined as the percentage of fully methylated molecules at a specific locus of the *SDC2* gene.[24] The PMR was calculated by dividing the *SDC2/ACTB* ratio in a sample by the *SDC2/ACTB* ratio in M.SssI-treated HCT116 DNA. The PMR for the *SDC2* gene in each sample was determined with the comparative C_T method instead of the relative standard curve method. The PMR value was calculated using the following formula[25]: $\text{PMR} = 2^{-\Delta\Delta Ct} \times 100$, where $\Delta\Delta Ct = [(C_{T(SDC2)} - C_{T(ACTB)})_{\text{sample}}] - [(C_{T(SDC2)} - C_{T(ACTB)})_{HCT116}]$. If the PMR value was >100%, it was considered to be 100%, and if methylation was not detected then the PMR value was considered to be 0%. The cutoff value of PMR for methylation positive was chosen on the basis of receiver operating characteristics (ROC) curve analysis to discriminate CRC patients from healthy normal individuals.

For each experiment, bisulfite-converted cell-line genomic DNA (Caco-2 and HCT116), bisulfite-unconverted placental

DNA, unmethylated (Qiagen) and bisulfite-treated DNA of HCT116 cells that was fully methylated by M.SssI methylase (New England Biolabs, Ipswich, MA) were used as methylation controls; nontemplate controls were also included. Given the limited amount of serum samples, qMSP was performed at one time. The amplification specificity and the limit of detection (LOD) for qMSP assay were evaluated before performance in serum DNA (Supplemental Figure S1). For PCR assay prequalification, before the study, qMSP specificity was determined by running PCR products on a 2% agarose gel. Primers demonstrated high specificity with single products of the desired length. Additionally, no bands were seen in the unconverted and the no template control lanes (Supplemental Figure S1A). *ACTB* was selected as a reference gene based on its stability in our assay, as evaluated by quantification cycle (C_T) variance, as well as the fact that our primers and probes for the *ACTB* assay did not cover any CpG sites, thus allowing unbiased amplification. The LOD was determined for *SDC2* qMSP assay by analysis of bisulfite-treated methylated (M.SssI-treated HCT116 DNA) DNA dilutions ranging from 0.0156 ng to 200 ng in triplicate (Supplemental Figure S1B). C_T calculations determined the LOD as 0.156 ng for both *ACTB* and *SDC2*. We assessed linear regression analysis output for statistical significance between the slopes, in conjunction with the magnitude of the coefficient of determination (R^2). C_T values were calculated using Rotor-Gene Q software version 2.0.2 (Qiagen). For LOD studies, C_T values were determined automatically using the same Rotor-Gene Q software. For serum testing, C_T values for each experimental set were determined using a cutoff value set manually; this value was established using unmethylated and unconverted DNA florescence levels as a baseline.

Statistical Analysis

All statistical analyses were performed using MedCalc software version 9.3.2.0 (MedCalc, Ostend, Belgium). A P value of <0.01 was considered statistically significant. The statistical significance for the trend of methylation positivity in the samples according to CRC tumor stage was assessed by χ^2 testing. The ROC curves and the area under the ROC curve (AUC) were generated to confirm the accuracy of diagnosis, as well as the sensitivity and specificity. Kruskal–Wallis test was performed to compare methylation levels and clinicopathological features.

Results

Identification of Novel DNA Methylation Candidate Genes for CRC

We performed CpG microarray analyses in conjunction with MeDIA to identify a subset of candidate genes with methylation patterns that clearly discriminate primary tumors from paired, adjacent nontumor tissues in CRC patients (Figure 1). Unsupervised hierarchical clustering of 43,097 reliable probes was performed to analyze overall methylation profiles across samples. The resulting dendrogram shows that the methylation patterns clearly segregated primary tumors from paired, adjacent nontumor tissues, indicating that our CpG microarray data are highly reliable and that the methylation patterns themselves can be used to discriminate tumor from its nontumor counterpart. The statistical analysis generated a list of 32 hypermethylated ($P < 0.01$) (Supplemental Table S1) and 41 hypomethylated genes (data not shown). Of the 32

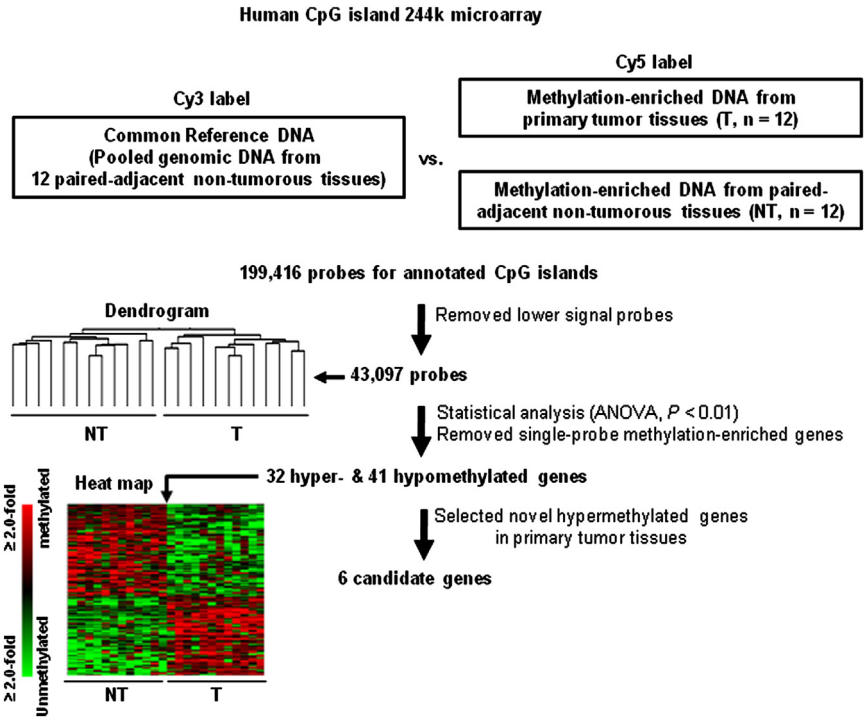

Figure 1 Stepwise filtering processes for candidate gene selection. The methylated DNA was separately enriched from 12 primary colorectal tumors (T) and paired, adjacent nontumor tissues (NT) by a MeDIA technique. The methylated DNAs (Cy5) were individually compared with amplified common reference DNA (Cy3) without methylation enrichment. The 43,097 reliable probes were selected and unsupervised clustering was performed based on Pearson's correlation. The heat map shows the methylation status of the statistically significant 32 hypermethylated and 41 hypomethylated genes. Six novel candidate genes hypermethylated in primary colorectal tumors were finally selected.

hypermethylated genes, we focused our analysis on *CHST11, KCNA1, IRX5, SIM1, SDC2,* and *SORCS3*, six genes that have not been previously reported as aberrantly methylated genes in CRC primary tumors.

Methylation Confirmation of Candidate Genes in CRC Cell Lines and Colorectal Tissues

Because most established cell lines derived from tumors have more malignant characteristics and are relatively less heterogeneous, relative to tumor tissue, we wanted first to verify the methylation status of CpG sites in the *CHST11, KCNA1, IRX5, SIM1, SDC2,* and *SORCS3* genes. Bisulfite pyrosequencing in the CRC cell lines Caco-2 and HCT116 showed that all but one of the tested genes had high levels of methylation as indicated by MtI $>$ 35% in both cell lines. The exception was *SDC2*, with MtI $>$ 35% in HCT116 cells but not in Caco-2 cells (Figure 2A).

To discover candidate genes that have potentially high specificity for CRC detection, we determined methylation levels of six genes in normal colon tissue samples from two disease-free healthy individuals (Figure 2B). The genes *CHST11*, *IRX5,* and *KCNA1* exhibited a relatively higher range of MtI $>$ 35.0% in both normal tissue samples, and these three genes were excluded from further analysis because they would not be able to meet a sufficient level of specificity when validated in a larger number of clinical samples. *SIM1* and *SORCS3* had relatively lower average methylation levels (MtI = 25.3% and MtI = 16.5%, respectively), and *SDC2* methylation had the lowest methylation levels (MtI $<$ 5.0%) in both normal samples. These three genes were therefore subjected to further verification.

To confirm that *SIM1, SORCS3,* and *SDC2* are indeed aberrantly methylated in the CRC tumor samples used in microarray analysis, we performed bisulfite pyrosequencing. Methylation target regions of all three genes exhibited a significantly higher methylation level in primary tumors, compared with paired, adjacent nontumor tissue ($P \leq 0.0011$) (Figure 2C). We defined a finding as methylation positive if the MtI of primary tumor tissue was greater than that of paired, adjacent nontumor tissue. For *SDC2*, *SIM1* and *SORCS3*, the frequency of methylation positivity was estimated as 100% (12/12), 100% (12/12), and 83% (10/12), respectively. Although each of these genes has the potential to be a methylation-specific marker for CRC tumors, we decided to pursue further validation only with *SDC2*, because this gene exhibited the most significant frequency of aberrant methylation in tumors and because it demonstrated sufficient specificity with only nominal levels of methylation in adjacent nontumor tissues.

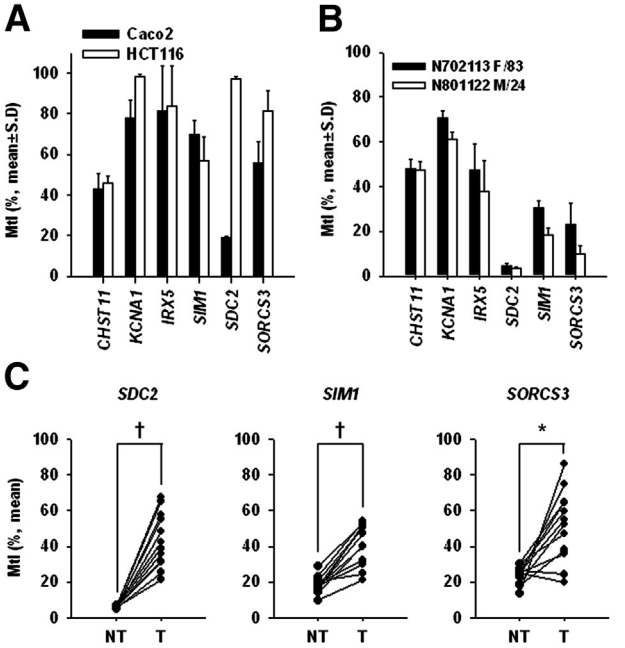

Figure 2 Quantitative bisulfite pyrosequencing assay of six candidate genes in cell lines and normal tissues from healthy individuals. The methylation levels were calculated for all examined CpGs in target regions. **A:** MtI values for each gene were plotted with results from pyrosequencing in two CRC cell lines, Caco-2 and HCT116. **B:** The methylation levels of the six genes were plotted in healthy normal tissues from an 83-year-old woman and a 24-year-old man. **C:** Methylation status of three of the genes was evaluated in primary tumors (T) and paired, adjacent nontumor tissues (NT) from 12 CRC patients from the initial CpG microarray analysis. MtI values were plotted from pyrosequencing results. Samples from the same patient are linked with a straight line. $^{*}P = 0.0011$; $^{\dagger}P < 0.0001$, Paired *t*-test.

Clinical Validation of Methylation Biomarker *SDC2* in CRC Tissues

To refine the best methylation target within CpG islands of the *SDC2* gene, we investigated the methylation status of CpG islands in the 5′ upstream promoter and the 5′ UTR (+1 to +618 bp). In addition to R3 (5′ UTR, +371 to +598 bp), the region used during candidate selection, two additional regions were selected for bisulfite pyrosequencing: R1 (−229 to −91 bp) and R2 (+299 to +399 bp) within the promoter and the 5′ UTR regions (Figure 3A). We examined the methylation status of these three regions in 32 independent, match-paired colorectal tumor tissues at different stages (stage I, $n = 5$; stage II, $n = 12$; stage III, $n = 11$; and stage IV, $n = 4$) (Figure 3B). Overall mean MtI values (± SD) for R1, R2, and R3 were estimated as 32.7 ± 17.4%, 22.6 ± 12.9%, and 47.9 ± 20.3%, respectively, in primary tumors ($P < 0.0001$) and as 9.3 ± 4.1%, 5.7 ± 2.6%, and 4.3 ± 4.0%, respectively, in adjacent nontumor tissues. Furthermore, the frequencies of methylation positivity were observed in 94% (30/32), 100% (32/32), and 100% (32/32) for regions R1, R2, and R3, respectively (Table 3). Taken together these results indicate that R3, which is in the 5′ UTR of the *SDC2* gene, has the greatest potential as a methylation target for the detection of CRC, because methylation levels of *SDC2* were shown to be the lowest in nontumor tissues and the highest in tumors ($P < 0.0001$).

To further confirm the differential levels of *SDC2* methylation in CRC compared with normal mucosa, we tested

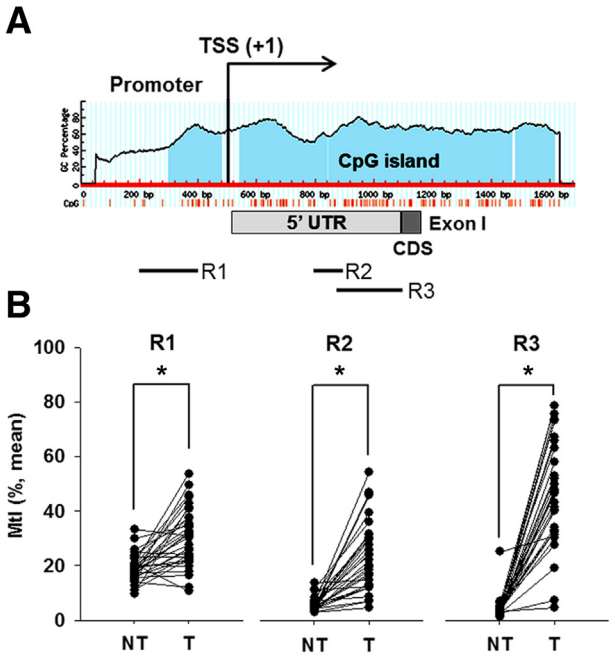

Figure 3 Refinement of the best methylation target and clinical verification of *SDC2* in match-paired colorectal tissues from CRC patients. **A:** Three pyrosequencing target regions were designed for CpG islands of the *SDC2* gene: R1 for promoter and R2 and R3 for the 5′ UTR. The transcription start site (TSS), 5′ UTR, and coding sequence (CDS) are indicated as such, and the three bisulfite-PCR target regions are indicated by horizontal bars. **B:** MtI values were plotted for 32 primary tumors (T) and paired, adjacent nontumor tissue samples (NT); samples from the same patient are linked with a straight line. *$P < 0.0001$, paired *t*-test.

SDC2 methylation status in primary tumors and in paired, adjacent nontumor tissue samples from 95 independent CRC patients. Region R3 of *SDC2* had significantly higher levels of methylation in tumor samples ($P < 0.0001$), compared with adjacent nontumor tissues. Importantly, *SDC2* methylation positivity was observed in 96% (22/23), 95% (19/20), 100% (38/38), and 93% (13/14) of stage I, II, III, and IV tissue samples, respectively (Figure 4). Additionally, there was no significant relationship between *SDC2* methylation and several clinicopathological features, including sex, age, survival, tumor stage, or recurrence (Table 4). *SDC2* exhibited a high methylation positivity (95.7%) in stage I, indicating that *SDC2* is suitable for early detection of CRC.

Table 3 Methylation Status of Three Regions of the *SDC2* Gene in 32 Paired Colorectal Tissues

	Methylation index (%)*		
SDC2	Paired-adjacent non-tumor tissue	Primary tumor tissue	Methylation positivity† [*n*/*N* (%)]
R1	9.3 ± 4.1	32.7 ± 17.4	30/32 (94)
R2	5.7 ± 2.6	22.6 ± 12.9	32/32 (100)
R3‡	4.3 ± 4.0	47.9 ± 20.3	32/32 (100)

*Average methylation level of two to four CpG sites in each assay. Data are expressed as mean MtI ± SD.

†Methylation positivity was defined as $MtI_{tumor} > MtI_{normal}$, where tumor is the primary tumor and normal is the adjacent-paired nontumor tissue.

‡The overall methylation level of R3 was significantly higher than that of R1 and R2 ($P < 0.0001$).

Diagnostic Feasibility Testing of *SDC2* for the Early Detection of CRC Using a Small Volume of Serum

For the feasibility test (whether qMSP with methylation-specific hydrolysis probe to *SDC2* methylation can sufficiently distinguish CRC patients from healthy subjects), we optimized qPCR conditions to measure methylated *SDC2* DNAs in serum. The primer set for qMSP was designed to cover CpG targets (121 bp; +378 to +498 bp) containing sequence associated with the region R3 tested in the pyrosequencing. We evaluated detection sensitivity by adjusting thermal cycling conditions (Supplemental Figure S1). In this PCR condition, we could not observe a measurable level of *SDC2* methylation in leukocyte DNA, indicating that any positive signal for *SDC2* methylation detected in the serum DNA test would not have originated from blood cells (data not shown). Because of limited serum volumes available for the test, we used only a small volume (1/3) of DNA extracted from 0.8 mL of serum DNA to determine methylation status of *SDC2* from independent groups of healthy control subjects ($n = 125$) and CRC patients ($n = 131$). PMR values for *SDC2* methylation were significantly elevated in CRC patients at all stages, compared with healthy control subjects ($P < 0.0001$) (Figure 5A).

To evaluate the diagnostic performance of methylated *SDC2* DNA in CRC detection using serum samples, an ROC curve was constructed by optimizing sensitivity and specificity (Figure 5B). Overall sensitivity and specificity were estimated as 87.0% (114/131; 95% CI, 80.0% to 92.3%) and 95.2% (6/125; 95% CI, 89.8% to 98.2%), respectively, using an optimal cutoff value at 0.936 of PMR. The AUC was determined at 0.927 (95% CI, 0.887 to 0.955; $P = 0.0001$) with high accuracy. Sensitivities for individual stages were calculated as 92% (24/26), 82% (47/57), 89% (32/36), and 92% (11/12) for stage I, II, III, and IV, respectively. Furthermore, statistical sensitivities ($P = 0.993$) and PMR values ($P = 0.850$) did not correlate with clinical stages of CRC. We also did not observe a significant difference in methylation positivity between Korean and European-origin patients ($P = 0.422$).

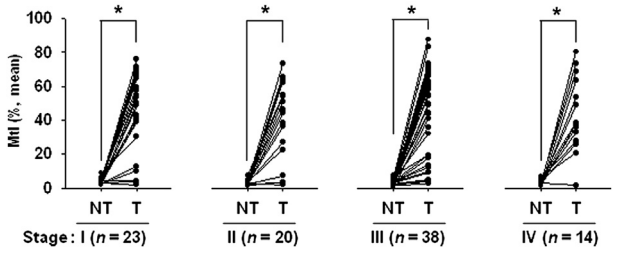

Figure 4 Assessment of methylation status for the *SDC2* gene in 95 match-paired CRC tissues by bisulfite pyrosequencing. MtI values were plotted for primary tumor tissue (T) and paired, adjacent nontumor tissues (NT); samples from the same patient are linked with a straight line. *$P < 0.0001$, paired *t*-test.

Table 4 The Relationship between Clinicopathological Parameters and MtI for the *SDC2* Gene

Features	Methylation index (%)*
Sex	
Male ($n = 56$)	42.8 ± 27.4
Female ($n = 39$)	45.3 ± 19.2
P value†	0.976
Age, years	
≤60 ($n = 42$)	45.1 ± 24.5
>60 ($n = 53$)	42.7 ± 24.3
P value†	0.481
Survival‡	
Survival ($n = 73$)	43.2 ± 23.9
Death ($n = 22$)	45.6 ± 26.2
P value†	0.798
TNM stage	
I, II ($n = 43$)	42.3 ± 24.1
III, IV ($n = 52$)	45.0 ± 24.7
P value†	0.590
Recurrence	
Yes ($n = 18$)	43.2 ± 23.6
No ($n = 77$)	43.9 ± 27.8
P value†	0.835

*Average methylation level of each CpG site. Data are expressed as mean MtI ± SD.

†Kruskal–Wallis test.

‡5-year survival and CRC-specific death.

Discussion

Although CRC screening has been found to be effective in reducing CRC-related deaths and costs,[18] detection of early-stage and precancerous lesions is hampered by poor performance of noninvasive screening tests.[26] Here, we have shown that aberrant methylation of *SDC2* can be significantly detected in early-stage CRC in both tissue and serum. Genome-scale discovery of molecular diagnostic markers often faces the challenge of false-positive selection of candidates evaluated in independent clinical samples. In the present study, in order to discover specific aberrant methylation sites in primary CRC tumor tissue, we performed a systematic CpG methylation microarray analysis coupled with enriched methylated DNA by MeDIA.[21] In comprehensive methylation profiling analysis, we confirmed that methylation patterns of primary tumor tissues from 12 patients with CRC at stages I through IV were correctly distinguished from those of corresponding nontumor tissues using unsupervised hierarchical clustering with all CpG probes with reliable signals (Figure 1); this finding indicates that subsequent statistical analysis would be reliable. For discovery of aberrantly methylated genes in tumor cells of CRC patients, we designed a study to indirectly compare methylated DNA pattern of primary tumors to nontumors through a common reference DNA consisting of pooled nontumor genomic DNA. We applied stringent filtering criteria at each step of candidate selection and emphasized specificity. Thus, only three genes (*SDC2*, *SIM1*, and *SORCS3*) were passed, because they exhibited less methylation in normal mucosa samples derived from disease-free individuals (Figure 2B) but significantly higher aberrant methylation across primary tumor tissues, compared with nontumor tissues (Figure 2C). Although each of these three genes was considered a reliable and robust methylation biomarker for CRC detection, we decided to continue only with *SDC2* in clinical validation studies with serum DNA, because of restricted availability of serum samples.

The syndecan-2 (SDC2) protein functions as an integral membrane protein and is known to participate in cell proliferation, cell migration, and cell–matrix interactions via its receptor for extracellular matrix proteins.[27,28] The *SDC2* gene is expressed in mesenchymal cells, but not in epithelial cells of normal colon tissues,[29] and the expression level of *SDC2* is moderate or strong in normal epithelial cells of pancreatic tissues.[30] Investigating epigenetic influence on expression of *SDC2* gene in specific cell types of tumor and microenvironment tissues during development of CRC would be very interesting, but is beyond the scope of the present study.

For the clinical validation of *SDC2* methylation status in independent set of primary tumors from 95 patients with CRC at stages I through IV, we used pyrosequencing, which comprises an in-built measurement for the completeness of bisulfite conversion and thus allows not only

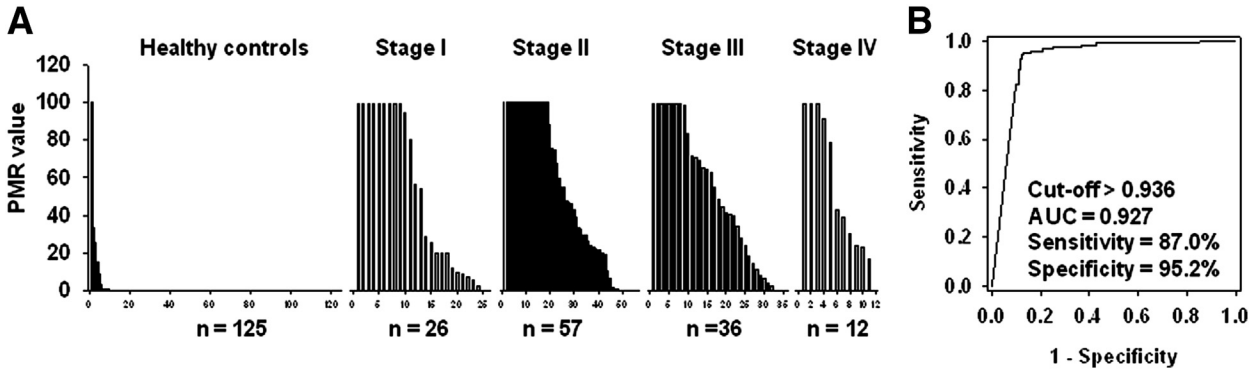

Figure 5 Methylation test results of *SDC2* marker for detecting CRC in serum DNA. **A**: Methylation status of *SDC2* gene is plotted as PMR values. **B**: ROC curves for *SDC2* gene for detecting CRC using serum DNA are depicted. AUC = 0.927 is significant at $P = 0.0001$.

precise evaluation of methylation but also estimation of the percentage of methylated allele for each CpG dinucleotide of interest. Across all stages, most patients exhibited higher aberrant levels and greater frequency of methylation of *SDC2* in tumor than in nontumor tissues. In addition, no significant correlation was found between the aberrant methylation of *SDC2* in primary tumors and any clinicopathological characteristics of CRC patients in the present study (Table 1). Both in tumor tissue harboring abnormal gene methylations and in normal tissue, cells can undergo apoptosis or necrosis that results in shedding of fragmented genomic DNA sequences into the bloodstream.[31,32] As a consequence, methylated target DNA of interest may be present at very low levels within a high background of unmethylated corresponding DNA.

For a feasibility test, determining whether *SDC2* methylation can be measured in a small volume of serum DNA of a CRC patient, we established fluorescence hydrolysis probe−based qMSP conditions[23] that meet the required analytic sensitivity for methylation assay in circulation cell-free DNA in blood.[33] Aberrant *SDC2* methylation was detected at high frequency in the serum of patients across CRC stages at high frequency, whereas it was rare in serum of healthy subjects, by one reaction of qMSP. Overall sensitivity was calculated at 87.0% [92.3% for stage I ($n = 26$), 82.5% for stage II ($n = 57$), 88.9% for stage III ($n = 36$), and 91.7% for stages IV ($n = 12$)], at a specificity of 95.2%. Notably, the sensitivity of *SDC2* for stage I CRC was as high as 92.3%, suggesting that *SDC2* methylation is a promising biomarker for the early detection of CRC. If it is true that prevalence of CRC in the screening population of subjects age 50 years or older is 0.5%,[34] then the serum DNA test for *SDC2* methylation in present study has a low positive predictive value (PPV = 8.3%), but a high negative predictive value (NPV = 99.9%). Normal serum samples for the control used in the present study were derived from apparently healthy individuals without any history of malignancy; however, these individuals did not undergo colonoscopy screening at the time of blood drawing. Specificity might be improved if the CRC-free status of healthy control subjects is confirmed by colonoscopy.

Other candidate genes have been reported as methylation markers for CRC detection.[18−20,26,35,36] Recently, Lange et al[20] identified two novel methylation biomarkers, *THBD* and *C9orf50* through a genome-wide discovery and verification study for blood-based detection (1 mL plasma) of CRC, with sensitivity of 71.0% and specificity of 80.0%. Among these various candidate genes, aberrant methylation has been most intensively studied in the septin 9 gene (*SEPT9*). The PRESEPT study, a multinational prospective screening study of nearly 8000 subjects, confirmed the utility of *SEPT9* as a biomarker for detecting CRC by plasma DNA test during routine screening, with a detection rate of 68.0% to 72.0% and a specificity of approximately 90.0%.[18] Our present findings for *SDC2* are highly similar to those for *SEPT9* in detection of CRC in both tissue and blood. More recently, after modification of qMSP testing conditions, results for *SEPT9* assay showed improved overall sensitivity (90.0%), with a specificity of 88%.[16] In that study, however, duplex PCR was used for methylation testing and each PCR reaction was run in triplicate, using 4.0 mL plasma samples. In the present study, we ran only a single reaction of qMSP, using a small volume (1/3 of 0.8 mL) of serum. It may be possible to improve the sensitivity of the *SDC2* methylation test if it is performed with multiple reactions of qMSP and in a larger volume of serum from each patient. Importantly, the high frequency *SDC2* methylation observed in patients at CRC stage I was similar to that of patients with later-stage disease.

In conclusion, we have successfully identified *SDC2* as a novel, sensitive, and specific marker for the remote detection of early-stage CRC in a small volume of serum. Although fecal occult blood testing is a valuable noninvasive screening method, it has limited sensitivity. Colonoscopy is a gold standard with high sensitivity and specificity for detection of CRC and large adenomas, but it requires bowel preparation that patients apparently find uncomfortable.[37] Thus, a noninvasive molecular diagnostic tool such as a DNA methylation assay in circulating cell-free DNA may contribute to development of a test to be used as an alternative to or in conjunction with colonoscopy. To this end, further study with larger sample sizes would be needed to validate *SDC2* marker for population-based screening of CRC. To extend the use of the methylation marker to preselection of asymptomatic individuals with precancerous lesions, the status of *SDC2* methylation needs to be further assessed in various polyps, including adenoma. Furthermore, because hypermethylation of *SDC2* has never been addressed in any type of cancer, to determine whether this methylation event is specific to CRC or is universal among cancers will require investigation of the methylation status of *SDC2* in other cancers. Finally, because *SDC2* methylation in serum is frequently detected across all CRC stages, this approach may also be useful for monitoring CRC.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2013.03.004*.

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