Genome-wide screening for methylation-silenced genes in colorectal cancer

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Abstract. Identification of methylation-silenced genes in colorectal cancer (CRC) is of great importance. We employed oligonucleotide microarrays to identify differences in global gene expression of five CRC cell lines (HCT116, RKO, Colo320, SW480 and HT29) that were analyzed before and after treatment with 5-aza-2'-deoxycitidine. Selected candidates were subjected to methylation-specific PCR and real-time quantitative reverse transcription-PCR using 15 CRC cell lines and 23 paired tumor and normal samples from CRC patients. After 5-aza-2'-deoxycitidine treatment, 139 genes were re-expressed in all 5 CRC cell lines collectively with a fold change of more than 1.5 in at least one cell line. These genes include known methylated and silenced genes in CRC. After applying study selection criteria we identified 20 candidates. The *GADD45B* and *THSD1* genes were selected for further analysis. Among 15 colon cancer cell lines, methylation was only identified in *THSD1* (27%). *THSD1* methylation was subsequently investigated in 23 colorectal tumors and methylation was detected in 9% of the analyzed samples; the observed promoter hypermethylation was cancer-specific. *THSD1* mRNA down-regulation was observed in tumor tissues. This genome-wide screening led to the identification of genes putatively affected by methylation in CRC. The *THSD1* gene may play a role in the tumorigenesis of CRC.

Introduction

The transcriptional inactivation of genes through CpG islands hypermethylation at their promoters is a widely reported mechanism in human malignancies, including colorectal cancer (CRC) (1,2). Reduced expression of genes, in particular the tumor suppressor genes, due to epigenetic masking can contribute to cancerous transformation by inducing selective growth advantages (3).

Microarray analysis can lead to the identification of changes in gene expression after the treatment of cells with 5-aza-2'-deoxycitidine (5-aza-dC), a methyltransferase inhibitor that can cause induction of epigenetically silenced genes (4-7).

Profiling of methylated genes is growing rapidly as a powerful diagnostic tool for the early detection, prognosis and even prediction of clinical response to treatment of various cancer types (8). This encourages the search for new candidate genes using global demethylation and microarray analysis that could effectively find genes silenced in association with promoter hypermethylation in several tumor types, including CRC, with a role in cell proliferation, tumor progression, apoptosis or angiogenesis (8-10). In CRC, the path of epigenetic silencing has not yet been fully explored and further searches for methylation-silenced genes are required. Despite the fact that hundreds of genes have been identified to be silenced through promoter CpG island methylation in CRC (11).

In this study, genome-wide demethylation and expression microarray screening were carried out to discover putative genes inactivated by methylation in CRC.

Materials and methods

Cell lines and 5-aza-dC treatment. Cell lines used in this study were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan) and the American Type Culture Collection (Manassas, VA, USA). Fifteen CRC cell lines (CCK81, CoCM1, Colo205, Colo320, ColoTC, DLD1, HCT116, HCT15, HT29, LoVo, RCM1, RKO, SW48, SW480 and WiDr) were cultured in appropriate medium and under conditions described by the providers, with 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 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA).

HCT116, RKO, Colo320, SW480 and HT29 CRC cell lines were treated with 5-aza-dC, each experiment was performed in triplicate. The five cell lines were cultured at 37˚C in 5% CO_2. Cells ($5x10^3$ per well) were plated in their respective culture

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media on day 1, on day 3 cultured cells were treated with PBS dissolved and filter-sterilized 0.5 μM 5-aza-dC (Sigma) for 72 h with media changed every 24 h, treatments were performed in parallel with drug-free phosphate-buffered saline (PBS) as a control. The dose of 5-aza-dC was administered on the basis of its pharmacological dose and the results of our preliminary experiments (12). On day 6, cells were harvested following incubation with trypsin-EDTA then stained with trypan blue and counted. For the analysis of mRNA expression total RNA was extracted using QIAshreder with RNeasy minikit (Qiagen, Hilden, Germany). Genomic DNA (gDNA) was extracted by proteinase K (Invitrogen) digestion, phenol/chloroform extraction and ethanol precipitation method for methylation analysis.

Clinical samples. Paired samples from 23 patients, who underwent surgical treatment for CRC in 2007 at Tokyo Medical and Dental University Hospital were included in this study. All specimens (macroscopically resected) were stored at -80˚C until further use. Mean age was 66.3±10.9 years (median 67.0 years; range 38-81 years) and male-to-female ratio was 1.3:1. The number of cases for stages I, II, III and IV was six, six, five and six, respectively. Written informed consent was obtained from all patients and the Institutional Review Board at Tokyo Medical and Dental University approved the study. All the samples were used in methylation analysis, of them 14 assigned to gene expression study.

Oligonucleotide microarray analysis. RNA was extracted from cell lines before and after treatment with 5-aza-dC. Contaminant DNA was removed by digestion with RNase-free DNase (Qiagen). The integrity of the total RNA obtained was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples had an RNA integrity number of ≥5.0 prior to gene expression analysis. Complementary RNA (cRNA) was prepared from 2 μg total RNA using one-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 array (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 no. 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 algorithm as implemented in the Affymetrix Expression Console™ software (version 1.1). Expression levels were $\log_2$-transformed, and 62 control probe sets were removed from subsequent analyses. R 2.11.1 statistical software together with a Bioconductor package (R Foundation for Statistical Computing, Vienna, Austria) was used to calculate fold-change (FC) values for each of the 54,613 probes on the HG-U133 Plus 2.0 array. FC ≥1.2 in all cell lines was selected as the threshold for up-regulation of gene expression. Genes with an FC difference greater than threshold in all of the five cell lines with FC >1.5 in at least one cell line were selected.

DNA extraction and methylation-specific PCR (MSP). gDNA was extracted using phenol/chloroform method. Bisulfite treatment was performed using EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. Sodium bisulfite treatment of gDNA converts unmethylated cytosine residues (but not methylated cytosine) to uracil, which is then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA.

The methylation status of the cyclin-dependent kinase inhibitor 2A (*CDKN2A/p14^{ARF}*), cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16^{INK4A}*), growth arrest and DNA-damage-inducible beta (*GADD45B*), protein-tyrosine phosphatase receptor type O gene (*PTPRO*) and thrombospondin, type I, domain containing 1 (*THSD1*) genes was determined by methylation-specific polymerase chain reaction (MSP) with 1 μl of bisulfite-treated DNA as template and AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) for amplification, as previously described (13). Bisulfite-converted gDNA, methylated and unmethylated human control DNA (EpiTect control DNA, Qiagen) was used as a positive control for the methylated and unmethylated experiments, respectively.

Methylated and unmethylated primer sequences (Life Technologies Inc., Rockville, MD, USA), amplicon lengths and PCR conditions have been previously reported (12,14-16). None of the primer sets amplified non-bisulfite-treated gDNA (data not shown), this eliminate the possibility of PCR product amplification from unconverted DNA. All reactions were performed in duplicate and 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 included as a positive control for target gene methylation analysis and verified the integrity of gDNA used in this study (i.e., showed unmethylated bands in both normal and tumor tissues) (17). Methylation status of *p14^{ARF}* and *p16^{INK4A}* gene was used to confirm the efficient demethylation of CpG-dinucleotides in the treated cell lines (17).

Quantitative real-time RT-PCR (qRT-PCR). Total RNA was extracted from primary CRC and adjacent normal tissues (14 pairs) using RNeasy minikit (Qiagen) and from 14 colon cancer cell lines. Total RNA (10 μg) was reverse-transcribed into complementary DNA (cDNA) samples using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. TaqMan gene expression assays (Applied Bioystems: *THSD1*, Hs00938785_m1, and β-actin, Hs99999903_m1) were used to determine the expression of *THSD1*. β-actin was used as an internal control. The PCR reaction was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems). Thermal cycling conditions (7300 ABI PRISM, TaqMan; Applied Biosystems) were as follows: 50˚C for 2 min, 95˚C for 10 min, 40 cycles of 15 sec denaturation at 95˚C and 1 min annealing at 60˚C. All calculated concentrations of target genes were normalized by the amount of the endogenous reference with the comparative Ct method for relative quantification (ΔΔCt method) using Relative Quantification Study software (7300 Sequence Detection System version 1.2.1, Applied Biosystems). Each assay was performed in 20 μl including 1 μl of cDNA and all assays were done in duplicate.

Statistical analysis. Differences between groups were estimated using; the χ^2 test, Fisher's exact test, Mann-Whitney U-test and Wilcoxon signed ranks test, where appropriate. A probability level of 0.05 was used for statistical significance. Statistical

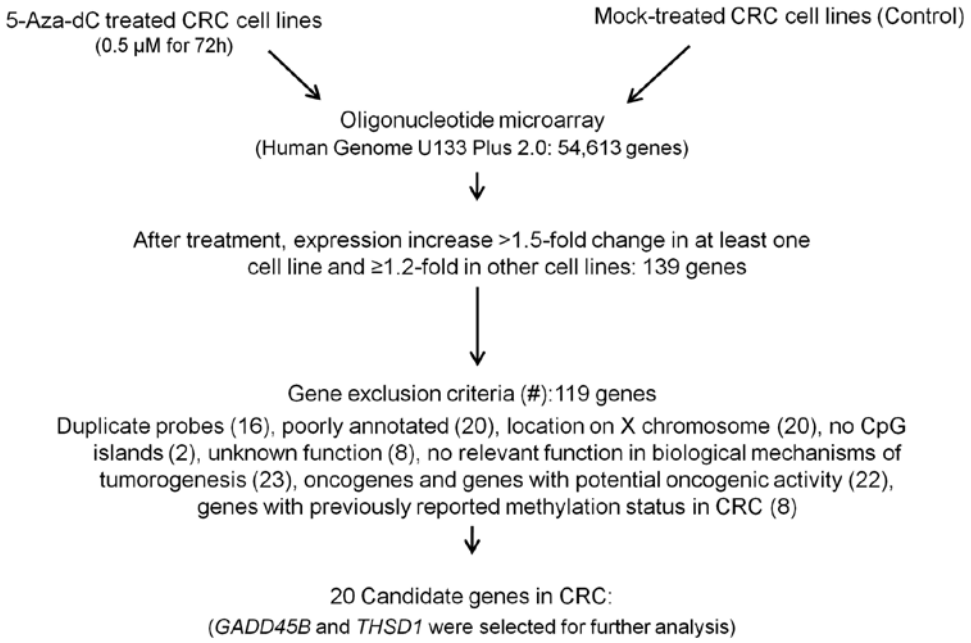

Figure 1. Schematic outline of selection of candidate genes in CRC. We used five CRC cell lines to screen for genes up-regulated after exposure to 0.5 μM 5-aza-2'-deoxycytidine (5-aza-dC) for 72 h followed by oligonucleotide microarray. We obtained 139 candidates, which showed up-regulation of >1.5-fold change in at least one cell line and no less than 1.2-fold change in other CRC cell lines.

analyses of gene expression were performed with SPSS (version 17.0, SPSS Inc., Chicago, IL, USA) for Windows software.

Results

Identification of 20 candidate genes up-regulated in 5-aza-dC-treated CRC cell lines. To find hypermethylated genes associated with CRC, microarray analysis comparison of the mock and 5-aza-dC treatment expression patterns in five colon cancer cell lines (HCT116, RKO, Colo320, SW480 and HT29) was done, this identified the reactivated genes expression profiles (method summarized in Fig. 1). Comparison of the resultant gene expression profiles revealed 139 genes that had FC >1.5 in signal intensities in at least one cell line with ≥1.2-fold change in the remaining four 5-aza-dC-treated cell lines collectively. Of note, this group of up-regulated transcripts contains several genes of cancer-germline antigen families (i.e., *GAGE, MAGE, PAGE* and *XAGE*), which is consistent with previous reports (18). Also the functional analysis by DAVID bioinformatics resource [(19); http://david.niaid.nih.gov] showed the significant enrichment (FDR <0.05%) of genes located on X chromosome (20 genes). In addition, the reactivation of gene transcripts known to be hypermethylated in CRC was also detected including DDB1 and CUL4 associated factor 4-like 1 (*DCAF4L1*), DEAD (Asp-Glu-Ala-Asp) box polypeptide 43 (*DDX43*), intercellular adhesion molecule 1 (*ICAM1*), msh homeobox 1 (*MSX1*), placental growth factor (*PGF*), *PTPRO* and zinc finger protein 42 homolog (*ZFP42*) (11,16,20-23).

In order to identify candidate genes potentially affected by methylation with a putative tumor suppressor activity, first we removed duplicated probes (16 genes), then we excluded genes with poor annotation (20 genes) and genes with a chromosomal location on X chromosome (20 genes). Genes with no CpG islands in the promoter (two genes), genes with unknown function (8 genes) and genes with no relevant function in biological mechanisms of tumorigenesis (23 genes), were also excluded. Oncogenes and genes with potential oncogenic activity (22 genes) were also removed. And lastly genes for which methylation status of their promoter CpG island loci has already been reported for CRC (8 genes) were eliminated. We identified 20 candidates (Fig. 2) that have not been reported to be affected by epigenetic mechanism in CRC (Table I) of which *GADD45B* and *THSD1* were selected for further analysis. Both genes have been recently reported to be frequently methylated in cancer types other than CRC (14,15). Also they were identified as having CpG-rich sequences fulfilling the criteria of CpG island (GC content ≥50%, CpG:GpC ratio ≥0.6, and minimum length 200 bp) in their 5' regions (24).

CpG island searcher (25) and UCSC Table Browser (26) using the current genome assembly (GRCh37/hg19), were used for the potential CpG islands screening. In addition, we searched the published literature for additional analysis of the up-regulated genes.

Methylation of THSD1 was observed in CRC cell lines. To identify novel targets for aberrant methylation in CRC, we further studied methylation status of two candidate genes, *GADD45B* and *THSD1*, in colon cancer cell lines. By using MSP, we determined their methylation status in 15 colon cancer cells. *GADD45B* showed no methylation in colon cancer cell lines and was excluded from further analysis, while *THSD1* was methylated in 27% (Table II; Fig. 3A).

THSD1 promoter region was demethylated in CRC cell lines after 5-aza-dC treatment. Methylation status of *THSD1* in five CRC cell lines before and after 5-aza-dC treatment was analyzed by MSP. 5-aza-dC treatment caused complete demethylation in the RKO cell line, partial demethylation in the Colo320 cell line and no changes in methylation were seen in SW480, HCT116 and

Table I. Twenty putative methylated genes in colorectal cancer up-regulated in 5-aza-2'-deoxycitidine-treated colorectal cancer cell lines compared to mock-treated cell lines by oligonucleotide microarray with a fold change >1.5 in at least one cell line.[a]

Gene symbol	Probes	Gene name	Locus	Function	Mean FC
CAMK2B	209956_s_at	Calcium/calmodulin-dependent protein kinase II beta	7p14.3-p14.1	Calmodulin-dependent protein kinase activity	2.52
CHAC1	219270_at	ChaC, cation transport regulator homolog 1 (*E. coli*)	15q15.1	Protein binding, proapoptotic component of the unfolded protein response	1.65
COL1A1	1556499_s_at	Collagen, type I, alpha 1	17q21.33	Extracellular matrix structural constituent, platelet-derived growth factor binding, transcription activator activity	2.47
COL6A1	212091_s_at	Pollagen, type VI, alpha 1	21q22.3	Platelet-derived growth factor binding	1.61
CSTA	204971_at	Cystatin A (stefin A)	3q21	Cysteine-type endopeptidase inhibitor activity	2.38
DMRTB1	240313_at	DMRT-like family B with proline-rich C-terminal 1	1p32.3	Metal ion binding, sequence-specific DNA binding	3.24
GADD45B	207574_s_at	Growth arrest and DNA-damage-inducible beta	19p13.3	Apoptosis, regulation of MAPKK activity	2.28
GAS5	224841_x_at	Growth arrest-specific 5 (non-protein coding)	1q25.1	Control of mammalian apoptosis and cell population growth	1.43
GPRC5A	203108_at	G-protein-coupled receptor, family C, group 5, member A	12p13-p12.3	G-protein coupled receptor activity	1.83
GPSM1	226043_at	G-protein signaling modulator 1 (AGS3-like, *C. elegans*)	9q34.3	G-protein alpha subunit binding, GTPase activator activity	1.33
KLHL35	1553611_s_at	Kelch-like 35 (*Drosophila*)	11q13.4	Protein binding	1.37
LTBP2	204682_at	Latent transforming growth factor beta binding protein 2	14q24	Calcium ion binding, growth factor binding	1.67
NAA11	210603_at	N(alpha)-acetyltransferase 11, NatA catalytic subunit	4q21.21	N-acetyltransferase activity	1.69
RBP4	219140_s_at	Retinol binding protein 4, plasma	10q23-q24	Retinol binding, retinol transporter activity	1.96
SEMA7A	230345_at	Semaphorin 7A, GPI membrane anchor (John Milton Hagen blood group)	15q22.3-q23	Integrin binding, receptor activity	1.4
SYCP3	1553599_a_at	Synaptonemal complex protein 3	12q	DNA binding	5.77
TBRG1	226318_at	Transforming growth factor beta regulator 1	11q24.2	DNA, protein binding	1.48
THSD1	219477_s_at	Thrombospondin, type I, domain containing 1	13q14.3	Extracellular matrix	1.46
TNFSF9	206907_at	Tumor necrosis factor (ligand) superfamily, member 9	19p13.3	Cytokine activity, tumor necrosis factor receptor binding	1.43
TXNIP	201009_s_at	Thioredoxin interacting protein	1q21.1	Enzyme inhibitor activity, ubiquitin protein ligase binding	3.04

[a]Gene symbols in bold indicate genes examined in this study. FC, fold change; MAPKK, mitogen-activated protein kinase kinase.

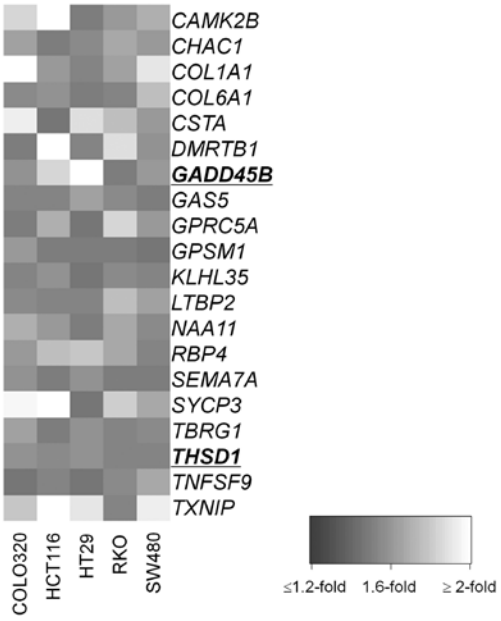

Figure 2. Heatmap showing expression based on microarray analysis. The panel shows the 20 candidate genes in CRC with increased expression in 5-aza-2'-deoxycytidine-treated CRC cell lines compared to mock-treated cells (fold change >1.5 in at least one cell line and fold change ≥1.2 in other cell lines, collectively). Columns represent individual CRC cell lines and rows represent individual genes. 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 and are underlined.

HT29 cell lines (Fig. 3B). This verified that *THSD1* promoter is affected by demethylation effect of 5-aza-dC and confirms the data obtained by oligonucleotide microarray analysis.

Table II. Methylation frequency of candidate genes in 15 CRC cell lines and 23 matched clinical CRC tissue samples.[a]

	GADD45B	*THSD1*
CRC cell lines (%)	0 (n=0)	4 out of 15 (n=27)
Primary CRC tissue (%)	np	2 out of 23 (n=9)
Normal colon tissue (%)	np	0 out of 23 (n=0)
P-value, tumor vs. normal (two-tailed Fisher's exact test)		0.49

[a]Methylation status was determined by MSP. No significant difference was found for *THSD1* in methylation between normal tissues and primary CRC. np, not performed.

Methylation of THSD1 was observed in tumor samples of CRC patients. To test whether the aberrant methylation identified in colon cancer cell lines was also present in primary colon cancer specimens, we studied the methylation status of *THSD1* gene in 23 matched CRC samples. Aberrant methylation in primary colon cancers was identified in 9% for *THSD1*. Distinctively, *THSD1* was completely unmethylated in all the samples from normal tissues (Table II; Fig. 3C). There was no significant difference in the methylation of *THSD1* between tumor and normal tissues (P=0.49; Table II).

Down-regulation of expression of THSD1 was found in tumor tissues of CRC patients. To investigate the relationship between gene expression and promoter hypermethylation, we analyzed *THSD1* mRNA expression in 14 CRC cell lines and 14 paired

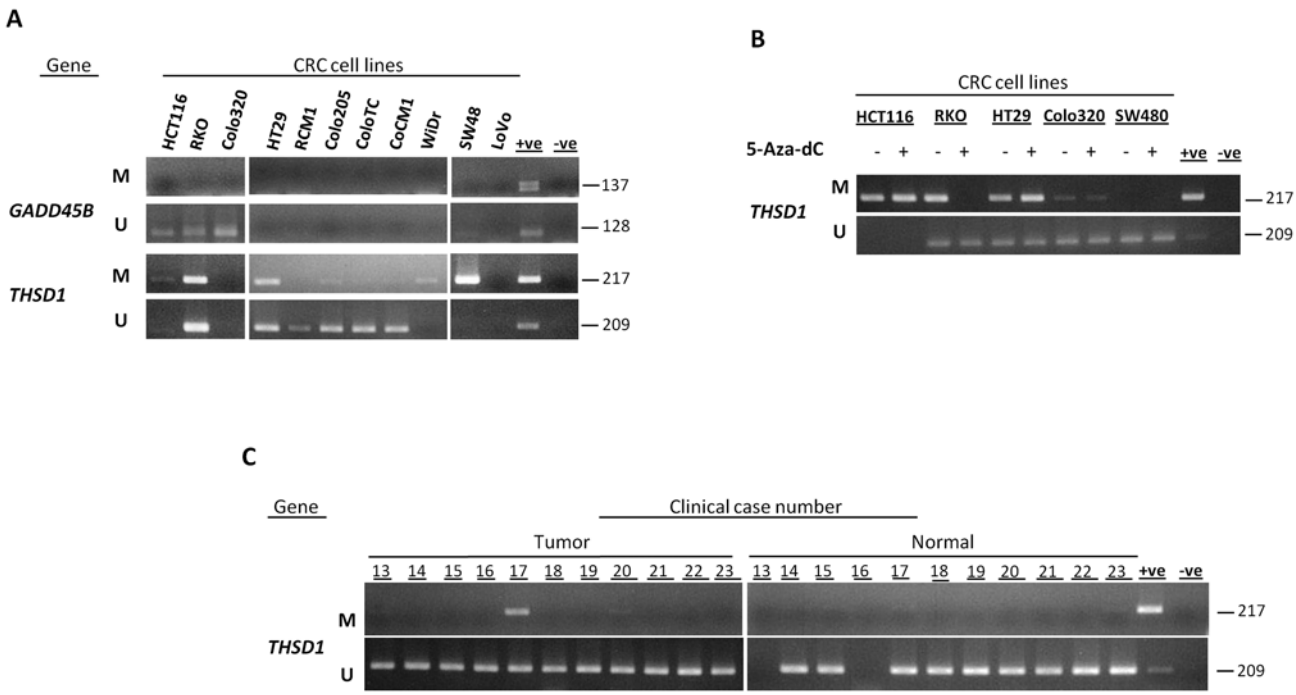

Figure 3. Representative MSP results in 15 CRC cell lines for *GADD45B* and *THSD1* (A), methylation status of *THSD1* in five CRC cell lines before and after 5-aza-dC treatment (B) and methylation status of *THSD1* in 23 pairs of primary colorectal tumor (Tumor) and normal epithelium (Normal) (C). Methylated (*M*) and unmethylated (*U*) denote the presence of PCR product using primers specific for methylated and unmethylated sequences, respectively. Fully methylated and unmethylated, human genomic DNA, 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 each panel.

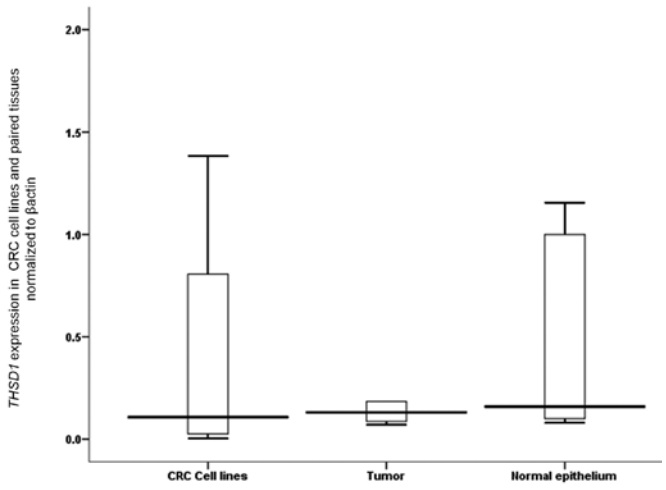

Figure 4. Boxplot showing reduced expression of *THSD1* in CRC. *THSD1* mRNA expression in 14 CRC cell lines and 14 paired tissue samples determined by RTQ-PCR. β-actin was used as a reference gene. Tumor tissue samples had lower *THSD1* expression than CRC cell lines and normal epithelium, however it did not reach a statistical significance (P=0.613 and 0.158, respectively). P-values were calculated by Mann-Whitney test for unpaired samples or Wilcoxon signed ranks test for paired samples.

CRC tissues. The level of *THSD1* mRNA was reduced (Fig. 4) in all tumor tissues relative to cancer cell lines and adjacent non-tumor tissues, however it did not reach statistical significance (P=0.613 and 0.158, respectively).

Discussion

In this study, we carried out a systematic global search for aberrantly methylated genes in CRC. 5-aza-dC was used to demethylate silenced genes in five colon cancer cell lines and then followed by expression microarray to identify overexpressed genes.

The exposure of cell lines to demethylating agent (5-aza-dC) induced expression changes in a group of genes in all treated cell lines. A total of 139 genes were up-regulated, including genes that have been previously reported to be up-regulated by demethylation in CRC (11,16,20-23).

Treatment of the cell lines with 5-aza-dC can induce genes without a direct consequence of CpG island demethylation (27). We examined the methylation status in two putative genes by MSP and one of them, *GADD45B*, was not methylated in any of the 15 CRC cell lines (Table II, Fig. 3A). In addition to the up-regulation of two genes with no CpG islands, up-regulation of such genes might be a secondary effect caused by an induced upstream factor (e.g. genes in the *p53* DNA damage pathway) (27).

THSD1 encodes a transmembrane molecule containing a thrombospondin type 1 repeat, which might be involved in cell adhesion and angiogenesis (15). Although *THSD1* has no clear function, other proteins possessing the thrombospondin type 1 repeat (TSR) have been shown to inhibit tumor angiogenesis and growth (28), such as thrombospondin 1 (*TSP1*), ADAM metallopeptidase with thrombospondin type 1 motif 1 (*ADAMTS1*) and ADAM metallopeptidase with thrombospondin type 1 motif 12 (*ADAMTS12*), all were reported as being methylated in CRC (29,30). High *THSD1* expression positively correlated with a better distant metastasis survival in breast cancer (15), hence its loss possibly is associated with metastatic tumor spread.

The chromosomal location of *THSD1* at 13q14.3 is similar to retinoblastoma 1 (*RB1*) gene region which has been reported with widely differing frequencies of allelic loss detected by LOH in CRC (31). This region is strongly associated with the progression of colorectal adenomas towards carcinomas (32).

THSD1 might have a role in radiation response as it was one of the consensus radiation response genes in primary human fibroblasts (33). The thrombospondins (TSPs) might act to support tumor progression to metastasis through their effects on the degradation of the extracellular matrix and the ability of tumor cells to invade the surrounding tissues, although few studies have addressed the role of the TSPs in metastasis *in vivo* (34).

Based on combined genomic and transcriptomic information from 6 patients, with tumor stage Dukes A, B, C and D, respectively (n=24), a recent study showed the presence of *THSD1* in 17 genes that have been expressed in Dukes D that may be relevant to tumor progression (35).

Silencing of *THSD1* involved LOH and promoter hypermethylation in esophageal cancer cell lines and tumor tissues with gene expression down-regulation (15). We identified *THSD1* as aberrantly methylated in CRC cell lines and CRC positive samples. *THSD1* showed a tumor specific methylation with no abnormal methylation in non-cancerous tissues (Table II, Fig. 3C). Moreover, low expression in tumor tissues rather than normal for *THSD1* besides no significant correlation between methylation and mRNA expression suggests that down-regulation of *THSD1* in CRC specimens could be attributed to allelic deletion, or epigenetic events other than methylation of the CpG islands.

GADD45B belongs to GADD45 genes family and proteins encoded by this family and recognized as stress sensors in signaling responses to various physiological or environmental stresses. GADD45 genes family mediates their activity via interactions with other cellular proteins (36) and can inhibit cell proliferation at different stages and induce cell apoptosis (37). Results from a mouse model showed that Gadd45b is involved in tumor surveillance by $CD8^+$ T cells and the Gadd45 signaling pathway is important in containing cancer (38). GADD45 genes become repressed in cancer by different mechanisms such as methylation, NF-κB activation or mutation through which deregulated expression may lead to tumorigenesis (36). Altogether, previous reports suggest that *GADD45B* is functionally a tumor suppressor gene.

Down-regulation due to promoter hypermethylation of *GADD45B* was reported in certain cancer types, such as hepatocellular carcinoma and non-small cell cancer (14,39). Na *et al* (39) showed no methylation of *GADD45B* promoter in five CRC cell lines in their efforts to investigate the GADD45 genes methylation profile in multiple tumors was consistent with our results.

A substantial number of putatively hypermethylated genes remain unexamined at present (Table I) and those remaining candidates may have a role in tumorigenesis of CRC.

In conclusion, this study adds an additional step in global profiling of gene promoter hypermethylation to identify novel aberrantly methylated genes in CRC. This may help in further understanding of CRC pathogenesis and may lead to

identification of diagnostic, prognostic methylation markers or therapeutic targets. Also our data showed that coherent mining of the microarray data can further widen the gene detection results. The current study is the first to identify *THSD1* down-regulation and methylation in primary CRC tissues. Further functional studies are required to clarify the role of *THSD1* in tumorigenesis and to examine its clinical significance in CRC.

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