

**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<sup>a</sup>, disialyl Lewis<sup>a</sup>, and so on.<sup>15,32-34</sup> In our results, the CpG islands of the *B4GALNT2* gene encoding Sd<sup>a</sup>-1,4GalNAcT, the enzyme responsible for the synthesis of the Sd<sup>a</sup> 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<sup>a</sup>. Our present study using freshly frozen samples reconfirmed our previous report that nearly 100% of gastric cancers showed loss of Sd<sup>a</sup> antigen.<sup>12</sup> We believe that the difference in frequency between DNA hypermethylation and loss of Sd<sup>a</sup> 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<sup>a</sup> 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 (%)		
	Total	<i>ST3GAL6</i>	
		Methylated	Unmethylated
Number of patients	63	28 (44.4)	35 (55.6)
<i>B4GALNT2</i> <sup>a</sup>			
Methylated	32 (50.8)	24 (85.7)	8 (22.9)
Unmethylated	31 (49.2)	4 (14.3)	27 (77.1)
Epstein-Barr virus <sup>a</sup>			
Positive	9 (14.3)	8 (28.6)	1 (2.9)
Negative	54 (85.7)	20 (71.4)	34 (97.1)
<i>p53</i> 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.

<sup>a</sup>Statistically significant ( $P$  .01).

BASIC-ALIMENTARY-TRACT

cancer cell lines including MKN28, MKN45, and KATO III cells.<sup>30</sup> We reported earlier that forced expression of Sd<sup>a</sup>-1,4GalNAcT resulted in a marked increase in cell-surface expression of Sd<sup>a</sup> along with a concomitant loss of cancer-associated sLe<sup>x/a</sup> carbohydrate antigens.<sup>15</sup> Of note, DNA hypermethylation of *B4GALNT2* gene may be associated with the concomitant increase in sLe<sup>x/a</sup> 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.<sup>28,29,35</sup> DNA hypermethylation of this gene in GI cancers may also lead to enhanced expression of sLe<sup>x/a</sup> 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<sup>INK4A</sup>), is absent significantly more often in EBV-associated gastric carcinomas than in EBV-negative ones and that their loss is associated with their methylation.<sup>36</sup> 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.<sup>37</sup> 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.<sup>38</sup> 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<sup>a</sup> 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](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2008.03.031.

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Address requests for reprints to: Dr Taeko Dohi, Department of Gastroenterology, Research Institute, International Medical Center of Japan, 1-2-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. e-mail: dohi@ri.imcj.go.jp; fax: 81-3-3202-7364.

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**Supplementary Table 1.** Human glyco-genes.

Genes	Accession No.	ID of TaqMan probe kits	Enzyme	References	
<i>FUT1</i>	NM000148	Hs00355741_m1	1,2-fucosyltransferase, H blood group	1,2-fucosyltransferase	1, 2
<i>FUT2</i>	NM000511	Hs00704693_s1	1,2-fucosyltransferase, Se blood group	1,2-fucosyltransferase	3
<i>FUT3</i>	NM000149	Hs00356857_m1	1,3/4-fucosyltransferase, Lewis blood group	1,3/4-fucosyltransferase	4
<i>FUT4</i>	NM002033	Hs00275643_s1	1,3-fucosyltransferase		5, 6
<i>FUT5</i>	NM002034	Hs00704908_s1	1,3-fucosyltransferase		7
<i>FUT6</i>	NM000150	Hs00173404_m1	1,3-fucosyltransferase		8
<i>FUT7</i>	NM004479	Hs00237083_m1	1,3-fucosyltransferase		9, 10
<i>FUT8</i>	NM178154	Hs00189535_m1	1,6-fucosyltransferase		11
<i>A3GALT/A3GALNT</i>	NM020469	Hs00220850_m1	1,3- <i>N</i> -galactosyltransferase,		12, 13
			1,3- <i>N</i> -acetylgalactosaminyltransferase		
<i>GBGT1</i>	NM021996	Hs00222752_m1	globoside 1,3- <i>N</i> -acetylgalactosaminyltransferase, Forssman synthetase		14
<i>B3GALNT1</i>	NM003781	Hs00364202_s1	1,3- <i>N</i> -acetylgalactosaminyltransferase 1, globoside synthase		15
<i>B3GALNT2</i>	NM152490	Hs00380823_m1	1,3- <i>N</i> -acetylgalactosaminyltransferase 2		16
<i>B4GALNT1</i>	NM001478	Hs00155195_m1	1,4- <i>N</i> -acetylgalactosaminyltransferase 1, GM2/GD2 synthase		17
<i>B4GALNT2</i>	NM153446	Hs00396440_m1	1,4- <i>N</i> -acetylgalactosaminyltransferase 2 Sd <sup>a</sup> synthase		18
<i>B4GALNT3</i>	NM173593	Hs00403843_m1	1,4- <i>N</i> -acetylgalactosaminyltransferase 3		19
<i>B4GALNT4</i>	NM178537	Hs00331790_m1	1,4- <i>N</i> -acetylgalactosaminyltransferase 4		20
<i>GCNT1</i>	NM001490	Hs00155243_m1	glucosaminyl ( <i>N</i> -acetyl) transferase 1, core 2 (1,6- <i>N</i> -acetylglucosaminyltransferase)		21
<i>GCNT3</i>	NM004751	Hs00191070_m1	glucosaminyl ( <i>N</i> -acetyl) transferase 3		22
<i>GCNT4</i>	NM016591	Hs00275464_s1	glucosaminyl ( <i>N</i> -acetyl) transferase 4, core 2 (1,6- <i>N</i> -acetylglucosaminyltransferase)		23
<i>ST3GAL1</i>	NM003033	Hs00161688_m1	CMP-NeuAc: -galactoside 2,3-sialyltransferase 1		24
<i>ST3GAL2</i>	NM006927	Hs00199480_m1	CMP-NeuAc: -galactoside 2,3-sialyltransferase 2		25
<i>ST3GAL3</i>	NM006279	Hs00196718_m1	CMP-NeuAc:Gal 1,3/4GlcNAc 2,3-sialyltransferase		26
<i>ST3GAL4</i>	NM006278	Hs00272170_m1	Gal 1,3/4GlcNAc 2,3-sialyltransferase		27
<i>ST3GAL5</i>	NM003896	Hs00187405_m1	CMP-NeuAc:lactosylceramide 2,3-sialyltransferase		28
<i>ST3GAL6</i>	NM006100	Hs00196086_m1	CMP-NeuAc: 2,3-sialyltransferase		29
<i>ST6GAL1</i>	NM003032	Hs00949382_m1	CMP-NeuAc:galactoside 2,6-sialyltransferase		30
<i>ST6GAL2</i>	NM032528	Hs00293264_m1	CMP-NeuAc:galactoside 2,6-sialyltransferase		31
<i>ST6GALNAC1</i>	NM018414	Hs00300842_m1	GalNAc 2,6-sialyltransferase 1		32
<i>ST6GALNAC2</i>	NM006456	Hs00197670_m1	GalNAc 2,6-sialyltransferase 2		33
<i>ST6GALNAC3</i>	NM152996	Hs00541761_m1	GalNAc 2,6-sialyltransferase 3		34
<i>ST6GALNAC4</i>	NM014403	Hs00205241_m1	GalNAc 2,6-sialyltransferase 4		35
<i>ST6GALNAC5</i>	NM030965	Hs00229612_m1	GalNAc 2,6-sialyltransferase 5		36
<i>ST6GALNAC6</i>	NM013443	Hs00203739_m1	GalNAc 2,6-sialyltransferase 6		37
<i>GALNAC4S-6ST</i>	NM015892	Hs00248144_m1	<i>N</i> -acetylgalactosamine 4-sulfate 6- <i>O</i> -sulfotransferase		38
<i>CHST1</i>	NM003654	Hs00186341_m1	galactose-6- <i>O</i> -sulfotransferase		39
<i>CHST2</i>	NM004267	Hs00358839_g1	carbohydrate <i>N</i> -acetylglucosamine-6- <i>O</i> -sulfotransferase 2		40
<i>CHST3</i>	NM004273	Hs00427946_m1	chondroitin 6-sulfotransferase		41
<i>CHST4</i>	NM005769	Hs00428480_m1	<i>N</i> -acetylglucosamine-6- <i>O</i> -sulfotransferase 2, HEC-specific <i>N</i> -acetylglucosamine-6- <i>O</i> -sulfotransferase		42
<i>CHST5</i>	NM012126	Hs00201677_m1	<i>N</i> -acetylglucosamine-6- <i>O</i> -sulfotransferase 3, intestinal <i>N</i> -acetylglucosamine-6- <i>O</i> -sulfotransferase		43
<i>NEU1</i>	NM000434	Hs00166421_m1	sialidase 1, lysosomal sialidase		44
<i>NEU2</i>	NM005383	Hs00193573_m1	sialidase 2, cytosolic sialidase		45
<i>NEU3</i>	NM006656	Hs00198406_m1	sialidase 3, plasma membrane-associated sialidase		46
<i>NEU4</i>	NM080741	Hs00293852_m1	sialidase 4, glyceraldehyde-3-phosphate dehydrogenase		47
<i>GAPDH</i>	NM002046	Hs00266705_g1			

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

YOKO ENDO,\* HIROYUKI MARUSAWA,\* TADAYUKI KOU,\* HIROSHI NAKASE,\* SHIGEHICO 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- $\alpha$  induced aberrant AID expression via I $\kappa$ B kinase-dependent nuclear factor (NF)- $\kappa$ B-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.<sup>1</sup> 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.<sup>2</sup> Thus, the relative risk of colorectal cancer in patients with UC was up to 20 times higher than that of the general population.<sup>3</sup> 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.<sup>4</sup> Mutations in the *TP53* gene appear to be an early event and are already present in dysplastic lesions associated with UC.<sup>5,6</sup> 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.<sup>7–9</sup> Under physiologic conditions, AID is required for somatic hypermutation and class switch recombination in immunoglobulin genes of activated B cells.<sup>10,11</sup> However, the inappropriate expression of AID could contribute to tumorigenesis via its DNA mutagenic activity.<sup>12</sup> 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.<sup>8,9,13,14</sup> Notably, ectopic AID expression is induced in response to tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), a proinflammatory cytokine that is important in the pathway leading to tumorigenesis.<sup>15</sup> These findings demonstrate a novel linkage between in-

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

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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.<sup>16</sup> Expression of most proinflammatory cytokines and chemokines, including TNF- $\alpha$ , is up-regulated in the colonic tissues of patients with IBD,<sup>17</sup> 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-I $\kappa$ B kinase (IKK)  $\alpha$ , IKK $\beta$ , and RelA (nuclear factor [NF]- $\kappa$ B) were as described.<sup>18</sup> The expression plasmids pcDNA3-I $\kappa$ B $\alpha$  $\Delta$ N, encoding the super-repressor form of I $\kappa$ B $\alpha$ , and pcDNA3-IKK $\beta$  (K44A), encoding the dominant negative mutant of IKK- $\beta$  have also been described.<sup>8</sup> Expression plasmid encoding the dominant negative form of STAT6 (STAT6 $\Delta$ C) lacking the C-terminal 186 amino acids<sup>19</sup> was generated from the STAT6 fragment using polymerase chain reaction (PCR) amplification. Recombinant human TNF- $\alpha$ , interleukin (IL)-4, IL-13, IL-1 $\beta$ , IL-12, and interferon (IFN)- $\gamma$  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'-TCG-GCGTGAGACCTACCTGTGCTAC-3'. Target complementary DNA (cDNA) were normalized to endogenous messenger RNA (mRNA) levels of the housekeeping reference gene *18s ribosomal RNA (18s rRNA)*.<sup>7</sup> 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 Bio-medicals, 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.<sup>20</sup> 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.<sup>21</sup>

### NF- $\kappa$ B 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).<sup>18</sup>

### 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](http://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.<sup>22</sup> A mouse monoclonal antibody against  $\alpha$ -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.<sup>23</sup>

### 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  $45.3 \pm 12.4$  years (mean  $\pm$  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.

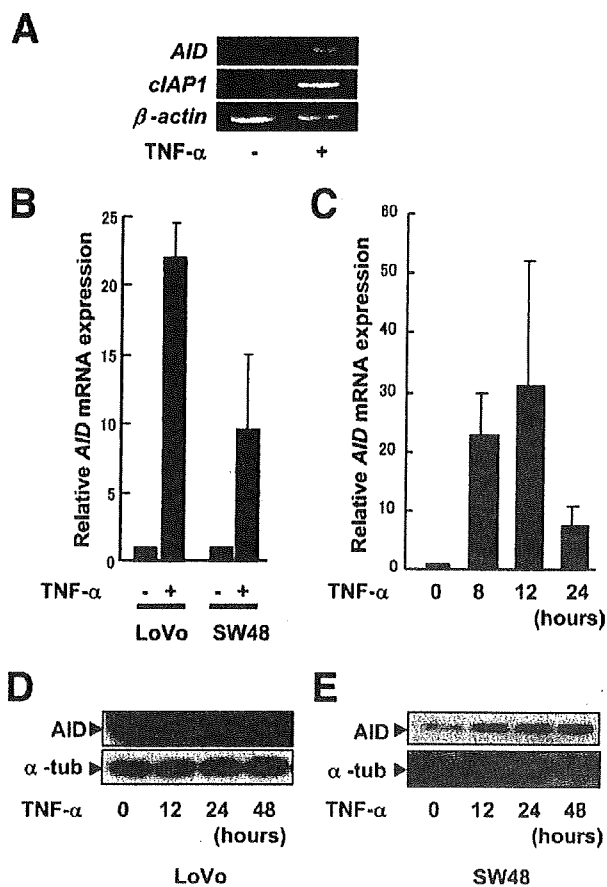
## Results

### Human AID Is Induced in Response to TNF- $\alpha$ 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- $\alpha$ -inducible gene in many types of cells, was increased in response to TNF- $\alpha$  stimulation in colonic cells (Figure 1A). Endogenous AID expression was markedly elevated after TNF- $\alpha$  treatment in both LoVo and SW48 cells (Figure 1A and B). TNF- $\alpha$  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 1C). Immunoblotting analysis using a specific antibody against human AID revealed that TNF- $\alpha$  induced a time-dependent up-regulation of AID protein in both cell types, with a peak level 12 to 24 hours after treatment (Figure 1D and E). RT-PCR analysis revealed that AID transcripts also increased in response to another proinflammatory cytokine, IL-1 $\beta$ , in LoVo cells (see Supplementary Figure 1A online at [www.gastrojournal.org](http://www.gastrojournal.org)). Taken together, these findings suggest that endogenous AID expression is induced by proinflammatory cytokine stimulation in human colonic cells.

### NF- $\kappa$ B Mediates AID Expression in an I $\kappa$ B Kinase-Dependent Manner in Colonic Epithelial Cells

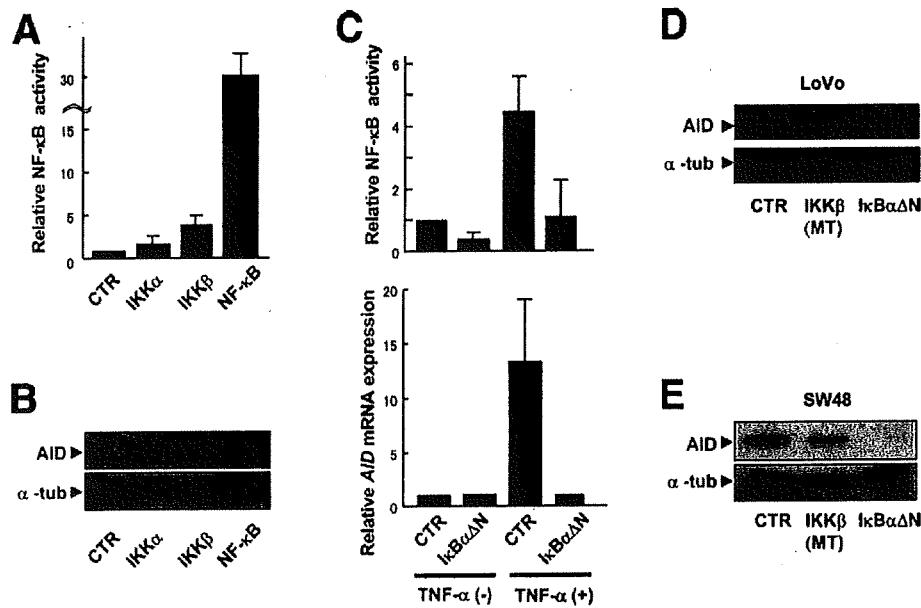
Transcription factor NF- $\kappa$ B is induced by TNF- $\alpha$  signaling, and NF- $\kappa$ B is frequently and constitutively activated in the colonic epithelia of patients with IBD.<sup>24</sup> Therefore, we examined whether AID expression would be regulated transcriptionally by NF- $\kappa$ B activity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



**Figure 1.** AID expression is induced in response to TNF- $\alpha$  stimulation in human colon cancer cells. (A) Total RNA was extracted from LoVo cells 12 hours after TNF- $\alpha$  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  $\beta$ -actin (lower panel). (B) Total RNA was isolated from LoVo and SW48 cells before and 12 hours after stimulation with TNF- $\alpha$  (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- $\alpha$  treatment (mean  $\pm$  SD; n = 3). (C) Total RNA from LoVo cells was isolated immediately before and 8, 12, and 24 hours after TNF- $\alpha$  treatment (100 ng/mL). (D) LoVo and (E) SW48 cells were treated with TNF- $\alpha$  (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- $\alpha$ -tubulin ( $\alpha$ -tub, lower panels).

(I $\kappa$ B $\alpha$  $\Delta$ N), would reduce AID production. We confirmed by reporter plasmid assay that TNF- $\alpha$ -mediated NF- $\kappa$ B activity was almost completely abolished by coproduction of I $\kappa$ B $\alpha$  $\Delta$ N (Figure 2C, upper graph). Up-regulation of AID after TNF- $\alpha$  treatment was reduced substantially in LoVo cells in which I $\kappa$ B $\alpha$  $\Delta$ N was coproduced (Figure 2C, lower graph). The coproduction of mutant IKK- $\beta$  or I $\kappa$ B $\alpha$  $\Delta$ N also reduced endogenous AID protein expression after TNF- $\alpha$  stimulation in both cells (Figure 2D and E). Because protein kinase C $\xi$  is required for NF- $\kappa$ B activation in several cells,<sup>25</sup> we also examined its involvement in TNF- $\alpha$ -mediated AID expression. We found that

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**Figure 2.** TNF- $\alpha$ -induced AID expression is achieved through the activation of NF- $\kappa$ B. (A) LoVo cells were transfected with NF- $\kappa$ B reporter plasmids together with expression plasmids encoding wild-type IKK- $\alpha$ , IKK- $\beta$ , and RelA (NF- $\kappa$ B) 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- $\alpha$ -tubulin ( $\alpha$ -tub, lower panel). (C) LoVo cells were transfected with a plasmid for the expression of the super-repressor form of I $\kappa$ B- $\alpha$  (I $\kappa$ B $\alpha$  $\Delta$ N) or with a control vector (CTR), followed by treatment with TNF- $\alpha$  (100 ng/mL) for 12 hours. Each sample was harvested, and luciferase activity was measured to quantify endogenous NF- $\kappa$ B 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  $\pm$  SD; n = 3). (D) LoVo cells and SW48 (E) cells were transfected with plasmids encoding dominant negative IKK $\beta$  (IKK $\beta$ MT), I $\kappa$ B $\alpha$  $\Delta$ N, or a CTR vector and then treated with TNF- $\alpha$  (100 ng/mL) for 24 hours. Cell lysates were immunoblotted with anti-AID antibody (upper panel) and anti- $\alpha$ -tubulin ( $\alpha$ -tub, lower panel).

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

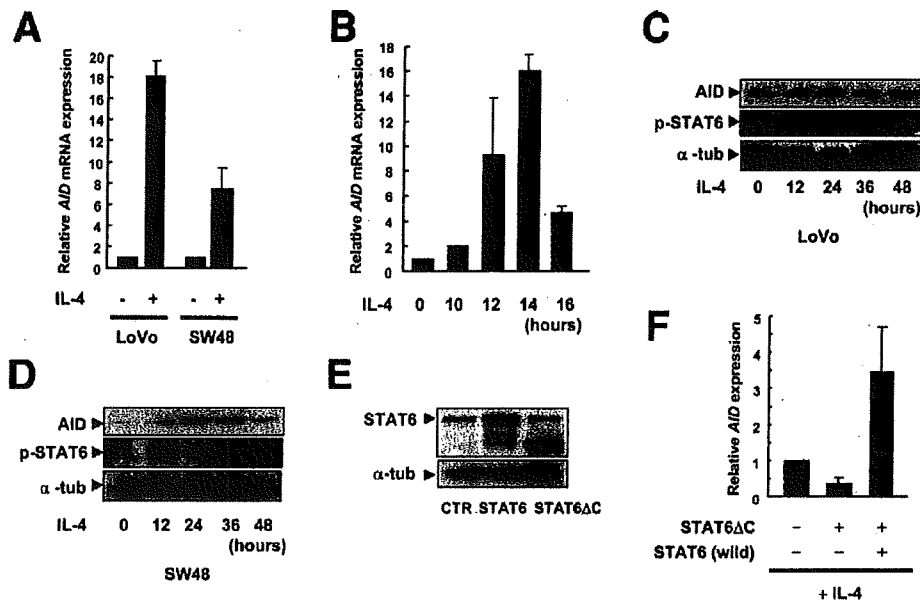
These findings indicate that AID expression in colonic epithelial cells is regulated through the IKK-dependent NF- $\kappa$ B-signaling pathway, suggesting a common mechanism for the regulation of AID gene expression in human epithelial cells in the colon, stomach, and liver under inflammatory conditions.<sup>8,9</sup>

#### *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,<sup>26</sup> 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,<sup>27</sup> 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 treat-

ment in both LoVo and SW48 cells (Figure 3A). AID transcripts were induced promptly, peaking at 12 to 14 hours after IL-4 treatment (Figure 3B). IL-4 activates STAT6 in a phosphorylation-dependent manner and contributes to the regulation of expression of various genes.<sup>28</sup> Immunoblotting analysis revealed that IL-4 treatment resulted in an increase in phosphorylated STAT6 protein expression (Figure 3C and 3D). 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 3C) and SW48 cells (Figure 3D). We next examined whether AID expression would be mediated by a STAT6-dependent mechanism using the dominant negative form of STAT6 (STAT6 $\Delta$ C) (Figure 3E). We found that STAT6 $\Delta$ C suppressed IL-4-mediated AID expression and that coexpression of the wild-type STAT6 reversed the suppression of AID expression caused by STAT6 $\Delta$ C in colonic cells (Figure 3F).

Another Th2 cytokine, IL-13, is a critical mediator of mucosal inflammation and could be a key molecule in the pathogenesis of human UC.<sup>29</sup> IL-13 shares many functional properties with IL-4, such as a common receptor subunit, the  $\alpha$ -subunit of the IL-4 receptor, and



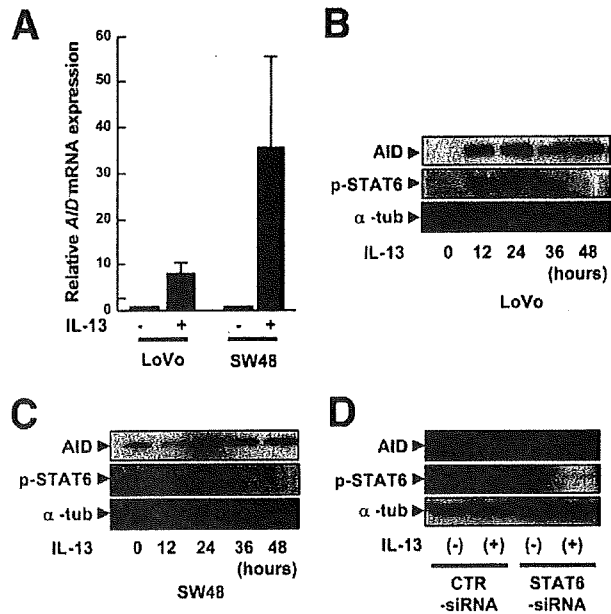
**Figure 3.** IL-4-mediated AID expression in human colonic epithelial cells. (A) LoVo and SW48 cells were treated with human recombinant IL-4 (100 ng/mL) for 12 hours. Quantitative RT-PCR was performed using FAM-labeled probes specific for human *AID*. (B) Time course of changes in *AID* mRNA expression 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- $\alpha$ -tubulin antibodies ( $\alpha$ -tub, lower panel). (E) LoVo cells were transfected with expression plasmids encoding wild-type STAT6, STAT6 $\Delta$ C, or control vector (CTR). After 48 hours, cell lysates were subjected to immunoblotting using anti-STAT6 (upper panel) or anti- $\alpha$ -tubulin ( $\alpha$ -tub, lower panel). (F) LoVo cells were transfected with STAT6 $\Delta$ C 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.<sup>30</sup> 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 4A). 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 4B 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 4D). 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- $\gamma$ , suggesting that some of the Th1 cytokines may also play roles in the aberrant expression of AID in colonic cells (see Supplementary Figure 1A online at [www.gastrojournal.org](http://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 mutagenic 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.<sup>8</sup> 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

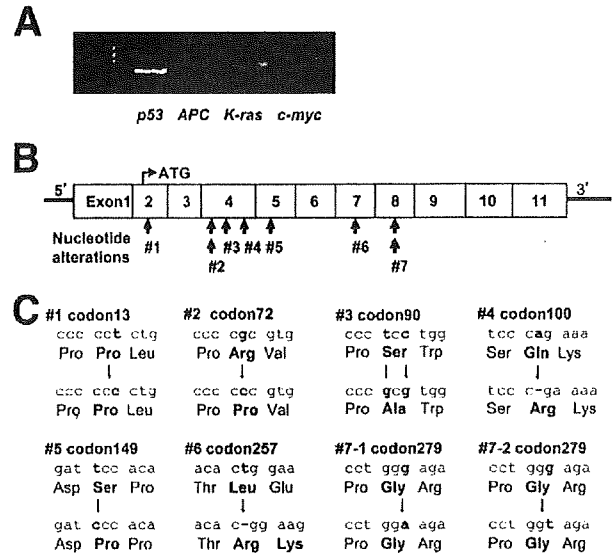


**Figure 4.** AID expression is regulated by IL-13 in human colonic epithelial cells. (A) Total RNA was isolated from LoVo and SW48 cells after treatment with human recombinant IL-13 (100 ng/mL) for 12 hours. The expression levels of *AID* mRNA were measured by quantitative real-time PCR using FAM-labeled 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- $\alpha$ -tubulin antibodies ( *$\alpha$ -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  $\alpha$ -tubulin ( *$\alpha$ -tub*, lower panel).

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 5B 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

**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)
<i>TP53</i> (exons 2-6)	2 weeks	1/40	1/22,000
	4 weeks	2/47	2/25,850
	8 weeks	6/48	7/26,400
<i>TP53</i> (exons 6-11)	2 weeks	1/43	1/23,650
	4 weeks	2/34	2/18,700
	8 weeks	3/38	3/20,900
<i>APC</i>	8 weeks	0/42	0/23,425
<i>K-ras</i>	8 weeks	0/40	0/18,760
<i>c-myc</i>	8 weeks	1/41	1/21,695



**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.

(<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.<sup>21</sup> 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](http://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.

**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-

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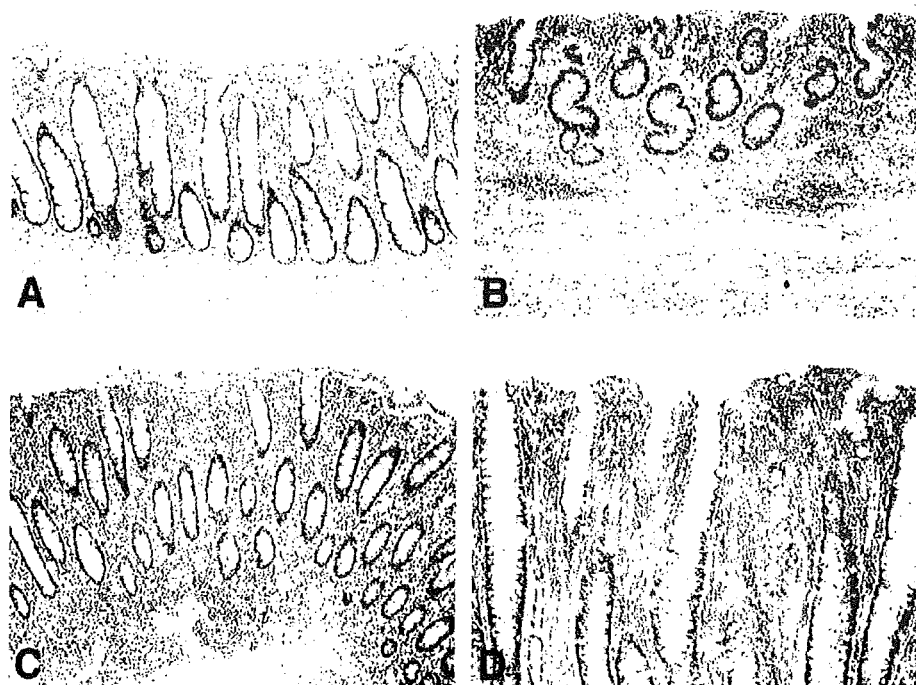
formed using a specific antibody against human AID in colonic tissue from 22 UC lesions, 15 colitis-associated neoplasms, and 5 nontumorous regions of the patients with sporadic colon cancers. Specificity of the antibody in immunostaining was confirmed by control staining performed on germinal centers of human mesenteric lymph nodes containing mostly activated B cells.<sup>7,9</sup> In normal colonic mucosa lacking inflammation, immunohistochemistry revealed no evidence of AID expression (Figure 6A). By contrast, endogenous AID immunoreactivity was detected in the colonic epithelium of 12 of 22 (54%) inflammatory lesions from patients with UC (Figure 6B–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 2A online at [www.gastrojournal.org](http://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 2B online at [www.gastrojournal.org](http://www.gastrojournal.org)).

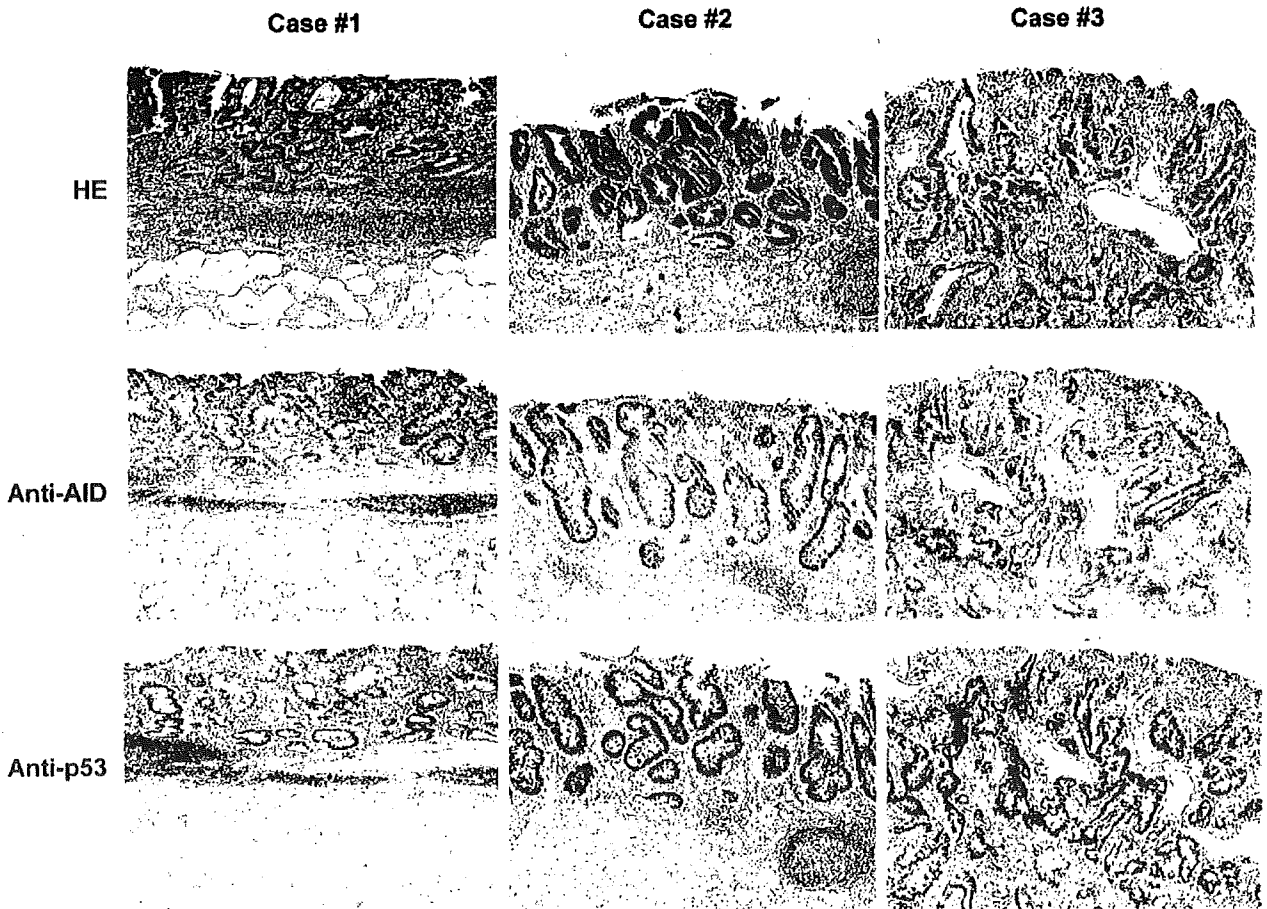
To test further for AID expression in inflamed colonic mucosa, we analyzed endogenous AID expression levels in the colon of T-cell receptor (TCR)  $\alpha$  mutant mice, an *in vivo* model of UC.<sup>31</sup> We found that AID transcript levels were up-regulated substantially in the inflamed colonic mucosa of the TCR- $\alpha$  mutant mice (see Supplementary Figure 3 online at [www.gastrojournal.org](http://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.<sup>32</sup> 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.<sup>8,9</sup> 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,  $\times 100$ ).



**Figure 7.** Expression of AID protein in colitis-associated neoplasia 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 $\times$ ).

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- $\gamma$  and IL-12 secretion or an excessive Th2 T-cell response associated with increased IL-4, IL-5, and IL-13 secretion.<sup>33,34</sup> Although the concentration of the Th2 cell-driven cytokine IL-4 varies in UC colon tissue, UC is considered to have a Th2 profile.<sup>33</sup> Indeed, a recent study in mice suggests that production of IL-13 is an important pathologic factor for UC.<sup>35</sup> Moreover, UC has an atypical Th2 response, mediated by natural killer T cells that secrete IL-13,<sup>36</sup> and markedly elevated levels of IL-13 production are observed in UC patients.<sup>29</sup> 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.<sup>37</sup> 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- $\kappa$ B transcription factors and the signaling pathways that activate NF- $\kappa$ B are central coordinators of inflammation-associated cancer development as well as immune responses.<sup>38</sup> NF- $\kappa$ B is activated in epithelial cells in the inflamed mucosa of patients with IBD.<sup>24,39</sup> Greten et al demonstrated that specific disruption of the IKK- $\beta$  gene within enterocytes leads to a significant decrease in colitis-associated cancer multiplicity, suggesting that IKK- $\beta$ -driven NF- $\kappa$ B contributes to the development of colitis-associated cancer.<sup>40</sup> It is thought that IKK- $\beta$ -dependent NF- $\kappa$ B 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- $\beta$ -dependent NF- $\kappa$ B activation pathway in human colonic cells. Our findings demonstrate a novel link between the IKK- $\beta$ -dependent NF- $\kappa$ B activation pathway and colitis-associated colorectal cancer development.

It is well established that several aspects of manifestation, including clinical features and genetic alteration characteristics, differ markedly between sporadic- and colitis-associated colorectal cancers.<sup>5,6,41</sup> Adenomatous polyps are considered to be the major precursor of sporadic colorectal cancers,<sup>42</sup> and inactivation of the *APC* gene is believed to be the initial event in sporadic colorectal cancer development, followed by changes in the *K-ras*, *DCC*, and *TP53* genes.<sup>43</sup> In contrast to sporadic cancer, an alteration in the *TP53* gene is usually an early event in the molecular pathogenesis of IBD-related colorectal cancer, whereas the mutation frequency of *APC* and *K-ras* is substantially lower than that in sporadic cancers. It is noteworthy that genetic alterations in the *TP53* gene likely precede dysplasia, the precancerous lesions in the inflammatory colon.<sup>44</sup> Moreover, there is an increased frequency of *TP53* mutations in noncancerous UC colon tissues.<sup>45</sup> The molecular mechanisms underlying the contribution of inflammatory conditions to the accumulation of *TP53* mutations are not well-defined. Here, we show that AID activation induced significant levels of *TP53* mutations in colonic mucosal cells. Thus, aberrant expression of AID might be involved in the generation of genetic alterations in the *TP53* gene in inflamed colonic epithelial cells.

An interesting point in our study is that the *APC*, *K-ras*, and *c-myc* genes were less frequently mutated by AID activation than the *TP53* gene in human colonic cells. These findings are consistent with previous observations that target gene selection of AID for somatic hypermutation varies among target cells.<sup>8,9</sup> Similar to its effect in colonic cells, aberrant AID expression in gastric epithelial cells induces nucleotide alterations in the *TP53* gene, whereas no mutations are induced in *c-myc*.<sup>9</sup> In contrast, AID expression in cultured hepatoma-derived cells results in the appearance of nucleotide alterations in the *c-myc* gene.<sup>8</sup> It is not clear why the *TP53* gene is more sensitive to AID activation in colonic epithelial cells. One possibility is that transcription levels of the genes targeted by AID are higher than other genes because AID-induced hypermutation depends on target gene transcription levels.<sup>46,47</sup> Consistent with this hypothesis, semiquantitative RT-PCR analysis revealed that *TP53* mRNA expression was higher than the *APC*, *K-ras*, and *c-myc* genes in LoVo cells. Further analysis is required to identify the specific target genes of AID-mediated mutagenesis in human colonic mucosal cells.

A previous study demonstrated that the development of hyperplasia of isolated lymphoid follicles in AID-deficient mice is associated with a 100-fold expansion of anaerobic flora in the small intestine.<sup>48</sup> On the other hand, oral administration of antigens from anaerobic bacterial flora of the intestine reduces the severity of experimental acute colitis.<sup>49</sup> Thus, further analyses are required to examine whether the AID expression level in the colon epithelium might influence the pattern of in-

testinal bacterial flora and the colonic inflammation in IBD patients.

In conclusion, we found that proinflammatory cytokines induced the aberrant expression of AID in human colonic cells, leading to the generation of somatic mutations in the host genome, including the *TP53* gene. Our findings provide a novel linkage between chronic inflammation and enhanced susceptibility to somatic mutations and an increased risk of colorectal cancers.

### Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2008.06.091.

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Address requests for reprints to: Hiroyuki Marusawa, MD, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. e-mail: maru@kuhp.kyoto-u.ac.jp; fax: (81) 75-751-4303.

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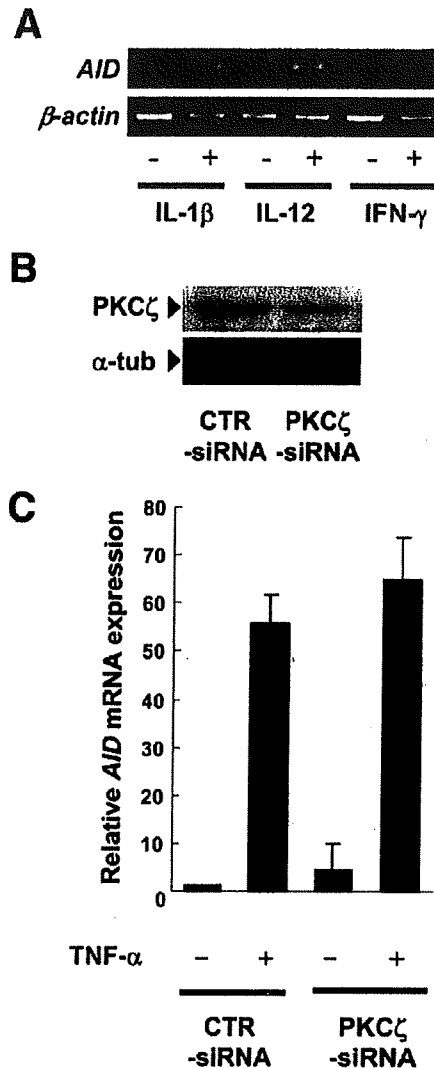
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Conflicts of interest: No conflicts of interest exist.



**Supplemental Table 1.** Oligonucleotides Used for Subcloning in the Current Study

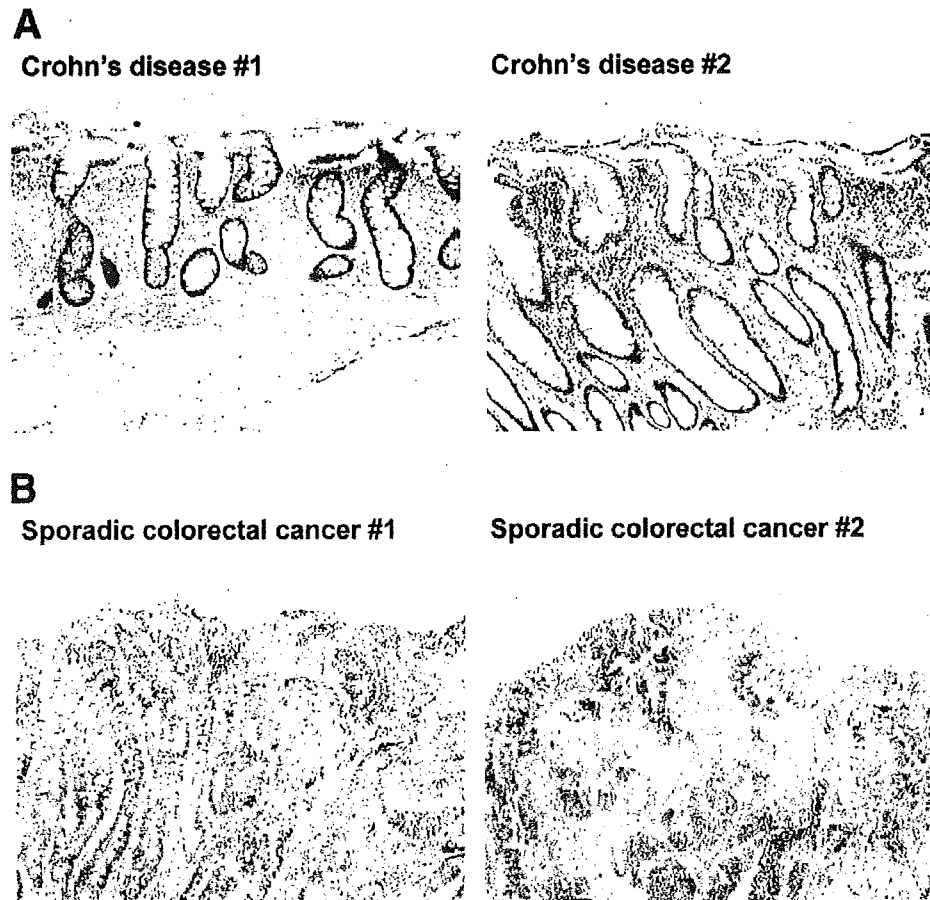
Primer	Nucleotide sequence
<i>TP53</i> -S (exons 2-6)	5'-GCCGAATTCATTGGCAGCCAGACTGCCTTC-3'
<i>TP53</i> -AS (exons 2-6)	5'-CCGCTCGAGAAATTCCTTCCACTCGGATA-3'
<i>TP53</i> -S (exons 6-11)	5'-CCGGAATTCAGTGGGAAAGGAAATTTGCGTGT-3'
<i>TP53</i> -AS (exons 6-11)	5'-ATCCTCGAGTCAGTGGGGAACAAGAAGTG-3'
<i>APC</i> -S	5'-GCCGAATTCCTTCTGCTAATACCCCTGCAA-3'
<i>APC</i> -AS	5'-ATCCTCGAGCAGCATCTGGAAGAACCT-3'
<i>K-ras</i> -S	5'-CGCGGATCCAACCTTGTGGTAGTTGG-3'
<i>K-ras</i> -AS	5'-CCGCTCGAGACCATCTTTGCTCATC-3'
<i>c-myc</i> -S	5'-GCCGAATTCGTAGTGGAAAACCAGCAGCC-3'
<i>c-myc</i> -AS	5'-ATCCTCGAGTCTGATGTGTGGAGACGTG-3'



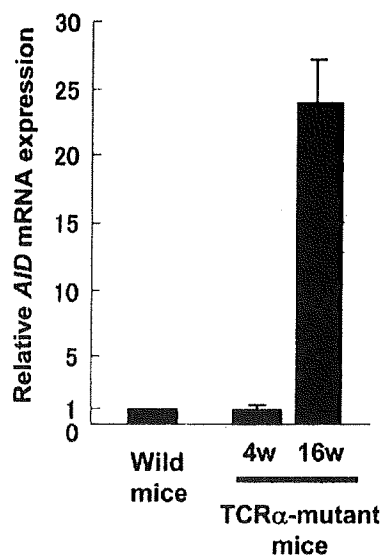
**Supplementary Figure 1.** Regulation of AID expression by cytokine stimulation in human colonic cells. (A) Semi-quantitative RT-PCR analyses for *AID* expression in LoVo cells treated with IL-1- $\beta$ , IL-12 or IFN- $\gamma$  treatment. Total RNA was extracted from LoVo cells after 12 h of treatment with IL-1 $\beta$  (25ng/ml), IL-12 (100ng/ml) or IFN- $\gamma$  (100ng/ml) treatment. Semi-quantitative RT-PCR was performed using oligonucleotide primer sets specific for human *AID* (upper panel) and *β-actin* (lower panel). (B) LoVo cells were transfected with siRNA targeting *PKC $\zeta$*  (In-vitrogen) for 48 h. Whole cell lysates were probed by anti-*PKC $\zeta$*  antibody (Cell Signaling Technology; upper panel) or anti- $\alpha$ -tubulin antibody (lower panel). (C) Effects of *PKC $\zeta$* -siRNA on TNF- $\alpha$ -induced *AID* gene expression. LoVo cells were transfected with siRNA targeting *PKC $\zeta$*  or control siRNA and lysates were prepared from the siRNA-treated cells after the stimulation with TNF- $\alpha$  (100ng/ml) for 12 h. Total RNA was extracted from each specimen and subjected to quantitative real-time RT-PCR analyses for *AID* expression. The data present the means of *AID* mRNA expression relative to the internal control *18S rRNA* (mean  $\pm$  SD; n=3).

**Supplemental Table 2.** Mutation Frequencies in the *TP53* Gene of LoVo Cells With the Wild or Mutant AID Expression

	Duration of AID activation	Mutated clones (n/total)	Mutation number (n/total bases)
AID (wild)	6 weeks	3/40	4/24200
AID (R35E)	6 weeks	2/42	2/25500
AID (R35E/R36D)	6 weeks	1/39	1/23600



**Supplementary Figure 2.** Expression of AID protein in various human colonic tissue specimens. Representative immunostaining for AID in the inflamed colonic epithelial mucosa of patients with Crohn's disease (A #1 and #2) and sporadic colorectal cancers (B #1 and #2). (Original magnification: A&B,  $\times 100$ ).



**Supplementary Figure 3.** Expression of AID in TCR $\alpha$ -mutant mice. Total RNA was collected from the colonic mucosa of 16-weeks-old of wild type and 4-weeks-old or 16-weeks-old TCR $\alpha$ -mutant mice. Expression levels of AID transcripts were determined by quantitative real-time RT-PCR analyses. Histological examination revealed that severe colitis was observed only in the colon of the 16-weeks-old TCR $\alpha$ -mutant mice.

# nature genetics

## A genome-wide association study identifies three new susceptibility loci for ulcerative colitis in the Japanese population

Kouichi Asano<sup>1-3</sup>, Tomonaga Matsushita<sup>1,2</sup>, Junji Umeno<sup>1,2</sup>, Naoya Hosono<sup>1</sup>, Atsushi Takahashi<sup>4</sup>, Takahisa Kawaguchi<sup>5</sup>, Takayuki Matsumoto<sup>2</sup>, Toshiyuki Matsui<sup>6</sup>, Yoichi Kakuta<sup>7</sup>, Yoshitaka Kinouchi<sup>7</sup>, Tooru Shimosegawa<sup>7</sup>, Masayo Hosokawa<sup>8</sup>, Yoshiaki Arimura<sup>8</sup>, Yasuhisa Shinomura<sup>8</sup>, Yutaka Kiyohara<sup>3</sup>, Tatsuhiko Tsunoda<sup>5</sup>, Naoyuki Kamatani<sup>4</sup>, Mitsuo Iida<sup>2</sup>, Yusuke Nakamura<sup>9</sup> & Michiaki Kubo<sup>1-3</sup>

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