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Expression of the human Sd^a β-1,4-*N*-acetylgalactosaminyltransferase II gene is dependent on the promoter methylation status

Hou-Ren Wang[2,3], Chuang-Yi Hsieh[3], Yuh-Ching Twu[3], and Lung-Chih Yu[1,2,3]

[2]Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan; and [3]Institute of Biochemical Sciences, National Taiwan University, Taipei 106, Taiwan

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It has been noted that the expression of Sd^a, including its antigenic structure, the β-1,4-*N*-acetylgalactosyltransferase II (β4GalNAcT-II) activity responsible for its formation, and the Sd^a β4GalNAcT-II mRNA transcript, is drastically reduced in oncogenetic processes in gastrointestinal tissues. Markedly reduced metastatic potential has been demonstrated in colon and gastric cancer cells transfected with the Sd^a β4GalNAcT-II gene. In this study, a putative CpG island encompassing the promoter and exon 1 regions in the human Sd^a β4GalNAcT-II gene was identified, and the investigation of DNA methylation of the Sd^a gene promoter region demonstrated a clear association between the methylation status of the CpG island promoter and expression of the Sd^a gene in gastrointestinal cancer cell lines. Hypomethylation of the promoter region of the Sd^a gene was shown in cells where this gene was expressed. By contrast, there was significant hypermethylation of the Sd^a gene promoter in cells that did not express the gene. A specific methylation profile in the Sd^a gene CpG island was demonstrated in KATO III gastric cancer cells. In colon cancer cells with the hypermethylated Sd^a gene promoter, treatment with the DNA methylation inhibitor, 5-aza-2′-deoxycytidine, resulted in demethylation of the promoter region and substantially induced the expression of the Sd^a gene and the Sd^a antigenic structure. These results strongly suggest that promoter DNA methylation plays a crucial role in the regulation of the Sd^a β4GalNAcT-II gene and Sd^a antigen expression.

Keywords: DNA methylation/gastrointestinal cancers/gene expression/*N*-acetylgalactosaminyltransferase/Sd^a antigen

Introduction

The Sd^a antigen was first reported as a human blood group antigen by Macvie et al. and Renton et al. simultaneously in 1967 (Macvie et al. 1967; Renton et al. 1967). The antigen was found in more than 90% of human red blood cells (RBCs), and also in several tissue types, including stomach, colon, and kidney (Morton et al. 1970; Capon et al. 2001; Robbe et al. 2003), and oocytes (Dell et al. 2003). Its presence was also demonstrated in various body fluids, such as saliva, milk, serum, and urine, with the greatest concentration occurring in urine (Morton et al. 1970; reviewed in Daniels 2002). The strength of the Sd^a antigen expression on Sd^a-positive RBCs varies greatly. RBCs with very strong Sd^a antigen expression, sufficient for agglutination by *Dolichos* lectin, have been defined as the Cad phenotype (Sanger et al. 1971). It has been revealed through various studies that the Sd^a/Cad antigenic epitopes are determined by the terminal trisaccharide structure, GalNAcβ1-4(NeuAcα2-3)Galβ, carried on glycoproteins and glycolipids (Blanchard et al. 1983, 1985; Donald et al. 1983), and that the final step in the formation of the Sd^a/Cad antigenic epitopes is determined by the activity of the Sd^a β-1,4-*N*-acetylgalactosaminyltransferase II (β4GalNAcT-II), which catalyzes the addition of GalNAc to the Gal of the NeuAcα2-3Galβ terminal structure in the β-1,4 linkage. This enzyme activity was first manifested in the guinea pig kidney (Serafini-Cessi and Dall'Olio 1983), with a subsequent investigation demonstrating the abundance of this enzyme in the human colon (Malagolini et al. 1989).

Following the first isolation of the Sd^a β4GalNAcT-II cDNA from murine cytotoxic T lymphocytes (Smith and Lowe 1994), a partial sequence of human Sd^a β4GalNAcT-II cDNA was cloned from the total RNA fraction of gastric mucosa (Dohi et al. 1996). The full coding sequence of human Sd^a β4GalNAcT-II cDNA was demonstrated by Montiel et al. in 2003 (Montiel et al. 2003). The human Sd^a gene is located on chromosome 17 and comprises at least 12 exon regions that span about 37 kb of genomic DNA. Notably, two transcript forms, which have different exon 1 (denoted as exon 1_S and exon 1_L), but identical exons 2–11, coding regions, are expressed from the human Sd^a gene locus through utilization of alternative promoters. The two transcripts, with respective open reading frames of 1521 and 1701 bp, encode the short- and long-form Sd^a β4GalNAcT-IIs with 506 and 566 amino acid residues, respectively. The two forms of Sd^a β4GalNAcT-II differ with respect to the lengths of their cytoplasmic tails, with the long-form unusually extended at 66 amino acid residues. An independent paper by Lo Presti et al. also reported the cDNA sequence for the long-form Sd^a β4GalNAcT-II (Lo Presti et al. 2003).

It has been noted that the expressions of Sd^a are developmentally regulated and increase as a function of age (Macvie et al. 1967; Renton et al. 1967; Dall'Olio et al. 1987, 1990). Increased Sd^a antigen expression and Sd^a β4GalNAcT-II transferase activity has been observed during differentiation of human colon and gastric cancer cell lines (Malagolini et al. 1991; Dohi et al. 1993). Especially, altered expression patterns of Sd^a antigen during oncogenetic processes have been noted. It was demonstrated that the Sd^a antigen and Sd^a β4GalNAcT-II activity were markedly diminished (Malagolini et al. 1989; Dohi et al. 1991), and the expression of the Sd^a gene transcript

[1]To whom correspondence should be addressed: Tel: +886-2-3366-4070; Fax: +886-2-2363-5038; e-mail: yulc@ntu.edu.tw

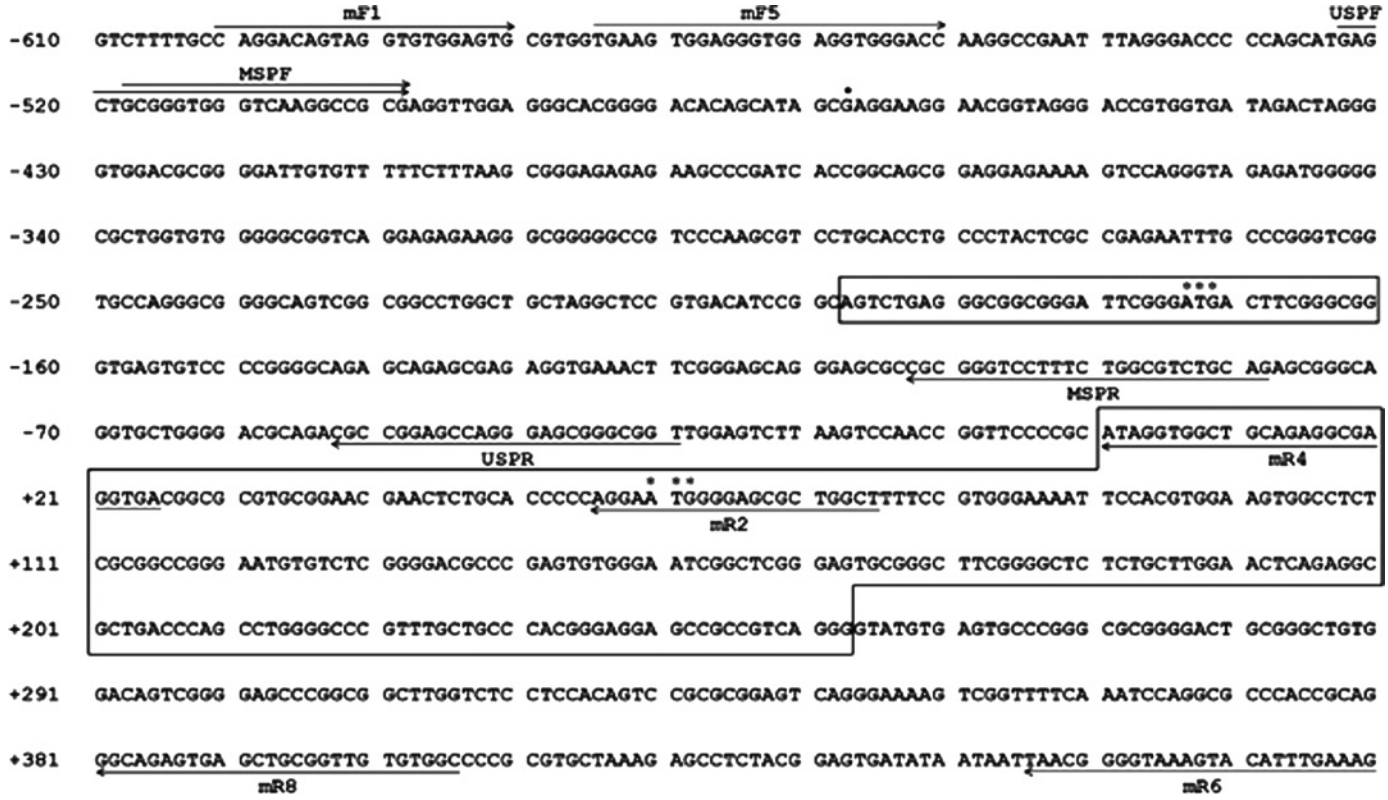

Fig. 1. Nucleotide sequence of the human Sd[a] β4GalNAcT-II gene encompassing the 5′ promoter, exon 1_S, and exon 1_L regions, and the primer locations for bisulfite genomic sequencing and methylation-specific PCR. The nucleotide sequence was derived from the human chromosome 17 genomic contig, deposited in GenBanK as accession number NT_010783. The transcription start nucleotide of exon 1_L is numbered +1. The regions of exon 1_S and exon 1_L are boxed, with asterisks indicating the translation start codons in exon 1_S and exon 1_L. Arrows represent the locations and orientations of the primers used in bisulfite genomic sequencing and methylation-specific PCR. It was determined that the −468 position (indicated by a dot) was the G nucleotide in KATO III and MKN45 cells, whereas it was the A nucleotide in the other eight cell lines assessed in this study.

significantly decreased in colon and gastric cancer tissues (Dohi et al. 1996). Although association between the downregulation of Sd[a] expression and malignant changes in the gastrointestinal tract mucosa has been noted, the biological implications of this phenomenon were not manifested until the study by Kawamura et al. (2005). It has been known that the expression of the E-selectin ligands, sialyl Lewis[a] (sLe[a]) and sialyl Lewis[x] (sLe[x]), in gastrointestinal cancer cells is associated with malignant metastasis. Kawamura et al. demonstrated that Sd[a] β4GalNAcT-II competes for the acceptor substrates with the α-1,3- and α-1,4-fucosyltransferases responsible for the formation of sLe[a] and sLe[x] carbohydrate structures, and that overexpression of Sd[a] β4GalNAcT-II eliminates the expression of sLe[a] and sLe[x] in colon and gastric cancer cells and, consequently, inhibits the metastasis of these cells. Their study suggests a potential for the development of antimetastatic gene therapy for gastrointestinal cancers. The relationship between the expression of Sd[a] and sLe[x] antigens in normal and cancerous colon tissues has been further elaborated in a recent study (Malagolini et al. 2007).

Although tissue- and cell-type specificities with respect to the distribution of Sd[a] antigen and its altered expression patterns during oncodevelopmental processes have long been noted, the control mechanism for the Sd[a] gene expression has never been elucidated. It is well known that the methylation of cytosine residue in the CpG dinucleotide-rich sequence provides one of the regulatory mechanisms for gene expression. The present study has demonstrated that the expression of the human Sd[a] β4GalNAcT-II gene is determined by the DNA methylation status of the CpG island in the promoter region.

Results

CpG island in the promoter and exon 1 regions of the Sd[a] β4GalNAcT-II gene

The short- and long-form human Sd[a] β4GalNAcT-II transcripts are composed of different exon 1, exon 1_S, and exon 1_L. Exon 1_S and exon 1_L contain 38 and 253 bp, respectively, and are separated by a 160-bp sequence (Figure 1) (Montiel et al. 2003). The sequence of nucleotides −2000 through +2000 (transcription start of exon 1_L as nucleotide +1) of the Sd[a] gene was analyzed using the CpGPlot utility from the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/) (Figure 2), and showing that the nucleotide region of −537 through +425 fulfilled the criteria for a CpG island defined by Gardiner-Garden and Frommer who first performed a large-scale analysis of CpG islands in vertebrate genomes (Gardiner-Garden and Frommer 1987). They defined a CpG island as a 200-bp (or longer) stretch of DNA with a G+C content greater than 50% and an observed CpG/expected CpG ratio (Obs/Exp CpG) greater than 0.6. Some of the human *Alu* sequences, which are highly repetitive short interspersed elements with a 280-bp consensus sequence

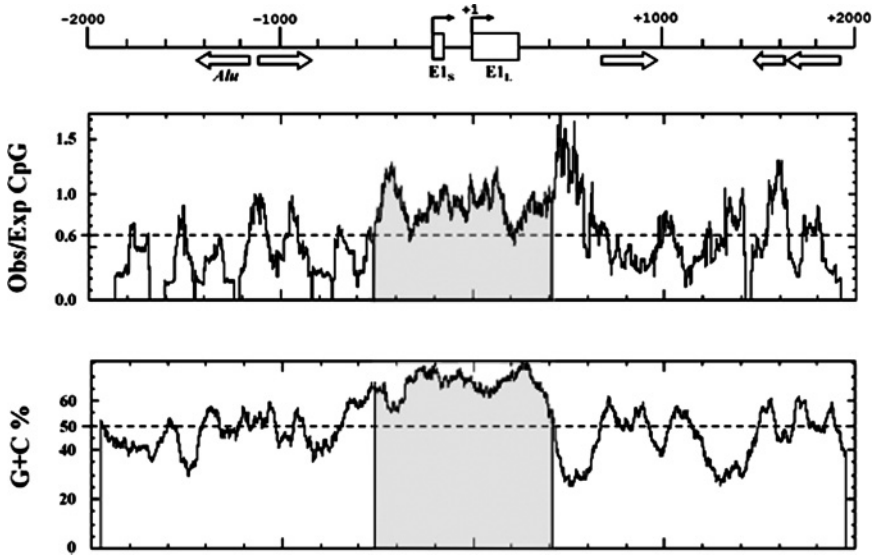

Fig. 2. CpG island in the Sd^a β4GalNAcT-II gene. The top diagram is a schematic representation of the nucleotide region −2000 through +2000 of the Sd^a gene (transcription start of exon 1_L as +1), with the locations of exon 1_S ($E1_S$) and exon 1_L ($E1_L$) shown as open boxes. Exon 2 locates about 8 kb downstream to exon 1_L, and thus is not shown in the diagram. Open arrows represent the positions and orientations of the *Alu* repetitive elements. The middle and bottom diagrams present the quantifications of the observed CpG/expected CpG ratio (Obs/Exp CpG) and the percentage of G+C nucleotides in the −2000 through +2000 nucleotide region, respectively. The values for Obs/Exp CpG and G+C% were plotted against the position in the analyzed sequence using the CpGPlot program, with a sliding window of 200 bp and a step increment of 1. The nucleotide region −537 through +425, indicated as gray areas, fulfils the criteria of a CpG island, Obs/Exp CpG greater than 0.6 and G+C content greater than 50%, as defined by Gardiner-Garden and Frommer (Gardiner-Garden and Frommer 1987).

(approximately), have relatively high G+C content and Obs/Exp CpG (Schmid 1998), and thus the nucleotide region from −2000 through +2000 of the Sd^a gene was analyzed by the RepeatMasker Web Server (http://repeatmasker.org) to detect the *Alu* repetitive elements. In total, five *Alu* elements were located in the region (Figure 2), but in the regions 5′ and 3′ sides of the CpG island, not within the putative CpG island. Takai and Jones have suggested that adoption of a more stringent definition for a CpG island, a DNA region of greater than 500 bp with a G+C content equal to or greater than 55% and Obs/Exp CpG of 0.65, is more likely to distinguish CpG islands associated with the 5′ regions of genes rather than analogs associated with intragenomic repetitive sequences (Takai and Jones 2002). The nucleotide region −537 through +425 of the Sd^a β4GalNAcT-II gene has a G+C content of 66.8% and an Obs/Exp CpG of 0.85, fulfilling these more stringent criteria.

Correlation of the methylation status of the CpG promoter with the Sd^a β4GalNAcT-II gene expression

Expression of the Sd^a gene in different human gastrointestinal cancer cell lines was analyzed using reverse transcription-polymerase chain reaction (RT-PCR). The expression profiles were analyzed for the Sd^a transcript (with the common region of the short- and long-form Sd^a transcripts PCR amplified), together with the short- (denoted as Sd^a-*S*) and long-form Sd^a (Sd^a-*L*) transcripts in seven colon cancer cell lines, two gastric cancer cell lines, and one pancreatic cancer cell line (Figure 3). Expression of the Sd^a transcript was demonstrated in Caco-2, Colo 205, SW1417, SW480, SW948, and MKN45 cells. Similar expression profiles for the short- and long-form Sd^a transcripts were revealed in these cell lines, except that only the short-form Sd^a transcript was detected in MKN45 cells. In contrast, the RT-PCR analysis did not detect any Sd^a transcript in the

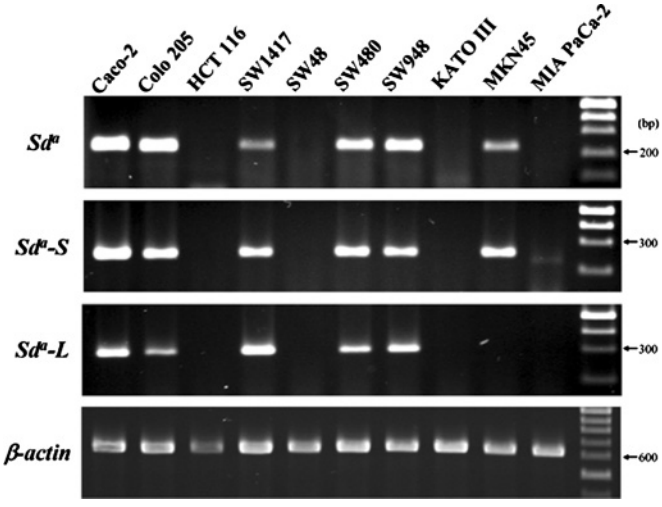

Fig. 3. Expression profiles of the Sd^a β4GalNAcT-II gene in various gastrointestinal cancer cell lines. Expression of the Sd^a β4GalNAcT-II gene in colon (Caco-2, Colo 205, HCT 116, SW1417, SW48, SW480, and SW948), gastric (KATO III and MKN45), and pancreatic (MIA PaCa-2) cancer cell lines was analyzed using RT-PCR. Total RNAs purified from these cells were primed using oligo-dT primer to synthesize the first-strand cDNAs, and PCR amplifications specific for the short-form Sd^a β4GalNAcT-II (denoted as Sd^a-*S*), long-form Sd^a β4GalNAcT-II (Sd^a-*L*), the common region (exon 4 to exon 6) of the two transcript forms (Sd^a), and β-*actin* cDNA were performed, as described in Materials and methods. The RT-PCR products were analyzed using 1.5% agarose gel electrophoresis. The expected sizes of the products from the Sd^a, Sd^a-*S*, Sd^a-*L*, and β-*actin* cDNAs were 228, 260, 310, and 626 bp, respectively. The rightmost lanes show the molecular mass standards of the 100-bp ladder.

RNA samples prepared from HCT 116, SW48, KATO III, and MIA Paca-2 cells. Nevertheless, a trace of expression of the short-form Sd^a, but not the long-form analog, was observed

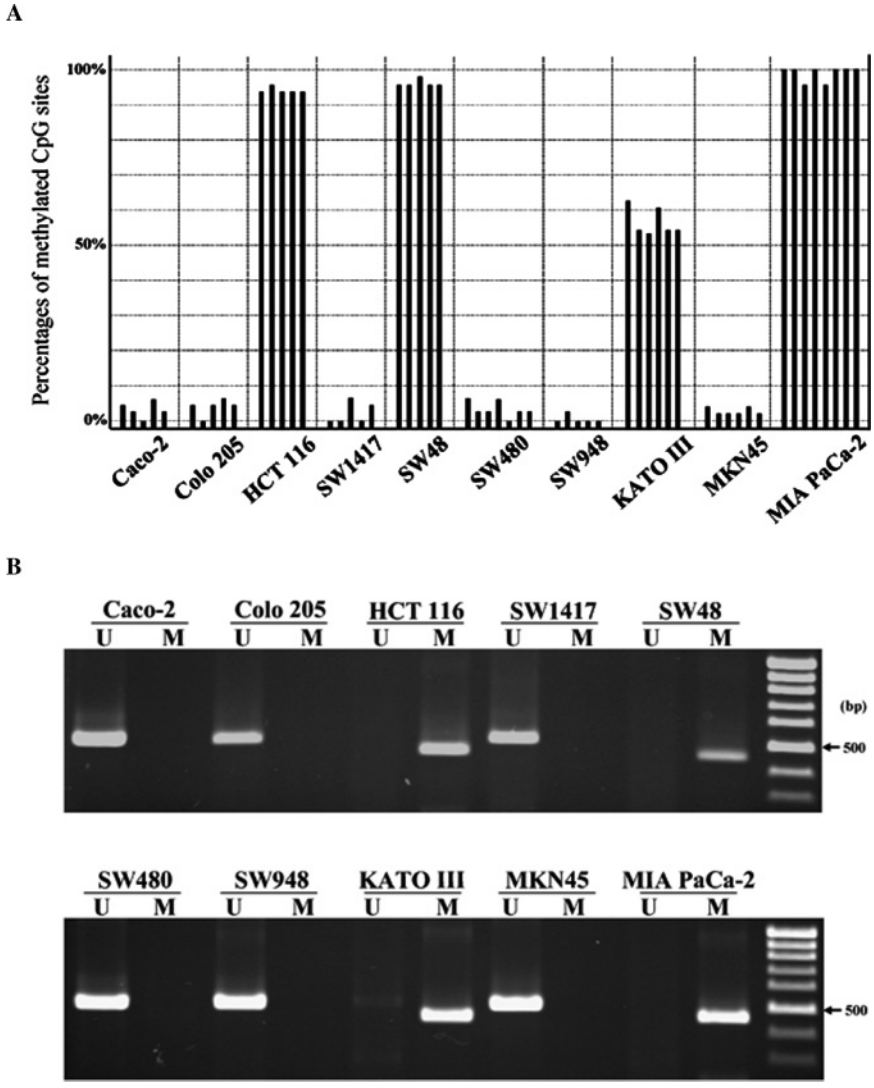

Fig. 4. Methylation status of the Sd^a β4GalNAcT-II gene promoter for different gastrointestinal cancer cell lines. (**A**) Percentage of methylated CpG sites in the −550 through −1 nucleotide region of the Sd^a gene. Each vertical line, illustrating the percentage of the methylated CpG site in the analyzed region, represents the result obtained from one individual clone. (**B**) Evaluation of the methylation status of the Sd^a promoter using methylation-specific PCR analysis. Genomic DNA samples from each cell line were bisulfite modified, and PCR amplifications with the primer pairs of USPF+USPR (lanes U) and MSPF+MSPR (lanes M), designed for the unmethylated and methylated status, respectively, were performed. The expected sizes of the PCR products from the USPF+USPR and MSPF+MSPR primer pairs were 494 and 443 bp, respectively. The rightmost lanes show the molecular mass standards of the 100-bp ladder.

in the KATO III cells with more PCR cycles (data not shown).

The methylation status of the Sd^a gene CpG island in these cells was analyzed through bisulfite genomic sequencing as described in the Materials and methods section. The region of nucleotide −550 through −1, which contains 47 CpG dinucleotides (48 in KATO III and MKN45 cells; see Figure 1 legend), was analyzed; 5–8 clones were sequenced per cell line. In the analyzed clones, almost all the cytosine residues not within the CpG dinucleotide were converted to thymidines, with only few exceptions (0–3 per clone), demonstrating that the bisulfite conversions were virtually complete. The percentage of methylated CpG in the analyzed region obtained from each clone is schematically represented in Figure 4A. The methylation status of the Sd^a CpG island in each cell line reveals a sharp contrast between cells with and without Sd^a gene expression. The CpG sites are barely methylated in Caco-2, Colo 205, SW1417, SW480, SW948, and MKN45 cells, which express the Sd^a gene. Only few sites (0–3 per clone) in the 47 (or 48) CpG dinucleotides were found to be methylated in these cells. The lowest methylation status was observed in SW948 cells as only one methylated CpG was located in the five analyzed clones. In contrast, hypermethylation of the Sd^a gene CpG island was observed in HCT 116, SW48, and MIA PaCa-2 cells, which do not express the Sd^a gene. In these three cell lines, almost all the CpG sites were methylated, with only 0–3 sites per clone unmethylated. MIA PaCa-2 cells showed the highest methylation percentage, with 100% methylation demonstrated for six of the eight analyzed clones. A different feature was observed in KATO III cells. The methylation percentages for the six clones from the KATO III cells were around 60%.

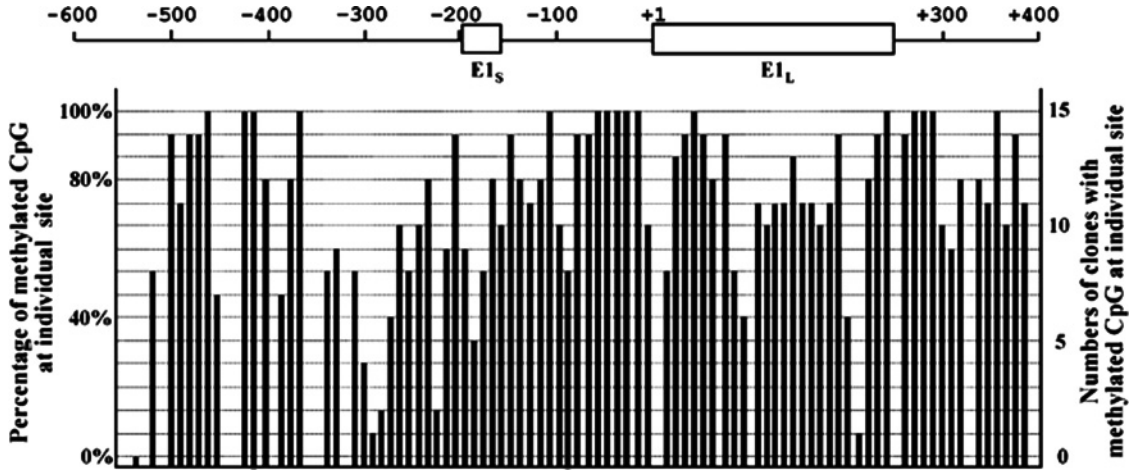

Fig. 5. Methylation profile for individual CpG dinucleotide in the Sd^a gene CpG island in KATO III cells. The methylation of individual CpG sites in the −550 through +380 nucleotide region of the Sd^a gene in KATO III cells was analyzed by bisulfite genomic sequencing, with the analyzed region encompassing 85 CpG dinucleotides. The top diagram is a schematic representation of the nucleotide region −600 through +400 of the Sd^a gene; the locations of exon 1_S ($E1_S$) and exon 1_L ($E1_L$) are shown as open boxes. Sequencing results were compiled for 15 clones, prepared from five separate batches of PCR products, and the methylation frequency for each individual CpG site, observed in the 15 analyzed clones, calculated. The methylation percentages (also the number of clones with methylated CpG) for each individual CpG site are illustrated and plotted against the position in the analyzed sequence.

Methylation-specific PCR was established to evaluate the methylation status of the Sd^a CpG promoter. In this analysis system, the primer pair designed to demonstrate the methylated status (lanes M, Figure 4B) yielded PCR products only from the bisulfite-treated genomic DNA samples prepared from HCT 116, SW48, KATO III, and MIA PaCa-2 cells, in which the Sd^a gene promoters were significantly methylated. When the primer pair designed to demonstrate the unmethylated status was used (lanes U, Figure 4B), only the samples prepared from the cells with the hypomethylated Sd^a promoter gave PCR products. However, bisulfite-treated genomic DNA from KATO III cells yielded, in addition to the obvious positive result from the methylated primer pair, a trace of PCR product from the unmethylated primer pair. This outcome may be caused by the semi-methylated status of the Sd^a promoter in KATO III cells. The results demonstrated from methylation-specific PCR were consistent with those obtained from bisulfite genomic sequencing, suggesting that this methylation-specific PCR is feasible for rapid analysis of the methylation status of the Sd^a gene CpG promoter.

The expression profiles for the Sd^a gene, revealed by RT-PCR, and the methylation status of the Sd^a gene CpG island, demonstrated by bisulfite genomic sequencing and methylation-specific PCR, display a definite inverse relationship between the methylation level in the Sd^a gene promoter region and gene expression level in these gastrointestinal cancer cell lines.

Methylation pattern of the Sd^a β4GalNAcT-II gene CpG island in KATO III cells

It has been shown in a number of genes that the methylation statuses of the individual CpG sites in the respective CpG islands are heterogeneous, and that the overall methylation profile in the CpG islands exhibits a specific pattern (Graff et al. 1997; Kominato et al. 1999; Pao et al. 2001). In the bisulfite genomic sequencing analysis described above, the methylation statuses of the individual CpG sites in the Sd^a promoter region were found to be heterogeneous in KATO III cells. Therefore, the methylation pattern in the KATO III cells was analyzed in detail extending from nucleotides −550 to +380, which nearly encompasses the whole putative CpG island of the Sd^a gene. Eighty-five CpG dinucleotides are included in the analyzed region. Fifteen clones, which were prepared from five separate batches of PCR products, were sequenced, and the results compiled to calculate the methylation percentage for each of the 85 CpG sites. The methylation percentages of individual CpG sites in the Sd^a CpG island in KATO III cells (as schematically represented in Figure 5) are highly heterogeneous. Many CpG sites were intensively methylated, including 16 sites showing methylated in all of the 15 analyzed clones, whereas several sites exhibited very low methylation frequencies.

Further, a specific methylation pattern in the CpG island was observed in KATO III cells. As shown in Figure 5, the methylation density in the region around nucleotides −350 through −200 (the 5′ region proximal to exon 1_S) is evidently lower than those in the other regions. The 15 CpG dinucleotides in the 5′ 150-bp region proximal to exon 1_L have an average methylation of 87%, while the 14 CpG sites in the 5′ 150-bp region proximal to exon 1_S have an average methylation of 49%. This lower methylation density in the region 5′ to exon 1_S may explain the trace expression of the short-form Sd^a transcript in KATO III cells.

Effect of the DNA methylation inhibitor on the expression of Sd^a β4GalNAcT-II gene and Sd^a antigen

The results described above demonstrate a good correlation between methylation in the promoter region of the Sd^a β4GalNAcT-II gene and restraint of Sd^a β4GalNAcT-II gene expression in gastrointestinal cancer cell lines. To discover whether methylation of the promoter region is itself inhibiting the expression of the Sd^a gene, the effects of the DNA methylation inhibitor on the expression of the Sd^a gene and Sd^a antigen in the cells with the hypermethylated Sd^a promoter were examined. To achieve this, 5-aza-2′-deoxycytidine (5-aza-CdR), which is a well-known reagent that specifically inhibits DNA

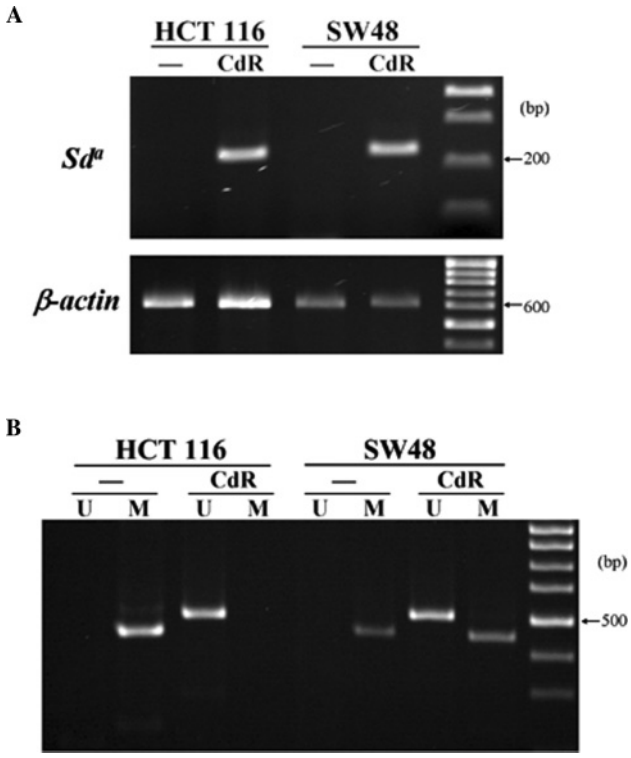

Fig. 6. (**A**) Induction of Sd^a β4GalNAcT-II gene expression by the DNA methylation inhibitor. Expression of the Sd^a transcript in HCT 116 and SW48 cells, with or without 5-aza-CdR treatment (5 μM of for 6 days), denoted as CdR and—, respectively, was analyzed using RT-PCR. The primer pair for the amplification of the common region of the short- and long-form Sd^a transcripts was used in the PCR. (**B**) Methylation status of the Sd^a promoter in the cells treated with the DNA methylation inhibitor. The methylation statuses of the Sd^a CpG promoter in HCT 116 and SW48 cells, with or without 5-aza-CdR treatment, was evaluated using methylation-specific PCR. The PCR results for the primer pairs designed for the unmethylated (USPF+USPR) and methylated statuses (MSPF+MSPR) are shown in lanes U and M, respectively. The molecular mass standards of the 100-bp ladder are shown in the rightmost lanes.

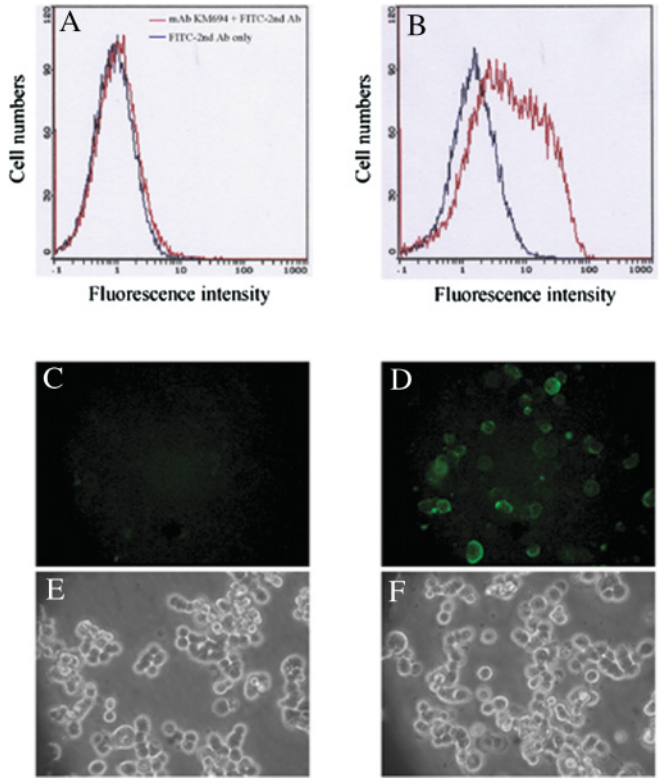

Fig. 7. Induction of Sd^a antigen expression by the DNA methylation inhibitor in SW48 cells. Expression of the Sd^a antigen on SW48 cells was analyzed using flow cytometry (upper panel; **A** and **B**) and fluorescence microscopy (lower panel; **C**–F). The cell surface Sd^a antigen was detected with mAb KM694, with the bound mAbs on the cell surface revealed by FITC-conjugated 2nd Ab, as described in Material and methods. The Sd^a antigen was not detected on the original SW48 cells (left panel; **A** and **C**), whereas, after 5-aza-CdR treatment (5 μM of for 6 days), the expression of the Sd^a antigen on SW48 cells was clearly demonstrated (right panel) using flow cytometry and fluorescence microscopy (**B** and **D**, respectively). Bright field micrographs of **B** and **D** are represented in **C** and **D**, respectively.

methylation (Momparler 2005), was employed to treat HCT 116 and SW48 cells. The results obtained from RT-PCR analysis showed that the transcription of the Sd^a gene was significantly induced by treatment of 5-aza-CdR in these cells (Figure 6A). Demethylation of the Sd^a promoter regions in HCT 116 and SW48 cells, after treatment with 5-aza-CdR, was demonstrated through bisulfite genomic sequencing; however, this demethylation was only partial. Methylation-specific PCR yielded positive results using the primer pair adopted for the unmethylated status (lanes U, Figure 6B), showing that the reagent treatment at least partially demethylated the promoter regions of the Sd^a genes. However, the failure of the primer pair for the methylated status to yield a positive result from the 5-aza-CdR-treated HCT 116 sample was unexpected.

The effect of 5-aza-CdR treatment in terms of the expression of Sd^a antigen on SW48 cells was investigated. As shown from flow cytometry analysis and fluorescence microscopy examination (Figure 7), there appeared to be no Sd^a epitope on the surfaces of the untreated SW48 cells; however, there was substantial elicitation of Sd^a antigen expression on SW48 cells with the 5-aza-CdR treatment. Fluorescence microscopy revealed that about 20% of the cells became positively bound by monoclonal antibody for the Sd^a antigen (mAB KM694) after treatment with a DNA methylation inhibitor.

These results demonstrate marked induction of Sd^a β4GalNAcT-II gene, and Sd^a antigen expression can be achieved in cells with the hypermethylated Sd^a gene promoter through inhibition of DNA methylation, indicating a direct relationship between the methylation status in the Sd^a promoter and Sd^a gene expression.

Discussion

Tissue- and cell-type specificities have been demonstrated in the expression of the Sd^a β4GalNAcT-II gene, and the alteration of the Sd^a β4GalNAcT-II gene transcription during the oncogenetic processes of gastrointestinal cancers has been noted. It is of interest, therefore, to improve understanding of the regulatory mechanism for Sd^a gene expression, and of significance to elucidate the mechanism leading to the cancer-associated down-regulation of the Sd^a gene transcription. In the present study, hypermethylation in the CpG island of the Sd^a gene promoter region was demonstrated in the gastrointestinal cancer cell lines without Sd^a gene expression, while the promoter region was

hypomethylated in the Sd^a gene-expressing cells. The results were consistent for all 10 cell lines assessed in this study. Furthermore, the suppression of the Sd^a gene transcription could be effectively relieved by the treatment of the DNA methylation inhibitor. These results demonstrate that the DNA methylation status of the CpG island in the promoter region determines the expression of the human Sd^a β4GalNAcT-II gene.

The connection between DNA methylation of the CpG-rich promoter and the transcriptional inactivation of the respective genes has been established (Scarano et al. 2005; Klose and Bird 2006). A recent systematic survey of tissue-specific differentially methylated regions of genes has demonstrated a correlation between the gene expression and the methylation status of the respective differentially methylated regions (Song et al. 2005), and the result is highly suggestive of a regulatory role for DNA methylation in gene expression. Mechanisms for methylation-induced gene suppression have been proposed, and binding of the methyl binding proteins to the methylated promoter regions appears to play a central role (Scarano et al. 2005; Klose and Bird 2006). Despite the proposed DNA methylation-triggered gene-silencing mechanisms, some studies have suggested that tissue- or cell-specific methylation may be the result of gene silencing rather than the controlling mechanism, and that the gene silencing occurs prior to DNA methylation, which takes place subsequently to stabilize the silencing (Song et al. 2002; Turker 2002). However, this proposed gene silencing prior to promoter methylation might not apply to the human Sd^a gene, as the suppression of this gene was effectively relieved, and re-expression of the Sd^a gene (Figure 6A), together with the Sd^a antigen (Figure 7), was efficiently elicited by singular treatment with a DNA methylation inhibitor in the cells with the hypermethylated Sd^a promoter. This demonstrates the crucial role of promoter-region DNA methylation in the downregulation of the Sd^a gene and Sd^a antigen expression.

Methylation of individual CpG sites in the Sd^a gene CpG island exhibits high heterogeneity in KATO III cells. Methylation heterogeneity has been described in the CpG islands of several genes (Kominato et al. 1999; Pao et al. 2001); however, the underlying mechanism leading to this phenomenon remains intangible. Except for the methylation heterogeneity, methylation in the Sd^a CpG island represents a specific pattern in KATO III cells. Relative to methylation frequencies in the other regions, methylation frequency in the 5′ region proximal to exon 1_S is lower. Differential methylation preference in specific regions of a CpG island has been demonstrated in a number of genes (Graff et al. 1997; Kominato et al. 1999; Pao et al. 2001), and the mechanism for the establishment of a specific methylation pattern in a CpG island has been an attractive subject. It has been demonstrated that a *cis*-acting sequence in the mouse adenine phosphoribosyltransferase (*aprt*) gene is responsible for the initiation of the conserved methylation pattern. The region was designated the methylation center for the *aprt* CpG island (Mummaneni et al. 1995). The methylation center sequence is derived from retrotransposons, which contribute to the human *Alu* repeats and homologous mouse B1 elements (Yates et al. 1999). Furthermore, it has been shown that the Sp1 element at the 5′ edge of the *aprt* CpG island is required for protecting the CpG island from de novo methylation (Brandeis et al. 1994; Macleod et al. 1994; Mummaneni et al. 1998). This intriguing model, which depicts the initiation of methylation spread by the *Alu* repeat-containing methylation center and the functioning of Sp1 elements as boundaries to protect the CpG island from methylation, has been suggested to be applicable to the establishment of methylation patterns of the CpG islands of other genes (Graff et al. 1997). The CpG island of the Sd^a β4GalNAcT-II gene is flanked by *Alu* elements (Figure 2), and multiple sites with the consensus sequence for the Sp1 binding are present in the Sd^a CpG island (not shown). Whether these elements play any role in the establishment of the methylation pattern in the Sd^a CpG island awaits further verification.

DNA methylation in CpG-rich promoters of genes is now recognized as a common feature of human neoplasia and cancers (Esteller 2005; Toyota and Issa 2005). Aberrant hypermethylation of gene promoters is known to be a major mechanism associated with inactivation of tumor-suppressor genes in cancers, and it is involved in almost all the critical steps of oncogenesis. In colorectal cancers, epigenetic changes in selected genes are tightly related to neoplastic transformation (Suzuki et al. 2002; Kondo and Issa 2004), and aberrant DNA methylation appears to arise very early in the colon (initially in mucosa of normal appearance), and may be part of the age-related defect in sporadic colorectal cancers (Ahuja et al. 1998; Toyota et al. 1999). It has been shown that the Sd^a antigen and Sd^a β4GalNAcT-II transferase activity are drastically diminished with the malignant changes in gastrointestinal tract mucosa (Malagolini et al. 1989; Dohi et al. 1991), and that the Sd^a β4GalNAcT-II mRNA disappears in most cancerous tissue specimens of colon and gastric cancers while it is detected in the majority of the paired tissue specimens from the normal portion (Dohi et al. 1996). Kawamura et al. showed that the forced expression of Sd^a β4GalNAcT-II suppressed the formation of the E-selectin ligands, sLe^a and sLe^x, in colon and gastric cancer cells and, consequently, reduced the metastasis potential of these cells (Kawamura et al. 2005). Thus, it can be conjectured that the downregulation of Sd^a gene expression in the oncogenetic processes facilitates the formation of the sLe^a and sLe^x antigens and, therefore, that it promotes metastasis in gastrointestinal cancers. In the present study, the results clearly indicate that DNA methylation in the promoter region plays a decisive role in the suppression of the Sd^a gene, and that the suppression can be substantially relieved by treatment with the DNA methylation inhibitor, 5-aza-CdR. 5-aza-CdR is one of the most extensively studied drugs of this type, and therapeutic efficacy has been demonstrated in several kinds of cancers (Momparler 2005). In the present study, however, the results were obtained using gastrointestinal cancer cell lines. It is of importance, therefore, to ascertain whether the downregulation of Sd^a β4GalNAcT-II gene expression during oncogenetic processes in gastrointestinal tissues occurs via this mechanism of DNA methylation in the promoter region, as demonstrated in cell lines. In addition, the possible existence of other control mechanisms, including those relative to positive and negative regulation, for expression of the Sd^a gene in gastrointestinal tissues should not be neglected, and elucidation of these mechanisms is expected.

Materials and methods

Cell culture

Human colon cancer cell lines Caco-2, Colo 205, HCT 116, SW1417, SW48, SW480, and SW948, gastric cancer cell line

KATO III, and pancreatic cancer cell line MIA PaCa-2 were purchased from the American Type Culture Collection (Manassas, VA). Human gastric cancer cell line MKN45 was purchased from the Japanese Collection of Research Bioresources (Health Science Research Resources Bank, Osaka, Japan). Colo 205 and KATO III cells were grown in 90% RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/mL of penicillin, and 50 μg/mL of streptomycin. The other eight cell lines were grown in 90% Dulbecco's modified eagle media with identical supplementations.

RT-PCR

Expression of the Sd^a β4GalNAcT-II gene in various gastrointestinal cancer cell lines was evaluated by RT-PCR. Total RNAs of the assessed cancer cell lines were prepared using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The first-strand cDNAs were primed by oligo-dT primer and synthesized by M-MLV reverse transcriptase (Promega Co., Madison, WI).

PCR amplifications specific for the short- and long-form Sd^a transcripts and the common region of the two transcript forms were performed to determine the Sd^a gene expression. The synthetic oligonucleotide primers, F41 (ACGGAGTGGAGGTGATGC, locating exon 4) and R64 (TGGTGTATGTCACGTGCTG, antisense sequence, locating exon 6), were used to amplify the common region of the short- and long-form Sd^a transcripts. The synthesized cDNA sample and 5 pmole of each primer were combined in 12.5 μL of PCR buffer containing 0.2 mM of dNTP and 0.625 U of Expand DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The PCR program included 2 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 62°C, and 1 min at 72°C. The forward primers of F3 (GGGATTCGGGATGACTT, locating exon 1_S) and F7 (CTCTGCTTGGAACTCAGA, locating exon 1_L) were used for specific amplification for the short- and long-form Sd^a cDNAs, respectively, with the reverse primer, R10 (TGGCTTCACATTTGCACTG, antisense sequence, locating exon 3), common for the two cDNA forms. The PCR conditions were similar to those mentioned above, except for annealing at 60°C. PCR for the β-*actin* cDNA was performed using the forward and reverse primers with sequences of CCTCGCCTTTGCCGATCC and GGATCTTCATGAGGTAGTCAGTC (antisense sequence), respectively, and 25 cycles of PCR at an annealing temperature of 58°C were performed. The PCR products were analyzed using 1.5% agarose gel electrophoresis.

Bisulfite genomic sequencing

Genomic DNAs of gastrointestinal cancer cell lines were prepared using the QIAamp DNA Blood Mini Kit (Qiagen). The bisulfite modification method was employed to determine the methylation status of cytosine residue in genomic DNA (Frommer et al. 1992). In this method, sodium bisulfite is used to convert cytosine residue to uracil in single-strand DNA under conditions whereby 5-methylcytosine remains nonreactive. In the following PCR amplification, the uracil and 5-methylcytosine residues in the sequence under investigation are amplified as thymine and cytosine, respectively. Bisulfite modification of the genomic DNA samples was achieved using the MethylEasy High Throughput DNA Bisulphite Modification Kit (Human Genetic Signatures Pty Ltd., Sydney, Australia).

The upper strand of the bisulfite-modified DNA segment encompassing the promoter and exon 1_S regions of the Sd^a gene was amplified using nested PCR. The first round PCR was performed with primers for the modified sequences, mF1 (TAGGATAGTAGGTGTGGAGTG, nucleotides −601 through −581, transcription start nucleotide of exon 1_L as +1) and mR2 (AACCAACRCTCCCCATTCCT, complementary of nucleotides +56 through +75) (Figure 1). Twenty nanograms of bisulfite-modified genomic DNA and 5 pmole of each primer were combined in 12.5 μL of PCR buffer containing 0.2 mM of dNTP and 0.25 U of *Taq* DNA polymerase (Promega). The PCR program consisted of 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The second round PCR was performed using the primers for the modified sequences, mF5 (TGAAGTGGAGGGTGGAGGTGGGATT, nucleotides −575 through −551) and mR4 (TCACCTCRCCTCTACAACCACCTAT, complementary of nucleotides +1 through +25). The conditions for the second round PCR were similar to those in the first PCR, except that annealing was at 65°C. The PCR products were cloned into the pGEM-T Easy vector (Promega). DNA sequences were determined using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

The methylation statuses of individual CpG sites in the Sd^a gene CpG island in KATO III cells were analyzed in detail. The upper strand of the bisulfite-modified DNA segment encompassing the majority of the putative CpG island of the Sd^a gene was PCR amplified. The first round PCR was performed using primers for the modified sequences, mF1 and mR6 (CTTTCAAATATACTTTACCCCRTTA, complementary of nucleotides +446 through +470). The PCR program consisted of 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C. The second round PCR was performed using the primers for the modified sequences, mF5 and mR8 (ACCACACAACCRCAACTCACTCTACC, complementary of nucleotides +381 through +406), at an annealing temperature of 68°C. Five separate PCR amplifications were performed, and the PCR products cloned. Fifteen clones from the five separate batches of PCR products were selected and the sequence determined.

Methylation-specific PCR

Methylation-specific PCR analysis was established to evaluate the DNA methylation status in the Sd^a gene promoter. The primer pair of USPF and USPR for the modified sequences, GAGTTGTGGGTGGGTTAAGGTTGTG (nucleotides −523 through −499) and ACCACCCACTCCCTAACTCCAACA (complementary of nucleotides −53 through −30) (Figure 1), respectively, was designed for the unmethylated status. The primer pair of MSPF and MSPR for the modified sequences, GTTGCGGGTGGGTTAAGGTCGCG (nucleotides −521 through −499) and CTACAAACGCCAAAAAAAACCCGCG (complementary of nucleotides −103 through −79), respectively, was designed for the methylated status. The primer pair of mF1 and mR2 was used for the first round PCR, with the bisulfite-modified genomic DNAs serving as templates. The primer pairs for the unmethylated and methylated status (USPF+USPR and MSPF+MSPR, respectively) were then used separately in the second round PCR. The PCR program in the second round PCR consisted of 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C. The products were analyzed using 1.5% agarose gel electrophoresis.

Treatment of cells with a DNA methylation inhibitor

HCT116 and SW48 colon cancer cells were split into six-well culture plates at a density of 1×10^5 cells/mL, and 5-aza-CdR (Sigma-Aldrich Co., St. Louis, MO), a potent and specific inhibitor of DNA methylation, was added to a final concentration of 5 μM. Cells were incubated for 6 days before harvesting total RNAs and genomic DNAs and examining cell surface Sd^a antigen expression.

Analysis of cell surface Sd^a antigen expression

The cell surface Sd^a antigen was detected using the monoclonal antibody (mAB) KM694, a generous gift from the Pharmaceutical Research Center, Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). The mAB KM694 was initially established as a mAB against GM2 ganglioside; however, greater binding affinity for the Sd^a structure was subsequently demonstrated (Kawamura et al. 2005). To examine the expression of the Sd^a antigen on the surfaces of the SW48 cells, the cells were incubated with 0.25 μg/mL of mAB KM694 after blocking with 5% bovine serum albumin in phosphate-buffered saline. The bound mABs were detected by incubation with 0.5 μg/mL of fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgM (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and then the cells were subjected to flow cytometry. If the FITC signal bound on the cell surfaces was to be examined by fluorescence microscopy, the cells were incubated with 5 μg/mL of mAB KM694.

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Conflict of interest statement

None declared.

Abbreviations

Aprt, adenine phosphoribosyltransferase; 5-aza-CdR, 5-aza-2′-deoxycytidine; β4GalNAcT, β-1,4-*N*-acetylgalactosaminyltransferas; FITC, fluorescein isothiocyanate; RBCs, red blood cells; RT-PCR, reverse transcription-polymerase chain reaction; sLe^a, sialyl Lewis^a; sLe^x, sialyl Lewis^x.

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