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Screening for Epigenetically Masked Genes in Colorectal Cancer Using 5-Aza-2'-deoxycytidine, Microarray and Gene Expression Profile

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Abstract. *Aim: Unearthing of silenced genes in colorectal cancer (CRC). Materials and Methods: Oligonucleotide microarray was used in order to find changes in gene expression in five CRC cell lines before and after 5-aza-2'-Deoxycitidine treatment. Up-regulated genes were integrated with expression profile of matched colorectal tissue samples. Methylation-specific polymerase chain reaction and Real-time quantitative reverse transcription polymerase chain reaction were used to further analyze candidates using 15 CRC cell lines and 23 paired samples. Results: After applying study selection criteria for 68 genes obtained from integrated arrays, we identified 16 genes; apoptosis-stimulating of p53 protein 1(ASPP1) and Scavenger receptor class A, member 5 (SCARA5) were selected for further analysis. Methylation was only identified for SCARA5 in 20% of the cell lines and in 17% of tumor the samples. Down expression of SCARA5 was observed in CRC cell lines and in tumor samples compared to normal (p<0.001 and p=0.001, respectively). Conclusion: Genome-wide screening identifies genes potentially affected by methylation in CRC. SCARA5 may have a role in tumorigenesis in CRC.*

Colorectal cancer (CRC) is the third most common type of cancer in males and the second in females, with new cancer cases worldwide exceeding 1.2 million and estimated deaths exceeded 600,000 in 2008 (1). The diagnosis and treatment of CRC has significantly improved during recent decades, leading to a substantial reduction in cancer-related mortality (2). In spite of this, CRC continues to be a major public health problem worldwide. This can be attributed, in part, to the lack of a complete understanding of the biological mechanisms behind carcinogenesis in CRC.

Genes, including tumor suppressors, can be inactivated by mutations, as well as by methylation of promoter CpG islands; both mechanisms play a crucial role in tumor progression. The transcriptional inactivation of genes through promoter methylation is a well documented mechanism in several human malignancies, including CRC (3). Epigenetic masking can affect gene expression, causing selective growth advantages that might participate in the cancerous transformation of colorectal epithelium (3).

Treatment of cancer cell lines with 5-Aza-2'-deoxycitidine (5-aza-dC) was shown to cause re-expression of epigenetically silenced genes in different types of cancer, in a dose- and duration-dependant manner, when investigated by expression microarray analysis (4). This represents one of the approaches used in genome-wide identification of cancer-associated methylated genes (5).

High-throughput profiling of genes affected by methylation can provide potential markers for early detection of CRC and prediction of prognosis and response to therapy (6). However, despite the fact that hundreds of genes have been found to be inactivated by methylation in CRC (5), the path of epigenetic alterations in CRC has still not yet been fully explored. To improve our understanding of carcinogenesis in CRC, further searches for methylation-silenced genes with critical role in cancer biology are required (5).

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In the current study, we carried out pharmacological unmasking in five CRC cell lines treated with 5-aza-dC followed by oligonucleotide microarray analysis to identify epigenetically silenced genes in CRC with putative tumor suppressor activity.

Materials and Methods

Clinical samples. Samples from 40 patients who underwent surgical treatment for CRC between 2005 and 2007 at Tokyo Medical and Dental University Hospital were included in this study. All macroscopically resected specimens were stored at −80˚C until use. The Institutional Review Board approved the study, and written informed consent was obtained from all patients. A total of 17 CRC patient matched samples (normal and tumor) were assigned to the microarray study. As for methylation analysis, matched samples from 23 CRC patients were used, with a male-to-female ratio of 1.3:1, with a mean age of 66.3±10.9 years (median=67.0 years; range=38 to 81 years). The number of cases for each stage was six, six, five and six for stage I, II, III and IV, respectively. Of these, 14 were assigned to the gene expression study. The samples used in the microarray analysis were excluded from additional validation studies.

Cell lines and 5-aza-dC treatment. Fifteen CRC cell lines (CCK81, CoCM1, Colo205, Colo320, ColoTC, DLD1, HCT116, HCT15, HT29, LoVo, RCM1, RKO, SW48, SW480 and WiDr) were used in this study. Cell lines were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan) and the American Type Culture Collection (Manassas, VA, USA). Cells were cultured under conditions described by the providers, with appropriate media obtained from Gibco (Grand Island, NY, USA) or Sigma (St Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Nichirei, Tokyo, Japan), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

Five CRC cell lines (Colo320, HCT116, HT29, RKO and SW480) were treated with 0.5 μM 5-aza-dC (Sigma) in triplicate as described previously (7). The dose of 5-aza-dC was administered on the basis of its pharmacological dose and our preliminary experiments (7). Total RNA and genomic DNA (gDNA) were separately isolated by using QIAshreder with RNeasy minikit (Qiagen, Hilden, Germany) and the phenol/chloroform method, respectively.

Oligonucleotide microarray analysis. RNA from cell lines was extracted before and after treatment with 5-aza-dC. Matched samples of primary cancer and adjacent normal tissues were obtained from 17 patients with CRC, and total RNA was extracted from each using RNeasy minikit (Qiagen). The integrity of the total RNA obtained was assessed by an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples had an RNA integrity number of at least 5.0 prior to gene expression analysis. Contaminant DNA was removed by digestion with RNase-free DNase (Qiagen, Hilden, Germany). Complementary RNA was prepared from cell lines (tissue samples) using 2 μg (100 ng) of total RNA by one-cycle (two-cycle) target labeling and a control reagents kit (Affymetrix, Santa Clara, CA, USA).

Hybridization and signal detection of the Human Genome (HG) U133 Plus 2.0 arrays (Affymetrix) were performed according to the manufacturer's protocol.

The gene expression data sets were submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE32323). To investigate the difference in expression patterns, the raw gene expression data were derived from each probe signal intensity (CEL file) using the model-based robust multi-array average method (RMA) algorithm. RMA performs normalization and background correction (8), as implemented in the Affymetrix Expression Console™ Software (Version 1.1). Expression levels were log2-transformed, and 62 control probe sets were removed from subsequent analyses. For each of the 54,613 probes on the HG-U133 Plus 2.0 array, fold-change (FC) values were calculated using R 2.11.1 statistical software together with a Bioconductor package (R Foundation for Statistical Computing, Vienna, Austria) as previously described (9).

Probe sets from cell lines were selected using the combination of the following criteria: i) up-regulation of gene expression in at least four CRC cell lines, and ii) FC >1.6 in at least one cell line. For the paired clinical samples, the false discovery rate (FDR) was used to correct multiple comparisons with microarray data analysis (10). FDR <0.05% (equivalent to p<0.000016 in this analysis) was used as the selection criteria with FC of normal compared to tumor (N/T) >1.6 (*i.e.* higher expression in normal tissue than tumor).

DNA extraction and methylation-specific polymerase chain reaction (MSP). Extraction of gDNA was carried out as described previously (11). DNA concentrations and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Bisulfite treatment was performed using EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The methylation status of the apoptosis-stimulating of p53 protein 1 (ASPP1), cyclin-dependent kinase inhibitor 2A (*CDKN2A/p14^{ARF}*), cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16^{INK4A}*), protein-tyrosine phosphatase receptor type O gene (*PTPRO*) and scavenger receptor class A, member 5 (*SCARA5*) genes was determined by MSP with 1 μl of bisulfite-treated DNA as template and AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) for amplification. All reactions were performed in duplicate. EpiTect control DNA (human), methylated (unmethylated) and bisulfite-converted gDNA (Qiagen, Hilden, Germany) was used as a positive control for the methylated (unmethylated) experiments. Methylated and unmethylated primer sequences (Life Technologies Inc., Rockville, MD, USA), were based on previous reports (12, 13, 7, 14). The primer sets were tested with non-bisulfite-treated DNA as a template to eliminate the possibility of PCR product amplification from unconverted DNA. None of the sets amplified non-bisulfite-treated gDNA (data not shown).

The PCR products were loaded onto a 2.0% agarose gel, stained with 0.5 μg/ml ethidium bromide and visualized under ultraviolet illumination. *PTPRO* gene was previously reported as being frequently methylated in right-sided tumors of the colon (14) and was included as a positive control for target gene methylation analysis (*PTPRO* had an increase in expression of >1.3 FC in all 5-aza-dC-treated CRC cell lines in our analyses).

Real-time quantitative RT-PCR (RTQ-PCR). Gene expression was quantified in duplicate as previously described (15). TaqMan gene expression assays (Applied Bioystems: SCARA5, Hs01073151_m1 and β-actin, Hs99999903_m1) were used to determine the expression of *SCARA5*. β-Actin was used as an internal control. Each assay was performed in 20 μl including 1 μl of cDNA. Relative gene expression data were obtained using the comparative ΔΔCt method.

Statistical analysis. Statistical analyses of gene methylation and expression data were performed with the SPSS software (Version 16.0, SPSS Inc, Chicago, IL, USA) for Windows. To estimate the differences between groups, the Chi-square test, Fisher's exact test, Mann-Whitney *U*-test and Wilcoxon signed ranks test were used where appropriate. Differences were considered statistically significant when $p<0.05$.

Results

p14^{ARF} and p16^{INK4a} genes were efficiently demethylated in 5-aza-dC-treated CRC cell lines. In order to determine whether the dose of 5-aza-dC efficiently demethylated the genes silenced by promoter methylation in CRC cell lines, assay of the methylation status of two control genes, *p14^{ARF}* and *p16^{INK4A}*, was carried out on treated and untreated CRC cell lines using MSP. Both genes were known to be affected by methylation in CRC cell lines (16). MSP analysis revealed a partial demethylating effect (detection of weak or no methylated band associated with increase in intensity of the unmethylated band) for *p14^{ARF}* in the fully methylated cell line RKO. Complete demethylation was observed for *p16^{INK4A}* gene in the HCT116 cell line. RKO, HT29 and Colo320 cell lines exhibited partial demethylation for *p16^{INK4A}* gene after treatment with 5-aza-dC (Figure 1A). These results confirm the efficient demethylation of CpG-dinucleotides in the treated CRC cell lines. Of note, the disappearance of an unmethylated band of *p14^{ARF}* gene in the HCT116 cell line after 5-aza-dC treatment could be part of the re-silencing phenomenon of the activated genes (17).

Screening for epigenetically regulated genes in CRC revealed 16 candidate genes. To identify genes silenced by methylation in CRC cell lines, we first treated five CRC cell lines (Colo320, HCT116, HT29, RKO and SW480) with 5-aza-dC. Microarray analysis, comparing the mock- and 5-aza-dC-treated cell lines, was then carried out to identify the reactivated genes (methodology outline summarized in Figure 2). After comparing the resultant gene expression profiles, 2808 genes (3283 probe sets, 6% of the total transcripts analyzed) had an increase in signal intensities after 5-aza-dC treatment. Of note, this group of up-regulated transcripts includes genes with known tumor suppressor activity identified through the Tumor-Associated Genes (TAG) database (18; URL:www.binfo.ncku.edu.tw/TAG/GeneDoc.php.). Some of these tumor suppressors were previously correlated and linked to CRC, such as cyclin-dependent kinase inhibitor 1A (*CDKN1A*), cyclin-dependent kinase inhibitor 1C (*CDKN1C*), neurofibromin 1 (*NF1*), protein phosphatase 2, regulatory subunit A, beta (*PPP2R1B*), serpin peptidase inhibitor, clade B, member 5 (*SERPINB5*) and SMAD family member 3 (*SMAD3*). We also found several genes of cancer-germline antigen families (*e.g. GAGE, MAGE* and *PAGE*); this was consistent with previous reports regarding genes induced by 5-aza-dC (19). Genes with methylation of the imprint control region, such as mesoderm specific transcript homolog (*MEST*) and paternally expressed 3 (*PEG3*) were also identified using the Imprinted Gene Catalogue (20; URL: www.otago.ac.nz/IGC). Moreover, gene transcripts known to be methylated and silenced in CRC and other types of cancer were detected including *PTPRO*, msh homeobox 1 (*MSX1*), suppressor of cytokine signaling 1 (*SOCS1*), Kruppel-like factor 4 (*KLF4*), and TIMP metallopeptidase inhibitor 3 (*TIMP3*) (3, 14, 21).

Functional analysis of the 2808 genes by the DAVID bioinformatics resource (22; http://david.niaid.nih.gov) disclosed the significant enrichment (FDR <0.05%) of genes expressed in colorectal adenocarcinoma (518 genes) and of genes involved in extracellular matrix (36 genes).

In order to identify genes specifically up-regulated in normal colorectal mucosa compared to primary CRC, we carried out gene expression profiling using array data obtained from gene expression changes in normal colonic mucosae *versus* their tumor counterpart for samples from 17 CRC patients. This identified 433 genes (533 probe sets, 0.9% of the total transcripts analyzed) that were up-regulated in normal tissues compared to primary cancer (FDR <0.05% and FC of N/T >1.6). The expression profiling array data were validated in clinical samples by previous studies (23, 24).

Sixty-eight genes (representing 75 probes) were determined by combining the cell lines and profiling array data sets. These genes were both responsive to 5-aza-dC treatment (the largest FC ranging between 1.6 and 5.5) and overexpressed in normal tissues (*i.e.* underexpressed in tumor tissues). Each probe set was matched to a single known human gene. The 68 genes were screened for having potential CpG islands by UCSC Table Browser (25) using the current genome assembly (GRCh37/hg19), in addition to CpG island searcher (26). Moreover, we examined the published literature for additional analysis of the enlisted genes.

In order to identify epigenetically affected genes with putative tumor suppressor activity, we first excluded genes with chromosomal location on X-chromosome (two genes). Oncogenes and genes with potential oncogenic activity (15 genes), along with genes with metastatic activity and genes with potential metastatic activity (3 genes), were also eliminated. In addition, we removed genes not affected by methylation in CRC (1 gene), genes with no relevant function in biological mechanisms of tumor suppression (26 genes), and lastly, genes reported previously to be methylated in CRC (5 genes). After exclusion of 52 genes, we obtained a final list of 16 genes with known or putative tumor suppressor function (Table I, Figure 3); these genes have not been reported to be epigenetically silenced in CRC.

Two genes, *ASPP1* and *SCARA5*, were selected for further analysis; both have been reported to be silenced by methylation in cancer types other than CRC (12, 13). In addition, investigation of the gene expression patterns between paired tumor and adjacent normal tissues for our

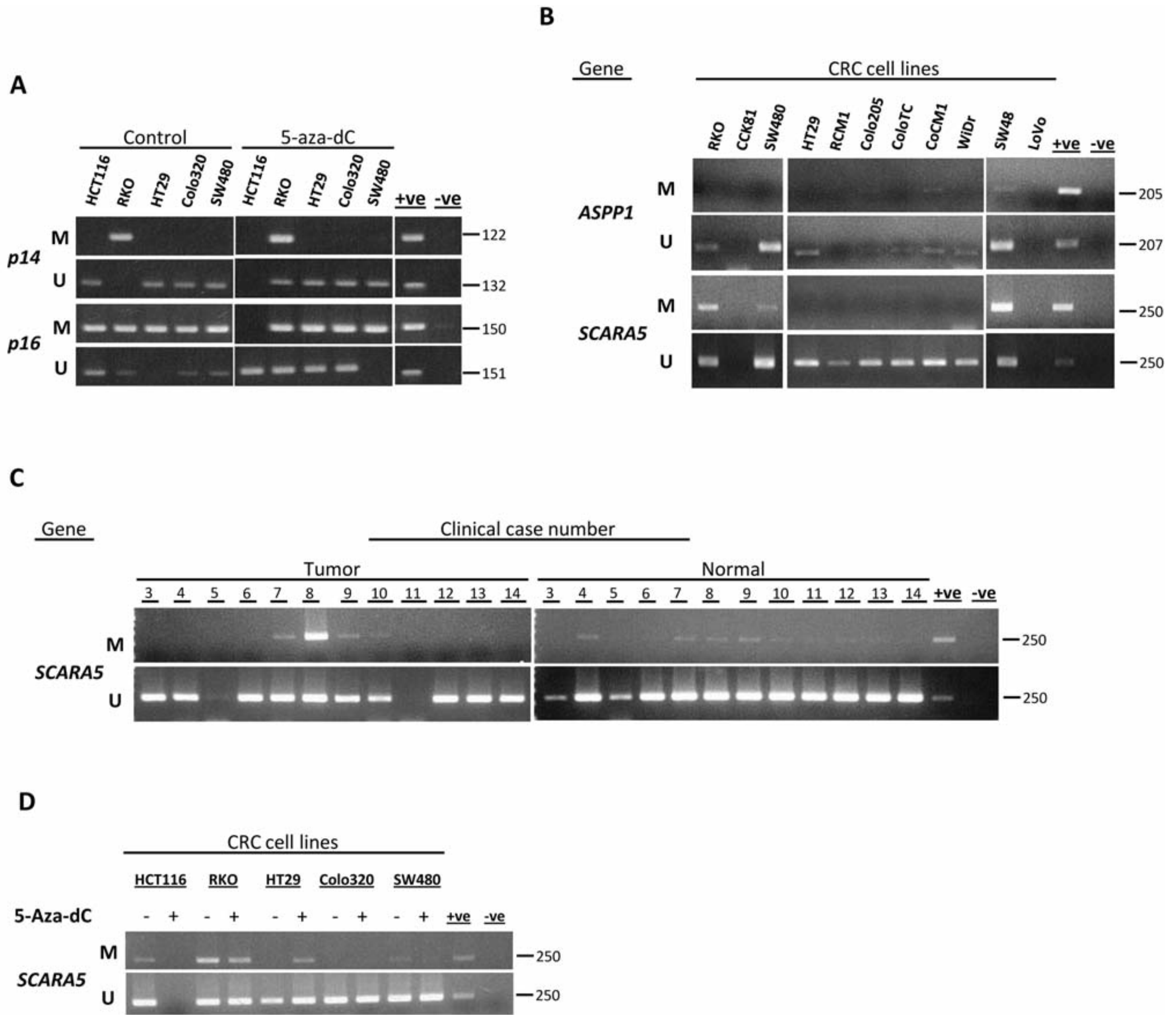

Figure 1. *Methylation specific polymerase chain reaction (MSP) analysis results. Methylation status of p14*^{ARF}*, p16*^{INK4A} *(A) and SCARA5 (D) in the five CRC cell lines before and after 5-aza-2' deoxycytidine (5-aza-dC) treatment, showing promoter demethylation after treatment. Representative MSP results in 15 CRC cell lines (B) and 23 pairs of primary colorectal tumor (Tumor) and normal epithelium (Normal) (C). M and U denote the presence of PCR product using primers specific for methylated and unmethylated sequences, respectively. Fully methylated and unmethylated human genomic DNA (HCT15 cell line and genomic DNA from whole blood in (A)) were used as positive controls (+ve) for methylated and unmethylated DNA, respectively. Reactions that contained no template DNA were used as a negative control (–ve). The expected size of the amplicon in base pairs is indicated on the right side of the panels.*

candidate genes revealed significant underexpression ($p<0.001$ by Wilcoxon signed rank test, FDR <0.05%) for *ASPP1* and *SCARA5* (Figure 3).

SCARA5 exhibited methylation in CRC cell lines and tumor samples of CRC patients. To identify genes affected by aberrant promoter methylation in CRC, we performed methylation analysis using MSP to determine the methylation status of the candidate genes in 15 CRC cell lines. The methylation incidences are shown in Table II. Figure 1B and C show representative examples of the MSP analysis.

ASPP1 exhibited no methylation in CRC cell lines and was excluded from further analysis. Six cell lines exhibited an unmethylated band of *ASPP1*. The methylation analysis for *SCARA5* gene showed methylation (either biallelic or monoallelic) in cell lines (20%).

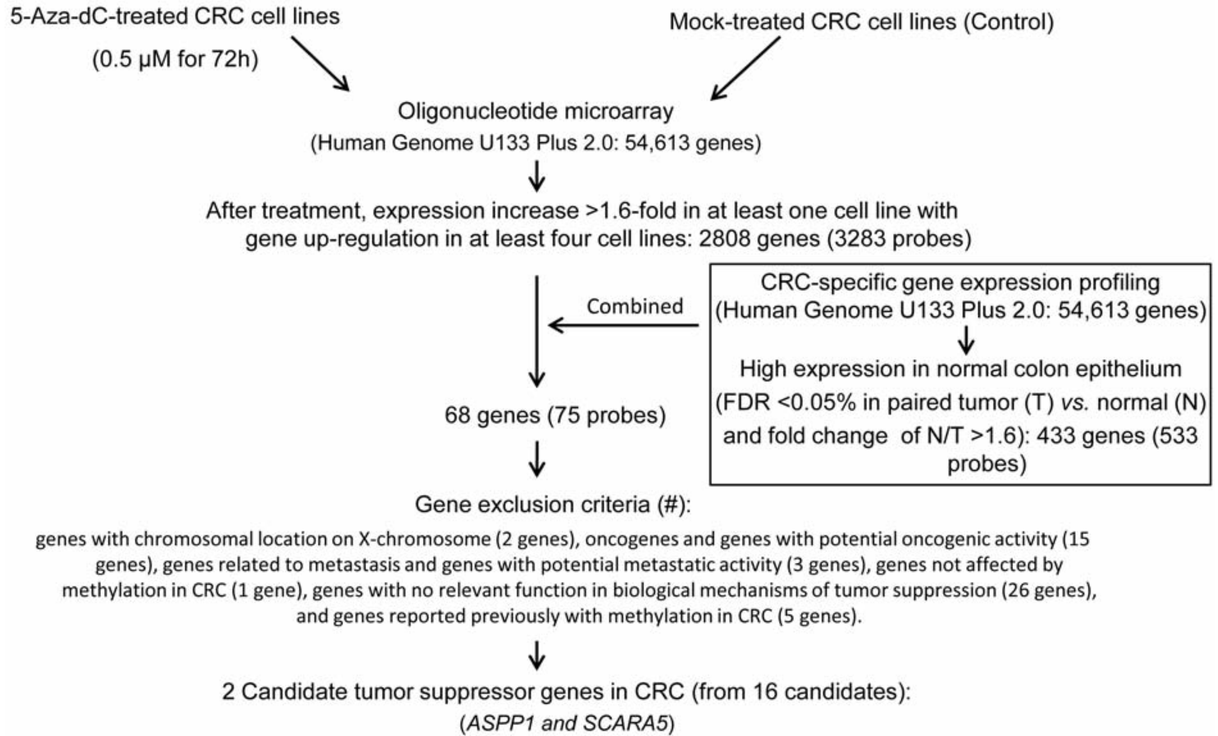

Figure 2. *Outline of selection of candidate genes in CRC.*

Table I. *Candidate genes in colorectal cancer. The table shows 16 genes up-regulated in 5-aza-dC treated CRC cell lines compared to mock-treated cell lines by oligonucleotide microarray (fold change, FC, >1.6 in at least one cell line and up-regulated in at least four CRC cell lines) with higher expression in normal (N) tissues compared to tumor (T) tissues identified by CRC-specific gene expression profiling (false discovery rate <0.05% and FC of N/T >1.6). Gene symbols in bold indicate genes examined in this study.*

Gene symbol	Probes	Gene name	Locus	Function
ASPP1 (PPP1R13B)	216347_s_at	Apoptosis-stimulating protein of p53 (protein phosphatase 1, regulatory (inhibitor) subunit 13B)	14q32.33	Protein binding, potent activator of p53
C17ORF91	214696_at	Chromosome 17 open reading frame 91	17p13.3	Unknown, regulating miR-22 expression
CASZ1	220015_at	Castor zinc finger 1	1p36.22	Probable transcription factor
CLEC3B	205200_at	C-Type lectin domain family 3, member B	3p22-p21.3	Protein binding
DUSP5	209457_at	Dual specificity phosphatase 5	10q25	Regulation of the MAP kinase superfamily
FOXO1	202723_s_at	Forkhead box O1	13q14.1	Transcription factor
GAS2L1	31874_at	Growth arrest-specific 2 like 1	22q12.2	Cross-linking of microtubules and microfilaments
KLF9	203542_s_at	Kruppel-like factor 9	9q13	Transcription factor
NKX2-3	1553808_a_at	NK2 homeobox 3	10q24.2	Transcription factor
PPP1R15A	37028_at	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	19q13.2	May promote apoptosis
SCARA5	229839_at	Scavenger receptor class A, member 5	8p21	Protein binding, scavenger receptor activity
SEMA3B	203071_at	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	3p21.3	Inhibition of axonal extension
SESN2	223196_s_at	Sestrin 2	1p35.3	Regulation of cell growth and survival
SMPD3	219695_at	Sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	16q22.1	Regulation of the cell cycle
WDR37	211383_s_at	WD repeat domain 37	10p15.3	Signal transduction, apoptosis, and gene regulation
ZFP36	201531_at	Zinc finger protein 36, C3H type, homolog (mouse)	19q13.1	Probable regulatory protein

MAP, Mitogen-activated protein.

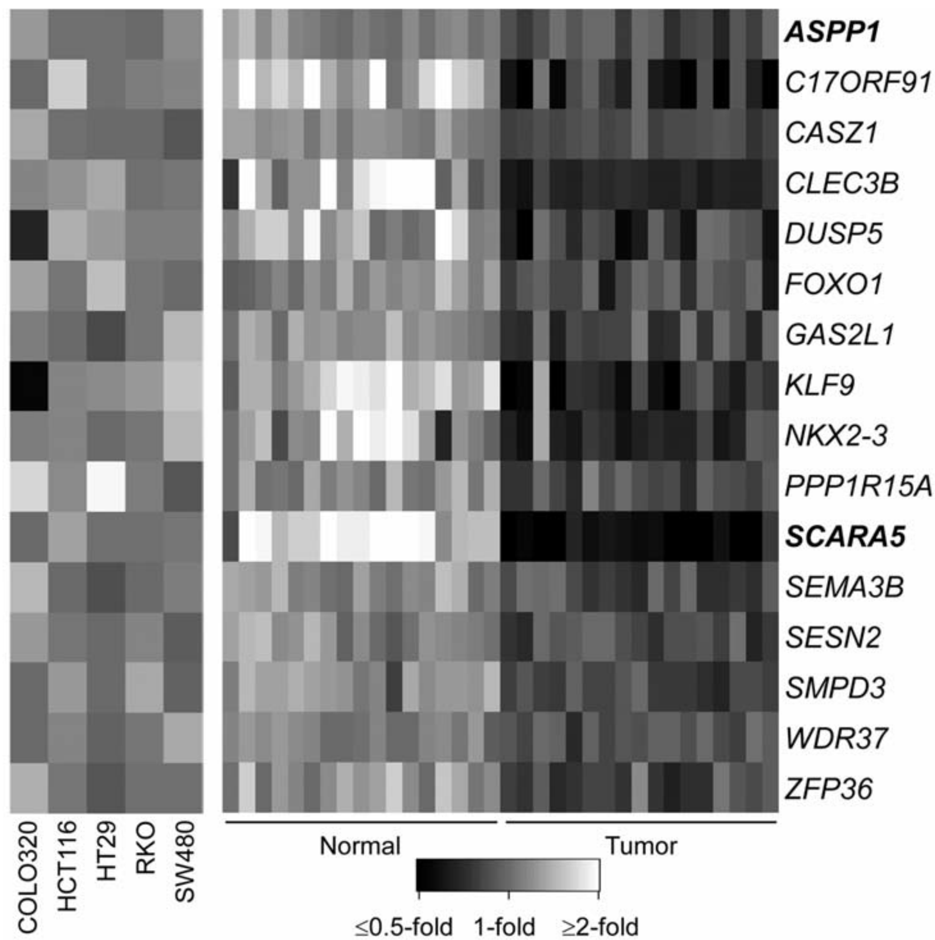

Figure 3. *Heatmap showing expression of the 16 candidate genes in CRC based on microarray analysis. The panels show fold change in gene expression after 5-aza-dC treatment compared to mock-treated cells (left), and overexpression in normal epithelium in comparison to CRC tumor tissues (right). Rows represent individual genes and columns represent individual CRC cell lines (left), and normal and tumor tissue samples (right). In each panel, the fold change is depicted according to the color score shown at the bottom right. Genes selected for validation in this study are indicated by bold letters.*

To test whether the aberrant methylation identified in CRC cell lines was also present in clinical colorectal samples, we evaluated the methylation status of *SCARA5* gene in 23 matched samples using MSP. Promoter methylation in primary CRC was identified in 17% for *SCARA5* (partially methylated). MSP analysis also showed a weak signal of methylated alleles in some tumor and normal samples.

As expected, the majority of clinical samples examined exhibited unmethylated bands, as they were macroscopically dissected. Additionally, *PTPRO* (positive control for methylation analysis) exhibited unmethylated bands in both normal and tumor tissues, hence verifying the integrity of the DNA in these samples (data not shown).

We found no significant differences in methylation frequency between primary CRC and normal tissues for *SCARA5* (p=0.699). Differences in methylation patterns according to patients' characteristics were not analyzed because of the limited number of clinical specimens.

Promoter region of SCARA5 gene was demethylated in CRC cell lines after 5-aza-dC treatment. *SCARA5* methylation status in five CRC cell lines before and after 5-aza-dC treatment was analyzed by MSP. As shown in Figure 1D, 5-aza-dC treatment was associated with partial demethylation in HCT116, RKO and HT29 cell lines. Complete demethylation was seen in the SW480 cell line. This analysis verified that SCARA5 promoter was affected by demethylation by 5-aza-dC. It also confirms the data obtained by oligonucleotide microarray analysis.

Significant down regulation of SCARA5 was found in CRC cell lines and tumor tissues of CRC patients. We analyzed SCARA5 mRNA expression to confirm the down-regulation

Table II. *Methylation frequency of candidate genes in CRC cell lines, primary CRC and normal colorectal tissues. Methylation in cell lines (n=15) and matched tissue samples (n=23) was determined by methylation specific polymerase chain reaction (MSP). SCARA5 showed no significant differences in methylation between normal tissue and primary CRC.*

	ASPP1	*SCARA5*
CRC cell lines, n (%)	0 (0)	3 out of 15 (20)
Primary CRC tissue, n (%)	ND	4 out of 23 (17)
Normal colon tissue, n (%)	ND	2 out of 23 (9)
P-value, tumor *vs*. normal (two-tailed Fisher's exact test)		0.699

ND, Not done.

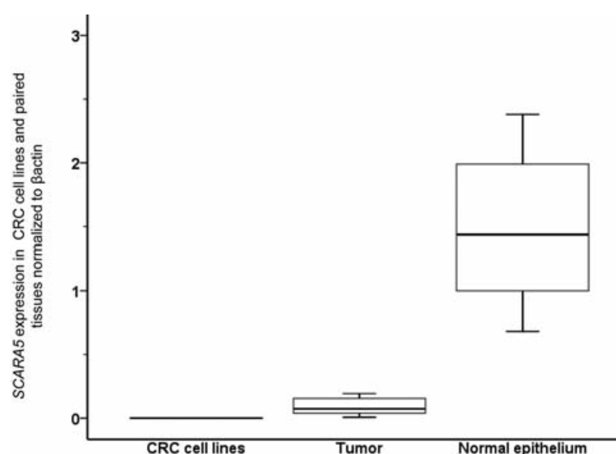

Figure 4. *Boxplot showing markedly reduced expression of SCARA5 in CRC. SCARA5 mRNA expression in 14 CRC cell lines and 14 paired tissue samples determined by RTQ-PCR. β-Actin was used as a reference gene. CRC cell lines and tumor tissue samples had lower SCARA5 expression than normal epithelium (p<0.001 and p=0.001, respectively); there was also a significant decrease in expression in CRC cell lines compared to tumor tissues (p<0.001). P-values were calculated by Wilcoxon signed ranks test for paired samples or Mann-Whitney test for unpaired samples.*

found in the microarray expression data (Figure 3). RTQ-PCR expression data were available for 14 CRC cell lines and 14 paired colorectal specimens. All were investigated earlier for methylation status by MSP. Figure 4 illustrates that the mRNA expression of *SCARA5* was significantly reduced in CRC cell lines and tumor tissue samples compared to normal adjacent samples ($p<0.001$ and $p=0.001$, respectively). There was also a significant difference in *SCARA5* expression between CRC cell lines and tumor tissues ($p<0.001$). The RTQ-PCR analysis confirms array expression data of *SCARA5* gene (Figure 3). Cell lines and tumor samples with partial methylation had low *SCARA5* expression compared to higher expression in normal tissues (data not shown). However, we found no significant correlation between methylation and mRNA expression for *SCARA5* ($p=0.855$).

Discussion

Despite significant improvements in CRC diagnosis and treatment, CRC is still considered as a major public health burden worldwide (1, 2). In this study, we carried out a genome-wide expression screening in order to identify potential genes with abnormal methylation in CRC and a potential role in tumorigenesis. 5-Aza-dC was used to reactivate silenced genes in five CRC cell lines followed by gene identification using microarray and then combined the data with the array of CRC-specific gene expression profiling. This well-established approach was effectively used in screening for candidate genes in a variety of human cancer types (4).

Treatment of cell lines with DNA methyltransferase inhibitor induced widespread expression changes in hundreds of genes in each cell line. Genes up-regulated by 5-aza-dC treatment include genes previously reported as being up-regulated by epigenetic reactivation in different cancer types, including CRC. By combining the cell lines and tissue profiling arrays, a list of 68 genes was determined, which includes genes previously reported as being hypermethylated in CRC (5 genes), some of which have a tumor suppressor function, such as DAB2 interacting protein (*DAB2IP*) (27) and *KLF4* (21).

The candidate genes (16 genes; Table I) identified in this study represent a variety of functions and some can be linked to pathways associated with apoptosis, cell cycle regulation and negative regulation of mitogen-activated protein kinase activity. Interestingly, we found four genes (*ASPP1, CASZ1, SCARA5* and *SEMA3B*) that were previously reported (12, 13, 28, 29) to be hypermethylated and to have a tumor suppressor function in cancer types other than CRC (two of them were analyzed in this study). Collectively, this confirms the integrity of our approach in finding genes with potential suppressor activity that might be affected by methylation in CRC.

ASPP1, also known as *PPP1R13B*, is a tumor suppressor and a potential activator of *p53*. Low *ASPP1* expression was reported in different types of cancer, in colorectal and gastric cancer, no frameshift mutation was found in *ASPP1* (30). Moreover, promoter hypermethylation with reduced expression of *ASPP1* was found in hepatocellular carcinoma (HCC) and acute lymphoblastic leukemia (12). We did not identify *ASPP1* promoter methylation in CRC cell lines (Figure 1B). These results imply that other mechanisms may be involved in the regulation of *ASPP1* gene in CRC. In addition, *ASPP1* up-regulation in 5-aza-dC-treated cell lines

might be a secondary effect caused by stress responses and activation of upstream factors, such as those in the *p53* DNA damage pathway (16).

SCARA5 belongs to the class A scavenger receptor family (31), located on chromosome 8p21, which is a region of tumor progression and metastasis-related loss of heterozygosity (LOH) in CRC and other types of cancer (32). Interestingly, functions of *SCARA5* are typical of tumor suppressors that include inhibiting tumorigenicity, colony formation, cell invasion, tumor metastasis and tyrosine phosphorylation of focal adhesion kinase (FAK), v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) (SRC) and Crk-associated substrate (p130CAS) (13).

Recently, inactivation of *SCARA5* due to LOH and DNA methylation with down-regulated mRNA levels has been reported in HCC (13). Methylation incidence in HCC cell lines and HCC specimens without LOH were 87% and 30%, respectively (13). Our data showed that *SCARA5* was methylated in 20% of CRC cell lines and 17% of primary CRC specimens (Figure 1B and C, Table II). A similar prevalence for methylation was observed in methylation profiles of 19 genes with tumor suppressor function in CRC (*e.g. RIZ1, RUNX3* and *TIMP3*), with frequency ranging from 11% to 68% (33).

Epigenetic changes may make cells liable to further genetic abnormalities that allow progression of the neoplastic process (34). In our data, the weak methylation band of SCARA5 in tissues (Figure 1C) may be attributed to the existence of fewer DNA methylated templates in these specimens and these lesions may represent precursors to tumorigenesis.

Significant down-regulated expression of *SCARA5* in CRC cell lines and tumor tissues compared to normal tissues (Figure 4), confirms the expression data obtained by gene expression array analysis (Figure 3). However, *SCARA5* showed no significant correlation between DNA methylation and mRNA expression, which might be the effect of our small sample size. Results from *SCARA5* methylation and expression analyses suggest that methylation has a minor role in *SCARA5* gene down-regulation in CRC specimens and the role of other mechanisms, such as allelic deletion, or other epigenetic events, such as histone modifications, need further analysis.

A set of other potential genes in CRC remain unexamined at present (Table I), although their reported functions in tumorigenesis of other cancer types signify that these candidates are likely to have a role in colorectal tumorigenesis.

To the best of our knowledge, the current study is the first to examine *SCARA5* methylation and expression in CRC; this study also carried out an additional step in global profiling of genes methylated in cancer.

In conclusion, the use of genome-wide screening led to the identification of candidate genes that may be affected by promoter methylation. Among them, *SCARA5* was methylated and markedly down-regulated in CRC. However, our findings are limited because of our small sample size. Further functional studies are required on *SCARA5*, along with large-scale validation on clinical CRC samples. This will improve our understanding of the role of *SCARA5* in tumorigenesis in CRC and its clinical relevance.

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