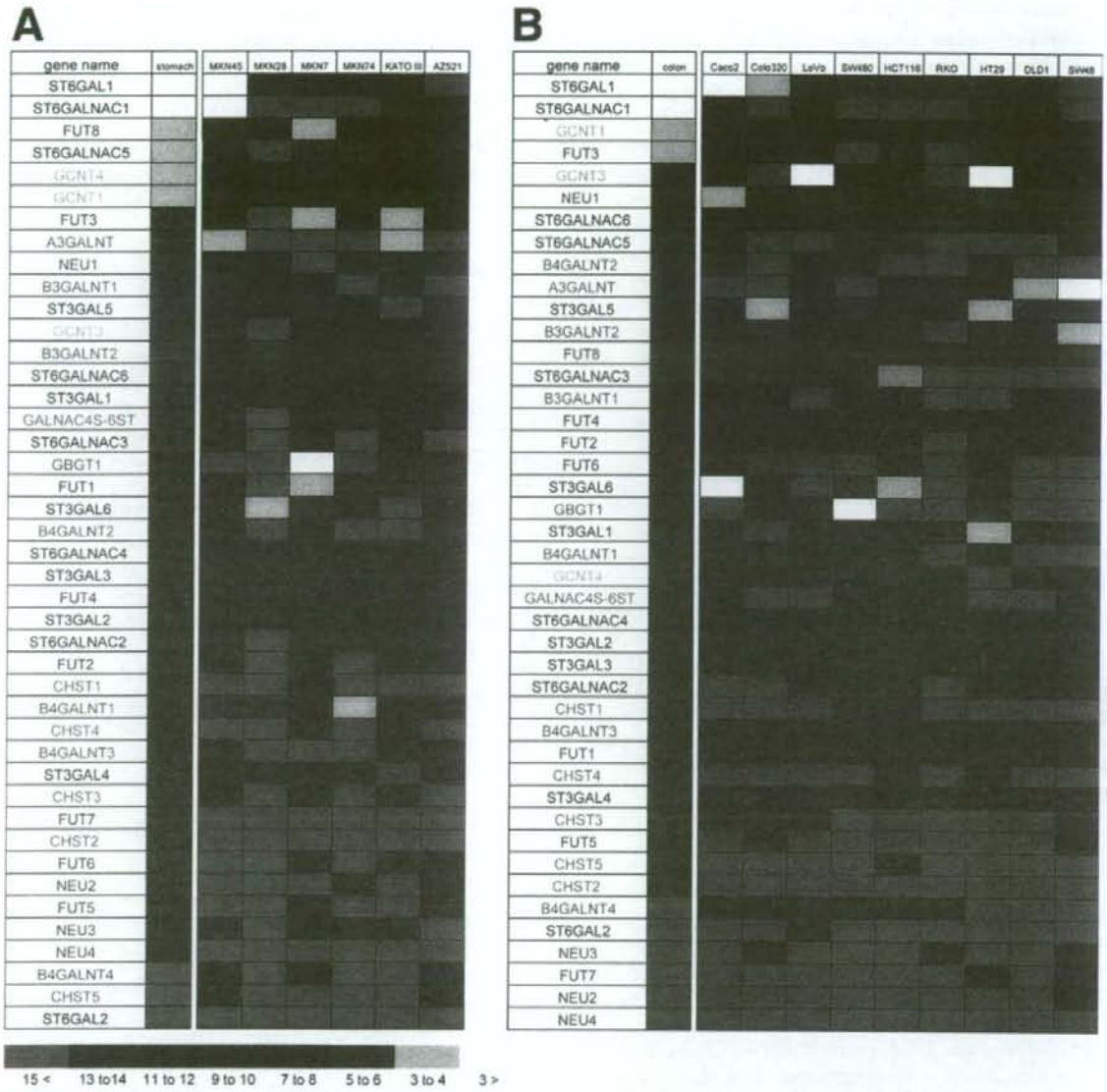

Figure 1. Profiles of expression of glycosylation-related genes in human GI tissues and cancer cell lines. Quantitative PCR analysis was carried out on normal human stomach and gastric cancer cell lines (*A*) and on normal human colon and CRC cell lines (*B*). Human glyco-genes, encoding 8 fucosyltransferases (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 Ct (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 1,4-*N*-acetylgalactosaminyl-transferase (1,4GalNAcT). The activity of the 1,4GalNAcT responsible for synthesizing the Sd^a determinant (Sd^a- 1,4GalNAcT) also dramatically decreases in gastric and colonic cancer tissue.[13,14] Recently we reported that forced expression of Sd^a- 1,4GalNAcT 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 Ct 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′-ATTGGTTTTTYGTATAGGTGGTTG-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′-RACTATCCACAACCCRCAATC-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- m 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).

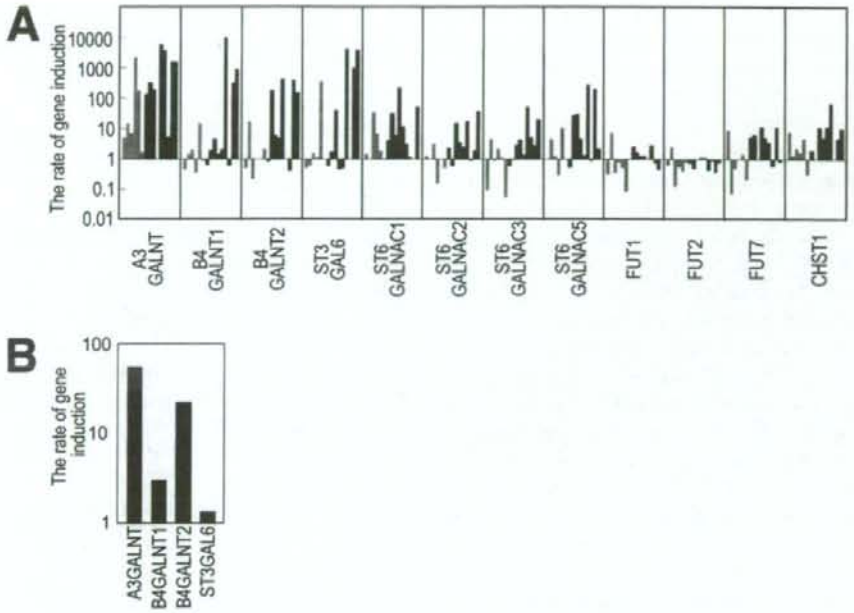

Figure 2. Expression of glycosylation-related genes in DNA methyltransferase-inhibited cells. (*A*) Six human gastric cancer (MKN45, MKN28, MKN7, MKN74, KATO III, and AZ521, as depicted in order from left to right by the *gray bars*) and 9 CRC (Caco2, Colo320, LoVo, SW480, HCT116, RKO, HT29, DLD1, and SW48, as depicted in order from left to right by the *black bars*) cell lines were treated with 2 mol/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 1*A* 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 2*A*). 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 2*B*). 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 1,4GalNAcT that is responsible for the synthesis of Sd^a carbohydrate antigen (Sd^a- 1,4GalNAcT). 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 3*A* 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 3*C*). 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 4*A*, *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 4*A*, *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 4*B*). We also examined the methylation status of the *B4GALNT2* gene in DNMT1/

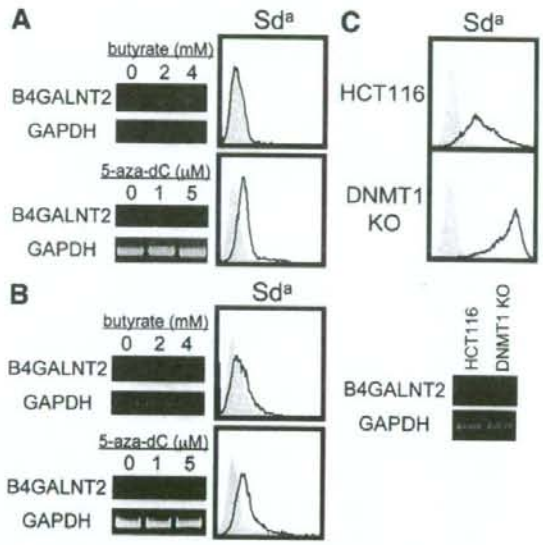

Figure 3. Effects of inhibitors on cell-surface Sd^a antigen and *B4GALNT2* mRNA expression in human CRC cells. Human CRC cell line T84 (*A*) and HT29 (*B*) were treated with the histone deacetylase inhibitor butyrate (*upper panels*) or the methyltransferase inhibitor 5-aza-dC at the concentrations 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 mol/L butyrate (*upper panels*) or 5 mol/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.

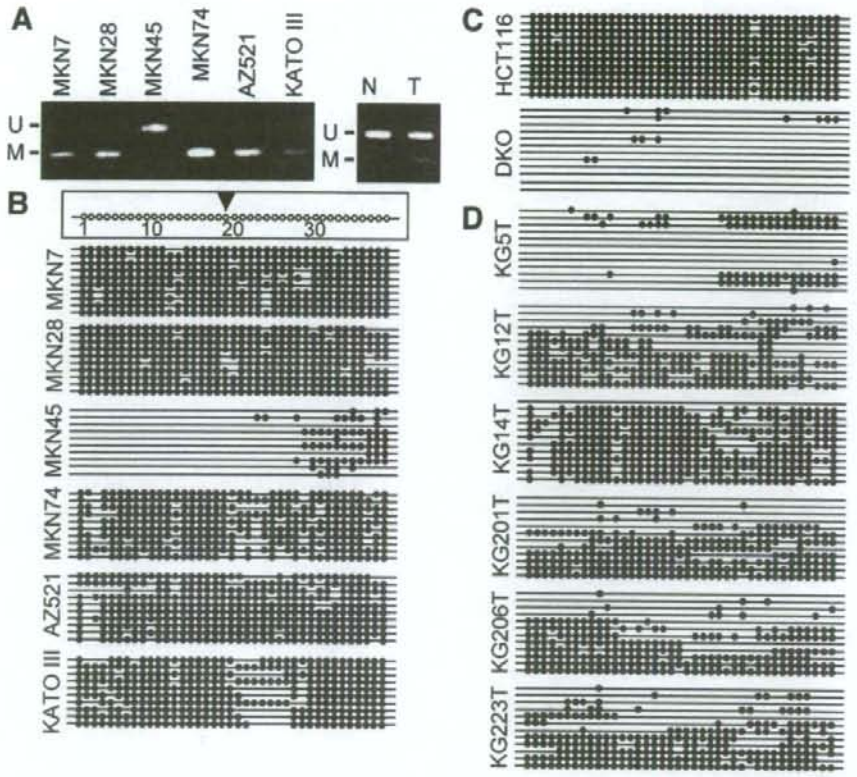

Figure 4. Methylation of the *B4GALNT2* gene in gastric cancer cells and primary gastric carcinomas. (*A*) Combined bisulfite restriction analysis (COBRA) of human gastric cancer cell lines (*left*) and representative results for a primary gastric carcinoma (*right*). M, methylated alleles; N, gastric 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 4*C*). Furthermore, it was clearly evident that the upstream of the *B4GALNT2* gene was frequently hypermethylated in human gastric cancer tissues (Figure 4*D*). 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).

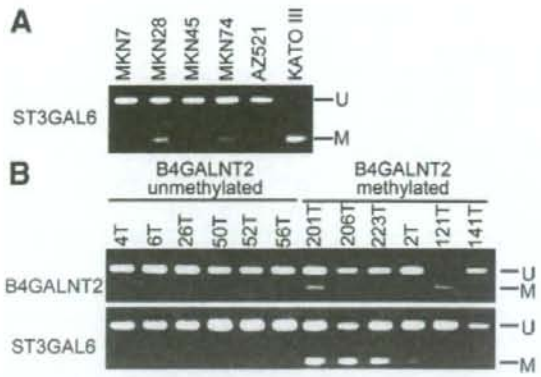

Figure 5. Methylation of *B4GALNT2* and *ST3GAL6* genes in gastric cancer cells and primary gastric carcinomas. Representative results are shown from COBRA using human gastric cancer cell lines (*A*) and primary tumors (*B*). M, methylated alleles; U, unmethylated alleles. The genes that were analyzed 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 4*A* 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- 1,4GalNAcT, 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 4*A*, *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*

	Number of patients (%)		
		ST3GAL6	
Characteristic	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- 1,4GalNAcT 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 2,3-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.

References

1. Hakomori S. Glycosylation defining cancer malignancy: New wine in an old bottle. Proc Natl Acad Sci U S A 2002;99:10231–10233.
2. Nakamori S, Kameyama M, Imaoka S, et al. Increased expression of sialyl Lewisx antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study. Cancer Res 1993;53:3632–3637.
3. Nakayama T, Watanabe M, Katsumata T, et al. Expression of sialyl Lewis^a as a new prognostic factor for patients with advanced colorectal carcinoma. Cancer 1995;75:2051–2056.
4. Futamura N, Nakamura S, Tatematsu M, et al. Clinicopathologic significance of sialyl Lex expression in advanced gastric carcinoma. Br J Cancer 2000;83:1681–1687.
5. Walz G, Aruffo A, Kolanus W, et al. Recognition by ELAM-1 of the sialyl- Lex determinant on myeloid and tumor cells. Science 1990;250:1132–1135.
6. Takada A, Ohmori K, Yoneda T, et al. Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. Cancer Res 1993;53:354–361.

7. Ito H, Hiraiwa N, Sawada-Kasugai M, et al. Altered mRNA expression of specific molecular species of fucosyl- and sialyl-transferases in human colorectal cancer tissues. Int J Cancer 1997; 71:556–564.
8. Salvini R, Bardoni A, Valli M, et al. 1,3-Galactosyltransferase 3Gal-T5 acts on the GlcNAc 1→3Gal 1→4GlcNAc 1→R sugar chains of carcinoembryonic antigen and other N-linked glycoproteins and is down-regulated in colon adenocarcinomas. J Biol Chem 2001;276:3564–3573.
9. Kumamoto K, Goto Y, Sekikawa K, et al. Increased expression of UDP-galactose transporter messenger RNA in human colon cancer tissues and its implication in synthesis of Thomsen-Friedenreich antigen and sialyl Lewis A/X determinants. Cancer Res 2001;61:4620–4627.
10. Hakomori S. Tumor-associated glycolipid antigens defined by monoclonal antibodies. Bull Cancer 1983;70:118–126.
11. Morton JA, Pickles MM, Terry AM. The Sda blood group antigen in tissues and body fluids. Immunol Invest 1988;17:217–224.
12. Dohi T, Ohta S, Hanai N, et al. Sialylpentaosylceramide detected with anti-GM2 monoclonal antibody. Structural characterization and complementary expression with GM2 in gastric cancer and normal gastric mucosa. J Biol Chem 1990;265:7880–7885.
13. Dohi T, Nishikawa A, Ishizuka I, et al. Substrate specificity and distribution of UDP-GalNAc:sialylparagloboside N-acetylgalactosaminyltransferase in the human stomach. Biochem J 1992; 288:161–165.
14. Dohi T, Hanai N, Yamaguchi K, et al. Localization of UDP-GalNAc: NeuAc 2,3Gal-R 1,4(GalNAc to Gal)N-acetylgalactosaminyltransferase in human stomach. Enzymatic synthesis of a fundic gland-specific ganglioside and GM2. J Biol Chem 1991;266: 24038–24043.
15. Kawamura YI, Kawashima R, Fukunaga R, et al. Introduction of Sda carbohydrate antigen in gastrointestinal cancer cells eliminates selectin ligands and inhibits metastasis. Cancer Res 2005;65:6220–6227.
16. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–428.
17. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002; 416:552–556.
18. Toyota M, Ho C, Ahuja N, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res 1999;59:2307–2312.
19. Suzuki H, Itoh F, Toyota M, et al. Inactivation of the 14-3-3 sigma gene is associated with 5′ CpG island hypermethylation in human cancers. Cancer Res 2000;60:4353–4357.
20. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 1997;25:2532–2534.
21. Okajima T, Fukumoto S, Miyazaki H, et al. Molecular cloning of a novel 2,3-sialyltransferase (ST3Gal VI) that sialylates type II lactosamine structures on glycoproteins and glycolipids. J Biol Chem 1999;274:11479–11486.
22. Lo YM, Chan LY, Lo KM, et al. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res 1999;59:1188–1191.
23. Clayton CL, Kleanthous H, Coates PJ, et al. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. J Clin Microbiol 1992;30:192–200.
24. Rhei E, Bogomolniy F, Federici MG, et al. Molecular genetic characterization of BRCA1- and BRCA2-linked hereditary ovarian cancers. Cancer Res 1998;58:3193–3196.
25. Japanese Gastric Cancer Association. Gastric Cancer 1998;1: 10–24.
26. Sobin LH, Wittekind C, eds. TNM classification of malignant tumors. 5th ed. New York: John Wiley & Sons; 1997.
27. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965; 64:31–49.
28. Chihara Y, Sugano K, Kobayashi A, et al. Loss of blood group A antigen expression in bladder cancer caused by allelic loss and/or methylation of the ABO gene. Lab Invest 2005;85:895–907.
29. Gao S, Worm J, Guldberg P, et al. Genetic and epigenetic alterations of the blood group ABO gene in oral squamous cell carcinoma. Int J Cancer 2004;109:230–237.
30. Kominato Y, Hata Y, Takizawa H, et al. Expression of human histo-blood group ABO genes is dependent upon DNA methylation of the promoter region. J Biol Chem 1999;274:37240–37250.
31. Yuyama Y, Dohi T, Morita H, et al. Enhanced expression of GM2/GD2 synthase mRNA in human gastrointestinal cancer. Cancer 1995;75:1273–1280.
32. Miyazaki K, Ohmori K, Izawa M, et al. Loss of disialyl Lewisa, the ligand for lymphocyte inhibitory receptor sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7) associated with increased sialyl Lewis^a expression on human colon cancers. Cancer Res 2004; 64:4498–4505.
33. Yago K, Zenita K, Ginya H, et al. Expression of -(1,3)-fucosyltransferases which synthesize sialyl Lex and sialyl Lea, the carbohydrate ligands for E- and P-selectins, in human malignant cell lines. Cancer Res 1993;53:5559–5565.
34. Majuri M-L, Niemelä R, Tiisala S, et al. Expression and function of 2,3-sialyl- and 1,,3/1,4-fucosyltransferases in colon adenocarcinoma cell lines: role in synthesis of E-selectin counter-receptors. Int J Cancer 1995;63:551–559.
35. Iwamoto S, Withers DA, Handa K, et al. Deletion of A-antigen in a human cancer cell line is associated with reduced promoter activity of CBF/NF-Y binding region, and possibly with enhanced DNA methylation of A transferase promoter. Glycoconj J 1999; 16:659–666.
36. Kang GH, Lee S, Kim WH, et al. Epstein-Barr virus-positive gastric carcinoma demonstrates frequent aberrant methylation of multiple genes and constitutes CpG island methylator phenotype-positive gastric carcinoma. Am J Pathol 2002;160:787–794.
37. Soejima K, Fang W, Rollins BJ. DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. Oncogene 2003;22:4723–4733.
38. Uemura N, Okamoto S, Yamamoto S, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–789.

Received November 15, 2007. Accepted March 13, 2008.

Address requests for reprints to: Dr Taeko Dohi, Department of Gastroenterology, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. e-mail: dohi@ri.imcj.go.jp; fax: 81-3-3202-7364.

Supported by grants and contracts from the International Health Cooperation Research; the Ministry of Health, Labor, and Welfare; the program Grants-in-Aid for Scientific Research on Priority Areas and Grants-in-Aid for Young Scientists from the Ministry of Education, Cultures, Sports, Science, and Technology; and the Japan Health Sciences Foundation and Organization.

The authors thank Drs Nobuo Hanai, Kenya Shitara, and So Ohta for providing us with monoclonal antibodies, and Ms Miyuki Nakasuji for her technical assistance.

Supplementary Table 1. Human glyco-genes.

Genes	Accession No.	ID of TaqMan probe kits	Enzyme	References
FUT1	NM000148	Hs00355741_m1	1,2-fucosyltransferase, H blood group 1,2-fucosyltransferase	1, 2
FUT2	NM000511	Hs00704693_s1	1,2-fucosyltransferase, Se blood group 1,2-fucosyltransferase	3
FUT3	NM000149	Hs00356857_m1	1,3/4-fucosyltransferase, Lewis blood group 1,3/4-fucosyltransferase	4
FUT4	NM002033	Hs00275643_s1	1,3-fucosyltransferase	5, 6
FUT5	NM002034	Hs00704908_s1	1,3-fucosyltransferase	7
FUT6	NM000150	Hs00173404_m1	1,3-fucosyltransferase	8
FUT7	NM004479	Hs00237083_m1	1,3-fucosyltransferase	9, 10
FUT8	NM178154	Hs00189535_m1	1,6-fucosyltransferase	11
A3GALT/A3GALNT	NM020469	Hs00220850_m1	1,3-*N*-galactosyltransferase, 1,3-*N*-acetylgalactosaminyltransferase	12, 13
GBGT1	NM021996	Hs00222752_m1	globoside 1,3-*N*-acetylgalactosaminyltransferase, Forssman synthetase	14
B3GALNT1	NM003781	Hs00364202_s1	1,3-*N*-acetylgalactosaminyltransferase 1, globoside synthase	15
B3GALNT2	NM152490	Hs00380823_m1	1,3-*N*-acetylgalactosaminyltransferase 2	16
B4GALNT1	NM001478	Hs00155195_m1	1,4-*N*-acetylgalactosaminyltransferase 1, GM2/GD2 synthase	17
B4GALNT2	NM153446	Hs00396440_m1	1,4-*N*-acetylgalactosaminyltransferase 2 Sd^a synthase	18
B4GALNT3	NM173593	Hs00403843_m1	1,4-*N*-acetylgalactosaminyltransferase 3	19
B4GALNT4	NM178537	Hs00331790_m1	1,4-*N*-acetylgalactosaminyltransferase 4	20
GCNT1	NM001490	Hs00155243_m1	gulcosaminyl (*N*-acetyl) transferase 1, core 2 (1,6-*N*-acetylglucosaminyltransferase)	21
GCNT3	NM004751	Hs00191070_m1	gulcosaminyl (*N*-acetyl) transferase 3	22
GCNT4	NM016591	Hs00275464_s1	gulcosaminyl (*N*-acetyl) transferase 4, core 2 (1,6-*N*-acetylglucosaminyltransferase)	23
ST3GAL1	NM003033	Hs00161688_m1	CMP-NeuAc: -galactoside 2,3-sialyltransferase 1	24
ST3GAL2	NM006927	Hs00199480_m1	CMP-NeuAc: -galactoside 2,3-sialyltransferase 2	25
ST3GAL3	NM006279	Hs00196718_m1	CMP-NeuAc:Gal 1,3/4GlcNAc 2,3-sialyltransferase	26
ST3GAL4	NM006278	Hs00272170_m1	Gal 1,3/4GlcNAc 2,3-sialyltransferase	27
ST3GAL5	NM003896	Hs00187405_m1	CMP-NeuAc:lactosylceramide 2,3-sialyltransferase	28
ST3GAL6	NM006100	Hs00196086_m1	CMP-NeuAc: 2,3-sialyltransferase	29
ST6GAL1	NM003032	Hs00949382_m1	CMP-NeuAc:galactoside 2,6-sialyltransferase	30
ST6GAL2	NM032528	Hs00293264_m1	CMP-NeuAc:galactoside 2,6-sialyltransferase	31
ST6GALNAC1	NM018414	Hs00300842_m1	GalNAc 2,6-sialyltransferase 1	32
ST6GALNAC2	NM006456	Hs00197670_m1	GalNAc 2,6-sialyltransferase 2	33
ST6GALNAC3	NM152996	Hs00541761_m1	GalNAc 2,6-sialyltransferase 3	34
ST6GALNAC4	NM014403	Hs00205241_m1	GalNAc 2,6-sialyltransferase 4	35
ST6GALNAC5	NM030965	Hs00229612_m1	GalNAc 2,6-sialyltransferase 5	36
ST6GALNAC6	NM013443	Hs00203739_m1	GalNAc 2,6-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*-acetylgulcosamine-6-*O*-sulfotransferase	42
CHST5	NM012126	Hs00201677_m1	*N*-acetylgulcosamine-6-*O*-sulfotransferase 3, intestinal *N*-acetylgulcosamine-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		

Supplementary References

1. Larsen RD, Ernst LK, Nair RP, et al. Molecular cloning, sequence, and expression of a human GDP-L-fucose: -D-galactoside 2- -L-fucosyltransferase cDNA that can form the H blood group antigen. Proc Natl Acad Sci USA 1990;87:6674–6678.
2. Kelly RJ, Ernst LK, Larsen RD, et al. Molecular basis for H blood group deficiency in Bombay (O_h) and para-Bombay individuals. Proc Natl Acad Sci USA 1994;91:5843–5847.
3. Kelly RJ, Rouquier S, Giorgi D, et al. Sequence and expression of a candidate for the human *secretor* blood group (1,2)fucosyltransferase gene (FUT2). J Biol Chem 1995;270:4640–4649.
4. Kukowska-Latallo J, Larsen RD, Nair RP, et al. A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group (1,3/1,4)fucosyltransferase. Genes Dev 1990;4:1288–1303.
5. Lowe JB, Stoolman LM, Nair RP, et al. ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. Cell 1990;63:475–484.
6. Goelz SE, Hession C, Goff D, et al. ELFT: a gene that directs the expression of an ELAM-1 ligand. Cell 1990;63:1349–1356.
7. Weston BW, Nair RP, Larsen RD, et al. Isolation of a novel human (1,3)fucosyltransferase gene and molecular comparison to the human Lewis blood group (1,3/1,4)fucosyltransferase gene. J Biol Chem 1992;267:4152–4160.
8. Weston BW, Smith PL, Kelly RJ, et al. Molecular cloning of a fourth member of a human (1,3)fucosyltransferase gene family. J Biol Chem 1992;267:24575–24584.
9. Sasaki K, Kurata K, Funayama K, et al. Expression cloning of a novel 1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis x carbohydrate determinants in leukocytes. J Biol Chem 1994;269:14730–14737.
10. Natsuka S, Gersten KM, Zenita K, et al. Molecular cloning of a cDNA encoding a novel human leukocyte -1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinants. J Biol Chem 1994;269:16789–16794.
11. Yanagidani S, Uozumi N, Ihara Y, et al. Purification and cDNA cloning of GDP-L-Fuc:*N*-acetyl- -D-glucosaminide: 1-6 fucosyltransferase (1-6 FucT) from human gastric cancer MKN45 cells. J Biochem 1997;121:626–632.
12. Yamamoto F, Marken J, Tsuji T, et al. Cloning and characterization of DNA complementary to human UDP-GalNAc: Fuc 1→2Gal 1→3GalNac transferase (histo-blood group A transferase) mRNA. J Biol Chem 1990;265:1146–1151.
13. Yamamoto F, Clausen H, White T, et al. Molecular genetic basis of the histo-blood group ABO system. Nature 1990;345:229–233.
14. Xu H, Storch T, Yu M, et al. Characterization of the human Forssman synthetase gene. J Biol Chem 1999;274:29390–29398.
15. Okajima T, Nakamura Y, Uchikawa M, et al. Expression cloning of human globoside synthase cDNAs. J Biol Chem 2000;275:40498–40503.
16. Hiruma T, Togayachi A, Okamura K, et al. A novel human 1,3-*N*-acetylgalactosaminyltransferase that synthesizes a unique carbohydrate structure, GalNAc 1-3GlcNAc. J Biol Chem 2004;279:14087–14095.
17. Nagata Y, Yamashiro S, Yodoi J, et al. Expression cloning of 1,4 *N*-acetyltransferase cDNAs that determine the expression of G_{M2} and G_{D2} gangliosides. J Biol Chem 1992;267:12082–12089.
18. Montiel MD, Krzewinski-Recchi MA, Delannoy P, et al. Molecular cloning, gene organization and expression of the human UDP-GalNAc:Neu5Ac 2-3Gal -R 1,4-*N*-acetylgalactosaminyltransferase responsible for the biosynthesis of the blood group Sd^a/Cas antigen: evidence for an unusual extended cytoplasmic domain. Biochem J 2003;373:369–379.
19. Sato T, Gotoh M, Kiyohara K, et al. Molecular cloning and characterization of a novel human 1,4-*N*-acetylgalactosaminyltransferase, 4GalNAc-T3, responsible for the synthesis of *N*, '-diacetyllactosediamine, GalNac 1-4GlcNAc. J Biol Chem 2003;278:47534–47544.
20. Gotoh M, Sato T, Kiyohara K, et al. Molecular cloning and characterization of 1,4-*N*-acetylgalactosaminyltransferases IV synthesizing *N,N'*-diacetyllactosamine. FEBS Lett 2004;562:134–140.
21. Bierhuizen MFA, Fukuda M. Expression cloning of a cDNA encoding UDP-GlcNAc:Gal 1-3-GalNAc-R (GlcNAc to GalNAc) 1-6GlcNAc transferase by gene transfer into CHO cells expressing polyoma large tumor antigen. Proc Natl Acad Sci USA 1992;89:9326–9330.
22. Yeh JC, Ong E, Fukuda M. Molecular cloning and expression of novel 1-6-*N*-acetylglucosaminyltransferase that forms core 2, core 4, and I branches. J Biol Chem 1999;274:3215–3221.
23. Schwientek T, Yeh JC, Levery SB, et al. Control of *O*-glycan branch formation. Molecular cloning and characterization of a novel thymus-associated core 2 1-6-*N*-acetylglucosaminyltransferase. J Biol Chem 2000;275:11106–11113.
24. Kitagawa H, Paulson JC. Differential expression of five sialyltransferase genes in human tissues. J Biol Chem 1994;269:17872–17878.
25. Kim YJ, Kin KS, Kim SH, et al. Molecular cloning and expression of human Gal 1,3GalNAc 2,3-sialyltransferase (hST3Gal II). Biochem Biophys Res Commun 1996;228:324–327.
26. Kitagawa H, Paulson JC. Cloning and expression of human Gal 1,3(4)GlcNAc 2,3-sialyltransferase. Biochem Biophys Res Commun 1993;194:375–382.
27. Sasaki K, Watanabe E, Kawashima K, et al. Expression cloning of a novel Galb(1-3/1-4)GlcNAc a2,3-sialyltransferase using lectin resistance selection. J Biol Chem 1993;268:22782–22787.
28. Ishii A, Ohta M, Watanabe Y, et al. Expression cloning and functional characterization of human cDNA for ganglioside G_{M3} synthase. J Biol Chem 1998;273:31652–31655.
29. Okajima T, Fukumoto S, Miyazaki, H, et al. Molecular cloning a novel 2,3-sialyltransferase (ST3Gal VI) that sialylates type II lactosamine structures on glycoproteins and glycolipids. J Biol Chem 1999;274:11479–11486.
30. Grundmann U, Nerlich C, Rein T, et al. Complete cDNA sequence encoding human -galactoside -2,6-sialyltransferase. Nucleic Acids Res 1990;18:667.
31. Takashima S, Tsuji S, Tsujimoto M. Characterization of the second type of human -galactoside 2,6-sialyltransferase (ST6Gal II), which sialylates Gal 1,4GlcNAc structures on oligosaccharides preferentially. J Biol Chem 2002;277:45719–45728.
32. Ikehara Y, Kojima N, Kurosawa N, et al. Cloning and expression of a human gene encoding an *N*-acetylgalactosamine-2,6-sialyltransferase (ST6GalNAc I): a candidate for synthesis of cancer-associated sialyl-Tn antigens. Glycobiology 1999;9:1213–1224.
33. Samyn-Petit B, Krzewinski-Recchi MA, Steelant WF, et al. Molecular cloning and functional expression of human ST6GalNAc II. Molecular expression in various human cultured cells. Biochim Biophys Acta 2000;1474:201–211.
34. Tsuchida A, Ogiso M, Nakamura Y, et al. Molecular cloning and expression of human ST6GalNAc III: restricted tissue distribution and substrate specificity. J Biochem 2005;138:237–243.
35. Harduin-Lepers A, Stokes DC, Steelant WFA, et al. Cloning, expression and gene organization of a human Neu5Ac 2-3Gal 1-3GalNAc 2,6-sialyltransferase: hST6GalNAc IV. Biochem J 2000;352:37–48.
36. Okajima T, Fukumoto S, Ito H, et al. Molecular cloning of brain-specific GD1 synthase (ST6GalNAc V) containing CAG/glutamine repeats. J Biol Chem 1999;274:30557–30562.
37. Okajima T, Chen HH, Ito H, et al. Molecular cloning and expression of mouse GD1 /GD1a /GQ1b synthase (ST6GalNAc VI) gene. J Biol Chem 2000;275:6717–6723.

38. Ohtake S, Ito Y, Fukuta M, et al. Human N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase cDNA is related to human B cell recombination activating gene-associated gene. J Biol Chem 2001;276:43894–43900.
39. Fukuta M, Inazawa J, Torii T, et al. Molecular cloning and characterization of human keratan sulfate Gal-6-sulfotransferase. J Biol Chem 1997;272:32321–32328.
40. Li X, Tedder TF. CHST1 and CHST2 sulfotransferases expressed by human vascular endothelial cells: cDNA cloning, expression, and chromosomal localization. Genomics 1999; 55:345–347.
41. Fukuta M, Kobayashi Y, Uchimura K, et al. Molecular cloning and expression of human chondroitin 6-sulfotransferase. Biochim Biophys Acta 1998;1399:57–61.
42. Bistrup A, Bhakta S, Lee JK, et al. Sulfotransferases of two specificities function in the reconstitution of high endothelial cell ligands for L-selectin. J Cell Biol 1999;145:899–910.
43. Lee JK, Bhakta S, Rosen SD, et al. Cloning and characterization of a mammalian *N*-acetylglucosamine-6-transferase that is highly restricted to intestinal tissue. Biochem Biophys Res Commun 1999;263:543–549.
44. Bonten E, van der Spoel A, Fornerod M, et al. Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. Genes Dev 1996;10: 3156–3169.
45. Monti E, Preti A, Rossi E, et al. Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. Genomics 1999;57:137–143.
46. Wada T, Yoshikawa Y, Tokuyama S, et al. Cloning, expression, and chromosomal mapping of a human ganglioside sialidase. Biochem Biophys Res Commun 1999;261:21–27.
47. Monti E, Bassi MT, Bresciani R, et al. Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene faminy. Genomics 2004;83:445–453.

GASTROENTEROLOGY 2008;135:889–898

Activation-Induced Cytidine Deaminase Links Between Inflammation and the Development of Colitis-Associated Colorectal Cancers

YOKO ENDO,* HIROYUKI MARUSAWA,* TADAYUKI KOU,* HIROSHI NAKASE,* SHIGEHIKO FUJII,‡ TAKAHIRO FUJIMORI,‡ KAZUO KINOSHITA,§ TASUKU HONJO,‖ and TSUTOMU CHIBA*

*Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto; ‡Department of Surgical and Molecular Pathology, Dokkyo University School of Medicine, Tochigi; §Evolutionary Medicine, Shiga Medical Center Research Institute, Moriyama; and ‖Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan

See editorial on page 736.

Background & Aims: Activation-induced cytidine deaminase (AID) was originally identified as an inducer of somatic hypermutations in the immunoglobulin gene. We recently revealed that ectopic AID expression serves as a link between the cellular editing machinery and high mutation frequencies, leading to human cancer development. In the current study, we investigated whether AID might contribute to the development of colitis-associated colorectal cancers. ***Methods:*** The expression and regulation of AID in association with proinflammatory cytokine stimulation were investigated in cultured colonic cells. Genotoxic activity of AID in colonic cells was analyzed using retroviral system. Immunohistochemistry for AID was carried out on various human colonic tissues specimens. ***Results:*** Tumor necrosis factor-α induced aberrant AID expression via IκB kinase-dependent nuclear factor (NF)-κB-signaling pathways in human colonic epithelial cells. Moreover, AID expression was also induced in response to the T helper cell 2-driven cytokines interleukin-4 and interleukin-13, which are activated in human inflammatory bowel disease. Aberrant activation of AID in colonic cells preferentially induced genetic mutations in the *TP53* gene, whereas there were no nucleotide alterations of the *APC* gene. Immunohistochemistry revealed enhanced expression of endogenous AID protein not only in the inflamed colonic mucosa of ulcerative colitis patients but also in tumor lesions of colitis-associated colorectal cancers. ***Conclusions:*** Our findings indicate that proinflammatory cytokine-mediated aberrant expression of AID in colonic epithelial cells is a genotoxic factor linking inflammation, somatic mutations, and colorectal cancer development.

Chronic inflammatory bowel diseases (IBD) are important etiologic factors in the development of colorectal cancers.[1] A cohort study of patients with ulcerative colitis (UC) revealed that extensive colitis increases the cumulative risk of colorectal cancer by 7.2% at 20 years and 16.5% at 30 years from disease onset.[2] Thus, the relative risk of colorectal cancer in patients with UC was up to 20 times higher than that of the general population.[3] Colon cancers arising in IBD patients have several distinct characteristics compared with sporadic colorectal cancers. There is a higher rate of multiple synchronous cancers and dysplastic lesions associated with cancer development.[4] Mutations in the *TP53* gene appear to be an early event and are already present in dysplastic lesions associated with UC.[5,6] These findings suggest that chronic inflammation of the colonic mucosa has a critical role in colon carcinogenesis, and the molecular processes leading to colitis-associated cancer development might differ from those of sporadic colorectal cancers. However, the mechanisms that account for the development of colon cancers via chronic inflammation remain unclear.

Recently, we demonstrated the possible role of activation-induced cytidine deaminase (AID) in linking chronic inflammation to the development of human gastric and liver cancers.[7–9] Under physiologic conditions, AID is required for somatic hypermutation and class switch recombination in immunoglobulin genes of activated B cells.[10,11] However, the inappropriate expression of AID could contribute to tumorigenesis via its DNA mutagenic activity.[12] In fact, constitutive and ubiquitous expression of AID in mice causes the development of neoplastic lesions including cancers in several organs in association with high mutation frequencies.[8,9,13,14] Notably, ectopic AID expression is induced in response to tumor necrosis factor α (TNF-α), a proinflammatory cytokine that is important in the pathway leading to tumorigenesis.[15] These findings demonstrate a novel linkage between in-

Abbreviations used in this paper: AID, activation-induced cytidine deaminase; IKK, IκB kinase; IL, interleukin; NF-κB, nuclear factor κB; PCR, polymerase chain reaction; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription; TCR, T-cell receptor; TNF, tumor necrosis factor.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.06.091

flammation and enhanced susceptibility to somatic mutations leading to tumor development in gastric epithelial cells and hepatocytes.

Excessive and chronically produced proinflammatory mediators are thought to contribute to tumor promotion and progression in colitis-associated cancers.[16] Expression of most proinflammatory cytokines and chemokines, including TNF-α, is up-regulated in the colonic tissues of patients with IBD,[17] suggesting that enhanced proinflammatory cytokine activity contributes to colitis-associated cancer development. Based on these findings, we speculated that AID might be involved in colon carcinogenesis within the background of chronic colitis, and we therefore investigated the proinflammatory cytokine stimulation-induced expression and regulation of endogenous AID in human colonic epithelial cells.

Materials and Methods

Plasmids and Reagents

The expression plasmids pcDNA3-IκB kinase (IKK) α, IKKβ, and RelA (nuclear factor [NF]-κB) were as described.[18] The expression plasmids pcDNA3-IκB$\alpha\Delta$N, encoding the super-repressor form of IκBα, and pcDNA3-IKKβ (K44A), encoding the dominant negative mutant of IKK-β have also been described.[8] Expression plasmid encoding the dominant negative form of STAT6 (STAT6ΔC) lacking the C-terminal 186 amino acids[19] was generated from the STAT6 fragment using polymerase chain reaction (PCR) amplification. Recombinant human TNF-α, interleukin (IL)-4, IL-13, IL1-β, IL-12, and interferon (IFN)-γ were obtained from PeproTech EC Ltd. (London, United Kingdom).

Quantitative Real-Time Reverse-Transcription PCR

Quantitative real-time reverse-transcription (RT) PCR (RT-PCR) for human *AID* amplification was performed using a 7300 Real-Time PCR System (PE Applied Biosystems, Foster City, CA). The 6-carboxyfluorescein (FAM)-labeled probe used for human *AID* was 5′-TCGGCGTGAGACCTACCTGTGCTAC-3′. Target complimentary DNA (cDNA) were normalized to endogenous messenger RNA (mRNA) levels of the housekeeping reference gene *18s ribosomal RNA (18s rRNA)*.[7] For simplicity, ratios are represented as relative values compared with expression levels in a lysate from control cells. The reproducibility of this quantification method was examined by comparing results obtained from replicate samples during the same reaction run with those from independent runs on different days. The PCR procedures were performed at least 3 times for each sample.

Cell Culture and Transfection

LoVo cells were cultured in Ham's F12 (MP Biomedicals, Solon, OH) containing 10% fetal bovine serum. SW48 human colonic cancer cells were grown in Dulbecco's modified Eagle medium (Gibco-BRL, Rockville, MD). Transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Small interfering RNA (siRNA) duplexes used for targeting signal transducers and activators of transcription (STAT) 6 were obtained from Invitrogen.

Recombinant Retrovirus Production and Infection of Colon Cancer Cells

The retroviral system for expression of AID was performed as described.[20] A full-length AID cDNA was subcloned into the *Eco*RI and *Xho*I restriction sites of the pFB vector (Stratagene, La Jolla, CA) with internal ribosome entry and a puromycin resistance gene. Viral vectors for the expression of the mutant AID (R35E, R35E/R36D) were constructed as described.[21]

NF-κB Luciferase Reporter Gene Assay

Luciferase assays were performed using the Dual-Luciferase TM Reporter assay system (Promega, Madison, WI). The transfection efficiency was normalized to cells cotransfected with pRL vector (Promega).[18]

Subcloning and Sequencing of Tumor-Related Genes

The oligonucleotide primers for the amplification of the human *TP53, APC, K-ras, and c-myc* genes are shown in Supplementary Table 1 (see Supplementary Table 1 online at www.gastrojournal.org). Amplification of the target sequences was performed using high-fidelity Phusion Taq polymerase (Finnzymes, Espoo, Finland), and the products were subcloned into a pcDNA3 vector (Invitrogen). The resulting plasmids were subjected to sequence analysis.

Immunoblotting and Immunohistochemistry

A polyclonal antibody against human AID was generated using purified recombinant AID protein as an immunogen.[22] A mouse monoclonal antibody against α-tubulin was purchased from Calbiochem (San Diego, CA), and a rabbit monoclonal antibody against human phospho-STAT6 was purchased from Cell Signaling Technology (Danvers, MA). Immunohistochemistry was performed as described protocol.[23]

Patients

The study group consisted of patients who had undergone colectomy because of severe UC or colitis-associated colon cancers at Dokkyo University or Kyoto University Hospitals between 2003 and 2005. Selection of patients enrolled in this study was based on the availability of a sufficient amount of tissue for analysis. The patients included 6 men and 6 women, with an average age at the time of surgery of 45.3 ± 12.4 years (mean ± SD; range, 22–72 years). As a control, 5 samples of nor-

mal colon tissues from the nontumorous region of patients with sporadic colon cancers were also examined. Biopsies of tumor tissue at the proximal edge of freshly resected specimens were obtained and immediately frozen in liquid nitrogen. Written informed consent for the use of resected tissue was obtained from all patients in accordance with the Declaration of Helsinki, and the Kyoto University Graduate School and Faculty of Medicine Ethics Committee approved the study.

Results

Human AID Is Induced in Response to TNF-α Signaling in Colonic Epithelial Cells

To gain preliminary insight into the role of human AID proteins in human colonic epithelial cells, the expression of AID was analyzed by quantitative RT-PCR in cultured human colon cancer cells. We first confirmed that *cIAP1*, a TNF-α-inducible gene in many types of cells, was increased in response to TNF-α stimulation in colonic cells (Figure 1*A*). Endogenous AID expression was markedly elevated after TNF-α treatment in both LoVo and SW48 cells (Figure 1*A* and *B*). TNF-α induced a time-dependent transcriptional up-regulation of *AID* with a peak level 8 to 12 hours after treatment, whereas expression of the internal control *18S rRNA* transcript was unchanged (Figure 1*C*). Immunoblotting analysis using a specific antibody against human AID revealed that TNF-α induced a time-dependent up-regulation of AID protein in both cell types, with a peak level 12 to 24 hours after treatment (Figure 1*D* and *E*). RT-PCR analysis revealed that *AID* transcripts also increased in response to another proinflammatory cytokine, IL-1β, in LoVo cells (see Supplementary Figure 1*A* online at www.gastrojournal.org). Taken together, these findings suggest that endogenous AID expression is induced by proinflammatory cytokine stimulation in human colonic cells.

NF-κB Mediates AID Expression in an IκB Kinase-Dependent Manner in Colonic Epithelial Cells

Transcription factor NF-κB is induced by TNF-α signaling, and NF-κB is frequently and constitutively activated in the colonic epithelia of patients with IBD.[24] Therefore, we examined whether AID expression would be regulated transcriptionally by NF-κB activity in cultured colonic cells. First, we examined whether induction of positive regulators of NF-κB signaling affected AID expression. A reporter plasmid assay revealed enhanced NF-κB activity in cells producing the wild-type IκB kinase (IKK)-α, IKK-β, or NF-κB (Figure 2*A*). Under these experimental conditions, the expression of these NF-κB-positive regulators substantially increased the expression of endogenous AID protein in LoVo cells (Figure 2*B*). Next, we examined whether negative regulators of NF-κB, the mutant IKK-β and the super-repressor form of IκB-α

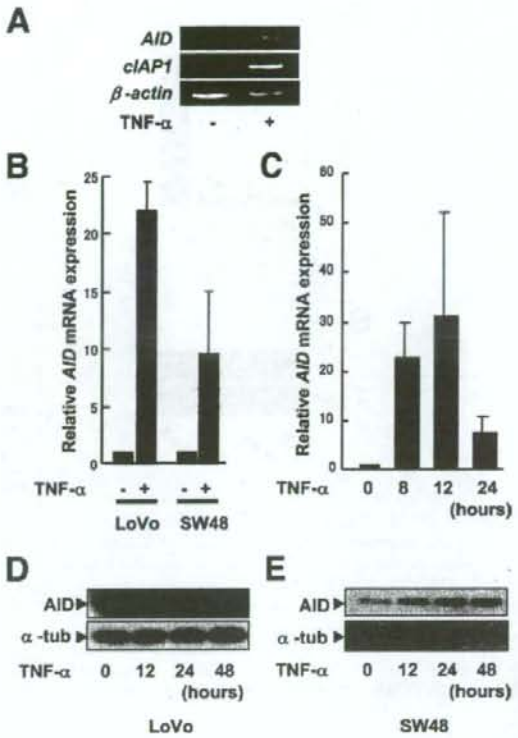

Figure 1. AID expression is induced in response to TNF-α stimulation in human colon cancer cells. (*A*) Total RNA was extracted from LoVo cells 12 hours after TNF-α treatment (100 ng/mL). Semiquantitative RT-PCR was performed using each RNA sample as a template and oligonucleotide primers specific for human *AID* (*upper panel*), *cIAP1* (*middle panel*), and β-*actin* (*lower panel*). (*B*) Total RNA was isolated from LoVo and SW48 cells before and 12 hours after stimulation with TNF-α (100 ng/mL). Real-time RT-PCR was performed using FAM-labeled probes and primers specific for human *AID*. *Values* shown in the *graphs* are normalized relative to specimens without TNF-α treatment (mean ± SD; n = 3). (*C*) Total RNA from LoVo cells was isolated immediately before and 8, 12, and 24 hours after TNF-α treatment (100 ng/mL). (*D*) LoVo and (*E*) SW48 cells were treated with TNF-α (100 ng/mL) for 0, 12, 24, and 48 hours. Total protein was isolated, and immunoblot analysis was performed using anti-human AID (*upper panels*) or anti-α-tubulin (*α-tub, lower panels*).

(IκBαΔN), would reduce AID production. We confirmed by reporter plasmid assay that TNF-α-mediated NF-κB activity was almost completely abolished by coproduction of IκBαΔN (Figure 2*C*, *upper graph*). Up-regulation of AID after TNF-α treatment was reduced substantially in LoVo cells in which IκBαΔN was coproduced (Figure 2*C*, *lower graph*). The coproduction of mutant IKK-β or IκBαΔN also reduced endogenous AID protein expression after TNF-α stimulation in both cells (Figure 2*D* and *E*). Because protein kinase Cξ is required for NF-κB activation in several cells,[25] we also examined its involvement in TNF-α-mediated AID expression. We found that

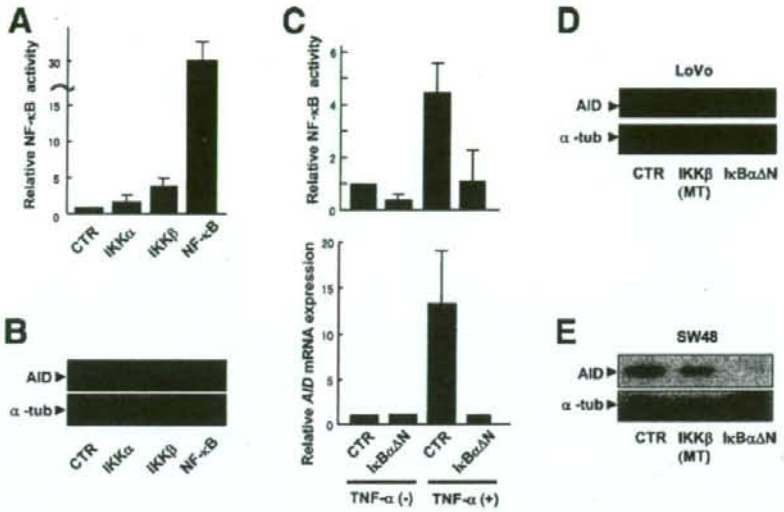

Figure 2. TNF-α-induced AID expression is achieved through the activation of NF-κB. (*A*) LoVo cells were transfected with NF-κB reporter plasmids together with expression plasmids encoding wild-type IKK-α, IKK-β, and RelA (NF-κB) or a control vector (CTR). After 48 hours, whole cell lysates were prepared, and luciferase activity was monitored in each extract. (*B*) Cell lysates were subjected to immunoblotting using anti-AID (*upper panel*) or anti-α-tubulin (*α-tub, lower panel*). (*C*) LoVo cells were transfected with a plasmid for the expression of the super-repressor form of IκB-α (IκBαΔN) or with a control vector (CTR), followed by treatment with TNF-α (100 ng/mL) for 12 hours. Each sample was harvested, and luciferase activity was measured to quantify endogenous NF-κB activity (*upper graphs*). Total RNA from each sample was isolated, and quantitative RT-PCR of endogenous *AID* expression was performed using each specimen as a template (*lower graphs*). The data present the means of *AID* mRNA expression relative to the internal control *18S rRNA* (mean ± SD; n = 3). (*D*) LoVo cells and SW48 (*E*) cells were transfected with plasmids encoding dominant negative IKKβ (IKKβMT), IκBαΔN, or a CTR vector and then treated with TNF-α (100 ng/mL) for 24 hours. Cell lysates were immunoblotted with anti-AID antibody (*upper panel*) and anti-α-tubulin (*α-tub, lower panel*).

knocking down the expression of endogenous protein kinase Cξ did not cause any significant changes in the levels of TNF-α-mediated AID expression in LoVo cells (see Supplementary Figure 1C online at www.gastrojournal.org).

These findings indicate that AID expression in colonic epithelial cells is regulated through the IKK-dependent NF-κB-signaling pathway, suggesting a common mechanism for the regulation of AID gene expression in human epithelial cells in the colon, stomach, and liver under inflammatory conditions.[8,9]

T Helper Cell 2 Cytokines IL-4 and IL-13 Are Involved in the Regulation of AID Expression in Colonic Epithelial Cells

In B cells, AID is regulated by IL-4 signaling in a STAT6-dependent manner,[26] suggesting that AID expression can be induced in human colonic epithelia by various inflammatory mediators produced in the human colon. Because IL-4 is involved in the T helper cell (Th) 2 cytokine response, which has a pivotal role in the pathogenesis of UC,[27] we examined whether IL-4 would contribute to the regulation of AID expression in colonic epithelial cells. Quantitative RT-PCR revealed marked up-regulation of *AID* transcripts in response to IL-4 treatment in both LoVo and SW48 cells (Figure 3*A*). *AID* transcripts were induced promptly, peaking at 12 to 14 hours after IL-4 treatment (Figure 3*B*). IL-4 activates STAT6 in a phosphorylation-dependent manner and contributes to the regulation of expression of various genes.[28] Immunoblotting analysis revealed that IL-4 treatment resulted in an increase in phosphorylated STAT6 protein expression (Figure 3*C* and 3*D*). Under these conditions, the expression of AID protein was increased substantially, peaking at 24 to 36 hours after IL-4 stimulation in both LoVo cells (Figure 3*C*) and SW48 cells (Figure 3*D*). We next examined whether AID expression would be mediated by a STAT6-dependent mechanism using the dominant negative form of STAT6 (STAT6ΔC) (Figure 3*E*). We found that STAT6ΔC suppressed IL-4-mediated AID expression and that coexpression of the wild-type STAT6 reversed the suppression of AID expression caused by STAT6ΔC in colonic cells (Figure 3*F*).

Another Th2 cytokine, IL-13, is a critical mediator of mucosal inflammation and could be a key molecule in the pathogenesis of human UC.[29] IL-13 shares many functional properties with IL-4, such as a common receptor subunit, the α-subunit of the IL-4 receptor, and

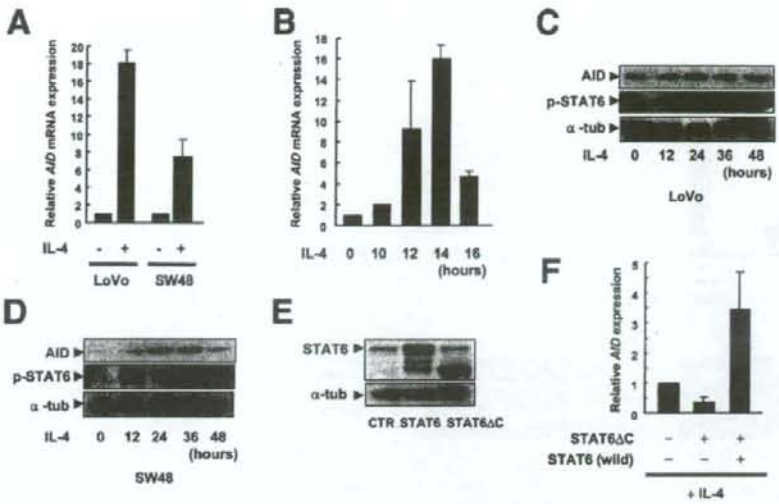

Figure 3. IL-4-mediated AID expression in human colonic epithelial cells. (*A*) LoVo and SW48 cells were treated with human recombinant IL-4 (100 ng/mL) for 12 hours. Quantitative RT-PCR was performed using FAM-labeled probes specific for human *AID*. (*B*) Time course of changes in *AID* mRNA expression after IL-4 stimulation. SW48 cells were harvested and subjected to total RNA isolation immediately before (0) and 10, 12, 14, and 16 hours after stimulation by IL-4 (100 ng/mL). (*C* and *D*) LoVo (*C*) and SW48 (*D*) cells were treated with human IL-4 (100 ng/mL) for the indicated times. Total protein was isolated, and immunoblot analysis was performed using anti-human AID (*upper panel*), anti-phospho-STAT6 (*p-STAT6, middle panel*), or anti-α-tubulin antibodies (*α-tub, lower panel*). (*E*) LoVo cells were transfected with expression plasmids encoding wild-type STAT6, STAT6ΔC, or control vector (CTR). After 48 hours, cell lysates were subjected to immunoblotting using anti-STAT6 (*upper panel*) or anti-α-tubulin (*α-tub, lower panel*). (*F*) LoVo cells were transfected with STAT6ΔC expression plasmid or control vector, followed by the cotransfection with pcDNA3-STAT6 encoding wild-type STAT6. Total RNA from each sample was isolated after IL-4 stimulation for 14 hours, and quantitative RT-PCR of endogenous *AID* expression was performed using each specimen as a template.

activates STAT6 by phosphorylation for further signal transduction.[30] Our finding that AID expression is regulated by IL-4 prompted us to test whether IL-13 would be also involved in the regulation of AID expression. Quantitative RT-PCR analysis clearly showed that IL-13 stimulation in both LoVo and SW48 cells substantially up-regulated *AID* transcripts (Figure 4*A*). A time course immunoblotting analysis revealed that AID protein expression in these cells increased in response to IL-13 and peaked at 24 hours after treatment (Figure 4*B* and *C*). Transfection of STAT6-specific siRNA, but not control siRNA, substantially suppressed phosphorylated STAT6 protein levels in LoVo cells. Under these conditions, IL-13 failed to induce AID expression in the cells treated with siRNA specific for STAT6 (Figure 4*D*). These findings support our conclusion that AID expression in human colonic epithelial cells is regulated by 2 Th2 cytokines, IL-4 and IL-13, through a STAT6-dependent pathway.

We also examined whether Th1 cytokines could trigger the expression of AID in colonic epithelial cells. We found that AID up-regulation was induced by treatment with IL-12, but not by IFN-γ, suggesting that some of the Th1 cytokines may also play roles in the aberrant expression of AID in colonic cells (see Supplementary Figure 1*A* online at www.gastrojournal.org).

AID Expression in Colonic Epithelial Cells Results in the Accumulation of TP53 Mutations

To clarify whether aberrant AID expression might be genotoxic in human colonic cells, we examined whether AID expression would cause somatic mutations in tumor-related genes. For this purpose, the mutagenetic activity of AID was determined by a retroviral vector-mediated AID expression system in LoVo cells. We investigated the overall mutation frequency in the *TP53*, *APC*, and *K-ras* genes because mutations of these genes are closely associated with the development of human colorectal cancers. In addition, we investigated the mutation occurrence in the *c-myc* gene, which is a target of AID for abnormal editing in cultured human hepatoma-derived cells.[8] Accordingly, multiple clones were picked randomly from cells at 2, 4, and 8 weeks after AID expression and subjected to sequence analysis. We first confirmed that either no changes or only a single nucleotide alteration was detected in all genes of 40 randomly picked clones from cells transfected with control vectors (data not shown). In contrast, nucleotide alterations in the *TP53* gene emerged in a time-dependent manner in cells producing AID, and a substantially higher number of nucleotide alterations appeared in cells 8 weeks after

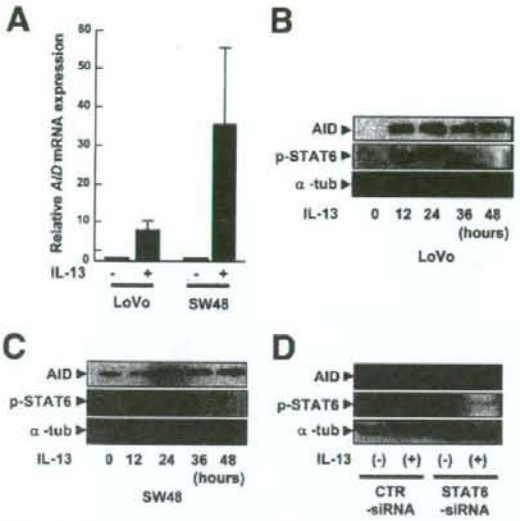

Figure 4. AID expression is regulated by IL-13 in human colonic epithelial cells. (*A*) Total RNA was isolated from LoVo and SW48 cells after treatment with human recombinant IL-13 (100 ng/mL) for 12 hours. The expression levels of *AID* mRNA were measured by quantitative real-time PCR using FAM-labeled probes specific for human *AID*. (*B* and *C*) Time course of changes in AID protein expression after IL-13 stimulation. LoVo (*B*) and SW48 (*C*) cells were treated with human IL-13 (100 ng/mL) for 0, 12, 24, 36, and 48 hours, followed by immunoblotting using anti-human AID (*upper panel*), anti-phospho-STAT6 (*p-STAT6, middle panel*), or anti-α-tubulin antibodies (*α-tub, lower panel*). (*D*) LoVo cells were transfected with siRNA targeting STAT6 or control RNA (CTR) for 24 hours, and lysates were prepared from siRNA-treated cells after stimulation with IL-13 (100 ng/mL) for 24 hours. Immunoblotting was performed using antibodies specific for human AID (*upper panel*), phospho-STAT6 (*p-STAT6, middle panel*), or α-tubulin (*α-tub, lower panel*).

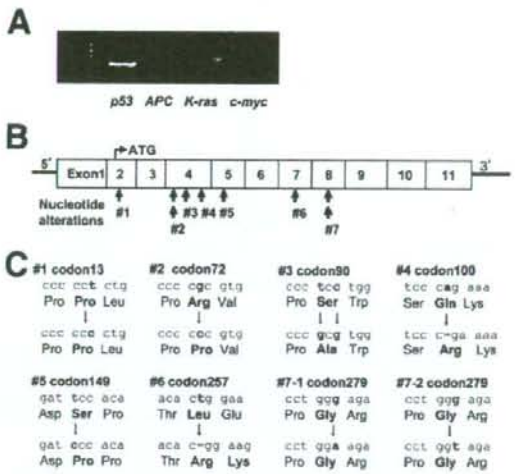

Figure 5. Distribution of mutations in the *TP53* sequence in AID-expressing LoVo cells. (*A*) Total RNA was extracted from LoVo cells and semiquantitative RT-PCR analyses were performed by using specific primers for *TP53, APC, K-ras,* and *c-myc*. (*B* and *C*) LoVo cells were infected with a retroviral vector for AID expression and cultured for 8 weeks. (*B*) Eight point mutations (numbers 1–3, 5, 7) and 2 deletions (numbers 4 and 6) appeared in the 48 (exons 2–6) and 38 (exons 6–11) sequenced *TP53* clones of LoVo cells with AID activation. (*C*) Point mutations that emerged in the coding region of the *TP53* gene resulted in amino acid substitutions.

AID activation, compared with control cells (Table 1). The nucleotide alterations induced by AID activation were distributed across the entire transcribed region of the *TP53* gene (Figure 5*B* and *C*). Notably, 6 of 10 nucleotide alterations that emerged in the *TP53* coding region resulted in amino acid substitutions with potential functional consequences. Compiled data in the International Agency for Research on Cancer *TP53* Mutation Database (http://www-p53.iarc.fr/index.html) revealed that 5 of the 10 nucleotide positions altered in the *TP53* gene after AID activation corresponded to alterations observed in clinical specimens of human malignancies. In contrast to the *TP53* gene, no somatic mutations appeared in the coding sequences of the *APC and K-ras* genes, even 8 weeks after AID activation. In the *c-myc* gene, only a single nucleotide alteration was observed in the presence of AID expression. A previous study demonstrated that N-terminal mutants R35E and R35E/R36D appear less processive and have altered mutational specificity compared with wild-type AID.[21] In colonic cells, R35E and R35E/R36D-AID induced *TP53* mutations less frequently than wild-type AID expression (see Supplementary Table 2 online at www.gastrojournal.org). In addition, the *TP53* gene mutation patterns observed in the R35E-AID-expressing cells did not show any target base preferences, an observation similar to that for the *TP53* gene mutations induced by wild-type AID expression in LoVo cells. Taken together, these findings suggest that aberrant AID expression preferentially induced nucleotide alterations in the *TP53* gene in human colonic epithelial cells.

Table 1. AID-Induced Mutagenesis in Various Tumor-Related Genes in LoVo Cells

Target gene	Duration of AID activation	Mutated clones (n/total)	Mutation number (n/total bases)
TP53 (exons 2–6)	2 weeks	1/40	1/22,000
	4 weeks	2/47	2/25,850
	8 weeks	6/48	7/26,400
TP53 (exons 6–11)	2 weeks	1/43	1/23,650
	4 weeks	2/34	2/18,700
	8 weeks	3/38	3/20,900
APC	8 weeks	0/42	0/23,425
K-ras	8 weeks	0/40	0/18,760
c-myc	8 weeks	1/41	1/21,695

Expression of Endogenous AID Protein in UC Mucosa and Colitis-Associated Cancers

To clarify the expression and localization of AID protein in human colonic tissues under physiologic and pathologic conditions, immunohistochemistry was per-

formed using a specific antibody against human AID in colonic tissue from 22 UC lesions, 15 colitis-associated neoplasms, and 5 nontumorous regions of the patients with sporadic colon cancers. Specificity of the antibody in immunostaining was confirmed by control staining performed on germinal centers of human mesenteric lymph nodes containing mostly activated B cells.[7,9] In normal colonic mucosa lacking inflammation, immunohistochemistry revealed no evidence of AID expression (Figure 6*A*). By contrast, endogenous AID immunoreactivity was detected in the colonic epithelium of 12 of 22 (54%) inflammatory lesions from patients with UC (Figure 6*B–D*). AID immunostaining was localized mainly in the cytoplasm of inflamed colonic epithelial cells. Strongly AID-positive cells were also observed in the lymphocytes that infiltrated the submucosa of colonic tissue from patients with UC. In the colitis-associated colon cancer tissues, AID protein expression was observed in neoplastic cells in 12 of 15 tumor lesions examined (Figure 7). Interestingly, tumor lesions with AID expression also exhibited staining for TP53 protein. Because the inflamed colon of patients with Crohn's disease is at risk for developing colon cancer, we also examined whether aberrant AID expression would be present in the inflamed colonic epithelium of 8 patients with Crohn's disease. Immunostaining for AID protein was detected in the inflamed regions of the colonic epithelium in all cases examined (see Supplementary Figure 2*A* online at www.gastrojournal.org). In the case of sporadic colon cancers, AID immunoreactivity was detected in 2 of 5 cancer tissue specimens examined (see Supplementary Figure 2*B* online at www.gastrojournal.org).

To test further for AID expression in inflamed colonic mucosa, we analyzed endogenous AID expression levels in the colon of T-cell receptor (TCR) α mutant mice, an in vivo model of UC.[31] We found that AID transcript levels were up-regulated substantially in the inflamed colonic mucosa of the TCR-α mutant mice (see Supplementary Figure 3 online at www.gastrojournal.org). These findings further supported the in vitro findings that inflammatory stimulation induced aberrant AID expression in colon epithelial cells.

Discussion

Emerging evidence suggests that human carcinogenesis is a multistage process resulting from the accumulation of genetic alterations.[32] Our recent studies highlighted the importance of the DNA editor, AID, in the cellular events leading to genetic mutations during the development of inflammation-associated human cancers.[8,9] Here, we demonstrated for the first time that the Th2 cytokines IL-4 and IL-13 can induce aberrant AID expression in human colonic cells, leading to the preferential accumulation of genetic mutations in the tumor suppressor gene *TP53*. Moreover, we detected endogenous AID expression in inflamed colonic mucosa of patients with UC and in colitis-associated colorectal neoplasms. Our findings suggest that proinflammatory cytokine-mediated AID expression has a key role in generating colonic mucosa *TP53* mutations underlying IBD,

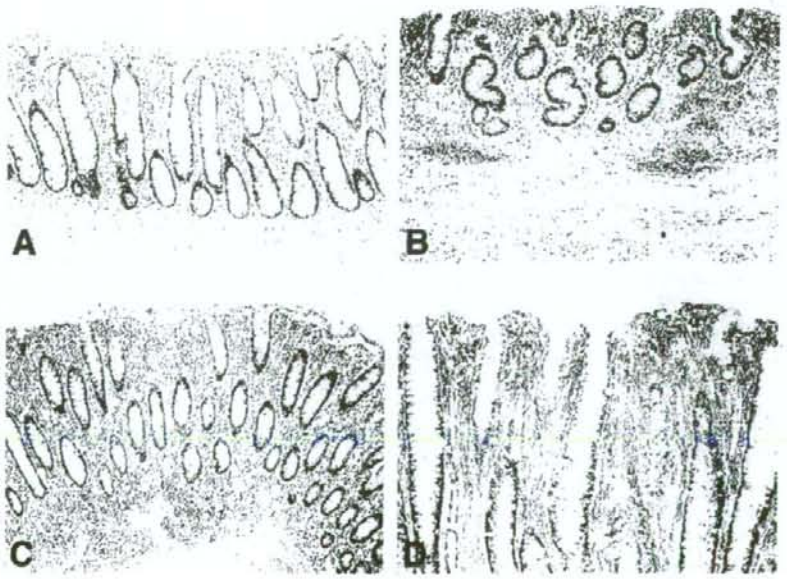

Figure 6. Expression of AID protein in UC tissue specimens. (*A*) No AID expression was observed in normal colonic tissue. (*B–D*) Strong AID immunoreactivity was present in colonic epithelial cells from patients with UC. Representative immunostaining results are shown for AID in the inflamed colonic mucosa from 3 UC cases. (original magnification, A–D, ×100).

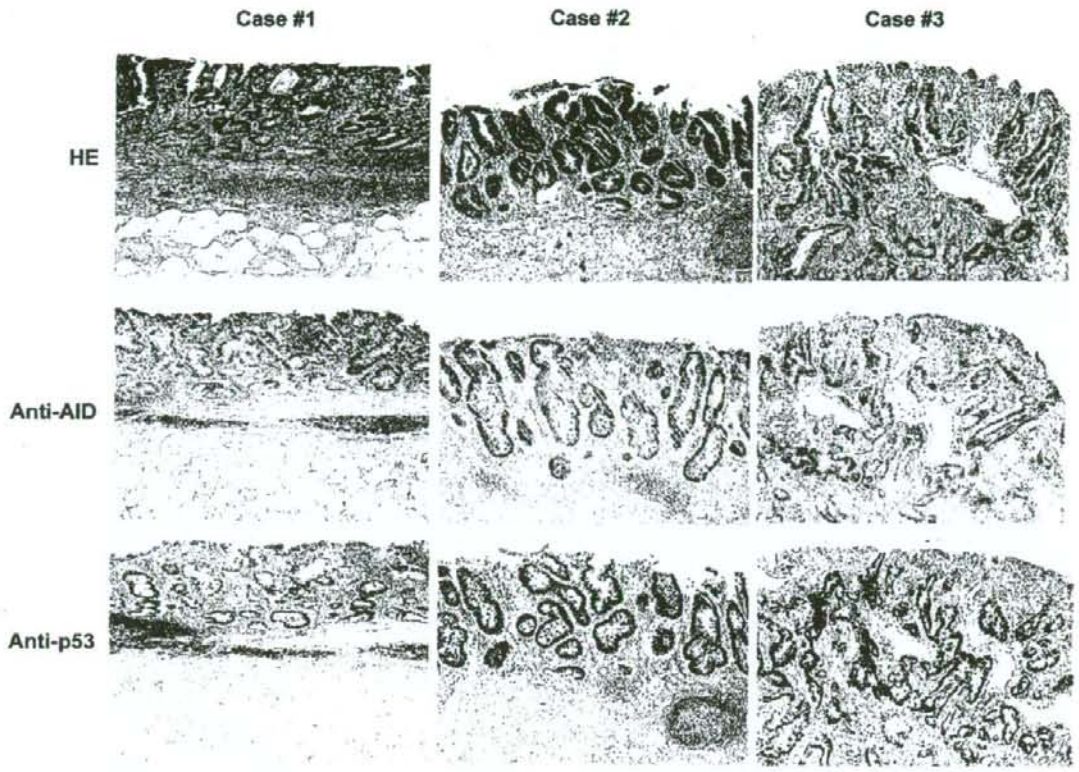

Figure 7. Expression of AID protein in colitis-associated neoplasma tissue specimens. Representative immunostaining for AID and TP53 protein is shown. Immunohistochemistry was performed on dysplasia (cases 1 and 2) or cancer (case 3) specimens from 3 patients with UC. (original magnification, 100×).

a well-known predisposing condition for colorectal cancer development.

Colonic mucosal inflammation is usually mediated by either an excessive Th1 T-cell response associated with increased IFN-γ and IL-12 secretion or an excessive Th2 T-cell response associated with increased IL-4, IL-5, and IL-13 secretion.[33,34] Although the concentration of the Th2 cell-driven cytokine IL-4 varies in UC colon tissue, UC is considered to have a Th2 profile.[33] Indeed, a recent study in mice suggests that production of IL-13 is an important pathologic factor for UC.[35] Moreover, UC has an atypical Th2 response, mediated by natural killer T cells that secrete IL-13,[36] and markedly elevated levels of IL-13 production are observed in UC patients.[29] In the current study, we demonstrated that 2 Th2 cytokines, IL-4 and IL-13, were capable of inducing endogenous AID expression in colonic epithelial cells. Interestingly, analysis using in animal models of colitis has shown that a predominance of Th2-type cytokines in inflamed colonic tissues, which mimics mucosal immunity in UC, enhances the development of colonic neoplasms.[37] Thus, we speculate that Th2 cytokine-mediated expression of AID in inflamed colonic epithelia might enhance the susceptibility to somatic mutations in tumor-related genes, leading to the formation of colonic neoplasms in patients with UC.

NF-κB transcription factors and the signaling pathways that activate NF-κB are central coordinators of inflammation-associated cancer development as well as immune responses.[38] NF-κB is activated in epithelial cells in the inflamed mucosa of patients with IBD.[24,39] Greten et al demonstrated that specific disruption of the IKK-β gene within enterocytes leads to a significant decrease in colitis-associated cancer multiplicity, suggesting that IKK-β-driven NF-κB contributes to the development of colitis-associated cancer.[40] It is thought that IKK-β-dependent NF-κB activation promotes the development of colorectal cancer via the transcriptional up-regulation of antiapoptotic target genes. In the present study, we identified AID as a target gene of the IKK-β-dependent NF-κB activation pathway in human colonic cells. Our findings demonstrate a novel link between the IKK-β-dependent NF-κB activation pathway and colitis-associated colorectal cancer development.