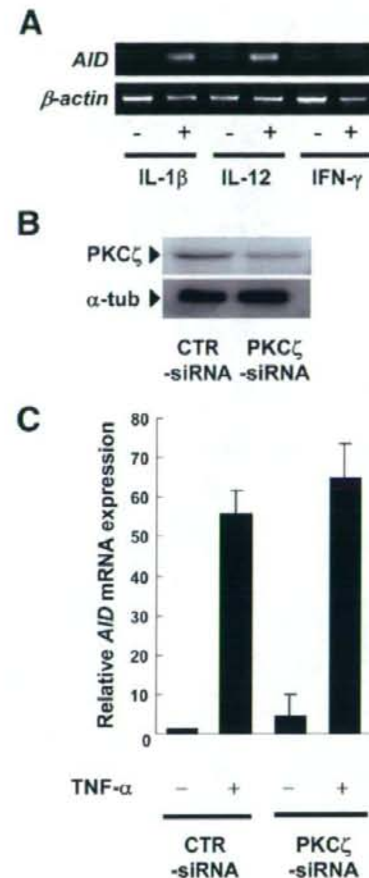
Supplemental Table 1. Oligonucleotides Used for Subcloning in the Current Study

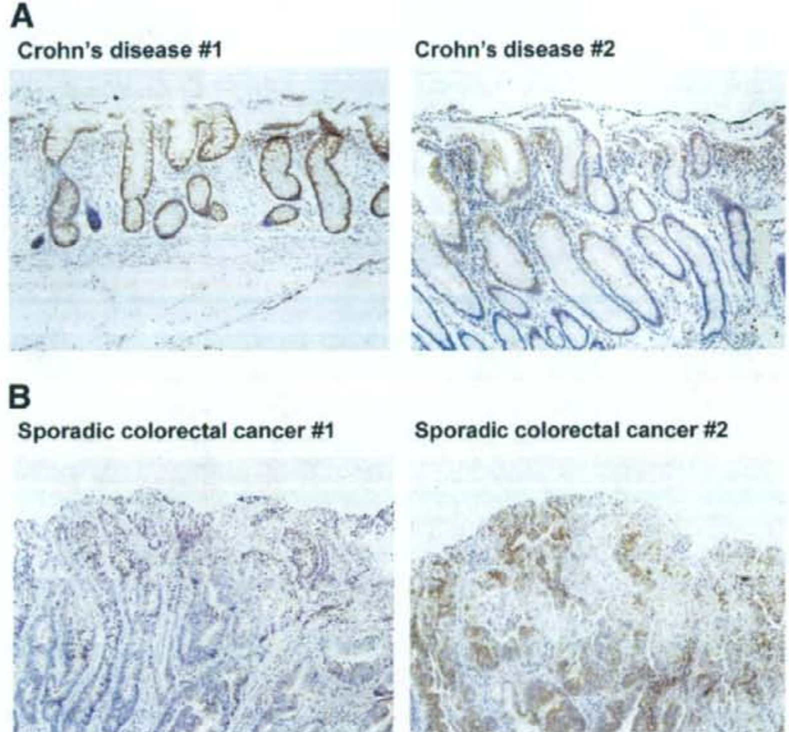
Primer	Nucleotide sequence
TP53-S (exons 2-6)	5'-GCCGAATTCATTGGCAGCCAGACTGCCTTC-3'
TP53-AS (exons 2-6)	5'-CCGCTCGAGAAATTTCCTTCCACTCGGATA-3'
TP53-S (exons 6-11)	5'-CCGGAATTCAGTGGAAGGAAATTTGCGTGT-3'
TP53-AS (exons 6-11)	5'-ATCCTCGAGTCAGTGGGGAACAAGAAGTG-3'
APC-S	5'-GCCGAATTCTTCTGCTAATACCCTGCAA-3'
APC-AS	5'-ATCCTCGAGCAGCATCTGGAAGAACCT-3'
K-ras-S	5'-CGCGGATCCAACTTGTGGTAGTTGG-3'
K-ras-AS	5'-CCGCTCGAGACCATCTTTGCTCATC-3'
c-myc-S	5'-GCCGAATTCGTAGTGGAAAACCAGCAGCC-3'
c-myc-AS	5'- ATCCTCGAGTGCTGATGTGTGGAGACGTG-3'

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

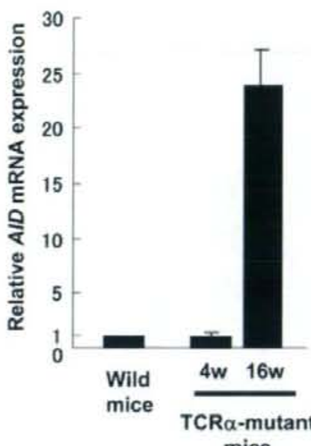
	Duration of AID activation	Mutated clones (n/total)	Mutation number (n/total bases)
AID (wild)	6 weeks	3/40	4/24200
AID (R35E)	6 weeks	2/42	2/25500
AID (R35E/R36D)	6 weeks	1/39	1/23600



Supplementary Figure 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 IL1-β, IL-12 or IFN-γ treatment. Total RNA was extracted from LoVo cells after 12 h of treatment with IL-1β (25ng/ml), IL-12 (100ng/ml) or IFN-γ (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 *PKCζ* (Invitrogen)for 48 h. Whole cell lysates were probed by anti-PKCζ antibody (Cell Signaling Technology; upper panel) or anti-α-tubulin antibody (lower panel). (*C*) Effects of PKCζ-siRNA on TNF-α-induced AID gene expression. LoVo cells were transfected with siRNA targeting *PKCζ* or control siRNA and lysates were prepared from the siRNA-treated cells after the stimulation with TNF-α (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 ± SD; n=3).



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*, ×100).



Supplementary Figure 3. Expression of AID in TCRα-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 TCRα-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 TCRα-mutant mice.

Activation-Induced Cytidine Deaminase Links Bile Duct Inflammation to Human Cholangiocarcinoma

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Chronic inflammation plays a critical role in oncogenesis in various human organs. Epidemiological studies have demonstrated that patients with primary sclerosing cholangitis have a predisposition to develop cholangiocarcinoma (CC). However, the molecular mechanisms that account for the development of bile duct carcinomas are not well defined. We recently provided evidence that activation-induced cytidine deaminase (AID), a member of the DNA/RNA editing enzyme family, is implicated in human tumorigenesis via its mutagenic activity. We found here that ectopic AID production is induced in response to tumor necrosis factor-α (TNF-α) stimulation via the IkappaB kinase-dependent nuclear factor-κB (NF-κB) activation pathway in human cholangiocarcinoma-derived cells. Aberrant expression of AID in biliary cells resulted in the generation of somatic mutations in tumor-related genes, including *p53*, *c-myc*, and the promoter region of the *INK4A/p16* sequences. In human tissue specimens, real-time reverse transcription polymerase chain reaction (RT-PCR) analyses revealed that *AID* was increased significantly in 28 of 30 CC tissues (93%), whereas only trace amounts of *AID* were detected in the normal liver. Immunohistochemistry showed that all of the CC tissue samples examined showed overproduction of endogenous AID protein in cancer cells. Moreover, immunostaining for AID was detectable in 16 of 20 bile epithelia in the tissues underlying primary sclerosing cholangitis. *Conclusion:* The proinflammatory cytokine-induced aberrant production of AID might link bile duct inflammation to an enhanced genetic susceptibility to mutagenesis, leading to cholangiocarcinogenesis.
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Cholangiocarcinoma (CC) is an epithelial neoplasm that originates from the bile duct and can occur at any level of the biliary tree.[1,2] The incidence CC is increasing worldwide; it is the second most common primary hepatobiliary malignancy.[2] Although most CC arise in the absence of apparent risk factors, chronic inflammation of the biliary epithelium plays a critical role for their development.[2] In fact, primary sclerosing cholangitis (PSC) is the commonest predisposing condition for cholangiocarcinogenesis, and the prevalence of CC in patients with PSC ranges from 9% to 23%, with a cumulative annual risk of 1.5% per year of the disease.[1] Other risk factors for cholangiocarcinogenesis are also associated with chronic biliary tract inflammation, including chronic choledocholithiasis, liver fluke infestation, hepatolithiasis, Caroli's disease, and hepatitis C viral infection.[1] It has been hypothesized that the increased risk of CC in these conditions occurs because of chronic epithelial inflammation leading to cell proliferation, along with enhanced production of endogenous mutagens in the bile.[1] However, the precise molecular mechanism that accounts for the development of CC on the basis of chronic biliary tract inflammation remains unsolved.

Abbreviations: AID, activation-induced cytidine deaminase; APOBEC, apolipoprotein B mRNA-editing enzyme catalytic-polypeptide; CC, cholangiocarcinoma; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; IKK, IκB kinase; mRNA, messenger RNA; NF-κB, nuclear factor kappa B; PSC, primary sclerosing cholangitis; RT-PCR, reverse transcription polymerase chain reaction; Tg, transgenic; TNF-α, tumor necrosis factor alpha.

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Activation-induced cytidine deaminase (AID) was originally identified as an inducer of somatic hypermutation, which diversifies the variable region of immunoglobulin genes in activated B cells in germinal centers.[3,4] However, animal models with constitutive expression of the gene for this enzyme revealed that aberrant AID production resulted in the accumulation of genetic mutations in various tumor-related genes, leading to lymphoid and nonlymphoid malignancies.[5] Indeed, most AID transgenic (Tg) mice developed microadenomas of the lung epithelium as well as T cell lymphomas exhibiting frequent point mutations in the *T cell receptor* and *c-myc* genes that appeared to be caused by AID activity.[5] Strikingly, we recently observed that constitutive expression of AID also caused the development of liver tumors with the morphological characteristics of hepatocellular carcinoma (HCC).[6] Although *AID* gene expression is restricted to the lymphoid organs under physiological conditions, we observed aberrant AID expression in both human hepatocytes and gastric epithelial cells underlying areas of chronic inflammation.[7,8] Consistent with these *in vivo* findings, we showed that endogenous *AID* gene expression was induced by proinflammatory cytokine stimulation in human hepatocytes as well as gastric epithelial cells.[6,8] These findings suggest a role for AID in the development of cancers in the setting of chronic inflammation in human epithelial organs.

Although the origin of CC is not well understood, it has been proposed that both HCC and CC could develop from a common origin, such as hepatic stem or progenitor cells.[9] The histogenesis of intestinal-type CC and combined hepatocellular and cholangiocellular carcinoma observed in experimental rodent models of liver carcinogenesis and in humans is consistent with the concept that at least some subtypes of CC derived from pluripotent liver stem cells.[10,11] Thus, as aberrant expression of AID in the liver can be genotoxic, leading to hepatocarcinogenesis, we were prompted to speculate that AID might be involved in cholangiocarcinogenesis. Therefore, in this study, we investigated the production and regulation of endogenous AID in human biliary epithelial cells in association with proinflammatory cytokine stimulation. We also examined whether there was aberrant AID production in human liver tissue specimens of PSC and bile duct cancers.

Patients and Methods

Patients. The study group consisted of 30 patients who had undergone potentially curative resection for a primary intrahepatic cholangiocarcinoma (ICC) at Kyoto University Hospital from 1995 to 2006. Selection of patients enrolled in this study was based on the availability of a sufficient amount of tissue for analysis. The patients included 15 men and 15 women, with a mean age at the time of surgery of 61.7 ± 12.3 years [mean ± standard deviation; range, 29-78 years; Table 1]. Moreover, the liver tissue specimens of 20 patients with PSC who received liver transplantation from 1999 to 2006 were examined for AID expression. As a control, 6 samples of normal liver tissues from patients with metastatic liver cancer were also examined. Biopsy specimens of tumor tissues at the proximal edge of freshly resected specimens were obtained and frozen immediately in liquid nitrogen. Written informed consent for the use of their resected tissues 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.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction. Total RNA was extracted from tissue specimens using the guanidinium–phenol–chloroform method (Sepasol; Nacalai Tesque, Kyoto, Japan).[12] Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) for human *AID* messenger RNA (mRNA) amplification was carried out using the 7300 Real-Time PCR System (PE Applied Biosystems, Foster City, CA). The 6-carboxyfluorescein–labeled probe used for human *AID* mRNA was 5′-TCG-

Table 1. Clinicopathological Features of Patients with ICC

Feature	Value
Age at surgery (years)	
Mean ± SD	61.7 ± 12.3
Gender	
Male	15
Female	15
Hepatitis	
HBV+	3
HCV+	3
HBV− HCV−	24
Noncancerous liver tissue	
Normal liver	22
Inflammatory liver	8
Number of tumor	
solitary	19
multiple	11
Tumor size (cm)	
Mean ± SD	6.0 ± 1.9
Tumor differentiation	
well	7
moderately	15
poorly	6
unknown	2
TNM staging	
I	0
II	8
III	12
IV	10

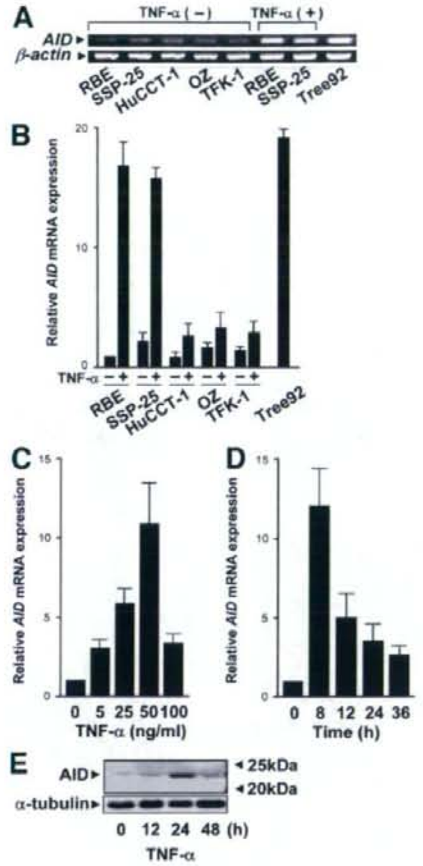

Fig. 1. Proinflammatory cytokine-mediated activation-induced cytidine deaminase (AID) expression in human bile duct-derived cells. (A) Two human bile duct-derived cell lines, SSP-25 and RBE, were treated with tumor necrosis factor-alpha (TNF-α) (50 ng/mL) for 12 hours. Total RNA was extracted and reverse transcription polymerase chain reaction (RT-PCR) amplification was performed using oligonucleotide primers specific for the human *AID* gene. B-cell lymphoma-derived Tree 92 cells were used as a positive control. (B) Five human bile duct-derived cells, RBE, SSP-25, HuCCT-1, OZ, and TFK-1, were stimulated with TNF-α (50 ng/mL) for 12 hours, and *AID* transcripts were measured by quantitative real-time RT-PCR. The expression levels were normalized to *18S ribosomal RNA (18S rRNA)* as an endogenous control. The ratios are shown as relative values compared with the AID expression levels in nonstimulated RBE cells. (C, D) Dose-dependent and time-dependent effects of TNF-α on *AID* gene expression. RBE cells were treated with various concentrations of TNF-α (0-100 ng/mL) for 12 hours (C) or with TNF-α (50 ng/mL) for the indicated times. (D) Total RNA was extracted from each specimen and subjected to quantitative real-time RT-PCR analyses. (E) RBE cells were treated with TNF-α (50 ng/mL) for 0, 12, 24, and 48 hours, followed by immunoblotting using anti-AID antibody (upper panel) or anti-α-tubulin antibody (lower panel).

GCGTGAGACCTACCTGTGCTAC-3′.[6] Standard curves were generated for every target using a 10-fold serial dilution series of 5 independent transcripts derived from BL2-lymphoma cells that contained a high endogenous level of *AID*.[8] Target complementary DNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene *18S ribosomal RNA (18S rRNA)*.[7] For simplicity, the ratios are given as relative values compared with the levels in a lysate from the nontreated cholangiocarcinoma-derived cells (RBE). Reproducibility was examined by comparing the results obtained from replicate samples during the same reaction run and those from independent runs on different days.[8] The PCR procedures were performed at least 3 times for each sample, and results are expressed as the mean ± standard deviation in Figs. 1B through D and 2B and as the mean ± SEM (standard error measurement) in Fig. 3A,B.

Cell Culture. The human CC cell lines HuCCT-1 and TFK-1 were obtained from the Cell Resource Center for Biochemical Research, Tohoku University; OZ was from the Japan Health Science Foundation (Tokyo); SSP-25 and RBE cells were from the RIKEN Bioresource Center (Tsukuba). These were cultured at 37°C in Dulbecco's modified Eagle's medium (Gibco-BRL, Tokyo, Japan) supplemented with 10% fetal bovine serum.

Plasmids and Reagents. The expression plasmids pcDNA3-IκB kinase (IKK) pcDNA3-α, pcDNA3-IKK-β, and pcDNA3-RelA [nuclear factor-κB (NF-κB)] were as described.[13] The expression plasmids pcDNA3-IκB-α-ΔN, pcDNA3-IKK-α (K44A), and pcDNA3-IKK-β (K44A), encoding the super-repressor form of IκB-α, and dominant negative mutants of IKK-α and IKK-β, respectively, were also as described.[6] Small interference RNA (siRNA) duplexes composed of 21-nucleotide sense and antisense strands used for targeting IKK-γ/NEMO and AID were obtained from Dharmacon Research (Lafayette, CO). Recombinant human tumor necrosis factor-α (TNF-α) was purchased from Peprotech EC Ltd. (London, UK).

Recombinant Retrovirus Production and Infection of Biliary Cells. The retroviral system for measuring the expression of the *AID* gene in cultured biliary cells was as described.[14] A full-length complementary DNA for *AID* was subcloned into the *Eco*RI and *Xho*I restriction sites of the pFB vector (Stratagene, La Jolla, CA). The plasmids and packaging plasmids, pCL-Ampho (Imgenex, San Diego, CA), were transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Preactivated cells were suspended in the medium containing retrovirus supplemented with 16 g/mL Polybrene (Sigma-Aldrich, St.

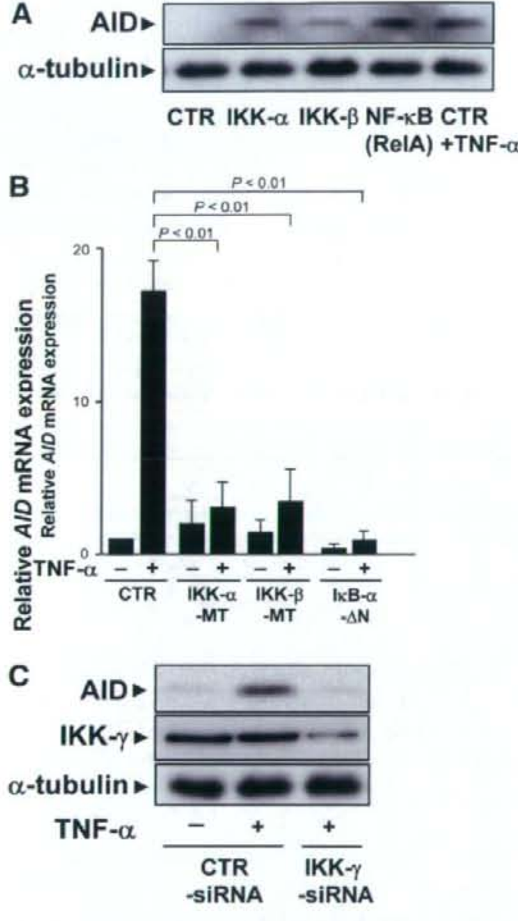

Fig. 2. Nuclear facter-κB (NF-κB)-dependent AID expression in biliary cells. (A) pcDNA3-IκB kinase (IKK)-α, -IKK-β, -NF-κB (RelA), or control vectors were transfected into RBE cells. The cell lysates were then analyzed by immunoblot analyses using anti-AID (upper panel) or anti-α-tubulin (lower panel) antibodies. (B) RBE cells were transfected with pcDNA3-IKK-α (K44A, IKK-α-MT), -IKK-β (K44A, IKK-β-MT), super-repressor form of IκB-α (IκB-α-ΔN) or control (CTR), followed by treatment with TNF-α for 12 hours. The cell lysates were subjected to real-time RT-PCR analyses to determine the expression levels of *AID* transcripts. These were normalized to an endogenous reference gene (*18S rRNA*), and values shown in the graphs were normalized to control specimens without TNF-α stimulation. (C) RBE cells were transfected with small interference (si) RNA targeting IKK-γ/NEMO or control (CTR) small interfering RNA for 24 hours, followed by treatment with TNF-α for an additional 12 hours. The cell lysates were subjected to immunoblot analyses to determine the protein production levels of AID (upper panel), IKK-γ (middle panel), or α-tubulin (lower panel).

Louis, MO), centrifuged for 40 minutes at 32°C, and incubated for 48 hours.

Subcloning and Sequencing of Tumor-Related Genes. The oligonucleotide primers for human *p53, c-myc, INK4A/p16,* and *k-ras* are shown in Supplementary Table 1. Amplification of these genes was carried out using high-fidelity Phusion polymerase (Finnzymes, Espoo, Finland), and the products were subcloned by insertion

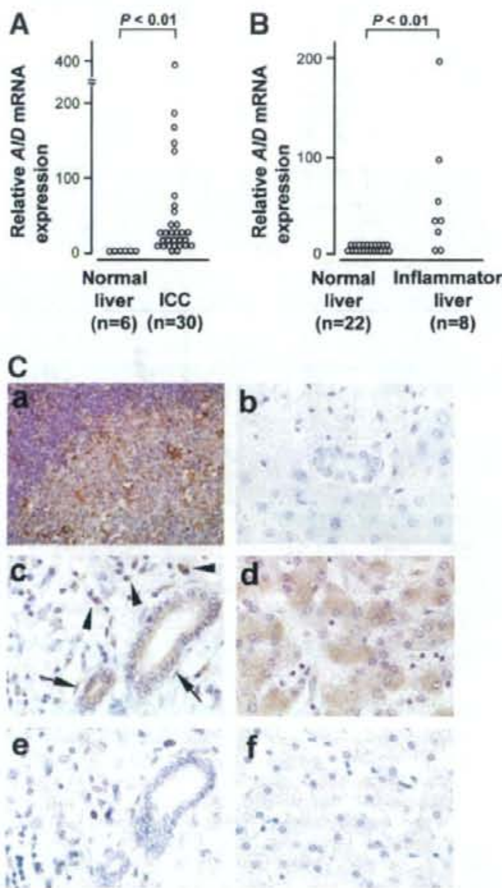

Fig. 3. *AID* mRNA and protein expression in human normal liver, intrahepatic cholangiocarcinoma (ICC), and its surrounding noncancerous liver tissues. (A) Comparison of *AID* transcript expression in intrahepatic cholangiocarcinoma (ICC) and normal liver tissues from a patient with a metastatic liver cancer. The amounts of *AID* mRNA were normalized to an endogenous reference gene (*18S rRNA*). (B) Comparison of *AID* mRNA expression in ICC-noncancerous livers exhibiting the features of chronic hepatitis or cholangitis (inflammatory liver) and those lacking any evidence of hepatic inflammation (normal liver). (C) AID immunostaining in the inflammatory liver tissues. AID immunoreactivity in a germinal center of an intra-abdominal lymph node (C-a). Normal liver tissue showed no AID immunostaining (C-b). The biliary epithelial cells (C-c, arrows) and hepatocytes (C-d) as well as the infiltrating lymphocytes (arrowheads) showed immunoreactivity for AID in the noncancerous region of liver tissue accompanying chronic inflammation. Negative control staining with nonimmunized serum in C-e and C-f correspond to specimens C-c and C-d, respectively (original magnifications: C-a, 400×; C-b-f, 800×).

into the pcDNA3 vector (Invitrogen).[15] The resulting plasmids were subjected to sequence analysis using a DY-Enamic ET terminator kit with AmpliTaq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ) on an automated sequencer (Applied Biosystems).

Immunoblotting and Immunohistochemistry. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12% (wt/vol) polyacrylamide gels and subjected to immunoblotting analyses. The polyclonal antibodies against human AID were used in this study as described.[6] Anti-α-tubulin antibodies were obtained from Calbiochem (San Diego, CA). Immunohistochemistry was carried out as described.[16] To semi-quantitate the immunostaining results, the slides were scored independently by two evaluators (J.K. and H.H.) for AID staining. Visual assessment based on degree and intensity of immunoreactivity was classified as no staining (−), weak positive staining (+), moderate positive staining (++), and strong positive staining (+++).

Statistical Analysis. Statistical differences in *AID* gene expression levels were analyzed using the Mann-Whitney nonparametric U test for real-time PCR results and the chi-squared test for immunohistochemical results. $P < 0.05$ was considered statistically significant.

Results

Aberrant AID Expression Is Induced by Proinflammatory Cytokine Stimulation in Human Cholangiocarcinoma-Derived Cells. To gain preliminary insights into the expression of the *AID* gene in human bile duct epithelium, expression of *AID* mRNA transcripts was first analyzed by RT-PCR in several cholangiocarcinoma-derived cells in the absence or presence of TNF-α, a proinflammatory cytokine that plays a central role in the pathogenesis of human sclerosing cholangitis.[17-19] We found that endogenous *AID* mRNA expression was enhanced by TNF-α stimulation in RBE and SSP-25 cells, whereas only small amounts were detectable in the quiescent cells (Fig. 1A). Quantitative RT-PCR analyses revealed that TNF-α stimulation induced up-regulation of *AID* gene expression in all the biliary cells examined, including HuCCT-1, OZ, and TFK-1 (Fig. 1B). Notably, the expression of *AID* mRNA in RBE cells was increased more than 15-fold after stimulation with TNF-α, a comparable level of *AID* expression to that in the B cell lymphoma-derived cells, Tree92. Real-time RT-PCR analysis with 6-carboxyfluorescein–labeled probes also revealed that TNF-α treatment induced a dose-dependent increase in *AID* mRNA expression in RBE cells (Fig. 1C). Moreover, TNF-α induced a time-dependent transcriptional upregulation of *AID* mRNA in RBE cells, peaking at 8 hours, whereas the expression of *18S rRNA* transcripts was unchanged (Fig. 1D). To confirm the TNF-α–mediated induction of *AID* mRNA expression in human bile duct–derived cells, we carried out immunoblotting analysis for the detection of endogenous AID protein. Only trace amounts were detectable in RBE cells without any stimulation. However, marked up-regulation of AID protein production was observed after treatment with TNF-α (Fig. 1E).

Because endogenous AID protein production was induced in response to TNF-α stimulation in biliary cells, we tested whether the *AID* gene would be regulated transcriptionally by the NF-κB. Activation of the NF-κB pathway converges on the IKK signals, a protein complex composed of 2 kinase subunits (IKK-α and IKK-β) and a noncatalytic subunit IκB kinase-γ (IKK-γ/NEMO). NF-κB is rendered inactive in unstimulated cells through binding of a specific NF-κB inhibitor, IκB-α protein. First, we examined whether synthesis of the positive regulators of NF-κB signaling affected AID expression and found that the expression of AID protein was substantially up-regulated by coproduction of the wild-type IKK-α, IKK-β or NF-κB itself (Fig. 2A). In contrast, the TNF-α–mediated *AID* mRNA expression was substantially reduced in biliary cells by coproduction of negative regulators of NF-κB, the dominant negative forms of IκB kinases or super-repressor form of IκB-α (Fig. 2B). Moreover, knockdown of endogenous IKK-γ by small interfering RNA resulted in the substantial reduction in the TNF-α–mediated AID expression (Fig. 2C). Taken together, these findings suggest that the proinflammatory cytokine TNF-α induces endogenous *AID* mRNA expression via NF-κB signaling in human bile duct–derived cells.

AID Activation Achieved Accumulation of Nucleotide Alterations in Tumor-Related Genes of the Human Cholangiocarcinoma-Derived Cells. We demonstrated previously that aberrant *AID* gene expression is capable of triggering the accumulation of genomic mutations in human hepatocytes.[6] To clarify whether proinflammatory cytokine-induced aberrant *AID* gene expression is genotoxic in biliary cells, we investigated whether AID caused somatic mutations in several tumor-related genes. For this purpose, the mutagenic effects of AID were determined using a retroviral vector-mediated *AID* gene expression system in HuCCT-1 cells. We investigated the overall somatic mutation frequencies in the *p53* gene and in the promoter region of *INK4A/p16*, both of which have been reported to contain nucleotide alterations in human CC tissues.[20,21] In addition, we also investigated mutations in *c-myc*, which is thought to be the common target for

Table 2. Genomic Mutations in HuCCT-1 Cells with AID Expression

Genome		Number of mutations*	Mutation frequency (10^4)**
p53	exon 5-6	4/93	0.71 (4/56480)
	exon 1	0/105	<0.18 (0/56860)
	exon 2-4	0/102	<0.19 (0/53554)
c-myc	exon 1	2/71	0.51 (2/38979)
INK4A/p16	promoter	4/90	0.72 (4/55535)
k-ras	exon 2	0/102	<0.20 (0/49164)

*Number of mutated clones/number of clones examined is shown.

**Values in parenthesis are number of mutated bases/number of total bases examined.

abnormal gene editing in lymphoma cells of AID Tg mice.[5] Accordingly, over 70 clones were randomly picked from the cells seven days after *AID* expression and subjected to sequence analyses. We first confirmed that no nucleotide alterations were detected in all of those tumor-related genes subcloned from the control cells (mutation frequencies less than 0.20 per 10^4 nucleotides). In contrast, nucleotide alterations appeared in both *c-myc* and the promoter region of the *INK4A/p16* gene of the cells with *AID* gene expression (mutation frequency 0.51 and 0.72 per 10^4 nucleotides; Table 2). Interestingly, the nucleotide alterations induced by *AID* gene activation were clustered in exons 5 to 6 of the *p53* gene, whereas exons 1 and 2 through 4 of the *p53* sequences had no mutation among the clones isolated from the same cells with *AID* gene expression. In contrast to those 3 genes, no somatic mutations emerged in *k-ras* sequences after *AID* activation. These findings suggest that aberrant *AID* gene expression plays a role as a DNA mutator for some of the tumor-related genes in human biliary epithelium cells.

Endogenous AID Expression Is Up-regulated in the Human Bile Duct Epithelium Underlying Sclerosing Cholangitis and Cholangiocarcinomas. The *in vitro* findings that endogenous *AID* gene expression was induced by proinflammatory cytokine stimulation prompted us to test whether aberrant AID expression is involved in human cholangiocarcinogenesis via bile duct inflammation. To examine *AID* gene expression in human bile ducts under physiological or pathological conditions, we first quantified the *AID* transcripts in normal liver and ICC tissues. Quantitative real-time PCR analyses revealed that 28 of the 30 ICC tissue samples (93%) showed up-regulation of *AID* gene expression, whereas it was transcribed only in trace amounts in the normal liver tissues (Fig. 3A). The mean *AID/18S rRNA* ratio in tumorous tissues (42.7 $\pm$ 15.6) was significantly higher than in normal liver (0.2 $\pm$ 0.1, $P < 0.01$). Next, we focused on the expression levels of endogenous *AID* mRNA in noncancerous liver tissues of the patients with ICC. Eight of 30 such samples (27%) exhibited the histological features of chronic cholangitis or hepatitis. In contrast, the remaining 22 showed no evidence of inflammatory changes in the liver tissue around the tumors. We found that the mean *AID* expression level of noncancerous tissues underlying chronic inflammation was 35.2 $\pm$ 23.9, significantly higher than those of the noncancerous tissue lacking inflammatory features ($P < 0.01$) (Fig. 3B).

To determine whether the increased AID expression in inflammatory liver tissues was derived from the biliary epithelial cells, hepatocytes, or infiltrating lymphocytes, we carried out immunostaining in various noncancerous liver specimens using antibodies specific for human AID. Specificity was confirmed by control blotting performed on AID-expressing lymphoid tissues (Fig. 3C-a). Immunoreactivity for endogenous AID was absent in the normal bile duct epithelium and hepatocytes in patients lacking hepatic inflammation (Fig. 3C-b). In contrast, AID protein expression was observed in both bile duct epithelium and inflammatory cells in the liver exhibiting chronic biliary inflammation (Fig. 3C-c). Immunoreactivity for AID was mainly detectable in hepatocytes as well as lymphocytes in the liver with underlying chronic hepatitis (Fig. 3C-d). Conversely, no immunoreactivity was detected when we used nonimmunized serum on those specimens (Fig. 3C-e, C-f). Taken together, these findings indicate that there was aberrant *AID* expression in bile duct epithelium in the liver with chronic inflammation and human CC tissues.

To further study the specific expression and precise localization of the AID protein in bile duct epithelium underlying chronic inflammation, we expanded the analyses regarding AID immunohistochemistry on liver tissue specimens of patients with PSC. We found that immunoreactivity for AID was detectable in the bile duct epithelium as well as in some of the lymphocytes infiltrating around the portal area in 16 of 20 (80%) liver specimens underlying sclerosing cholangitis (Table 3; Fig. 4). In

Table 3. Semiquantitation of AID Immunoreactivity in Normal Liver, Primary Sclerosing Cholangitis (PSC) and Intrahepatic Cholangiocarcinoma (ICC)

Condition	Specimens analyzed (n)	Specimens with AID immunoreactivity (n)				Frequency of ++ to +++*
		−	+	++	+++	
Normal liver	5	5	0	0	0	0/5
PSC	20	4	6	7	3	10/20†
ICC	20	0	6	9	5	14/20‡

+++, strong positive; ++, moderate positive; +, weak positive; −, not detectable.

*Number of specimens with ++ to +++ AID immunoreactivity/number of specimens analyzed is shown.

†$P < 0.05$, PSC versus normal liver. ‡$P < 0.01$, ICC versus normal liver.

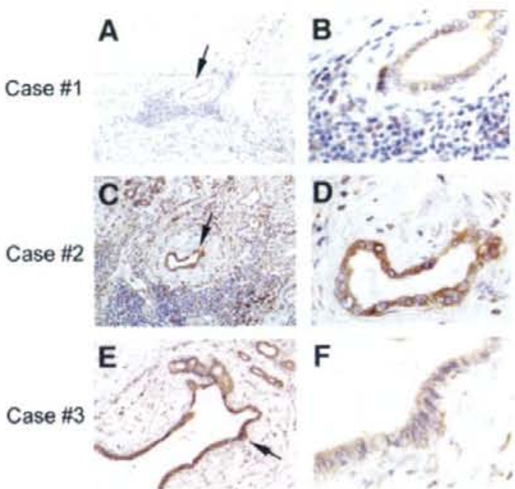

Fig. 4. Production of endogenous AID protein in human liver tissues underlying primary sclerosing cholangitis (PSC). Representative moderate to strong AID immunostaining is shown in the liver tissues from patients with PSC. Case 1 showed the moderate AID immunoreactivity in the bile ducts (A and B). Cases 2 and 3 had strong staining for AID in the bile epitheliums (C and D for Case 2, E and F for Case 3). Arrows show the AID gene overexpressing bile duct under the inflammatory condition (original magnification: A, C, and E, 200×; B, D, and F, 800×).

contrast, no immunostaining for AID was observed in any of the normal liver tissues. Thus, the frequency of expression of moderate to strong positive immunostaining for AID protein in bile duct epithelium was significantly higher in the PSC livers than in the normal liver tissues ($P < 0.05$; Table 3). In CC tissues, all 20 tumor specimens examined showed positive AID immunostaining, and AID protein was observed in neoplastic cells mainly in the cytoplasm (Table 3; Fig. 5). We also confirmed that no immunostaining was obtained when nonimmunized serum or phosphate-buffered saline were used instead of the antibodies against AID in any of the tissue specimens (Fig. 5D,H,L). Taken together, these findings revealed that the AID protein is produced aberrantly in a substantial proportion of human bile epithelial cells with chronic inflammation and cholangiocarcinoma cells.

Discussion

Various molecular alterations in relation to dysregulation of cell growth and survival pathways, invasion and metastasis, and tumor microenvironment have been reported to occur during the development of CC.[10] In fact, many mutations in oncogenes and tumor suppressor genes have been identified in human CC tissues, suggest-

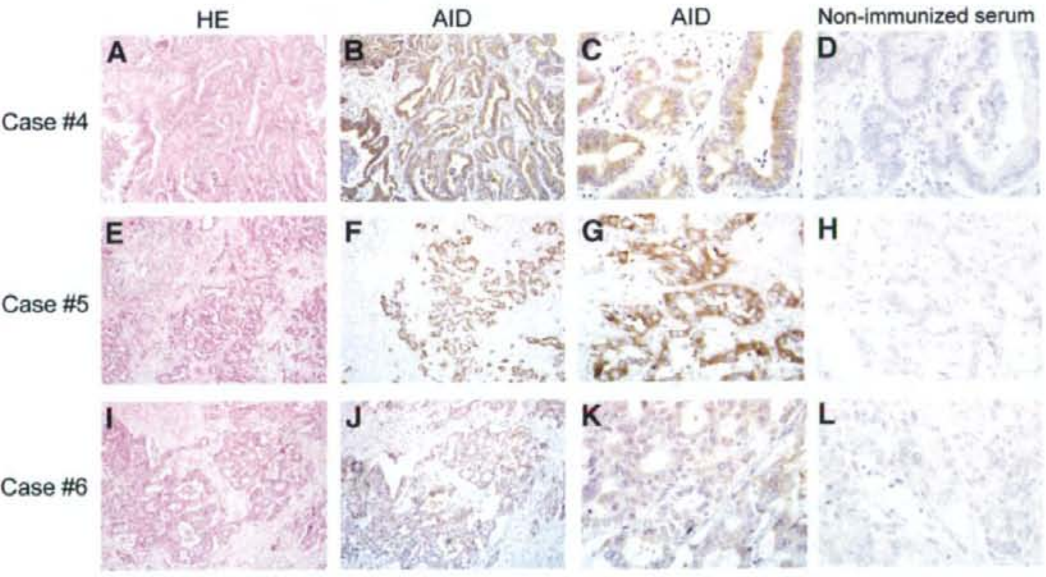

Fig. 5. Aberrant AID protein production in human cholangiocarcinoma (CC) tissues. Representative moderate to strong AID immunostaining is shown in the tumor tissues of intrahepatic cholangiocarcinomas (Case 4, A-D; Case 5, E-H; Case 6, I-L). Cases 4 and 5 showed the strong AID immunoreactivity in tumor cells (B and C for Case 4, F and G for Case 5). Case 6 had moderate staining for AID in tumor cells (J and K). The cholangioma cells show cytoplasmic staining for AID, whereas stromal cells surrounding neoplastic cholangiocytes lack immunoreactivity for AID. A, E, and I show hematoxylin-eosin staining; B, C, F, G, J, and K show anti-AID immunohistochemistry; D, H, and L show negative control with nonimmunized serum (original magnifications: A, B, E, F, I, and J, 100×; C, D, G, H, K, and L, 400×).

ing that biliary neoplastic cells may arise from cellular and consequent DNA injury.[22] However, how somatic mutations accumulate through the process of human cholangiocarcinogenesis is unknown. In the current study, we demonstrated that a recently identified DNA editing enzyme, AID, is induced by proinflammatory cytokine stimulation in biliary epithelial cells. Moreover, AID production caused multiple somatic mutations, which accumulated in some genes possibly involved in oncogenic pathways of the biliary cells. These findings suggest the involvement of aberrant *AID* gene expression in biliary epithelial cells in causing a high susceptibility to somatic mutations, which may lead to the development of bile duct neoplasms.

AID is a member of the DNA/RNA-editing cytidine deaminase, apolipoprotein B mRNA-editing enzyme catalytic-polypeptide (APOBEC) family that includes APOBEC-1, APOBEC-2, APOBEC-3A, APOBEC-3B, APOBEC-3C, APOBEC-3DE, APOBEC-3F, APOBEC-3G, APOBEC-3H, and APOBEC-4.[23] The inappropriate expression of APOBEC family molecules could act as a DNA/RNA mutator and thus contribute to tumorigenesis.[24] The first evidence for the oncogenic potential of the APOBEC family was shown using animal models with constitutive expression of the gene for APOBEC-1. APOBEC-1 Tg animals developed HCC via APOBEC-1–induced mutagenesis of inappropriate target genes including *NAT-1*.[25] However, more remarkable phenotypical changes were observed in mice producing AID. Interestingly, AID Tg mice developed various forms of neoplasia, including T cell lymphomas, lung cancers, and HCC,[5,6] suggesting that AID acts as a genome mutator in various tissues including the liver and that aberrant *AID* gene expression might play a role in producing neoplastic cells in these organs.

One of the intriguing findings in the current study is that AID production was significantly up-regulated in human biliary epithelium cells in the setting of PSC as well as in CC tumor cells. PSC is characterized by chronic inflammatory damage of the biliary tree, and patients with PSC have a predisposition to develop CC.[19,26] How biliary epithelia underlying chronic inflammation develop cancers remains unclear. One hypothesis is that chronic biliary inflammation leads to the generation of cytokines and reactive oxygen species, causing irreversible DNA damage.[2] Our current data showing that *AID* mRNA expression is mediated by proinflammatory cytokine stimulation via NF-κB in biliary epithelium could provide a link between chronic biliary inflammation and the development of CC. In fact, proinflammatory cytokine levels including TNF-α are up-regulated in patients with PSC.[27,28] It has been shown that proinflammatory cytokine-mediated NF-κB signaling pathways play a critical role in tumorigenesis. The mechanism by which IKK-β–dependent NF-κB activation drives tumor promotion is thought to be due to the transcriptional upregulation of anti-apoptotic target genes or cyclin D1 and other growth factors such as interleukin-6.[29-31] In this study, we identified *AID* as a target gene of the IKK-β–dependent NF-κB activation pathway in bile epithelial cells. Thus, 1 possible mechanism for increased susceptibility to CC development under chronic inflammation is due to the aberrant expression of DNA mutator, AID, in the bile tract via NF-κB activation.

Various molecular alterations have been described during the development of CC.[10] Among them, *p53* is the most commonly mutated tumor suppressor gene and was shown to be implicated in CC developing in patients with PSC.[20,32] For example, mutated p53 protein was detectable in 31% of the CC tumors in patients with PSC, as opposed to negative findings in the control bile duct specimens.[33] Another study revealed the accumulation of p53 protein in 79% of patients with CC, most of whom had underlying PSC.[32] Our findings that aberrant expression of AID in biliary cells resulted in the emergence of nucleotide alterations in the *p53* gene suggest that AID production might lead to the generation of a mutated *p53* gene that plays a critical role of tumorigenesis. Alterations in the INK4A/p16 signaling pathway by homozygous deletions, exon mutations, promoter mutations, and methylation were also shown in CC, and more importantly in PSC.[21] *AID* gene expression in biliary cells induced the nucleotide alterations in the promoter region of the *INK4A/p16* gene preferentially, whereas the *k-ras* gene was not mutated at all. It is unclear why the *p53* and *INK4A/p16* genes were more sensitive to AID activation compared with the *k-ras* gene in cholangiocarcinoma-derived cells. However, our current findings may be consistent with a previous observation that target gene selection for AID-mediated somatic hypermutation is variable among target cells.[6]

In conclusion, we demonstrated that proinflammatory cytokine stimulation is responsible for the aberrant *AID* gene expression in human biliary epithelial cells, providing a possible link between chronic biliary inflammation and the development of CC. Further analyses will be necessary to determine the significance of AID production on leading precancerous cells to acquire a critical number of genetic changes.

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Medicine in focus

Aberrant AID expression and human cancer development

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Abstract

Cancer develops via a multistep process that occurs through the accumulation of somatic mutations of tumor-related genes that govern cell proliferation, regeneration, and apoptosis. The question how normal cells acquire the genetic changes that lead to malignant transformation is, however, unknown at present. Activation-induced cytidine deaminase (AID) produces immune-diversity by inducing somatic hypermutations and class-switch recombinations in human immunoglobulin genes. Unfortunately, this function of AID as a genome mutator could aim at the generation of somatic mutations in various host genes of non-lymphoid tissues and contribute to tumorgenesis. Notably, aberrant AID expression can be triggered by several pathogenic factors, including *Helicobacter pylori* infection and proinflammatory cytokine stimulation, in human epithelial cells, whereas AID expression is absent in those cells under physiologic conditions. Thus, aberrant AID activity in epithelial tissues may provide the critical link between inflammation, somatic mutations, and cancer development.
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Keywords: Activation-induced cytidine deaminase; Mutation; Cancer

1. Introduction

In contrast to normal human cells that replicate their DNA with exceptional accuracy, most cancer cells arise from a stepwise accumulation of genetic changes. The genomes of incipient cancer cells acquire alterations in the nucleotide sequences of proto-oncogenes, tumor-suppressor genes, and other genes that control cell proliferation, regeneration, and apoptosis (Hahn & Weinberg, 2002). Because normal mutation rates cannot account for the accumulation of multiple mutations in tumor cells (Loeb, Loeb, & Anderson, 2003), certain molecular mechanisms must be present to account for the nucleotide alterations observed in most human cancer cells. One mechanism that may account for the enhanced susceptibility to mutagenesis is a genetic defect in the DNA repair pathways. For example, impairments of the mismatch repair system result in a familial colorectal cancer syndrome, defects in nucleotide excision repair are associated with skin cancer, and defects in homologous recombination and double-strand break repair are associated with breast cancer and lymphoma (Hoeijmakers, 2001). The frequency of such defects in the DNA repair system, however, is generally low among human cancers. Thus, how a large number of genetic mutations arise during the course of cancer development remains a fundamental question. We provide an overview of the novel molecular mechanism

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by which normal epithelial cells acquire DNA mutations under pathologic conditions, including chronic inflammation, and pathogenic viral or bacterial infections.

2. Nucleotide-editing enzymes that can induce mutations in DNA and/or RNA

To maintain homeostasis and conserve genetic information, cells have several systems to prevent mutations, and repair any changes in nucleotide sequences, thus avoiding harmful sporadic nucleotide alterations, so-called "somatic mutations". In contrast to normal cells, however, cancer cells usually acquire a variety of somatic mutations during the transformation process. Recently, a novel enzyme family was highlighted in association with the mechanism of mutagenesis. Cytidine deaminases are enzymes involved in DNA and/or RNA editing by converting cytosine to uracil, resulting in nucleotide alterations in target sequences. Among them, the apolipoprotein B-editing catalytic polypeptide (APOBEC) represents a clustered family characterized by a zinc-binding catalytic domain with the consensus amino acid sequences (Cascalho, 2004). The human APOBEC family comprises a series of molecules, including APOBEC1, -2, -3A, -3B, -3C, -3DE, -3F, -3G, -3H -4 and activation-induced cytidine deaminase (AID) (Conticello, Thomas, Petersen-Mahrt, & Neuberger, 2005). APOBEC family member-induced cytidine deamination has a critical role in mediating subtle changes in the DNA or RNA sequences that can produce diverse physiologic functions of target genes (Pham, Bransteitter, & Goodman, 2005). For example, APOBEC1 induces a mutation in apoB mRNA at a specific site that results in the generation of a premature stop codon, thereby producing a truncated form of apoB (Chen et al., 1987; Powell et al., 1987). A full-length apoB mRNA product, apoB-100, is a component of very-low-density and low-density lipoprotein, whereas the truncated apoB is secreted in the triglyceride-rich chylomicrons that carry dietary fat (Chan, 1992). In contrast to APOBEC1, APOBEC-3G is involved in cellular defense against retroviruses by inducing mutations into the viral genome. It has been shown that deamination activity of APOBEC-3G contributes to its antiviral activity against human immunodeficiency virus (HIV)-1 and restricts viral growth through a massive deamination of cytosines in the viral-minus DNA strands (Goff, 2003; KewalRamani & Coffin, 2003). Interestingly, more recent study reported that a deamination-independent mechanism might also be involved in APOBEC-3G antiviral activity (Iwatani et al., 2007).

3. AID is capable of inducing mutations in DNA sequences

Among the APOBEC family, AID has a unique ability with favorable function. Antigen stimulation of activated B lymphocytes triggers somatic hypermutations, which diversifies the variable region of the immunoglobulin genes, and AID expression is essential for this process. The finding that AID induces the production of somatic hypermutations in the immunoglobulin gene indicates that AID can induce nucleotide alterations in human DNA sequences. The activity of AID as a genome mutator leads to the question of whether AID induces inappropriate mutations in non-immunoglobulin genes.

The link between AID expression and unfavorable consequences in various organs was first revealed by phenotypic analyses of a transgenic mouse model with AID expression. Constitutive and ubiquitous AID expression in transgenic mice induced the development of lymphomas (Okazaki et al., 2003). Moreover, point mutations are massively introduced in various non-immunoglobulin genes, including the proto-oncogene *c-myc* in lymphoma cells. Interestingly, those mice also develop epithelial tumors including micro-adenomas and dysgenetic lesions of the respiratory bronchioles in the lung. Further phenotypic analyses revealed that AID-transgenic mice develop neoplasia in other epithelial tissues, including liver and stomach (Endo et al., 2007; Matsumoto et al., 2007). These findings indicate that aberrant AID expression might cause tumorigenesis in both lymphoid and non-lymphoid organs, via the accumulation of somatic mutations in tumor-related genes (Fig. 1). Fortunately, AID transcription is restricted to activated B lymphocytes and thus almost no AID expression is observed in most human tissues under physiologic conditions.

4. Aberrant AID expression in gastric epithelial cells in association with *Helicobacter pylori* infection

The majority of human gastric cancers arise in the stomach with clinical features of chronic gastritis (Aoi, Marusawa, Sato, Chiba, & Maruyama, 2006). The most important causative pathogen for chronic gastric inflammation and a class one carcinogen for human gastric cancer is *H. pylori* infection (Chiba, Seno, Marusawa, Wakatsuki, & Okazaki, 2006). The mechanisms that link *H. pylori*-induced chronic gastric inflammation and cancer development remain unclear, but it is thought to involve a multistep process of genetic alterations. Indeed, several studies have reported various nucleotide alter-

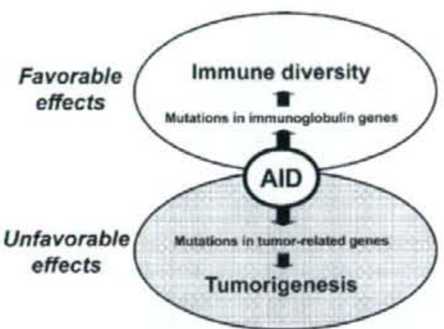

Fig. 1. Dual effects induced by AID activation. A schematic depicting the favorable and unfavorable effects of AID. AID acts as a cytidine deaminase that is capable of inducing nucleotide alterations in human DNA sequences. Under the physiological condition, AID is a protein indispensable for the diversification of immunoglobulin genes by somatic hypermutation and class-switch recombination. On the other hand, AID is able to induce genome-wide mutations in a variety of mammalian non-lymphoid cells, and thus can contribute to the production of unfavorable genetic changes in the tumor-related genes leading to carcinogenesis.

ations in tumor-related genes, including *TP53* in gastric cancer cells (Fenoglio-Preiser, Wang, Stemmermann, & Noffsinger, 2003). These somatic mutations are also observed in non-cancerous stomach tissues with *H. pylori* infection, suggesting that nucleotide alterations accumulate in gastric epithelial cells during the course

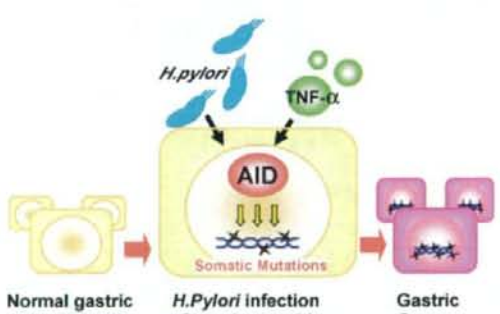

Fig. 2. AID links chronic inflammation to gastric cancer development via its mutagenic activity. This figure presents a model that depicts how AID plays a role in the development of human gastric cancer. Human gastric epithelium lacks endogenous AID expression under the physiological condition. *Helicobacter pylori* infection and the resultant inflammatory stimulation, however, trigger the aberrant AID expression in gastric epithelial cells. Constitutive AID activation in gastric epithelium results in the accumulation of somatic mutations in various target genes. If critical nucleotide changes in the tumor-related genes may be induced by AID activity, the resultant gastric epithelial cell can acquire the transformation, leading to the development of gastric cancer cells.

of chronic inflammation. The *in vivo* findings that AID-transgenic mice develop gastric neoplasms suggest that aberrant AID expression in gastric epithelial cells contributes to cancer development via the accumulation of somatic mutations (Matsumoto et al., 2007). Surprising findings were obtained by analyses of AID expression in human stomach tissue specimens with *H. pylori*-related chronic gastritis and gastric cancers. In contrast to normal gastric mucosa, aberrant AID expression is present in gastric epithelial cells of the stomach tissues with *H. pylori*-positive chronic gastritis (Matsumoto et al., 2007). In addition, AID protein is expressed in neoplastic cells in approximately 80% of *H. pylori*-infected gastric cancer tissues. Because AID expression is specifically upregulated in human gastric epithelial cells and neoplastic cells with *H. pylori*-induced chronic inflammation, the relationship between *H. pylori* infection, proinflammatory cytokine stimulation, and AID expression was further investigated *in vitro*. Although AID expression is low in cultured human gastric epithelial cells, marked upregulation of AID is induced in response to either *H. pylori* infection or tumor necrosis factor (TNF)-α stimulation. AID expression in human gastric epithelial cells by *H. pylori* infection or TNF-α is induced by the activation of the transcription factor NF-κB, indicating that AID expression is regulated through an NF-κB activation pathway in human gastric epithelial cells. Notably, aberrant AID expression triggers the accumulation of nucleotide alterations in the *TP53* gene in human gastric epithelial cells. Taken together, these findings provide evidence that AID is induced in response to *H. pylori* infection or proinflammatory cytokine stimulation via the NF-κB signaling pathway and is capable of contributing to the generation of somatic mutations in tumor-related genes in gastric epithelial cells. Thus, inflammation-mediated AID expression might underlie the development of human gastric cancer via *H. pylori*-associated chronic gastritis (Fig. 2).

5. Conclusion and future aspects

Proinflammatory cytokine induction of AID expression via the NF-κB activation pathway is not limited to gastric epithelial cells. Indeed, AID expression is also mediated by TNF-α or interleukin-1β in human hepatocytes (Endo et al., 2007). More importantly, hepatitis C virus (HCV) strongly triggers AID expression in hepatocytes in collaboration with proinflammatory cytokines (Endo et al., 2007), and ectopic AID expression is observed in human liver specimens with chronic hepatic inflammation caused by HCV infection (Kou et

al., 2007). Thus, AID possibly has a role in enhancing genetic susceptibility to mutagenesis, leading to the development of hepatocellular carcinoma in the setting of HCV-related chronic liver disease.

In conclusion, these recent findings support the idea that aberrant expression of the endogenous DNA mutator AID in epithelial cells provides a novel link between inflammation, mutagenesis, and cancer development. There might be more examples of human cancers that arise due to chronic inflammation that causes mutational accumulation mediated by AID activity.

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Organ-specific profiles of genetic changes in cancers caused by activation-induced cytidine deaminase expression

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Various molecular changes characterizing organ-specific carcinogenesis have been identified in human tumors; however, the molecular mechanisms of the genomic changes specific for each cancer are not well defined. A transgenic (Tg) mouse model with constitutive expression of the nucleotide-editing enzyme, activation-induced cytidine deaminase (AID), develops tumors in various organs as a result of the mutagenic activities of AID. This phenotypic character of AID Tg mice allowed us to analyze the organ-specific genetic changes in tumor-related genes commonly triggered by AID-mediated mutagenesis. Among the 80 AID Tg mice analyzed, 11 mice developed hepatocellular carcinomas, and 7 developed lung cancers. In addition, 1 developed the gastric cancer and 3 developed gastric adenomas. Organ-specific preferences for nucleotide changes were observed in some of the tumor-related genes in each epithelial tissue of the AID Tg mice. Of note, the *c-myc* and *K-ras* genes were the preferential targets of the mutagenic activity of AID in lung and stomach cancers, respectively, whereas mutations in the *p53* and *β-catenin* genes were commonly observed in all 3 organs. Quantitative RT-PCR analyses revealed that *alpha-fetoprotein, insulin-like growth factor2* and *cyclin D1* genes were specifically upregulated in HCC, whereas upregulation of the *matrix metalloproteinase7* gene was more marked in lung cancer. Our findings suggest that AID, a DNA mutator that plays a critical role linking inflammation to human cancers, might be involved in the generation of organ-specific genetic diversity in oncogenic pathways during cancer development.
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Key words: AID; transgenic mouse; gastric cancer; hepatocellular carcinoma; mutation

Cancer develops in various organs as a consequence of a series of genetic changes, including nucleotide mutations and chromosomal rearrangement.[1–3] A growing number of mutations in oncogenes and tumor suppressor genes have been identified in human cancer tissues.[4,5] However, it is widely recognized that the prevalence of mutations and the patterns of nucleotide changes in tumor-related genes differ among individual cancers. For example, the *c-myc* gene is a frequent target for genetic changes in human lung cancers and lymphomas, whereas changes in this oncogene are rarely detectable in hepatocellular carcinoma (HCC).[5–7] Similarly, almost all pancreatic cancers contain the *K-ras* gene mutations,[8] whereas the mutation frequencies in the *K-ras* gene are usually low in other types of tumors. These findings suggest that organ-specific genetic changes might be induced or maintained in the pathway to cancer development in each tissue. However, the molecular mechanisms underlying the accumulation of tissue-specific genomic changes in oncogenic pathways are not well defined.

Activation-induced cytidine deaminase (AID), an enzyme with homology to members of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) family,[9,10] is required for germinal center B-cells to undergo somatic hypermutation and class switch recombination in immunoglobulin genes.[11–13] However, recent studies have revealed that the inappropriate expression of *AID* could act as a DNA mutator that contributes to tumorigenesis *via* its mutagenic activity.[14–16] We recently demonstrated aberrant *AID* expression in inflammatory tissues, as well as in tumor cells, during the development of both human gastric cancer and HCC.[16,17] Moreover, *AID* expression is induced in response to proinflammatory cytokine stimulation, leading to the accumulation of genetic changes in several tumor-related genes in both human gastric epithelial cells and hepatocytes.[17,18] These findings suggest that *AID* is involved in the development of human gastric cancers and HCC through the accumulation of genetic changes.[16–18] Consistent with this hypothesis, the constitutive and ubiquitous expression of *AID* caused the development of neoplasia *in vivo*.[19] It is noteworthy that AID transgenic (Tg) mice develop tumors in several organs, including the liver, lung and lymphoid tissues, through the accumulation of genetic changes induced by the genotoxic effects of AID.[18–20] In fact, somatic mutations in the *c-myc* and *T-cell receptor* genes were frequently detectable in the lymphoma tissues of AID Tg mice.[19,20] However, it remains unclear whether the carcinogenesis pathway triggered by the accumulation of genetic changes is common to the different types of epithelial tissues with AID expression. Therefore, in this study, we analyzed the mutational spectra and expression profiles of various tumor-related genes in 3 types of epithelial tissues, including the stomach, liver and lung, as well as gastric, liver and lung cancers that developed in AID Tg mice in an identical genetic background.

Material and methods

Generation of AID Tg mice

The generation of Tg mice with constitutive and ubiquitous expression of AID has been described previously.[19] Tissue samples from Tg mice were removed and fixed in 4% (w/v) formaldehyde, embedded in paraffin, stained with hematoxylin and eosin (H&E) and examined for histological abnormalities. Tissue samples were also frozen immediately in liquid nitrogen for nucleotide extraction. The mice received humane care according to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health, USA (NIH publication 86-23).

Subcloning and sequence analysis of the p53, c-myc, K-ras *and β-*catenin *genes*

Genomic DNA was extracted with DNeasy purification (Qiagen, Hilden, Germany). The *p53*, *c-myc*, *K-ras* and β-*catenin*

Additional Supporting Information may be found in the online version of this article.

Abbreviations: 18s rRNA, 18s ribosomal RNA; Afp, alpha-fetoprotein; AID, activation-induced cytidine deaminase; APOBEC, apolipoprotein B mRNA-editing enzyme catalytic polypeptide; HCC, hepatocellular carcinoma; RT-PCR, reverse transcription polymerase chain reaction; Tg, transgenic.

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TABLE I – MUTATIONAL PROFILE OF THE *p53*, *c-myc*, *K-ras* AND β-*catenin* GENES IN THE THREE TYPES OF CANCERS DEVELOPED IN AID Tg MOUSE

Gene	Organ	Clone			Mutation frequency per 10^4 [1]
		Cell source	Mutated/total	Mutations	
p53 (exon7 and exon8)	Liver	Tumor	2/89	2	0.42
		Nontumor	1/89	1	0.21
	Stomach	Tumor	2/86	2	0.44
		Nontumor	1/82	1	0.23
	Lung	Tumor	1/113	1	0.16
		Nontumor	1/84	1	0.22
c-myc (exon1)	Liver	Tumor	1/89	1	0.22
		Nontumor	1/90	1	0.22
	Stomach	Tumor	3/76	3	0.79
		Nontumor	1/91	1	0.22
	Lung	Tumor	11/67	15	4.47
		Nontumor	7/80	9	2.25
K-ras (exon2)	Liver	Tumor	0/82	0	<0.33
		Nontumor	1/89	1	0.30
	Stomach	Tumor	3/86	4	1.28
		Nontumor	0/85	0	<0.32
	Lung	Tumor	0/110	0	<0.21
		Nontumor	0/89	0	<0.31
β-*catenin* (exon3)	Liver	Tumor	2/88	2	0.58
		Nontumor	0/88	0	<0.29
	Stomach	Tumor	0/85	0	<0.30
		Nontumor	1/88	1	0.29
	Lung	Tumor	1/110	1	0.20
		Nontumor	2/86	2	0.60

[1]Mutational frequency is expressed as the number of mutated nucleotide per 1×10^4 nucleotide in the each target gene.

genes were amplified by genomic PCR with Phusion High-Fidelity DNA Polymerase (FINNZYMES, Espoo, Finland). The primer sets for the amplification of those genomic sequences are shown in Supplemental Table I. After 35 cycles of PCR, the PCR products of the *p53*, *K-ras and* β-catenin genes were subcloned into the *Eco*RI-*Xho*I sites of the pcDNA3 vector (Invitrogen, Carlsbad, CA). The PCR product of the *c-myc* gene was also subcloned into the *Hind*III-*Xho*I sites of the pcDNA3 vector, followed by sequence analyses.

Quantitative real-time reverse transcription (RT)-PCR

Total RNA was extracted from tissues using the RNeasy Mini Kit (Qiagen). Gene expression was quantified by real-time RT-PCR using the 7300 Real-Time PCR System (PE Applied Biosystems, Foster City, CA). The sequences of the oligonucleotide primers used in this experiment are shown in Supplemental Table II. cDNA was synthesized using the oligo-dT primer and SuperScript III (Invitrogen). Real-time PCRs were set up in 50 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with the RT product and the forward and reverse primers, as described previously.[21] Standard curves were generated for every target using a 10-fold serial dilution series of 7 independent transcripts derived from the normal liver tissues of wild-type mice. To assess the quantity of isolated RNA and the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene for *18S ribosomal RNA* (*18S rRNA*).[16] For simplicity, the ratios are presented as values relative to the expression levels in lysate from control specimens.

Immunohistochemistry

Immunostaining for alpha-fetoprotein (Afp) was carried out according to a previously described protocol.[22] The polyclonal antibody directed against Afp was purchased from Dako Cytomation (Glostrup, Denmark).

Statistical analysis

Results were analyzed using Student's t test. A value of $p < 0.05$ was deemed significant. Statistical analysis was performed with StatView 5.0 (Abacus Concepts, Berkeley, CA).

TABLE II – MUTATIONS ACCUMULATED IN THE *c-myc* GENE IN NONTUMOR TISSUES OF SEVERAL LITTERMATES OF AID Tg MICE

Gene	Organ	Clone	Mutations	Mutation frequency per 10^4
		Mutated/total		
c-myc (exon1)	Liver	0/29	0	<0.68
	Stomach	2/38	2	1.05
	Lung	3/38	4	2.10
		4/41	4	1.95
		5/45	5	2.22
		5/42	5	2.38

Results

Development of gastric cancer in AID Tg mice

In our previous analyses, we observed that AID Tg mice developed 3 types of tumors, including lymphoma, HCC and lung cancer.[18,19] In this study, we focused on the development of nonlymphoid tumors and analyzed the overall phenotypes of the epithelial organs in AID Tg mice (B2 line).[19] Among the 80 AID Tg mice analyzed, most developed T-cell lymphomas and 8 had sarcomas of the skin. Furthermore, 11 AID Tg mice developed HCC, 7 developed lung cancers and 4 developed gastric tumors (Fig. 1*a*). Histological examination revealed that the 1 gastric tumor had the morphological appearance of typical human gastric cancers (Fig. 1*b*), and the remaining 3 tumors showed the pathological characteristics of gastric adenomas. These findings suggest that constitutive expression of *AID* could cause the development of neoplasia in 3 types of epithelial organs: the liver, stomach and lung.

Organ-specific mutational profiles in liver, stomach and lung cancers that developed in AID Tg mice

The finding that the constitutive expression of AID caused cancer development in different types of epithelial organs prompted us to ask whether *AID* could induce tissue-specific genotoxic effects in oncogenic pathways during the process of each carcinogenesis. To determine the tissue-specific mutational profiles of tumor-related genes, we took the advantage of 1 AID Tg mouse that developed both HCC and gastric cancer simultaneously and its littermate with lung cancer, and investigate the occurrence of

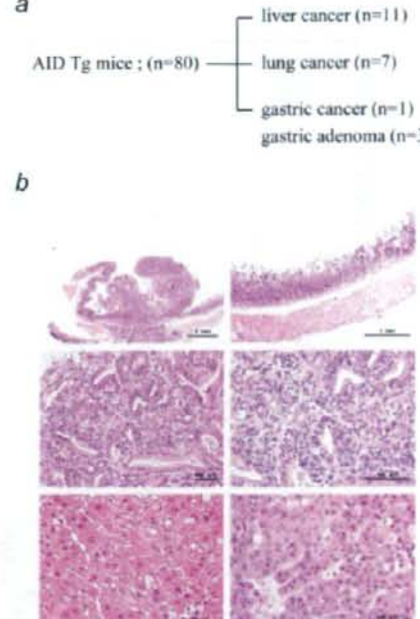

FIGURE 1 – Epithelial neoplasia developed in AID Tg mice. (*a*) Numbers of epithelial tumors developed in AID Tg mice are shown in this figure. (*b*) Representative histological findings for the gastric, liver, and lung tumors developed in AID Tg mice. Top left, macroscopic view of a representative gastric carcinoma in a 53-week-old AID Tg mouse. Top right, macroscopic view of nontumor gastric tissues in the AID Tg mouse with gastric carcinoma. Middle left, hematoxylin and eosin (H&E)-stained section of gastric carcinoma (magnification ×200). Middle right, H&E-stained section of gastric carcinoma (magnification ×400), showing a typical histological pattern of gastric adenocarcinoma. Bottom left, histological pattern (H&E stained; magnification ×400) of a hepatocellular carcinoma in a 53-week-old AID Tg mouse. Bottom right, histological pattern (H&E stained; magnification ×400) of a lung adenocarcinoma in a AID Tg mouse.

genetic mutations in several tumor-related genes in both nontumor and tumor tissues of the liver, stomach and lung. For this purpose, we analyzed the genetic changes in the *c-myc* gene, which is the common target for abnormal editing in the lymphoma cells of AID Tg mice.[20] In addition, we also analyzed the nucleotide changes in the *p53*, β-*catenin* and *K-ras* genes, all of which are thought to be involved in human carcinogenesis.[3] First, we confirmed by sequencing that no mutations were present in those tumor-related genes in 32 randomly selected clones amplified from normal liver, stomach and lung tissue specimens obtained from the non-Tg littermates of the same mouse line. In contrast, several nucleotide changes had accumulated in the nontumor tissues as well as in the tumor tissues of the liver, stomach and lung of AID Tg mice (Table I). The most marked mutations had accumulated in the *c-myc* gene in the lung cancer developed in the AID Tg mouse, with a mutation frequency of 4.48 substitutions per 1×10^4 nucleotides. Notably, nucleotide changes in the *c-myc* gene had also accumulated in the noncancerous lung tissues (2.25 substitutions per 1×10^4 nucleotides). In contrast to the lung, far fewer or no mutations were observed in the *c-myc* gene in the stomach and liver tissues. To examine whether AID preferentially induces somatic mutations in the *c-myc* gene of the lung tissues, we analyzed the mutation frequencies of the *c-myc* gene in the nontumor tissues of the lung, liver and stomach of several AID Tg mouse littermates. Interestingly, we found that the incidence of nucleotide alterations in the *c-myc* gene of the lung tissues was substantially higher than that of the liver or stomach tissues in all the AID Tg mice examined (Table II). These findings suggest that the *c-myc* gene might be one of the lung-specific target genes in AID-mediated genotoxicity. In contrast to the *c-myc* gene, several nucleotide changes in the *K-ras* gene were observed in the gastric cancer tissues of AID Tg mice, whereas no mutations had accumulated in the lung and liver cancers. On the other hand, the *p53* and β-*catenin* genes had acquired mutations in both the tumor and the nontumor tissues of all 3 organs, although the mutation frequencies were low compared to those observed in the *c-myc* gene of the lung or the *K-ras* gene of the stomach in AID Tg mice. Interestingly, 4 of 6 (67%) nucleotide changes in the *p53* coding region observed in the AID Tg mice tissues resulted in amino acid substitutions with potential functional consequences, whereas all the mutations occurred in the *c-myc* gene were synonymous. These findings suggest that the mutational profiles induced by aberrant AID expression could differ in the liver, stomach and lung tissues.

Organ-specific activation of signaling pathways in the cancer tissues of AID Tg mice

The tissue-specific target preference of AID-induced mutagenesis in 3 types of epithelial organs prompted us to speculate that there might be differences in the expression profiles of tumor-related genes in each epithelial organ. Therefore, we analyzed the gene expression profiles in the liver, stomach and lung cancer tissues of the AID Tg mice. In the current study, we selected the following 7 tumor-related genes and analyzed their expression by quantitative real-time RT-PCR assay using primers and probes specific for each gene (Supplemental Table II): the human HCC tumor marker *alpha-fetoprotein* (*Afp*), cell-proliferation related *insulin-like growth factor2* (*IGF2*), the cell-cycle regulator *cyclin D1*, *matrix metalloproteinase7* (*MMP7*), *c-myc*, *K-ras* and β-*catenin* gene. We found that the expression of *Afp*, *IGF2* and *cyclin D1*, all of which are closely associated with the development of human HCC,[7,23,24] which were markedly enhanced in all the HCC tissues examined, compared to their expression in the stomach and lung cancers that developed in AID Tg mice ($p < 0.01$) (Fig. 2). Immunohistochemical analysis confirmed that *Afp* was expressed only in HCC cells, and not in lung or gastric cancer cells (Fig. 3). This indicates that the upregulation of *Afp* expression occurs only in the process of hepatocarcinogenesis, even though the mutagenic impact was common to every epithelial organ. Interestingly, the expression of *IGF2* and *cyclin D1* genes was also upregulated in the nontumor liver tissues comprising the constitutive expression of *AID* (Fig. 2). *In vitro* analysis showed that the transient expression of *AID* in cultured hepatoma-derived cells did not cause any changes in the transcription levels of *cyclin D1* and *Afp* genes (data not shown), suggesting that the changes in the expression of these genes in the livers of AID Tg mice might be triggered by the accumulation of genetic changes, not by their transcriptional upregulation caused by *AID* expression in hepatocytes. In contrast, the expression of the *c-myc* and *MMP7* genes, implicated in various types of carcinogenesis in human lung cancer,[25] was significantly upregulated in the lung cancer tissues compared with that in the liver and stomach tumors ($p < 0.01$) (Fig. 2). In gastric cancer tissue, the *K-ras* gene expression was relatively high compared to that in the liver and lung, although not significantly so. Taken together, these findings indicate that organ-specific genetic changes in oncogenic pathways occur during the processes of cancer development in AID Tg mice and suggest the possibility that the gene expression profiles differ strikingly between liver, stomach and lung cancers, even though the causative genotoxic effect and the genetic background of the cancers are completely identical.

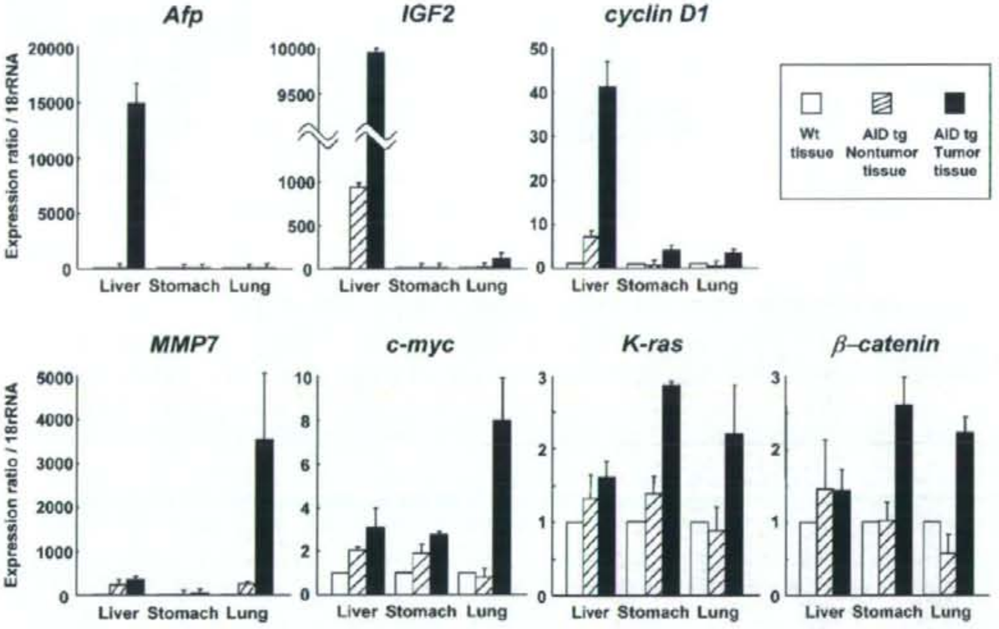

FIGURE 2 – Relative expression levels of 7 tumor-related genes in 3 types of epithelial tissues of the representative AID Tg mice. Relative mRNA levels of each gene were measured by quantitative RT-PCR assay. Values shown in the graphs are normalized relative to the specimens of wild-type (Wt) mice (mean ± SD of 3 mice).

Discussion

It is well recognized that human cancer is caused by genetic changes including the accumulation of various mutations in tumor-related genes.[1–3] In fact, a large number of nucleotide alterations have been identified in various types of human cancers.[26–28] Although all cancer cells seem to share a common set of genetic changes required for carcinogenesis,[29] organ-specific genetic changes and gene expression signatures of oncogenic pathways are present in each human malignancy.[30,31] A recent study defined the genetic landscape of 2 human cancer types, breast and colorectal cancers, with a systematic analysis of their mutational spectra.[32] Moreover, Greenman *et al.*[33] demonstrated that mutational signatures differed between cancer types by surveying the numbers and patterns of somatic mutations in a diverse set of human cancer genomes. These tissue-specific expression profiles and somatic mutations would provide clues to understand the cellular processes involved in tumorigenesis. However, the molecular mechanisms underlying these organ-specific genomic changes are unknown. In the current study, we took advantage of AID Tg mice, which develop various types of solid tumors through the accumulation of genetic alterations, and investigated the mutational profiles and changes in expression profiles in 3 types of epithelial neoplasia: liver, stomach and lung cancers. We demonstrated that the constitutive genotoxic effects of AID resulted in the appearance of tissue-specific mutational spectra and gene expression profiles, possibly leading to the activation of organ-specific oncogenic pathways in liver, stomach and lung tissues.

AID is a unique cellular enzyme that can trigger point mutations and chromosomal translocations, both of which potentially lead to the initiation and progression of human lymphoid malignancies.[15,34–37] A causal relationship between the ectopic expression of *AID* and epithelial tumorigenesis has been suggested from the results of analyses of clinical specimens and an *in vitro* study of AID-mediated mutagenesis in human hepatocytes and gastric epithelial cells.[16–19] These findings suggested that the inappropriate expression of *AID* acts as a DNA mutator, which enhances the genetic susceptibility to mutagenesis in various human epithelial cells.[16] Consistent with these findings, the analyses described here provides the first evidence that AID Tg mice, in which the inappropriate expression of *AID* was driven under the control of an ubiquitous promoter, caused carcinogenesis in the stomach as well as in the liver, with the accumulation of genomic mutations in these cancer tissues. These findings indicate that aberrant *AID* expression can lead to carcinogenesis in the liver and stomach, in which organs the expression of *AID* is absent under physiological conditions.

We have shown here that organ-specific changes in mutational and gene expression profiles are present in liver, stomach and lung tissues, caused by the common genotoxic effects of aberrant *AID* activation. Indeed, the *c-myc* gene was the preferential target of AID-induced mutagenesis in the lung cancers of AID Tg mice. In addition, mutational changes in the *c-myc* gene also accumulated in the nontumor lung tissues of AID Tg mice. In contrast, mutations in the *K-ras* gene were frequently observed in the gastric cancer. These findings suggest that the *c-myc* and *K-ras* genes could be the preferential targets of AID-mediated genotoxic effects in the lung and stomach, respectively, and that the target selection of AID-mediated mutagenesis might be associated with organ-specific genetic changes in oncogenic pathways. However, it is not clear why the *c-myc* and *K-ras* genes are more sensitive to AID-mediated mutagenesis in the lung and stomach tissues, respectively, compared to the other organs during tumorigenesis. One possible explanation for the selective mutations of the *c-myc*