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Nano-Particle Materials Prepared From a Synthetic Antigenic Sequence of *Plasmodium falciparum* Enolase

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*Malaria is the major cause of mortality and morbidity in the tropical and
subtropical regions in the world. Our previous studies have shown that a
series of partial peptides of a Plasmodium falciparum enolase have antigenic
reactivity against patients' sera. In this paper, we wish to report
nano-encapsulation of a synthetic antigen into bioabsorbable polymer particles
and their releasing studies in vitro and in vivo.*

*Keywords: Plasmodium falciparum, enolase, malaria, peptide, antigen,
nanosphere, fluorescence spectroscopy, in vivo imaging.*

Introduction

The newest survey, *World Malaria Report 2008* [1] has described that half of
the world's population is at risk of malaria, and an estimated 247 million cases led to
nearly 881,000 deaths in 2006 especially caused by falciparum malaria caused by
the infection one of in 2006. In Japan, although indigenous malaria was eradicted
in 1961, an increasing number of grobal travel has resulted 100-170 cases of
imported malaria per year [2].

We have studied a glycolytic enzyme, enolase [3] from *Plasmodium
falciparum* (*P.f.*), which is the most lethal among four malarial parasites caused
human infection. In 1990, an antigen toward *P.f.* enolase was found in patients'

sera based on the field studies by Kano and Suzuki [4]. This is a probable evidence to support an epidemiological view of which residents in an endemic area are often immune to malaria. To prove this finding, we have started synthetic studies of partial peptides based on the amino acid sequence of $P.f.$ enolase, and successfully found similar antigenic property [5]. Among several sequences, we have especially focused on a sequence Ala^{256}-Asp^{277} (AD22, Figures 1c) by using solid and solution phase methods in our laboratory [6]. Our reaserch goal is to make a vaccination materials to realize the immunity condition in endemic area.

For the development of effective malaria control methods, micro- and nano-particles made of polymers and inorganic materials have been intensively investigated for *in vivo* vaccine formulation systems [7] and *in vitro* diagnostic devices [8]. In this paper, we wish to report nano-encapsulation and slow releasing studies *in vitro* and *in vivo* of the synthetic enolase antigen in bioabsorbable polymer particles.
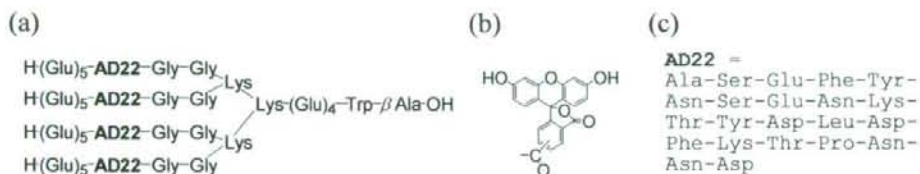
(a)

H(Glu)₅–**AD22**–Gly–Gly
H(Glu)₅–**AD22**–Gly–Gly⟩Lys
H(Glu)₅–**AD22**–Gly–Gly⟩Lys–(Glu)₄–Trp–β-Ala-OH
H(Glu)₅–**AD22**–Gly–Gly⟩Lys
H(Glu)₅–**AD22**–Gly–Gly

(b)

(c)

AD22 =
Ala-Ser-Glu-Phe-Tyr-
Asn-Ser-Glu-Asn-Lys-
Thr-Tyr-Asp-Leu-Asp-
Phe-Lys-Thr-Pro-Asn-
Asn-Asp

Figure 1. (a) Antigenic peptide (1) and (b) a fluorescent label, 5(6)-carboxyfluorescein, used in this study. (c) A potential antigenic sequence, Ala^{256}-Asp^{277} (AD22) in P.f. enolase is incorporated in this peptide.

Results and Discussion
The peptide antigen **1** was constructed by solid-phase synthesis using Fmoc-amino acids bearing benzyl-type protecting groups on the side chain functional groups. For amino acid analysis of **1**, acid hydrolysis was carried out with a 4 M CH_3SO_3H aqueous solution at 110°C for 24 h. The observed (calculated) composition of amino acids are follows: Asp and Asn, 28.8 (28); Glu, 33.1 (32); Ser 7.9 (8); Thr, 7.7 (8); β-Ala, 1.9 (1); Ala, 3.9 (4); Pro, 4.2 (4); Tyr, 7.8 (8); Leu, 4.0 (4); Phe, 8.0 (8); Trp 1.4 (1); Lys 23.6 (22). **1** was also labeled with 5(6)-carboxyfluorescein (CF) and used for the nano-encapsulation in poly(lactic acid-*co*-glycolytic acid) (PLGA).

PLGA nanospheres were formulated using an oil/water emulsion technique. For example, 2 mg of CF-**1** in 2 mL of acetic acid was emulsified in 7.0 mL $CH2Cl2$ containing 375 mg PLGA (75:25 for lactic and glycolytic acid, mw~20,000) using a probe type ultrasonic homogenizer. The resulted primary emulsion was added into 180 mL of 0.5% poly(vinylalcohol) and was further homogenized to form the secondary emulsion. The secondary emulsion was continued stirring for 5h to complete hardning. The produced microspheres were then collected by centrifugation (2,000g). Particle size was determined with a scanning electron microscope, presented a distribution between 0.3 and 1.5 mm as shown in Figure 2a.

Fluorescence intensity has shown the loading of CF-**1** was determined as 4.0
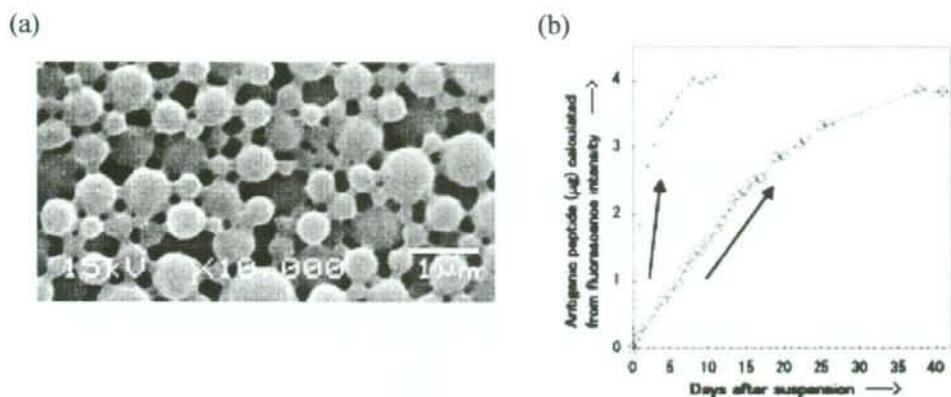
(a)   (b)

Figure 2.   (a) Scanning electron micrograph of antigen loaded microspheres. (b) Cumulative in vitro release profiles of CF-1 from nano-spheres (-O-) in 0.2% SDS containing PBS at 37°C.   As a comparison, the fluorescence intensity changes were plotted for the suspension of CF-1 (-x-).   From this profile, CF-1 dissolves slowly in SDS-PBS solvent due to difficult solubility of the antigenic sequence. Conditions: fluorescent spectrophotometer (JASCO FP6200); excitation at 490 nm; detection at 500-650 nm; slit width 5 nm.

(a)   (b)
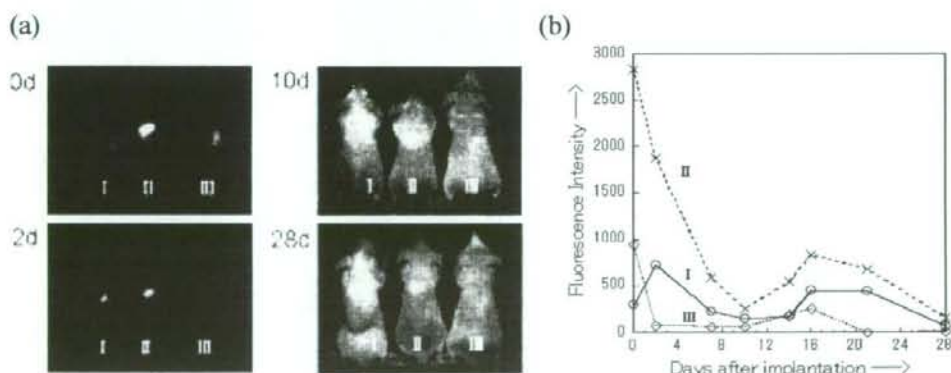
Figure 3.   (a) Fluorescence images after subcutaneous implantation of biodegradable nano-particles containing a carboxy-fluorescein labeled synthetic peptide.   Conditions: fluorescence imager (CRi Maestro FL500); excitation 445-490 nm (band-path filtered); detection 500-720 nm (spectral images taken every 10 nm).   (b) Fluorescence intensity changes after implantation were plotted from the resolved images of fluorescein spectrum.

μg in 1.0 mg of nanospheres.   The release profile of the antigen CF-1 from the particles evidenced nearly zero-order kinetics (Figure 2b).   Therefore, with this

preparation method, the nanospheres did not show first release ("initial burst") property.

Understanding the *in vivo* behavior of polymeric nano-spheres is crucial for the successful application of this antigen delivery system. Therefore, 1.0 mg nanospheres containing fluorescein-labeled antigen (4.0 µg) were suspended in physiological saline and implanted subcutaneously in each nude mouse. As shown in Figure 3b, obtained images of experimental mice showed that the fluorescence intensity gradually decreased to minimum level until 12 days. Interestingly, the intensity was increasing at 12~16 days and then decreasing again at 16~28 days. This first intensity change is attributable to the degradation or phagocytosis. The second intensity changes are due to the fluorescein uptake by adipocytes which is actually observed in tissue sections. This result shows that antigen containing nanospheres are a viable candidate for long-acting nano-particular protein delivery.

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BASIC—ALIMENTARY TRACT

DNA Hypermethylation Contributes to Incomplete Synthesis of Carbohydrate Determinants in Gastrointestinal Cancer

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See editorial on page 305.

Background & Aims: It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants. The aim of this study was to clarify the cause of cancer-associated abnormal glycosylation in gastrointestinal (GI) cancers. ***Methods:*** We compared the expression levels of "glyco-genes," including glycosyltransferases and glycosidases, in normal GI mucosa and in gastric and colorectal cancer cells. To examine the possibility that DNA hypermethylation contributed to the down-regulation of these genes, we treated GI cancer cells with 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase. ***Results:*** The silencing of some of these glyco-genes, but not up-regulation of certain molecules, was observed. The Sd^a carbohydrate was abundantly expressed in the normal GI mucosa, but its expression was significantly decreased in cancer tissues. When human colon and gastric cancer cells were treated with 5-aza-dC, cell surface expression of Sd^a and the transcription of B4GALNT2, which catalyzes the synthesis of the Sd^a, were induced. The promoter region of the human B4GALNT2 gene was heavily hypermethylated in many of the GI cancer cell lines examined as well as in gastric cancer tissues (39 out of 78 cases). In addition, aberrant methylation of the B4GALNT2 gene was strongly correlated with Epstein-Barr virus-associated gastric carcinomas and occurred coincidentally with hypermethylation of the ST3GAL6 gene. ***Conclusions:*** Epigenetic changes in a group of glycosyltransferases including B4GALNT2 and ST3GAL6 represent a malignant phenotype of gastric cancer caused by silencing of the activity of these enzymes, which action may eventually induce aberrant glycosylation and expression of cancer-associated carbohydrate antigens.

It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants.[1] Many glycosyl epitopes such as sialyl Tn, Tn, T, and sialyl Lewis x/a (sLe$^{x/a}$) have been reported to be cancer-associated antigens. Some of them show statistically significant correlations between the degree of their expression in cancer tissues and the postoperative prognosis of patients with many types of human cancers.[2–4] In addition, sLe$^{x/a}$ determinants are known to serve as ligands for E-selectin, which is inducibly expressed by endothelial cells, in hematogenous metastasis of cancers.[5,6] A long-standing debate is which is more important in understanding cancer-associated carbohydrate antigens, "neo-synthesis" or "incomplete synthesis." To verify the former hypothesis, the levels of many glycosyltransferases involved in "neo-synthesis" of tumor-related glycosyl epitopes and their mRNA expression have been studied; however, no conclusive results have been obtained to date.[7–9]

On the other hand, there is a group of carbohydrate determinants that is less expressed in cancer tissues when compared with their level in normal tissues. Because their structures are commonly more complicated, the concept of "incomplete synthesis," that the synthesis of complex carbohydrate determinants in nonmalignant cells might be impaired upon malignant transformation, has been proposed as an important cause of cancer-associated abnormal glycosylation.[10] The blood group Sd^a carbohydrate antigen serves as a typical example among the latter
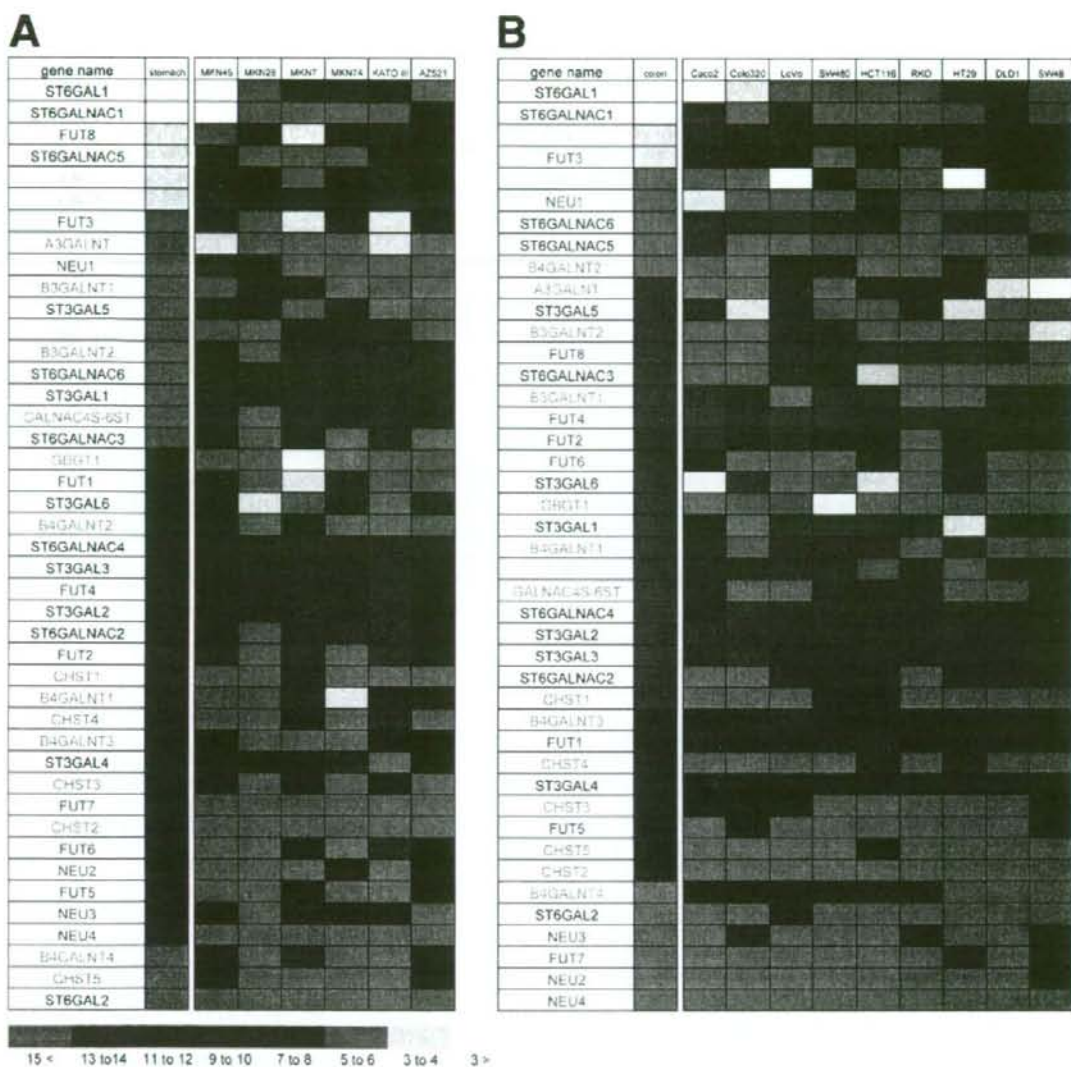

Figure 1. Profiles of expression of glycosylation-related genes in human GI tissues and cancer cell lines. Quantitative PCR analysis was carried out on normal human stomach and gastric cancer cell lines (A) and on normal human colon and CRC cell lines (B). Human glyco-genes, encoding 8 fucosyltransferases (classified by *blue*), 8 N-acetylgalactosaminyltransferase genes (*red*), 3 N-acetylglucosaminyltransferase genes (*orange*), 14 sialyltransferase genes (*black*), 6 sulfotransferase genes (*green*), and 4 sialidase genes (*purple*) were examined. Expression levels of each gene were sorted according to ΔCt (see Materials and Methods) calibrated by using GAPDH and visualized by color as indicated by the bar below.

group. This carbohydrate determinant is abundantly expressed on glycolipids and glycoproteins in the normal gastrointestinal (GI) tract mucosa in the majority of humans; however, its expression in cancer tissue is strikingly reduced or absent.[11,12] The last step in the biosynthesis of Sd[a] is catalyzed by β1,4-N-acetylgalactosaminyl-transferase (β1,4GalNAcT). The activity of the β1,4GalNAcT responsible for synthesizing the Sd[a]

determinant (Sd[a]-β1,4GalNAcT) also dramatically decreases in gastric and colonic cancer tissue.[13,14] Recently we reported that forced expression of Sd[a]-β1,4GalNAcT in GI cancer cells reduced their expression of sLe[x/a] carbohydrates and decreased their metastatic potential in nude mice, probably owing to competition with sLe[x/a] synthases for acceptor carbohydrate.[15] Thus, the lack of Sd[a] antigens in cancer cells is functionally important;

however, very little is known about the molecular mechanism underlying the regulation of Sd^a expression.

In line with these hypotheses of "neo-synthesis" and "incomplete synthesis," we compared the expression of "glyco-genes," including glycosyltransferases and glycosidases, in normal GI mucosa with that in gastric and colorectal cancer (CRC) cells in this study. Recently, epigenetic changes, such as DNA hypermethylation, have been recognized as one of the important mechanisms for gene inactivation.[16] In this study, we investigated the possible role of aberrant methylation in the glycosyltransferase gene promoter region in human GI cancer cells. We also examined epigenetic changes in a group of glycosyltransferases in human gastric cancer tissues and analyzed their relation to clinicopathologic features of the cases.

Materials and Methods

Cell Lines and Specimens

The gastric and colon carcinoma cell lines that were used in this study were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) or the American Tissue Type Collection (Manassas, VA). Human CRC cell line HCT116 with genetic disruption of DNMT1 (DNMT1 KO) or both DNMT1 and DNMT3b (DKO) were established as described previously.[17] The 78 gastric tumor specimens and their paired normal tissue specimens were obtained from 78 randomly selected Japanese patients. Informed consent was obtained from all patients before the samples were collected.

Reverse Transcription-Polymerase Chain Reaction

Quantitative polymerase chain reaction (PCR) of glyco-genes was performed by using ABI TaqMan probes (Applied Biosystems, Foster City, CA) as described previously.[18,19] Threshold cycle numbers (Ct) were determined with Sequence Detector software and transformed by using the ΔCt method as described by the manufacturer, with *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) used as the calibrator gene. Human glyco-genes examined in this study, 8 genes encoding fucosyltransferases (*FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7,* and *FUT8*), 8 N-acetylgalactosaminyltransferase genes (*A3GALNT, GBGT1, B3GALNT1, B3GALNT2, B4GALNT1, B4GALNT2, B4GALNT3,* and *B4GALNT4*), 3 N-acetylglucosaminyltransferase genes (*GCNT1, GCNT3,* and *GCNT4*), 14 sialyltransferase genes (*ST3GAL1, ST3GAL2, ST3GAL3, ST3GAL4, ST3GAL5, ST3GAL6, ST6GAL1, ST6GAL2, ST6GALNAC1, ST6GALNAC2, ST6GALNAC3, ST6GALNAC4, ST6GALNAC5,* and *ST6GALNAC6*), 6 sulfotransferase genes (*GALNAC4S-6ST, CHST1, CHST2, CHST3, CHST4* and *GCNT5*), and 4 sialidase genes (*NEU1, NEU2, NEU3,* and *NEU4*), and TaqMan probe kits used in this study are summarized in Supplementary Table 1 (see supplementary material online at www.gastrojournal.org). Human stomach and colon total RNA (BioChain, Hayward, CA) were used as

normal controls; they were prepared from normal stomachs and colon mucosae pooled from healthy subjects.

Flow Cytometry

Flow cytometry was performed with a FACScan (BD Bioscience, Franklin Lakes, NJ). Monoclonal antibody (mAb) KM694 (directed against Sd^a) was provided by Tokyo Research Laboratories (Kyowa Hakko Kogyo Co, Ltd. Tokyo, Japan).

Combined Bisulfite Restriction Analysis and Bisulfite Sequencing

We assessed gene methylation by using primers that were designed to amplify both the methylated and unmethylated alleles.[20] Bisulfite modification was carried out by using an EpiTect Bisulfite Kit (Qiagen, Tokyo, Japan). For combined bisulfite restriction analysis (COBRA), the PCR primers used for B4GALNT2 were 5'-ATTGGTTTTTTYGTATAGGTGGTTG-3' and 5'-CCRAACCRATTCCCACACTC-3', yielding a PCR product of 174 bp. Primers for ST3GAL6[21] were 5'-GTTTGTTATATYGGGTYGTAGAAG-3' and 5'-AATTAAAACTAACRAAAACCTAAAACT-3' (162 bp). The products were then digested with the restriction endonuclease HhaI (for *B4GALNT2*) or AfaI (for *ST3GAL6*), which cleave only methylated CpG sites. For bisulfite sequencing, the PCR primers used for B4GALNT2 were 5'-GAGAGGTGAAATTTYGGGAGTA-3' and 5'-RAC-TATCCACAACCCRCAATC-3' (430 bp). For sequencing of the bisulfite-PCR product, the DNA fragment was purified and cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Clones for subsequent sequencing were randomly picked up.

Detection of the Epstein–Barr Virus Genome and Helicobacter pylori

To detect the Epstein–Barr virus (EBV) genome in gastric tumors, we performed real-time PCR using 2 sets of primers as described previously.[22] Consistent results were obtained with both systems. *Helicobacter pylori* (HP) infection was identified by conducting histologic review of hematoxylin and eosin-stained tissue specimens and PCR assays as described by Clayton et al.[23]

Mutation Analysis

Genomic DNA was amplified by using exon-specific primers for p53 exons 2–11 and the mutations were examined as described previously.[24]

Immunohistochemical Analysis

Frozen sections of 8-μm thickness were prepared from a surgical specimen. After blocking sections with 3% bovine serum albumin in phosphate-buffered saline and then incubating them with mAb KM694, bound mAbs were detected with fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin M (Southern Biotechnology Associates, Inc, Birmingham, AL).
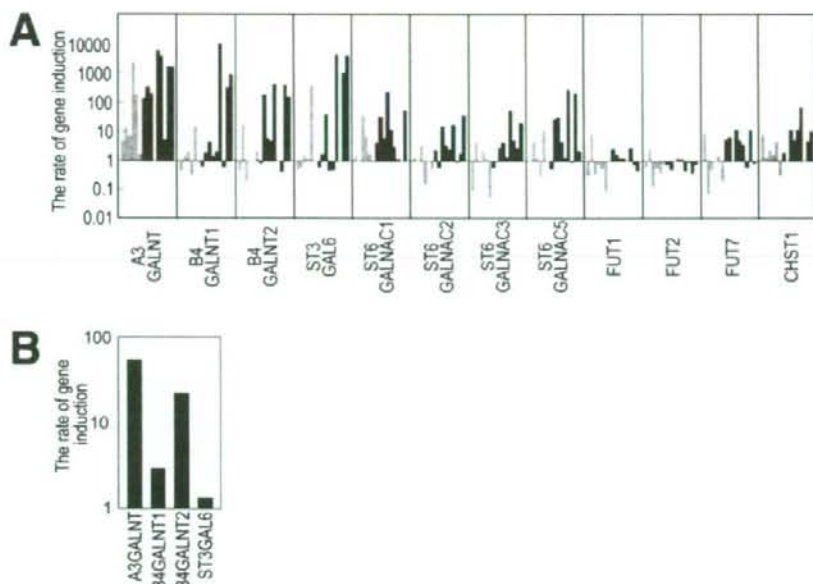
Figure 2. Expression of glycosylation-related genes in DNA methyltransferase-inhibited cells. (A) Six human gastric cancer (MKN45, MKN28, MKN7, MKN74, KATO III, and AZ521, as depicted in order from left to right by the *gray bars*) and 9 CRC (Caco2, Colo320, LoVo, SW480, HCT116, RKO, HT29, DLD1, and SW48, as depicted in order from left to right by the *black bars*) cell lines were treated with 2 μmol/L 5-aza-dC for 72 hours, and the expression level of each gene was then assessed by RT-PCR. The rate of induction is expressed as the ratio of treated to untreated cells. The genes that were analyzed are shown at the bottom of the bar graph. (B) RNA was harvested from HCT116 and DKO cells, and the expression level of each of the indicated genes was assessed by RT-PCR. The rate of induction is expressed as the ratio of induction in DKO cells to that in the parental HCT116 cells.

Statistical Analysis

Each tumor was classified based on tumor location; macroscopic type; lymphatic invasion; venous invasion (Japanese Gastric Cancer Association)[25]; pathologic tumor, lymph node, metastasis (pTMN) classification[26]; and the Lauren classification.[27] Methylation of *B4GALNT2* was compared by using the Student *t*-test for age; the Mann–Whitney *U* test for tumor size, pT status, pN status, and disease stage; and the Fisher exact test for gender, tumor location, macroscopic type, histology, lymphatic invasion, venous invasion, pM status, EBV association, HP status, p53 mutation, and methylation of ST3GAL6. The Fisher exact test was carried out by using SAS (SAS Institute Inc, Cary, NC), and other statistical analyses were made with SPSS software (version 11.0; SPSS Inc, Chicago, IL). All tests were 2-tailed, and values of $P < .05$ were considered significant.

Results

Expression of Genes Involved in the Synthesis and Modification of Carbohydrate Determinants in Human GI Tissue and Cancer Cells

It has been known that carbohydrate structures in GI cancers are quite different from those in normal GI epithelium. To clarify the cause of this abnormal glycosylation in GI cancer cells, we first examined the expression levels of 43 "glyco-genes," including 8 genes encoding fucosyltransferases, 14 sialyltransferase genes, 8 N-acetylgalactosaminyltransferase genes, 3 N-acetylglucosaminyltransferase genes, 6 sulfotransferase genes, and 4 sialidase genes (Supplementary Table 1). There was no gene whose expression was universally up-regulated in the GI cancer cell lines examined when compared with normal tissues. On the other hand, we found approximately one third of glycosyltransferase genes that were expressed in normal GI mucosa but whose expression levels were decreased in many GI cancer cell lines (Figure 1A and B). This silencing of glycosyltransferases was the major cancer-associated change detected in glyco-gene expression. To examine the possibility that DNA methylation contributed to the low expression levels of these genes, we chose 12 genes containing CpG islands in their promoter region from among cancer-associated down-regulated glyco-genes. When GI cancer cells were treated with 5-aza-2'-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, the mRNA expression of glycosyltransferases was significantly induced in many of them (Figure 2A). On the other hand, the expression of ≥2 glycosyltrans-

ferases (*FUT1* and *FUT2*) was not recovered by the 5-aza-dC-treatment, implying there might be certain glyco-genes whose expression was not controlled by DNA hypermethylation despite the presence of CpG islands. In the human CRC cell line HCT116 with genetic disruption of both *DNMT1* and *DNMT3b*,[17] in which genomic DNA methylation was nearly eliminated, the expression of *A3GALNT* and *B4GALNT2* was rescued (Figure 2B). Because it has been reported that promoter hypermethylation of the *A3GALNT* gene is associated with the loss of blood group A antigen expression in bladder cancer, oral squamous cell carcinoma, and gastric cancer cell lines,[28–30] our results suggest that aberrant methylation of the *A3GALNT* gene may lead to a cancer-associated reduction in the level of A antigen in colon cancers. Although remarkable induction of *B4GALNT1* mRNA was observed after 5-aza-dC-treatment, we excluded the *B4GALNT1* gene from subsequent analysis; because the expression of *B4GALNT1* and GM2 gangliosides synthesized by *B4GALNT1* is already known to be increased in GI cancers.[31] In any case, these results strongly suggest that down-regulation of glycosyltransferases might be the leading cause of cancer-associated abnormal glycosylation and that the *B4GALNT2* gene is a good representative of gene silencing by hypermethylation.

Recovery of Sd^a Carbohydrate Determinant in CRC Cells by Suppression of DNA Methyltransferases

The human *B4GALNT2* gene encodes a β1,4Gal-NAcT that is responsible for the synthesis of Sd^a carbohydrate antigen (Sd^a-β1,4GalNAcT). A noteworthy characteristic of the Sd^a carbohydrate determinant is that its expression is restricted to normal GI mucosa and is strikingly reduced or absent in GI cancer tissue.[11,12] So we asked if the membrane Sd^a structure could be detected in human CRC cell lines in which DNA methylation was suppressed. Treatment of T84 and HT29 human colonic cancer cell lines, which originally lacked the Sd^a carbohydrate, with 5-aza-dC resulted in an obvious increase in cell-surface expression of Sd^a along with the concomitant induction of *B4GALNT2* expression (Figure 3A and B). When these cells were treated with butyrate, a histone deacetylase inhibitor, neither expression of Sd^a antigen nor *B4GALNT2* mRNA was induced. We found that DNMT1 KO cells strongly expressed Sd^a determinants, whereas the parental HCT116 cells only weakly expressed it (Figure 3C). Furthermore, transcripts of *B4GALNT2* were detected in DNMT1 KO cells, but not in the parental HCT116 cells. These results suggest collectively that DNA hypermethylation rather than histone deacetylation may contribute to the down-regulation of *B4GALNT2* expression in cancer cells.

Methylation Status of B4GALNT2 Gene Promoter Region in GI Cancer Cell Lines and Primary Gastric Carcinomas

Next, we examined the methylation status of the upstream of the *B4GALNT2* gene in gastric cancer cell lines by COBRA. Hypermethylation in the *B4GALNT2* gene was detected in 5 of 6 human gastric cancer cell lines tested (Figure 4A, *left*). Atypical methylation in the *B4GALNT2* in a primary gastric carcinoma but not in the normal gastric mucosa adjacent to it was also found (Figure 4A, *right*). Because COBRA reflects the methylation status of only 2 adjoining CpG motifs, PCR products, extending from 169 bp upstream to 217 bp downstream from the translation start site and containing 39 CpGs, were subjected to bisulfite sequencing. Most of the CpGs examined were methylated in gastric cancer cells except in MKN45 cells, which were methylation negative by COBRA (Figure 4B). We also examined the methylation status of the *B4GALNT2* gene in DNMT1/
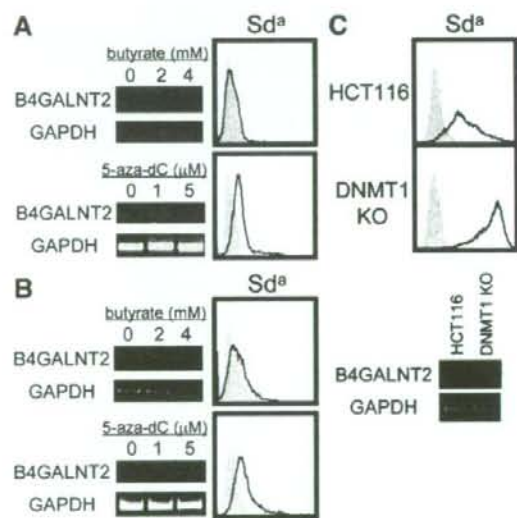

Figure 3. Effects of inhibitors on cell-surface Sd^a antigen and *B4GALNT2* mRNA expression in human CRC cells. Human CRC cell line T84 (A) and HT29 (B) were treated with the histone deacetylase inhibitor butyrate (*upper panels*) or the methyltransferase inhibitor 5-aza-dC at the concentrations indicated (*lower panels*). For RT-PCR analysis, cells were collected after 3 days of treatment, and expression levels of *B4GALNT2* and *GAPDH* were then assessed. For flow cytometric analysis, cells were treated with 4 μmol/L butyrate (*upper panels*) or 5 μmol/L 5-aza-dC (*lower panels*) for 6 days and then were stained with mAb KM694 (specific for Sd^a). Filled histograms represent the control staining of untreated cells. (C) Human CRC cell line HCT116 and DNMT1 KO cells were stained with mAb KM694 and then analyzed by flow cytometry. Filled histograms represent the control staining without mAb (*upper panels*). HCT116 and DNMT1 KO cells were also assessed for expression levels of *B4GALNT2* and *GAPDH* by RT-PCR (*lower panels*). The data are representative of 3 separate experiments, which gave similar results.
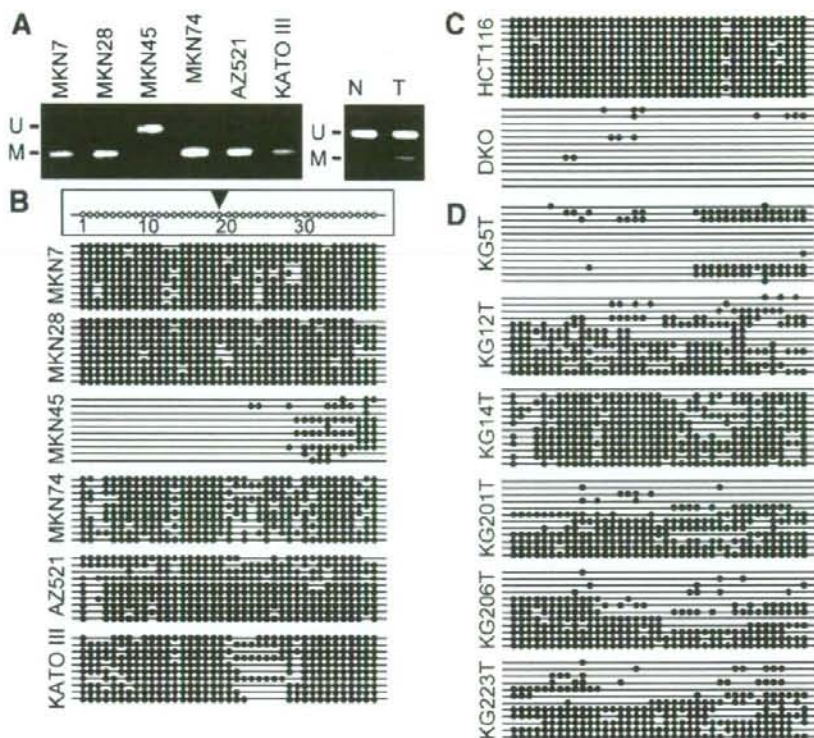
Figure 4. Methylation of the *B4GALNT2* gene in gastric cancer cells and primary gastric carcinomas. (*A*) Combined bisulfite restriction analysis (COBRA) of human gastric cancer cell lines (*left*) and representative results for a primary gastric carcinoma (*right*). M, methylated alleles; N, gastric normal mucosa adjacent to the tumor; T, gastric tumor; U, unmethylated alleles. (*B, C* and *D*) Methylation status of individual CpG residues in the *B4GALNT2* gene in human gastric cancer cell lines (*B*), human CRC cell line HCT116 and DKO cells (*C*), and primary gastric carcinomas (*D*) assessed by bisulfite sequencing. Bisulfite-PCR products cloned into the pCR4-TOPO vector were randomly picked up for sequencing. As illustrated in the box at the top of *B*, the line indicates an independent clone of bisulfite-PCR products; it contains 39 consecutive CpGs (*open circles*). For sequencing of the bisulfite–PCR product, the DNA fragment was purified and cloned into the pCR4-TOPO vector (Invitrogen). The start site of translation is indicated by the *arrowhead*. In the results shown below this box, the *filled circles* on the lines for each clone appear only when CpGs are methylated. Cell lines and case ID of tumors are shown at the left in *B* and *C*, respectively.

DNMT3b DKO cells. As expected, methylated CpGs were hardly seen in DKO cells, whereas most of the CpGs examined were methylated in the parental HCT116 cells (Figure 4C). Furthermore, it was clearly evident that the upstream of the *B4GALNT2* gene was frequently hypermethylated in human gastric cancer tissues (Figure 4D). Sample KG5T, methylation negative by COBRA, and MKN45 cells looked less methylated but included apparently hypermethylated clones. These results imply that DNA hypermethylation in the promoter region of the *B4GALNT2* gene may have contributed to the down-regulation of *B4GALNT2* expression in gastric cancers.

Methylation Status of B4GALNT2 Gene and Clinicopathologic Characteristics in Primary Gastric Carcinomas

To understand the significance of hypermethylation in the *B4GALNT2* gene, we analyzed the methylation status

of the *B4GALNT2* gene and clinicopathologic characteristics of patients with gastric carcinomas. We deemed that the *B4GALNT2* gene was methylated when the percentage of methylated DNA was ≥10% by COBRA. Of the 78 primary gastric tumors studied, 39 were classified as methylation positive (Table 1). Univariate analysis revealed no difference between the methylation-positive and -negative groups with respect to age, gender, tumor location, macroscopic type, lymphatic invasion, venous invasion, or pT, pN, or pM status. However, there were significant differences between patients in the methylation-positive and -negative groups with respect to histology ($P = .012$) and EBV status ($P = .001$). EBV was detected in 10 of the 78 tumors, and all EBV-associated tumors were methylation-positive ones. No difference was noted in the frequency of *p53* mutation or the infection of HP between the methylation-positive and -negative groups. To examine the correlation between the

Table 1. Clinicopathologic Features of Gastric Cancer With or Without Methylation of *B4GALNT2*

Characteristics	Total	Number of patients (%)		P-value
		Methylated	Unmethylated	
Number of patients	78	39 (50.0)	39 (50.0)	
Mean age ± SD (y)		63.6 ± 13.6	65.4 ± 10.3	.531
Gender				
Male	52 (66.6)	24 (61.5)	28 (71.8)	.472
Female	26 (33.3)	15 (38.5)	11 (28.2)	
Tumor location				
Upper one third	22 (28.2)	14 (35.9)	8 (20.5)	.279
Middle one third	23 (29.5)	9 (23.1)	14 (35.9)	
Lower one third	33 (42.3)	16 (41.0)	17 (43.6)	
Macroscopic type				
0	4 (5.1)	2 (5.1)	2 (5.1)	.98
1	6 (7.7)	3 (7.7)	3 (7.7)	
2	30 (38.5)	14 (35.9)	16 (41.0)	
3	30 (38.5)	15 (38.5)	15 (38.5)	
4	8 (10.3)	5 (12.8)	3 (7.7)	
Histology (Lauren)				
Intestinal	36 (46.2)	12 (30.8)	24 (61.5)	.012
Diffuse	42 (53.8)	27 (69.2)	15 (38.5)	
Lymphatic invasion				
Negative	20 (25.6)	9 (23.1)	11 (28.2)	.78
Positive	58 (74.4)	30 (76.9)	28 (71.8)	
Venous invasion				
Negative	37 (47.4)	20 (51.3)	17 (43.6)	.651
Positive	41 (52.6)	19 (48.7)	22 (56.4)	
Pathologic tumor classification				
pT1	5 (6.4)	3 (7.7)	2 (5.1)	.407
pT2	43 (55.1)	19 (48.7)	24 (61.5)	
pT3	28 (35.9)	15 (38.5)	13 (33.3)	
pT4	2 (2.6)	2 (5.1)	0 (0.0)	
Pathologic lymph node status				
pN0	22 (28.2)	9 (23.1)	13 (33.3)	.373
pN1	28 (35.9)	15 (38.5)	13 (33.3)	
pN2	16 (20.5)	8 (20.5)	8 (20.5)	
pN3	12 (15.4)	7 (17.9)	5 (12.8)	
Pathologic metastasis status				
pM0	66 (84.6)	36 (92.3)	30 (76.9)	.114
pM1	12 (15.4)	3 (7.7)	9 (23.1)	
Stage (pTNM)				
I	18 (23.1)	8 (20.5)	10 (25.6)	.804
II	16 (20.5)	8 (20.5)	8 (20.5)	
III	21 (26.9)	12 (30.8)	9 (23.1)	
IV	23 (29.5)	11 (28.2)	12 (30.8)	
Helicobacter pylori				
Positive	65 (83.3)	33 (84.6)	32 (82.1)	.999
Negative	13 (16.7)	6 (15.4)	7 (17.9)	
Epstein–Barr virus				
Positive	10 (12.8)	10 (25.6)	0 (0.0)	.001
Negative	68 (87.2)	29 (74.4)	39 (100.0)	
p53 mutation				
Positive	19 (24.4)	6 (15.4)	13 (33.3)	.112
Negative	59 (75.6)	33 (84.6)	26 (66.7)	

SD, standard deviation; pTNM, pathologic tumor, lymph node, metastasis status according to the International Union Against Cancer classification system.

Sd^a expression and DNA hypermethylation of *B4GALNT2*, we determined the expression levels of Sd^a carbohydrates in freshly frozen gastric cancers, because Sd^a antigen is expressed as a glycolipid in the stomach; its reactivity to antibodies was lost in formalin-fixed paraffin-embedded samples that we used for our clinicopathologic analysis. Of the 15 freshly frozen gastric cancers studied, the expression of Sd^a determinants was totally lost in all cases as determined by immunohistologic staining; 7 cases were methylation positive by COBRA (data not shown).
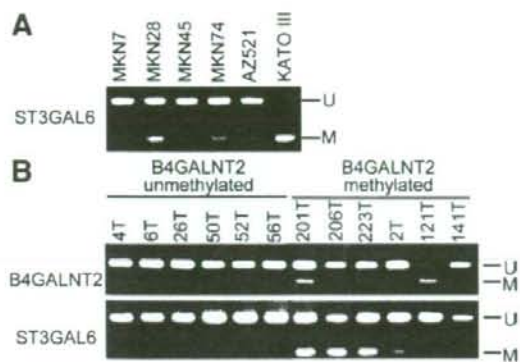
A

B

Figure 5. Methylation of *B4GALNT2* and *ST3GAL6* genes in gastric cancer cells and primary gastric carcinomas. Representative results are shown from COBRA using human gastric cancer cell lines (*A*) and primary tumors (*B*). M, methylated alleles; U, unmethylated alleles. The genes that were analyzed are shown on the left.

Methylation Status of Glyco-Genes in Gastric Cancer Cell Lines and Primary Gastric Carcinomas

Finally, we examined whether epigenetic changes occurred in the *ST3GAL6* gene together with those in the *B4GALNT2* gene in human gastric cancer cells. Of the cancer-associated down-regulated glyco-genes that we found in the present study, the *ST3GAL6* was hypermethylated in concurrence with methylation of the *B4GALNT2* in many of the gastric cancer cell lines as well as in gastric cancer tissues (Figure 4A and Figure 5). As shown in Table 2, aberrant methylation in the *ST3GAL6* was detected in 24 of 32 primary gastric tumors with statistically significant correlation with the methylation of *B4GALNT2* and EBV status ($P <$ .01). No difference was noted in the frequency of p53 mutation between the *ST3GAL6*-methylated and -unmethylated groups. These results strongly suggest that epigenetic changes may occur in a group of glyco-genes including *B4GALNT2* and *ST3GAL6* in gastric cancer tissues, which may eventually induce aberrant glycosylation and expression of cancer-associated carbohydrate antigens by silencing the enzyme activity responsible for antigen expression.

Discussion

Aberrant glycosylation, which would be expected to eventually induce the expression of cancer-associated carbohydrate antigens, has been observed in many types of tumors. In the aspect of carbohydrate synthesis, here we clearly demonstrated that the down-regulation of a set of glyco-genes involved in carbohydrate biosynthetic pathways is a major event in the cancer, rather than the up-regulation of certain glycosyltransferases. Cancer-specific DNA hypermethylation played a significant role in

this gene silencing. We further extended the analysis to 93 gastric cancer tissues and, for the first time, found a high frequency of DNA hypermethylation in glyco-genes. The significance of these issues is discussed below.

Recent studies including ours suggest clearly that the precise mechanism for up-regulation of cancer-associated carbohydrate antigens revolves not necessarily around the enhancement of glycosylation in tumors, but rather around the down-regulation of glyco-genes that are involved in the synthesis of normally expressed determinants, such as Sd[a], disialyl Lewis[a], and so on.[15,32–34] In our results, the CpG islands of the *B4GALNT2* gene encoding Sd[a]-β1,4GalNAcT, the enzyme responsible for the synthesis of the Sd[a] structure, were densely methylated; and this methylation was closely correlated with the transcriptional silencing of the *B4GALNT2* gene. Because DNA hypermethylation of the *B4GALNT2* gene was detected in 50% of our gastric cancer cases examined by COBRA, this hypermethylation seems to be an important molecular mechanism well explaining the down-regulation of Sd[a]. Our present study using freshly frozen samples reconfirmed our previous report that nearly 100% of gastric cancers showed loss of Sd[a] antigen.[12] We believe that the difference in frequency between DNA hypermethylation and loss of Sd[a] may be attributed to the rather dull sensitivity of COBRA. For example, MKN45 cells were methylation negative by COBRA, despite the apparent hypermethylation of certain areas in the promoter region as assessed by bisulfite sequencing (Figure 4A, B). Besides, in the case of the *B4GALNT2* gene and Sd[a] antigens, the methylation status of the *A3GALNT* gene, encoding the enzyme responsible for the synthesis of the blood group A and B determinants, correlates well with the expression of the blood group A and B determinants in gastric

Table 2. Methylation Status of *B4GALNT2*, EBV Infection, and *P53* Mutation of Gastric Cancer With or Without Methylation of *ST3GAL6*

Characteristic	Number of patients (%)		
		ST3GAL6	
	Total	Methylated	Unmethylated
Number of patients	63	28 (44.4)	35 (55.6)
B4GALNT2[a]			
Methylated	32 (50.8)	24 (85.7)	8 (22.9)
Unmethylated	31 (49.2)	4 (14.3)	27 (77.1)
Epstein-Barr virus[a]			
Positive	9 (14.3)	8 (28.6)	1 (2.9)
Negative	54 (85.7)	20 (71.4)	34 (97.1)
p53 mutation			
Positive	15 (23.8)	6 (21.4)	9 (25.7)
Negative	48 (76.2)	22 (78.6)	26 (74.3)

NOTE. Methylation of *ST3GAL6* was compared by using the Fisher exact test for methylation of *B4GALNT2*, EBV association, and p53 mutation.
[a]Statistically significant ($P <$ .01).

cancer cell lines including MKN28, MKN45, and KATO III cells.[30] We reported earlier that forced expression of Sd^a-β1,4GalNAcT resulted in a marked increase in cell-surface expression of Sd^a along with a concomitant loss of cancer-associated sLe$^{x/a}$ carbohydrate antigens.[15] Of note, DNA hypermethylation of *B4GALNT2* gene may be associated with the concomitant increase in sLe$^{x/a}$ in GI cancers as a typical example of "incomplete synthesis" for abnormal expression of carbohydrate determinants. In addition, we reaffirmed that DNA methylation contributes to the cancer-associated silencing of the *A3GALNT* gene in GI cancer cells. In bladder cancers and oral squamous cell carcinomas, a relationship between decreased expression of the blood group A and B determinants and allelic loss and/or hypermethylation of the *A3GALNT* gene has been reported.[28,29,35] DNA hypermethylation of this gene in GI cancers may also lead to enhanced expression of sLe$^{x/a}$ consequent to a reduction in blood group A antigen.

Further, our report provides the first description of a relationship between the methylation status of glycosyltransferase and clinicopathologic features. Throughout the present study, we observed a strong correlation between promoter methylation of the *B4GALNT2* gene and EBV-associated gastric carcinoma (Table 1). The frequency of *B4GALNT2* methylation in EBV-associated tumors was 100%. EBV is a ubiquitous herpes virus that infects most children during early childhood and is involved in a subset of gastric carcinomas, although its specific role in carcinogenesis remains unclear. It has been shown that the expression of tumor-suppressor genes, such as p16 cyclin-dependent kinase 4A inhibitor (p16^{INK4A}), is absent significantly more often in EBV-associated gastric carcinomas than in EBV-negative ones and that their loss is associated with their methylation.[36] Our finding is consistent with former reports indicating that carcinogenesis of EBV-associated gastric tumors commonly involves hypermethylation of multiple genes. Extensive studies on the molecular mechanism underlying oncogenic virus-related aberrant methylation have been carried out. It was reported that oncogenic virus-related aberrant methylation was caused by DNMT3b up-regulation via Ras activation.[37] It is plausible that aberrant methylation seen in EBV-associated gastric tumors may be based on the same molecular machinery. Furthermore, in the present study, approximately three fourths of the *B4GALNT2* methylation-positive carcinomas were EBV negative; therefore, increased methylation of this gene in tumors without EBV association might be mediated by some different, as yet unknown mechanism. HP infection of the stomach is also a significant factor related to carcinogenesis.[38] No correlation was observed between hypermethylation of *B4GALNT2* and HP status, although HP infection is extraordinarily common (83.3% in Table 1). These results suggest collectively that aberrant methylation of *B4GALNT2* might be induced by factors independent of those related to HP infection.

Another important finding of this study was that the hypermethylation occurred coincidentally in *B4GALNT2* and *ST3GAL6* genes, as was clearly shown in Figure 5 and Table 2. Because the human *ST3GAL6* gene encodes the α2,3-sialyltransferase responsible for the synthesis of type II precursor, the suppression of this gene seems to result in a lesser amount of the precursor for the biosynthesis of the Sd^a determinant. These observations allow us to suppose that epigenetic suppression of multiple glyco-genes, including glycosyltransferases, glycosidases, and mucins in tumors, may not occur in a random manner but in a certain set of them and other genes. We can add on yet the fact that some glyco-genes, whose expression is decreased in cancers and whose promoter regions contain CpG islands, seemed to be controlled epigenetically. Taken together, our data suggest that there might be a certain group of glyco-genes whose expression in cancers is controlled together by DNA hypermethylation. Although more studies on individual glyco-genes are required to support this hypothesis, simultaneous silencing of glycosyltransferases might eventually result in the induction of aberrant glycosylation and expression of cancer-associated carbohydrate antigens by inactivating their enzyme activity. In conclusion, we propose that an epigenetic change such as DNA hypermethylation is one of the major mechanisms causing cancer-associated changes in carbohydrate determinants by silencing normal glycosylation, especially being a part of the mechanism referred to previously as incomplete synthesis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.03.031.

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Supplementary Table 1. Human glyco-genes.

Genes	Accession No.	ID of TaqMan probe kits	Enzyme	References
FUT1	NM000148	Hs00355741_m1	α1,2-fucosyltransferase, H blood group α1,2-fucosyltransferase	1, 2
FUT2	NM000511	Hs00704693_s1	α1,2-fucosyltransferase, Se blood group α1,2-fucosyltransferase	3
FUT3	NM000149	Hs00356857_m1	α1,3/4-fucosyltransferase, Lewis blood group α1,3/4-fucosyltransferase	4
FUT4	NM002033	Hs00275643_s1	α1,3-fucosyltransferase	5, 6
FUT5	NM002034	Hs00704908_s1	α1,3-fucosyltransferase	7
FUT6	NM000150	Hs00173404_m1	α1,3-fucosyltransferase	8
FUT7	NM004479	Hs00237083_m1	α1,3-fucosyltransferase	9, 10
FUT8	NM178154	Hs00189535_m1	α1,6-fucosyltransferase	11
A3GALT/A3GALNT	NM020469	Hs00220850_m1	α1,3-N-galactosyltransferase, α1,3-N-acetylgalactosaminyltransferase	12, 13
GBGT1	NM021996	Hs00222752_m1	globoside α1,3-N-acetylgalactosaminyltransferase, Forssman synthetase	14
B3GALNT1	NM003781	Hs00364202_s1	β1,3-N-acetylgalactosaminyltransferase 1, globoside synthase	15
B3GALNT2	NM152490	Hs00380823_m1	β1,3-N-acetylgalactosaminyltransferase 2	16
B4GALNT1	NM001478	Hs00155195_m1	β1,4-N-acetylgalactosaminyltransferase 1, GM2/GD2 synthase	17
B4GALNT2	NM153446	Hs00396440_m1	β1,4-N-acetylgalactosaminyltransferase 2 Sdª synthase	18
B4GALNT3	NM173593	Hs00403843_m1	β1,4-N-acetylgalactosaminyltransferase 3	19
B4GALNT4	NM178537	Hs00331790_m1	β1,4-N-acetylgalactosaminyltransferase 4	20
GCNT1	NM001490	Hs00155243_m1	gulcosaminyl (N-acetyl) transferase 1, core 2 (β1,6-N-acetylglucosaminyltransferase)	21
GCNT3	NM004751	Hs00191070_m1	gulcosaminyl (N-acetyl) transferase 3	22
GCNT4	NM016591	Hs00275464_s1	gulcosaminyl (N-acetyl) transferase 4, core 2 (β1,6-N-acetylglucosaminyltransferase)	23
ST3GAL1	NM003033	Hs00161688_m1	CMP-NeuAc:β-galactoside α2,3-sialyltransferase 1	24
ST3GAL2	NM006927	Hs00199480_m1	CMP-NeuAc:β-galactoside α2,3-sialyltransferase 2	25
ST3GAL3	NM006279	Hs00196718_m1	CMP-NeuAc:Galβ1,3/4GlcNAc α2,3-sialyltransferase	26
ST3GAL4	NM006278	Hs00272170_m1	Galβ1,3/4GlcNAc α2,3-sialyltransferase	27
ST3GAL5	NM003896	Hs00187405_m1	CMP-NeuAc:lactosylceramide α2,3-sialyltransferase	28
ST3GAL6	NM006100	Hs00196086_m1	CMP-NeuAc:α2,3-sialyltransferase	29
ST6GAL1	NM003032	Hs00949382_m1	CMP-NeuAc:galactoside α2,6-sialyltransferase	30
ST6GAL2	NM032528	Hs00293264_m1	CMP-NeuAc:galactoside α2,6-sialyltransferase	31
ST6GALNAC1	NM018414	Hs00300842_m1	GalNAc α2,6-sialyltransferase 1	32
ST6GALNAC2	NM006456	Hs00197670_m1	GalNAc α2,6-sialyltransferase 2	33
ST6GALNAC3	NM152996	Hs00541761_m1	GalNAc α2,6-sialyltransferase 3	34
ST6GALNAC4	NM014403	Hs00205241_m1	GalNAc α2,6-sialyltransferase 4	35
ST6GALNAC5	NM030965	Hs00229612_m1	GalNAc α2,6-sialyltransferase 5	36
ST6GALNAC6	NM013443	Hs00203739_m1	GalNAc α2,6-sialyltransferase 6	37
GALNAC4S-6ST	NM015892	Hs00248144_m1	N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase	38
CHST1	NM003654	Hs00186341_m1	galactose-6-O-sulfotransferase	39
CHST2	NM004267	Hs00358839_g1	carbohydrate N-acetylgulcosamine-6-O-sulfotransferase 2	40
CHST3	NM004273	Hs00427946_m1	chondroitin 6-sulfotransferase	41
CHST4	NM005769	Hs00428480_m1	N-acetylgulcosamine–6-O-sulfotransferase 2, HEC-specific N-acetylglucosamine–6-O-sulfotransferase	42
CHST5	NM012126	Hs00201677_m1	N-acetylgulcosamine–6-O-sulfotransferase 3, intestinal N-acetylglucosamine–6-O-sulfotransferase	43
NEU1	NM000434	Hs00166421_m1	sialidase 1, lysosomal sialidase	44
NEU2	NM005383	Hs00193573_m1	sialidase 2, cytosolic sialidase	45
NEU3	NM006656	Hs00198406_m1	sialidase 3, plasma membrane-associated sialidase	46
NEU4	NM080741	Hs00293852_m1	sialidase 4, glyceraldehyde-3-phosphate dehydrogenase	47
GAPDH	NM002046	Hs00266705_gl		