Table 5. The prevalence of M204VI mutation at YMDD site in patients before and after entecavir administration.

	Entecavir treatment				
	Before		After		
	Prevalence of the mutated clones		Prevalence of the mutated clones		Period of NA treatment
Serum #3	222/32,238	(0.7%)	2,284/23,791	(9.6%)	2w
Serum #2	401/34,041	(1.2%)	266/25,301	(1.1%)	24w
Serum #5	521/48,723	(1.1%)	245/25,521	(1.0%)	56w
Serum #8	748/65,573	(1.1%)	336/28,702	(1.2%)	48w
Serum #9	312/30,599	(1.0%)	169/14,172	(1.2%)	56w
Serum #1	9/22,843	(-)	2,839/34,162	(8.3%)	8w
Serum #7	26/65,564	(-)	923/66,458	(1.4%)	4w
Serum #12	91/65,616	(-)	258/27,958	(0.9%)	24w
Serum #13	11/23,209	(-)	206/64,747	(0.3%)	32w
Serum #4	3/7,923	(-)	39/65,575	(-)	12w
Serum #6	52/65,582	(-)	77/55,273	(-)	16w
Serum #10	38/22,522	(-)	8/21,053	(-)	8w
Serum #11	47/43,853	(-)	5/16,520	(-)	16w
Serum #14	42/42,784	(-)	40/36,668	(-)	12w

Mutation frequency (%): the ratio of total mutant clones to total aligned coverage at each nucleotide sites.
(-): mutant clones less than 0.3% among total clones at each nucleotide sites.
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and the elucidation of other unknown mutations involved in HBe seroconversion are necessary for a better understanding of the underlying mechanisms of HBe seroconversion.

One thing to be noted is that the majority of the chronic-NA cases had extremely low levels of the G1896A pre-C mutant in their liver tissues, even though those cases were serologically positive for anti-HBe and negative for HBeAg. Moreover, entecavir administration significantly reduced the proportion of the G1896A pre-C mutant in the serum of the majority of patients irrespective of their HBeAg serostatus, while the G1896A pre-C mutant clones were detectable in a substantial proportion before treatment in all cases. These findings suggest that the G1896A pre-C mutant have higher sensitivity to NA than the wild-type viruses. Consistent with this hypothesis, several previous studies reported that NA is effective against acute or fulminant hepatitis caused by possible infection with the G1896A pre-C mutant [34,35]. Based on these findings, early administration of NA might be an effective strategy for treating patients with active hepatitis infected predominantly with the G1896A pre-C mutant.

Ultra-deep sequencing has a relatively higher sensitivity than conventional direct population sequencing and is thus useful for detecting drug-resistant mutations not detected by standard sequencing [20,21]. Recently, we revealed that drug-resistant mutants were widely present in treatment-naïve HCV-infected patients, suggesting a putative risk for the expansion of resistant clones to anti-viral therapy [19]. Here, we demonstrated that various drug-resistant HBV variants are present in a proportion of chronically HBV-infected, NA-naïve patients. Several studies using ultra-deep sequencing provided evidence that naturally-occurring drug-resistant mutations are detectable in treatment-naïve individuals with human immunodeficiency virus-1 infection [30,36,37]. Consistent with the cases of human immunodeficiency virus-1 infection, a few studies detected minor variants resistant to NA in the plasma of treatment-naïve patients with chronic HBV infection [20,21]. It remains unclear, however, whether these minor drug-resistant mutations have clinical significance. Our observation of the relative expansion of viral clones with the M204VI mutation during entecavir therapy in some cases indicates the possibility that preexisting minor mutants might provide resistance against NA through the selection of dominant mutant clones. Future studies with a larger cohort size are required to clarify the clinical implications of the latently existing low-abundant drug-resistant mutations.

The current ultra-deep parallel sequencing technology has limitations in the analyses of viral quasispecies. First, because the massively-parallel ultra-deep sequencing platform is based on a multitude of short reads, it is difficult to evaluate the association between nucleotide sites mapped to different genome regions in a single viral clone. Indeed, potential mutational linkages between the pre-C and reverse transcriptase regions were difficult to elucidate due to the short read length of the shotgun sequencing approach. Second, accurate analysis of highly polymorphic viral clones by ultra-deep sequencing is also difficult because the identification of mutations depends strongly on the mapping to the reference genome sequences.

In conclusion, we demonstrated that the majority of patients positive for anti-HBe and negative for HBeAg lacked the predominant infection of the G1896A pre-C mutant in the presence of NA treatment, suggesting that the G1896A pre-C mutant have increased sensitivity to NA therapy compared with wild-type HBV. We also revealed that drug-resistant mutants are widely present, even in the liver of treatment-naïve HBV-infected patients, suggesting that the preexisting low-abundant mutant clones might provide the opportunity to develop drug resistance against NA through the selection of dominant mutations. Further analyses utilizing both novel and conventional sequencing technologies are necessary to understand the significance and clinical relevance of the viral mutations in the pathophysiology of various clinical settings in association with HBV infection.

Supporting Information

Figure S1 Comparison of the viral complexity between the liver and serum of the same individual. Shannon entropy values throughout the whole viral genome of the liver and serum of the representative two cases are shown. (upper two panels, case #11; lower two panels, case #14). preC-C: pre-core~core, preS: pre-surface, P: polymerase.
(TIF)

Table S1 The oligonucleotide primers for amplifying HBV sequences in each clinical specimen.
(DOCX)

Table S2 Error frequency of Ultra-deep sequencing for the expression plasmid encoding wild-type genotype C HBV genome sequences by the three control experiments.
(DOCX)

Table S3 The sensitivity and accuracy of detecting the low abundant minor clones in association with the different coverage numbers.
(DOCX)

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Author Contributions

Conceived and designed the experiments: NN HM. Performed the experiments: NN HM. Analyzed the data: NN HM YU AN TF ST KS TC. Contributed reagents/materials/analysis tools: NN HM YU YO TK SY SU. Wrote the paper: NN HM YU KT TC.

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Original Article

Efficacy and safety of prophylaxis with entecavir and hepatitis B immunoglobulin in preventing hepatitis B recurrence after living-donor liver transplantation

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Aim: Hepatitis B recurrence after liver transplantation can be reduced to less than 10% by combination therapy with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). The aim of this study was to evaluate the efficacy and safety of prophylaxis with entecavir (ETV), which has higher efficacy and lower resistance rates than LAM, combined with HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

Methods: Twenty-six patients who received ETV plus HBIG (ETV group) after LDLT for hepatitis B virus (HBV)-related end-stage liver disease were analyzed by comparing with 63 control patients who had received LAM plus HBIG (LAM group).

Results: The survival rates of the patients treated with ETV plus HBIG was 73% after both 1 and 3 years, and there was no statistical difference between the patients in the ETV group and LAM group. No HBV recurrence was detected during the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 4% at 3 years and 6% at 5 years in the LAM group. No patients had adverse effects related to ETV administration.

Conclusion: ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Key words: entecavir, hepatitis B, liver transplantation, living donor

INTRODUCTION

THE RECURRENCE OF hepatitis B virus (HBV) infection after liver transplantation for HBV-related diseases resulted in poor outcomes before the development of effective prophylaxis with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). Without the prophylaxis, the majority of patients developed recurrent infections due to HBV in the early phases after liver transplantation, and the recurrence resulted in rapidly progressive liver injury, early graft loss and reduced survival.[1–3] The development of prophylaxis dramatically reduced the post-transplant recurrence of hepatitis B and markedly improved prognosis. The most widely used prophylaxis so far has been a combination therapy of LAM and i.v. HBIG.

In the non-transplant setting, the long-term use of LAM resulted in high rates of emergence of resistance to the drug, with rates ranging 14–32% after 1 year and 60–70% after 5 years of treatment. In most cases, the resistance was the result of selection of LAM-resistant mutations in the YMDD motif of the DNA polymerase domain of HBV.[4] Moreover, the emergence of HBV strains with mutations that allow escape from hepatitis B surface antibody (anti-HBs) recognition has been reported in patients vaccinated for HBV,[5,6] in patients with chronic hepatitis B[7,8] and in liver transplant recipients after HBIG administration.[9–11] Therefore, the emergence of LAM resistance and HBIG resistance might

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increase the risk of recurrence during long-term administration of LAM and HBIG, although the rate of HBV recurrence in liver transplant recipients who received prophylaxis with LAM and HBIG for more than 10 years has not been reported to date. At present, several nucleoside analogs are available for the treatment of chronic hepatitis B[4]. Among them, there is entecavir (ETV), a carbocyclic analogue of 2′-deoxyguanosine, which has been shown to have higher efficacy than LAM in patients with chronic hepatitis B. In addition, ETV has a higher genetic barrier to resistance than LAM. The resistance to ETV requires at least three mutations including rtM204V/I, which causes LAM-resistance, rtL180M, and a mutation at one of the following codons: rtT184, rtS202 or rtM250.[4] Therefore, ETV is now used as a first-line therapy in the treatment of chronic hepatitis B worldwide. Data available in the published work suggest that, in transplant recipients, ETV plus HBIG represents a better prophylaxis protocol than LAM plus HBIG for long-term prevention of HBV recurrence after liver transplantation. However, the efficacy and safety of this treatment is largely unknown.

The aim of this study was to evaluate the efficacy and safety of prophylaxis with ETV and HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

METHODS

Patients

WE RETROSPECTIVELY ANALYZED the medical records of 97 patients who underwent LDLT for HBV-related end-stage liver diseases from September 2002 to December 2010. Of these, eight patients were excluded from our study because they had breakthrough hepatitis due to HBV with LAM-resistant mutations and were prescribed LAM plus adefovir before liver transplantation. Accordingly, 89 patients were enrolled in this study.

Prophylaxis with ETV or LAM combined with HBIG

Lamivudine plus HBIG therapy was given to all recipients with HBV-related end-stage liver diseases from September 2002 to November 2006, as reported previously.[12] From December 2006, we changed the protocol for prophylaxis to ETV plus HBIG. ETV at a dose of 0.5 mg/day or LAM at a dose of 100 mg/day was given before transplantation, usually when the patient was referred to the hospital and scheduled for transplantation. Preoperative ETV or LAM prophylaxis was followed by combination with HBIG after transplantation. The first application of HBIG at a dose of 200 IU/kg body mass was administrated i.v. during the anhepatic phase of LDLT, and repeated every day for the first 5 days post-surgery. HBV serological markers were examined at weekly intervals for the first 2 months after the transplant, then at monthly intervals, and 1000 IU of HBIG was periodically administrated to maintain the serum anti-HBs titers at more than 500 IU/L during the first 6 months and 200 IU/L thereafter throughout the follow-up period.[12]

Immunosuppression

Tacrolimus and low-dose steroid therapy were administrated to induce immunosuppression in most patients.[13] Mycophenolate mofetil was administrated to patients who experienced refractory rejection or required reduction of tacrolimus dose due to adverse events. Patients who received ABO blood-type-incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone.[14]

Diagnosis of HBV activation

Activation of HBV was diagnosed when hepatitis B surface antigens (HBsAg) and/or HBV DNA became positive in the serum of the patients. After LDLT, HBsAg, anti-HBs and serum HBV DNA were measured at least at 3 monthly intervals. Serological HBV markers, including HBsAg, anti-HBs, hepatitis B core antibody, hepatitis B e antigen (HBeAg) and antibodies to HBeAg (anti-HBe), were measured by chemiluminescent enzyme immunoassay (Fuji Rebio, Tokyo, Japan). Serum HBV DNA titer was analyzed using a commercial polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche, Branchburg, NJ, USA). LAM-resistant YMDD mutant virus was detected by the PCR enzyme-linked mini-sequence assay.[15]

Statistical analysis

Baseline characteristics are shown in Table 1. For continuous variables, medians and ranges are given, and the significance of the data was analyzed with the Wilcoxon rank sum test. For categorical variables, counts are given, and the data were analyzed with the χ^2-test. Survival rates and the rates of patients who showed HBV activation after LDLT were estimated using the Kaplan–Meier method and compared using log–rank tests. $P < 0.05$ was considered significant.

Table 1 Baseline characteristics of 90 patients

	Entecavir + HBIG ($n = 26$)	Lamivudine + HBIG ($n = 63$)	*P*-value
Age (years)	55 (33–68)	53 (26–64)	0.062†
Men/women	19/7	46/17	0.995‡
Primary disease			0.595‡
Acute liver failure	6 (23%)	9 (14%)	
Liver cirrhosis, HCC^-	6 (23%)	20 (32%)	
Liver cirrhosis, HCC^+	14 (54%)	34 (54%)	
HBV markers before LDLT			
$HBsAg^+$	24 (92%)	61 (97%)	0.350‡
$HBeAg^+$	6 (23%)	18 (29%)	0.595‡
HBV DNA before LDLT	<2.6 (<2.6–7.6<)	3.7 (<2.6–7.6<)	0.010†
<2.6 log IU/mL	14 (54%)	19 (30%)	0.024‡
Follow-up period (months)	25.1 (0.2–58.6)	70.6 (0.5–109.2)	<0.001†

Qualitative variables are shown in number; and quantitative variables expressed as median (range).
†Wilcoxon rank sum test.
‡χ^2-Test.
HBeAg, hepatitis B e antigen; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LDLT, living-donor liver transplantation.

RESULTS

Patient characteristics

TWENTY-SIX PATIENTS who received ETV plus HBIG (ETV group) after LDLT for HBV-related end-stage liver disease were included in this study. Baseline characteristics of these patients are listed in Table 1 and compared with those of 63 control recipients who received LAM plus HBIG (LAM group) at our institute already present in our database. The two groups of patients did not differ significantly by age, sex, primary diseases or serological markers for HBV before LDLT. Serum HBV DNA levels before LDLT were significantly lower in the ETV group than in the LAM group. Fourteen of 26 patients (54%) showed less than 2.6 log IU/mL of serum HBV DNA in the ETV group. Median follow-up period was 25.1 months (range, 0.2–58.6) in the ETV group, whereas it was 70.6 months (range, 0.5–109.2) in the LAM group.

Efficacy and safety of prophylaxis with ETV plus HBIG

Survival rates of the patients treated with ETV plus HBIG estimated by Kaplan–Meier analysis was 73% at both 1 and 3 years (Fig. 1a). There was no difference between the ETV group and the LAM group, in which survival rates were 81% at 1 year, 78% at 3 years and 73% at

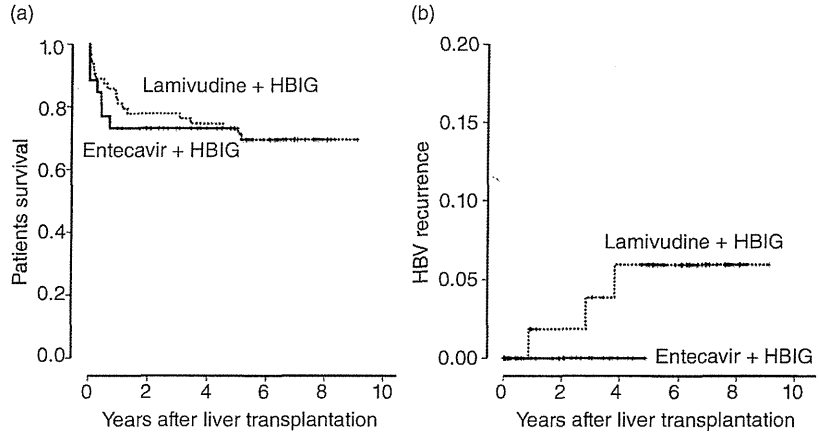

Figure 1 (a) Post-transplantation survival rates and (b) hepatitis B virus (HBV) recurrence after living-donor liver transplantation in HBV positive recipients who received entecavir and hepatitis B immunoglobulin (HBIG) (solid line), or lamivudine and HBIG (dotted line), estimated by Kaplan–Meier method.

5 years. Causes of death in patients in the ETV group were pneumonia ($n = 2$), sepsis ($n = 1$), pulmonary hemorrhage ($n = 1$), cerebral hemorrhage ($n = 1$), graft liver failure ($n = 1$) and multiple organ failure ($n = 1$), none of which were related to ETV. No HBV recurrence was detected in the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 2% at 1 year, 4% at 3 years and 6% at 5 years in the LAM group (Fig. 1b). Three patients in the LAM group had HBV recurrence at 10, 34 and 46 months after LDLT. The emergence of HBV with LAM-resistant mutations in the YMDD motif was confirmed in two of the three patients. HBV mutations of another patient could not be determined because of the low level of serum HBV DNA. As the follow-up period of the ETV group was shorter than that of the LAM group and the HBV recurrence in the LAM group occurred in long-term follow-up after LDLT, the rate of HBV recurrence was not significantly different between the ETV and LAM groups. No patients had adverse events due to ETV administration.

DISCUSSION

IN THIS STUDY, we demonstrated that ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Two studies of patients receiving a combination of ETV and HBIG after liver transplantation have been previously reported.[16,17] One study demonstrated that 30 recipients who received ETV plus HBIG prophylaxis had no recurrence of HBV and no adverse effect relating to ETV.[17] The other study showed that no HBV recurrence was observed in two recipients with HBV-associated cirrhosis receiving ETV, tenofovir and HBIG.[16] Both studies showed the efficacy and safety of prophylaxis with ETV and HBIG in preventing short-term recurrence of HBV after liver transplantation. The current study confirmed their results for longer follow-up periods. Our results showed that prophylaxis with ETV and HBIG has similar efficacy and safety to that with LAM and HBIG, but did not show any further advantage of ETV compared to LAM treatment. Longer follow up might be needed to reveal the difference of HBV recurrence rate. One characteristic of our present report is that all patients in this study underwent LDLT. Our results suggest that prophylaxis with ETV and HBIG in patients after LDLT has similar efficacy and safety to patients after deceased-donor liver transplantation demonstrated in the previous reports.[16,17] More recently, efficacy of ETV monotherapy in preventing recurrence of HBV for liver transplant recipients with chronic hepatitis B was reported.[18] The study demonstrated that most patients showed disappearance of HBsAg and undetectable serum HBV DNA after liver transplantation without HBIG. Although long-term efficacy of ETV monotherapy needs be confirmed, both our data and previous reports suggest that ETV is an effective and safe antiviral agent in the post-transplant setting.

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Excessive activity of apolipoprotein B mRNA editing enzyme catalytic polypeptide 2 (APOBEC2) contributes to liver and lung tumorigenesis

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Apolipoprotein B mRNA editing enzyme catalytic polypeptide 2 (APOBEC2) was originally identified as a member of the cytidine deaminase family with putative nucleotide editing activity. To clarify the physiologic and pathologic roles, and the target nucleotide of APOBEC2, we established an APOBEC2 transgenic mouse model and investigated whether APOBEC2 expression causes nucleotide alterations in host DNA or RNA sequences. Sequence analyses revealed that constitutive expression of APOBEC2 in the liver resulted in significantly high frequencies of nucleotide alterations in the transcripts of eukaryotic translation initiation factor 4 gamma 2 (*Eif4g2*) and phosphatase and tensin homolog (*PTEN*) genes. Hepatocellular carcinoma developed in 2 of 20 APOBEC2 transgenic mice at 72 weeks of age. In addition, constitutive APOBEC2 expression caused lung tumors in 7 of 20 transgenic mice analyzed. Together with the fact that the proinflammatory cytokine tumor necrosis factor-α induces ectopic expression of APOBEC2 in hepatocytes, our findings indicate that aberrant APOBEC2 expression causes nucleotide alterations in the transcripts of the specific target gene and could be involved in the development of human hepatocellular carcinoma through hepatic inflammation.

Cancer Cell Biology

The number of coding sequences in the genome is limited, but the genomic information encoded in DNA or RNA sequences can be manipulated to produce a wide range of expression products in cells.[1] Apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC) family members are nucleotide-editing enzymes capable of inserting somatic mutations in DNA and/or RNA through their cytidine deaminating activity.[2] The APOBEC family comprises APOBEC1, -2, -3A, -3B, -3C, -3DE, -3F, -3G, -3H, -4, activation-induced cytidine deaminase (AID) in humans, and APOBEC1, -2, -3, and AID in mice, and contribute to producing various physiologic outcomes by modifying target gene sequences.[3–5] For example, APOBEC1 participates in lipid metabolism by deaminating a specific cytidine to uridine in Apolipoprotein (Apo-) B transcript sequences. The nucleotide change induced by APOBEC1 activity results in the formation of a termination codon in an Apo-B48 mRNA, leading to the production of molecules about half the size of a full-length genomically encoded Apo-B100.[6,7] APOBEC3G is a cytidine deaminase that induces hypermutation in viral DNA sequences and acts as a host defense factor against various viruses, including HIV-1 and hepatitis B viruses.[8–15] On the other hand, AID is expressed in germinal center B-cells and induces somatic hypermutation and class switch recombination of the immunoglobulin genes encoded in human DNA sequences, resulting in the amplification of immune diversity.[16,17] APOBEC1, APOBEC3G and AID thus create nucleotide changes in their preferential target DNA or RNA structures. In contrast to these APOBEC proteins, little is known about the function and editing activity of APOBEC2. Although previous reports indicate that murine APOBEC2 mRNA and protein are expressed exclusively in heart and skeletal muscle, the substrate and function of APOBEC2 and whether APOBEC2 has nucleotide editing activity remain unknown.[18,19]

Accumulating evidence suggests that excessive or aberrant activity of APOBEC family members leads to tumorigenesis through their nucleotide editing of tumor-related genes.

Key words: APOBEC2, hepatocellular carcinoma, lung cancer

Abbreviations: APOBEC: Apolipoprotein B mRNA editing enzyme catalytic polypeptide; EIF4G2: Eukaryotic translation initiation factor 4 gamma 2; AID: activation-induced cytidine deaminase; Apo-: Apolipoprotein; Tg: transgenic; NF-κB: nuclear factor-κB; HCC: hepatocellular carcinoma; TNF: tumor necrosis factor; cDNA: Complimentary DNA; RT-PCR: real-time reverse-transcription polymerase chain reaction; ER: estrogen receptor

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Transgene expression of APOBEC1 causes dysplasia and carcinoma in mouse and rabbit liver due to its aberrant editing of the eukaryotic translation initiation factor 4 gamma 2 (Eif4g2).[20,21] A more striking tumor phenotype is observed in mice with constitutive and ubiquitous AID expression. We previously demonstrated that AID transgenic (Tg) mice developed tumors in various organs, including liver, lung, stomach and lymphoid organs, accompanied by the accumulation of somatic mutations on several tumor-related genes such as *Tp53* and *Myc*.[22,23] Interestingly, we also found that proinflammatory cytokine stimulation induces a substantial upregulation of APOBEC2 transcription *via* the activation of the transcriptional factor nuclear factor-κB (NF-κB) in hepatoma-derived cells, whereas only trace amounts of endogenous APOBEC2 expression are detectable in normal hepatocytes.[24] On the basis of the fact that most human hepatocellular carcinoma (HCC) arises in the setting of chronic liver disease with the features of chronic hepatitis or liver cirrhosis, we hypothesized that APOBEC2 enzyme activity has a role in the accumulation of genetic alterations in tumor-related genes under conditions of hepatic inflammation, thereby contributing to the development of HCC. In this study, we investigated the putative nucleotide editing ability of APOBEC2 on the host genes in hepatocytes, and its relevance to carcinogenesis by establishing Tg mice that constitutively express APOBEC2.

Material and Methods

APOBEC2 Tg mice

Total RNA was extracted from murine liver using Sepasol-RNA 1 Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Complimentary DNA (cDNA) was synthesized from total RNA with random hexamer primers using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). After amplification of the murine APOBEC2 gene using high-fidelity Phusion Taq polymerase (Finnzymes, Espoo, Finland) with oligonucleotide primers, 5′-GCAGAATTCCACCATGGCTCAGAAGGAAG AGGC-3′(forward) and 5′-ACTCTCGAGCCTACTTCAGGA TGTCTGCC-3′ (reverse), murine APOBEC2 cDNA (1.2 kbp) was cloned downstream of the chicken β-actin (CAG) promoter. The purified fragment of the CAG promoter and APOBEC2 transgene was microinjected into fertilized eggs of the Slc:BDF1, the hybrid of C57BL/6CrSlc and DBA/2CrSlc (Japan SLC, Shizuoka, Japan), to generate APOBEC2 Tg mice. Tg mice were maintained in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University. Control mice were littermates carrying no transgene. Tissue samples from Tg mice were removed and fixed in 4% (w/v) formaldehyde, embedded in paraffin, stained with hematoxylin and eosin and examined for histologic 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).

Quantitative real-time reverse transcription PCR

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) for murine *APOBEC1* and *APOBEC2* amplification was performed using a LightCycler® 480 instrument (Roche). cDNA was synthesized from 1 μg of total RNA isolated from the cells with random hexamer primers in a total volume of 20 μL using Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCRs were set up in 20 μL of FastStart Universal SYBR Green (Roche) with the RT product and the following oligonucleotide primers: APOBEC1, 5′-CGAAGCTTATTGGCCAAGGT-3′ (forward) and 5′-AAGGAGATGGGGTGGTATCC-3′ (reverse); APOBEC2, 5′-CCCTTCGAGATTGTCACTGG-3′ (forward) and 5′-TGTTCATCCTCCAGGTAGCC-3′ (reverse). Target cDNAs were normalized to the endogenous RNA levels of the housekeeping reference gene for *18S ribosomal RNA* (*18S rRNA*).[25] For simplicity, the expression levels of *APOBEC2* are represented as relative values compared with the control specimen in each experiment.

Immunoblotting

Homogenates of murine specimens were diluted in 2× sodium dodecyl sulfate sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 5% β-mercaptoethanol; 10% glycerol, and 0.002% bromophenol blue) and boiled for 5 min. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% (w/v) polyacrylamide gels and subjected to immunoblotting analysis.[26] A polyclonal antibody against human and murine APOBEC2 was generated using purified recombinant APOBEC2 protein as an immunogen. A mouse monoclonal antibody against α-tubulin was purchased from Calbiochem (San Diego, CA).

Cell culture and transfection

Human hepatoma-derived cell lines HepG2 and Huh7 were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% fetal bovine serum. Trans-IT 293 transfection reagent (Mirus Bio Corporation, Madison, WI) was used for plasmid transfection. To generate stable cell lines, pcDNA3-ERT2 was made by inserting the ERT2 fragment, which was cut out from pERT2[27] with *Bam*HI and *Eco*RI. pcDNA3-APO2-ERT2 was made by inserting the PCR-amplified coding sequence of human *APOBEC2*, which was synthesized by RT-PCR with the oligonucleotide primers 5′-ATAGG TACCATGGCCCAGAAGGAAGAGGC-3′ (forward) and 5′-ATAGGATCCAGCTTCAGGATGTCTGCCAAC-3′ (reverse), into the *KpnI-BamHI* site of pcDNA3-ERT2. HepG2 cells were transfected with a *ScaI*-linearized pcDNA3-APO2-ERT2 vector encoding the active form of APOBEC2 fused with the hormone-binding domain of the human estrogen receptor (ER), designated APOBEC2-ER, and cultured in medium

containing G418 (Roche) until colonies of stably transfected clones arose.

Subcloning and sequencing of the target genes

The oligonucleotide primers for the amplification of the human *EIF4G2*, *PTEN*, and *TP53*, and murine *Eif4G2*, *Pten*, *Bcl6* and *Tp53*, genes are shown in Supporting Information Table S1. 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, Carlsbad, CA) using pGEM(R) -T Easy Vector System (Promega, Madison, WI) according to the manufacture's instruction. The resulting plasmids were subjected to sequence analysis as described.[28]

Results

Detection of endogenous APOBEC2 protein expression in hepatocytes

We previously reported that transcription of *APOBEC2* is induced by the proinflammatory cytokine tumor necrosis factor (TNF)-α through the activation of NF-κB. To confirm whether endogenous APOBEC2 protein is elevated in response to TNF-α stimulation in human hepatocytes, we generated a rabbit polyclonal antibody against a common amino-acid sequence to human and murine APOBEC2. Using this anti-APOBEC2 antibody, we first confirmed that plasmid-derived exogenous APOBEC2 protein was efficiently detected by immunoblotting analysis (Fig. 1*a*). We then examined whether endogenous APOBEC2 protein was upregulated by TNF-α stimulation in Huh-7 cells. Immunoblotting analysis using the APOBEC2 antibody revealed that endogenous APOBEC2 protein expression was strongly induced after TNF-α stimulation, suggesting that APOBEC2 protein has a role in hepatocyte function under inflammatory conditions (Fig. 1*b*).

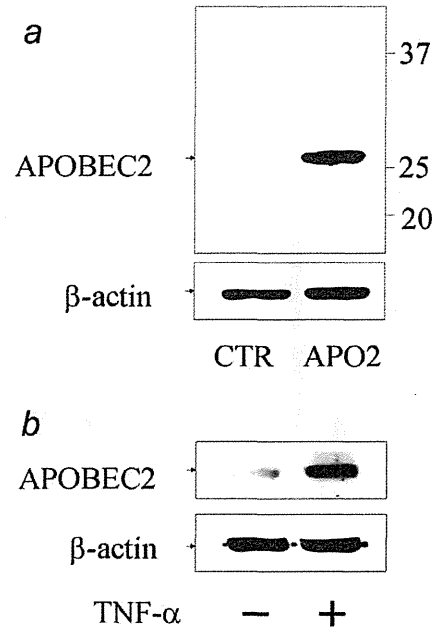

Figure 1. Detection of human APOBEC2 protein in hepatocytes by a specific anti-APOBEC2 antibody. (*a*) Huh7 cells were transfected with plasmid to induce the expression of human APOBEC2 (APO2) or control vector (CTR). After 48 hr, lysates of transfected cells were immunoblotted with anti-APOBEC2 antibody (upper panel) or anti-β-actin antibody (lower panel). (*b*) Huh7 cells were treated with tumor necrosis factor-α (100 ng/ml) for 48 hr followed by immunoblotting using anti-APOBEC2 antibody (upper panel) or anti-β-actin antibody (lower panel).

Establishment of a Tg mouse model constitutively expressing APOBEC2

To investigate the enzymatic activity of APOBEC2 *in vivo*, we generated a Tg mouse model with constitutive and ubiquitous expression of APOBEC2 under the control of CAG promoter. APOBEC2 Tg mice were born healthy and with a body weight similar to that of their wild-type littermates. The expression level of APOBEC2 in various organs of the Tg mice was examined by quantitative RT-PCR and compared with that in the wild-type mice. In wild-type mice, endogenous APOBEC2 transcript was expressed at high levels in heart and skeletal muscle, whereas little or no APOBEC2 expression was detected in the liver, gastrointestinal tracts, lung, spleen and kidney. In contrast, high expression of *APOBEC2* mRNA was ubiquitously detected in the Tg mice, but the expression levels of *APOBEC2* in the liver or lung of the Tg mice were relatively lower than those of the wild-type heart or skeletal muscle (Fig. 2*a*). Immunoblotting analysis using the specific antibodies against APOBEC2 also revealed widespread expression of APOBEC2 protein in various epithelial organs of the Tg mice, with relatively low expression in kidney and spleen (Fig. 2*b*).

Constitutive expression of APOBEC2 resulted in the accumulation of nucleotide alterations in RNA sequences of Eif4g2 and Pten genes in hepatocytes

To clarify whether APOBEC2 targets DNA or RNA, we first extracted total RNA from the nontumor liver tissues of 2 APOBEC2 Tg mice that developed HCC (described below) and their 3 APOBEC2 Tg littermates without any tumor phenotypes, and subjected them to sequence analyses. We chose 2 representative tumor-suppressor genes that are frequently mutated in human cancers, *Pten*, and *Tp53*. The *Bcl6* and *Eif4g2* genes were also included because they are the preferential targets for AID- and APOBEC1-mediated mutagenesis, respectively. We first confirmed that the transcription levels of the genes analyzed for RNA sequencing did not differ between the liver tissues of APOBEC2 Tg mice and wild-type littermates (Supporting Information Fig. S1). In addition, there was no difference in the quantitative levels of APOBEC1 expression between the APOBEC2-expressing liver and

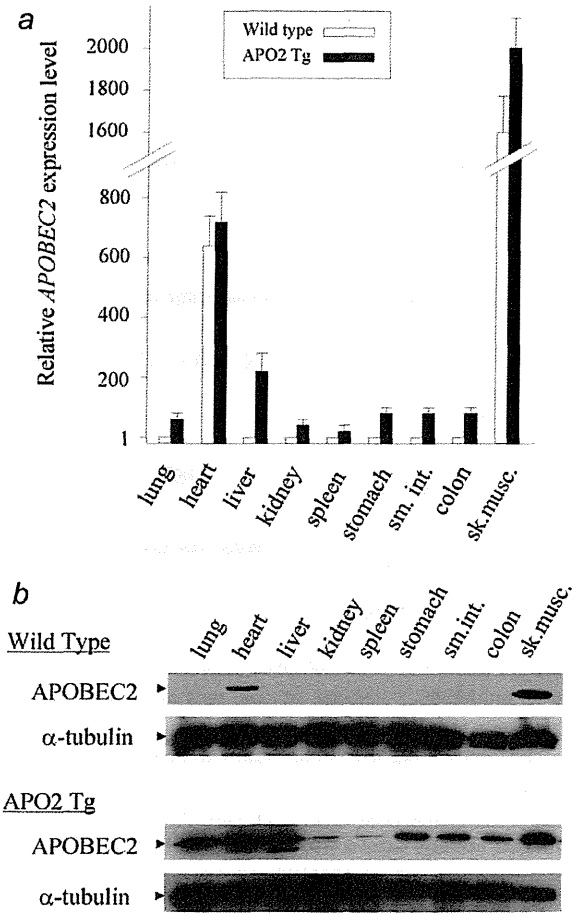

Figure 2. Expression analyses of APOBEC2 Tg mice. (*a*) Relative expression levels of *APOBEC2* transcripts calibrated by the amount of *18S rRNA* for indicated organs of adult APOBEC2 Tg mice (48-week-old) and their wild-type littermates. Data shown are mean results of quantitative real-time RT-PCR analyses for the indicated mouse groups ($n = 6$). Filled bar, APOBEC2 Tg mice; open bar, wild-type mice; sm.int, small intestine; sk.musc, skeletal muscle. (*b*) Results of immunoblot analysis using anti-APOBEC2 (upper panel) or anti-α-tubulin (lower panel) antibody for the lysates of the indicated organs of 48-week-old APOBEC2 Tg mice and their littermates.

normal liver of the wild-type mice (Supporting Information Fig. S2). Sequence analysis revealed a mean of 98,000 and 55,400 base reads per each gene transcript derived from the nontumor liver tissues of the APOBEC2 Tg and control mice, respectively. The total number of amplified clones and RNA sequence reads, and the frequency of nucleotide alterations detected in the nontumor liver tissues of 2 APOBEC2 Tg mice with HCC and the wild-type littermate of the same mouse line are shown in Table 1. The mutation frequencies were highest in the *Eif4g2* transcripts among the genes analyzed in APOBEC2-Tg mice, and were significantly greater compared with those in control tissues (mutation frequencies were 2.75 and 2.36 *vs.* 0.58 substitutions per 1×10^4 nucleotides; $p < 0.05$). Moreover, the nucleotide alteration frequency was significantly higher in the *Pten* gene transcripts from a APOBEC2-expressing liver (Tg-1) than in the control tissues (mutation frequencies were 2.43 *vs.* 0.44 substitutions per 1×10^4 nucleotides, respectively; $p < 0.01$). The *Pten* mRNA of a liver derived from another APOBEC2 Tg mouse (Tg-2; mutation frequency was 1.36 substitutions per 1×10^4 nucleotides) also had a higher nucleotide alteration frequency than that in the control mice, although the difference was not statistically significant ($p = 0.16$ *vs.* control). For the *Eif4g2* and *Pten* transcripts, nucleotide alterations were distributed over the sequences examined and all the alterations detected were different among clones (Fig. 3). Similar results were obtained from the analyses on the liver of 3 APOBEC2 Tg mice that lacked any tumor phenotypes. Indeed, several nucleotide changes had accumulated in both *Eif4g2* and *Pten* transcripts in the liver of all 3 APOBEC2 Tg mice examined (Supporting Information Table S2). In contrast, the mutation frequencies of *Tp53* and *Bcl6* genes of the liver of the APOBEC2 Tg mice were comparable with those of the wild-type mice.

APOBEC2 expression in the liver induced no nucleotide changes in DNA sequences

To clarify whether the nucleotide alterations that emerged in *Eif4g2* and *Pten* transcripts were due to DNA or RNA sequence changes, we determined the DNA sequences of both genes derived from the liver tissues of APOBEC2 Tg and control mice. DNA sequences with an average base length of 0.7 k containing exonic and intronic sequences were amplified, followed by sequence analyses. The total number of amplified clones and DNA sequences read, and the frequency of nucleotide alterations are shown in Supporting Information Table S3. In contrast to the analyses on the RNA sequences, there were no significant differences between the mutation frequency of APOBEC2 Tg mice and that of the wild-type mice of the DNA sequences of the *Eif4g2* and *Pten* genes in the liver. Indeed, no nucleotide alterations were observed in the DNA sequences of the *Eif4g2* gene in the liver of the APOBEC2 Tg mice. Similarly, no mutation was accumulated in the *Pten* DNA sequences of the APOBEC2-expressing liver, suggesting that constitutive expression of the APOBEC2 transgene had no effect on the DNA sequences of the examined regions in the *Eif4g2 and Pten* genes in hepatocytes.

APOBEC2 transgenic mice developed liver and lung tumors

Although most Tg mice were viable at 72 weeks, macroscopic liver and lung tumors developed in some of the APOBEC2 Tg mice. At 72 weeks of age, liver tumors were observed in 2 of 20 Tg male mice, and lung nodules were detected in 7 Tg mice. In contrast to the APOBEC2 Tg mice, none of the wild-type mice developed any tumors at the same age, except 1 with a very small adenoma in the lung. Histopathologic

Table 1. Summary of sequence analysis on the RNA extracted from the liver of the wild-type and APOBEC2 Tg mice

Gene	Mice	Clone	Sequence reads	Nucleotide alterations		
				Number	Frequency(/10^4)	APO2/Wt*
Eif4g2	Wt	82	50,949	3	0.58	
	Tg-1	83	50,835	14	2.75	4.7**
	Tg-2	90	54,986	13	2.36	4.1**
Pten	Wt	92	67,352	3	0.44	
	Tg-1	79	57,599	14	2.43	5.5***
	Tg-2	69	51,323	7	1.36	3.1
Bcl6	Wt	48	41,776	3	0.72	
	Tg-1	59	51,414	1	0.19	0.3
	Tg-2	48	42,413	4	0.94	1.3
Tp53	Wt	84	61,705	2	0.32	
	Tg-1	51	42,285	3	0.71	2.2
	Tg-2	50	40,880	3	0.73	2.3

*Frequency of nucleotide alteration in APOBEC2 Tg mice / in wild type mice. **$p < 0.05$, *vs.* Wt. ***$p < 0.01$, *vs.* Wt.
Abbreviations: Tg, APOBEC2 Tg mice; WT, wild type mice.

analysis of hepatic tumors developed in the APOBEC2 Tg mice revealed nodular aggregates of neoplastic hepatocytes and permeation of tumor cells into residual normal lobules (Fig. 4). Tumor cells had enlarged and hyperchromatic nuclei with chromatin clumping and occasional prominent nucleoli, which were similar to the morphologic characteristics of typical human HCC. On the other hand, lung tumors showed various degrees of cellular atypia, from adenoma to adenocarcinoma (Fig. 5*a*). In addition, monotonous atypical lymphocytes with cytologic features of lymphoblastic lymphoma, such as enlarged round nuclei, irregular nuclear contours, and frequent mitotic figures, massively invaded the spleens of 2 Tg mice (Fig. 5*b*). These findings suggest that constitutive expression of APOBEC2 causes the development of neoplasia in the epithelial organs, including the liver and the lung.

APOBEC2 induced the accumulation of nucleotide alterations of specific target RNA sequences in hepatocytes in vitro

To confirm whether APOBEC2 exerts genotoxic effects on RNA transcripts of the specific target genes, we investigated the alteration frequencies of RNA sequences in cells with constitutive APOBEC2 expression. For this purpose, we established a conditional expression system that allowed for APOBEC2 activation in the cells in response to an estrogen analogue, 4-hydroxytamoxifen (OHT). OHT treatment triggered a posttranslational conformational change and prompt activation of APOBEC2 in APOBEC2-ER expressing cells.[29] We analyzed 3 genes including *PTEN*, *TP53* and *EIF4G2* for the sequence analysis of APOBEC2-mediated mutagenesis *in vitro*. Total RNA was extracted from the APOBEC2-ER expressing HepG2 cells treated with OHT for 8 weeks and the coding RNA sequences of the selected genes were determined by sequence analyses. The total number of amplified clones and RNA sequence reads, and the frequency of nucleotide alterations are shown in Supporting Information Table S4. We found that the emergence of nucleotide alterations in the *PTEN* and *EIF4G2* transcripts was detected at higher frequencies in the cells with APOBEC2 activation compared with control cells treated with OHT, while these differences were not statistically significant ($p = 0.23$ *vs.* control, and $p = 0.39$ *vs.* control, respectively). In contrast, the frequency of nucleotide alterations in the transcripts of the *TP53* in the cells with APOBEC2 activation was comparable with that in the control cells. Similar to the findings obtained from the APOBEC2 Tg mice liver tissues, there were no significant differences between APOBEC2-expressing hepatocytes and control cells in the incidence of nucleotide alterations in the *PTEN* and *EIF4G2* genes (Supporting Information Table S5). These data further suggest that APOBEC2 exerts mutagenic activity in hepatocytes and preferentially achieves nucleotide substitutions in the coding sequences of the specific target genes.

Discussion

Among the APOBEC family members, APOBEC2 and AID homologs can be traced back to bony fish, whereas APOBEC1 and APOBEC3s are restricted to mammals.[30,31] The broad preservation of the APOBEC2 homolog among vertebrates suggests that APOBEC2 has a critical role in the physiology of many species. Little is currently known, however, about the biologic activity of APOBEC2 in any type of cells. Moreover, it is not known whether APOBEC2 possesses nucleotide editing activities like other APOBEC family member proteins. In the present study, we demonstrated for the first time that APOBEC2 expression triggered nucleotide alterations in RNA sequences of the specific genes in hepatocytes. In addition, our findings suggest that APOBEC2 could

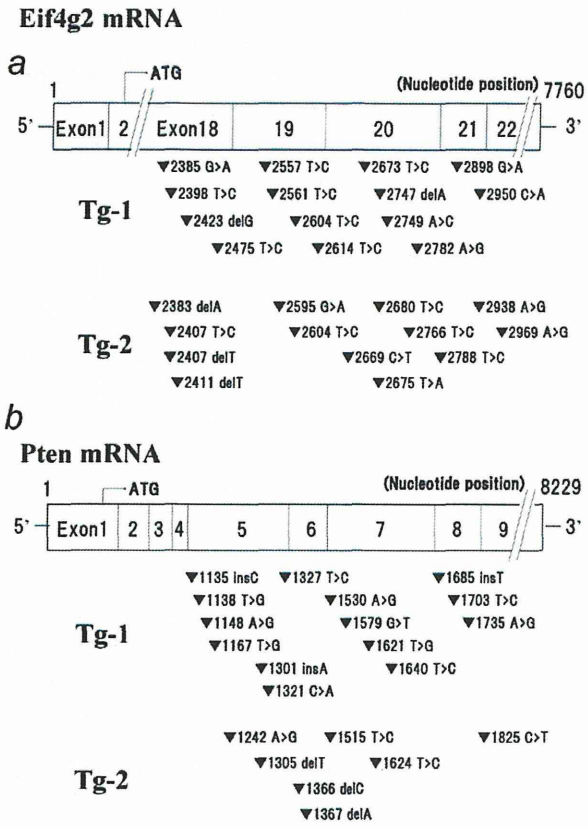

Figure 3. Distribution of nucleotide alterations in the Eif4g2 and Pten trascripts in the APOBEC2-expressing hepatocytes. The mRNA sequences between exon 18 and exon 21 of the Eif4g2 gene (*a*), and the mRNA sequences between exon 5 and exon 8 of the Pten gene (*b*) were determined in the nontumor liver tissues of 2 APOBEC2 Tg mice. The nucleotide positions of the mutations emerged in the Eif4g2 and Pten mRNA of APOBEC2-expressing liver are shown.

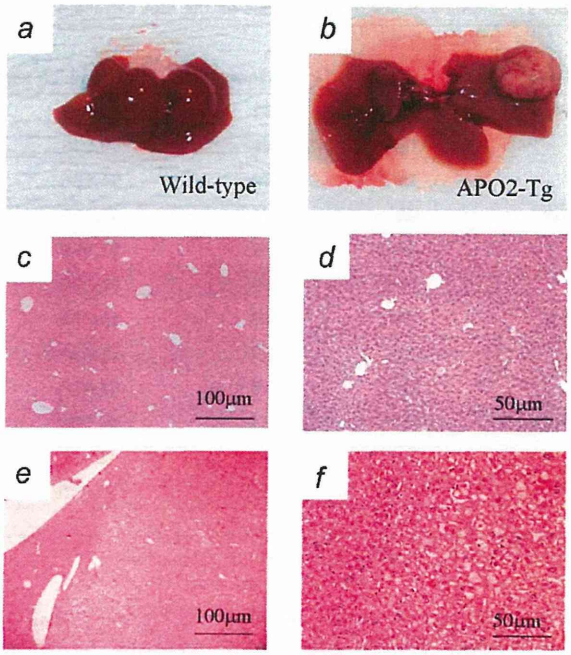

Figure 4. Tumors developed in the liver of APOBEC2 Tg mice. Macroscopic (*b*) and microscopic (haematoxylin and eosin) images (*e, f*) of the HCC that developed in a 72-week-old APOBEC2 Tg mouse and the non-cancerous liver of the same animal (*c,d*). Macroscopic image of the liver of a wild-type littermate is also shown (*a*). (Original magnifications: 3*c,e* ×40; 3*d,f* ×100).

contribute to tumorigenesis via the nucleotide alterations of RNA sequences of the target genes.

On the basis of the close sequence homology of APOBEC2 with other APOBEC proteins, APOBEC2 is thought to exhibit deamination activity to achieve nucleotide editing. Indeed, crystal structure analysis indicates that APOBEC2 contains amino acid residues with 4 monomers in each asymmetric unit that form a tetramer with an atypical elongated shape, and this prominent feature of the APOBEC2 tetramer suggests that the active sites are accessible to large RNA or DNA substrates.[32] In the present study, in a mouse model with constitutive APOBEC2 expression, nucleotide alterations were induced in RNA sequences of the *Eif4g2* and possibly the *Pten* genes in hepatocytes. Similar to its effect *in vivo*, aberrant APOBEC2 expression in cultured hepatocyte-derived cells induced nucleotide alterations in the *EIF4G2* transcripts. Although our findings demonstrate potential mutator activity of the APOBEC2 protein, it is unclear why the *EIF4G2* transcripts were more sensitive to APOBEC2 activity than other genes in hepatocytes. APOBEC1 expression in hepatocytes also induced somatic mutations in the transcripts of the *EIF4G2* gene.[21] Thus, the sequences of the *EIF4G2* gene might be a common target for the nucleotide editing effects of both the APOBEC1 and APOBEC2 proteins. Further analysis is required to identify the specific target genes of APOBEC2-mediated nucleotide editing in hepatocytes.

An intriguing finding was that the mouse model with constitutive and ubiquitous APOBEC2 expression spontaneously developed epithelial neoplasia in the lung and liver tissues as well as lymphoma. Similar phenotypic findings are observed in mouse models expressing APOBEC1 or AID. Tg mice with RNA-editing enzyme APOBEC1 expression develop HCC at high frequencies with an accumulation of somatic mutations at multiple sites on *Eif4g2* mRNA.[20,21] We also demonstrated that AID Tg mice develop tumors in several organs, including the liver, lung, stomach, and the lymphoid tissues through the accumulation of genetic changes induced by the genotoxic effect of AID.[22,23,28] The molecular mechanisms underlying the contribution of constitutive APOBEC2

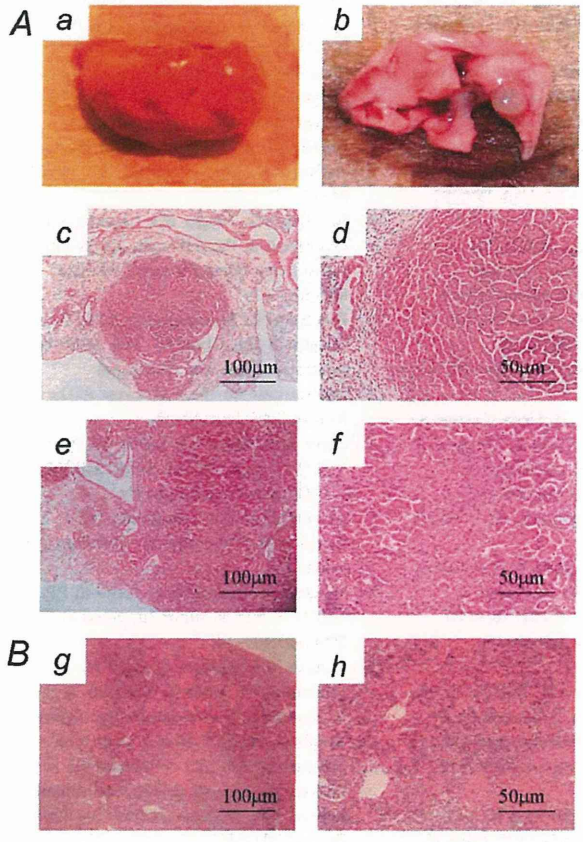

Figure 5. Lung tumors and lymphoma developed in APOBEC2 Tg mice. (*A*) Macroscopic view of a lung tumor that developed in a 72-week-old APOBEC2 Tg mouse (*b*). Microscopic view of a lung adenoma (*c,d*) and adenocarcinoma (*e,f*) that developed in a 72-week-old APOBEC2 Tg mouse. Macroscopic view of the lung of the wild-type littermate (*a*). (*B*) Histologic findings for lymphoma detected in the spleen of APOBEC2 Tg mice. (Original magnifications: 4*c,e,g* ×40; 4*d,f, h* ×100).

expression to tumorigenesis remain unknown. The number of mRNA mutations observed in the *Eif4g2 and Pten* genes in the liver of APOBEC2 Tg mice suggests that these genetic alterations by APOBEC2 have a role in the development of HCC. Indeed, the *EIF4G2* gene is a candidate molecule responsible for oncogenesis caused by the overexpression of APOBEC1,[21] and is frequently downregulated in human cancer tissues.[33] In addition, *PTEN* is one of the most frequently mutated tumor-suppressor genes in human cancers.[34] Thus, the tumorigenesis caused by constitutive APOBEC2 expression might be a consequence of promiscuous nucleotide editing.

Recent studies revealed that the expression of a subset of APOBEC family members is induced by cytokine stimulation in liver tissues. For example, we and other investigators demonstrated that APOBEC3G expression is triggered by interferon-α in hepatocytes, suggesting that APOBEC3G acts as a host defense in response to interferon signaling against viral infection.[35–37] In this study, we showed that TNF-α induced APOBEC2 protein expression in human hepatocytes. Considering the fact that chronic inflammation has important roles in human HCC development,[38,39] the finding that APOBEC2 is induced by proinflammatory cytokine stimulation and induces nucleotide alterations in tumor-related genes in hepatocytes provides a novel idea that aberrant expression of APOBEC2 in epithelial cells acts as a genotoxic factor linking inflammation and cancer development. The tumorigenic phenotype of the APOBEC2-Tg mice further suggests that APOBEC2 is involved in carcinogenesis of the liver tissue under conditions of chronic inflammation, the typical procancerous background of human HCC.

In conclusion, our findings provide the first direct evidence that APOBEC2 induces nucleotide changes preferentially in the *Eif4g2* and possibly the *Pten* genes, and the constitutive expression of APOBEC2 in epithelial tissues contributes to the development of various tumors including HCC and lung cancers. Understanding the pathologic role of APOBEC2 provides new insight into the mechanisms of cancer development in the liver underlying chronic inflammation. During our manuscript preparation, Sato *et al.* reported that they could not find the evidence of APOBEC2's affinity for RNA or high-stoichiometry association with a partner which usually associated with the known RNA editing enzymes.[40] Thus, further analyses would be required to clarify whether APOBEC2 dose possess an RNA-editing activity against specific target genes or overexpression of APOBEC2 causes nucleotide alterations in genome senquences in a promiscuous manner in hepatocytes.

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REVIEWS AND PERSPECTIVES

REVIEWS IN BASIC AND CLINICAL GASTROENTEROLOGY AND HEPATOLOGY

Robert F. Schwabe and John W. Wiley, Section Editors

Inflammation-Associated Cancer Development in Digestive Organs: Mechanisms and Roles for Genetic and Epigenetic Modulation

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Chronic inflammation, regardless of infectious agents, plays important roles in the development of various cancers, particularly in digestive organs, including *Helicobacter pylori*–associated gastric cancer, hepatitis C virus–positive hepatocellular carcinoma, and colitis-associated colon cancers. Cancer development is characterized by stepwise accumulation of genetic and epigenetic alterations of various proto-oncogenes and tumor-suppressor genes. During chronic inflammation, infectious agents such as *H pylori* and hepatitis C virus as well as intrinsic mediators of inflammatory responses, including proinflammatory cytokines and reactive oxygen and nitrogen species, can induce genetic and epigenetic changes, including point mutations, deletions, duplications, recombinations, and methylation of various tumor-related genes through various mechanisms. Furthermore, inflammation also modulates the expressions of microRNAs that influence the production of several tumor-related messenger RNAs or proteins. These molecular events induced by chronic inflammation work in concert to alter important pathways involved in normal cellular function, and hence accelerate inflammation-associated cancer development. Among these, recent studies highlighted an important role of activation-induced cytidine deaminase, a nucleotide-editing enzyme essential for somatic hypermutation and class-switch recombination of the immunoglobulin gene, as a genomic modulator in inflammation-associated cancer development.

Keywords: H pylori; HCV; Mutation Induction; Epigenetics.

Nearly 150 years ago, Rudolf Virchow noted that inflammatory cells are present in tumor tissues and that tumors develop at sites of chronic inflammation; he suggested that chronic inflammation plays important roles in cancer development. Since then, many clinical and epidemiologic studies have confirmed a strong association between inflammation and cancer (Table 1).[1,2] For instance, epidemiologic studies have shown that approximately 10%–15% of cancers were related to chronic infections with viruses, bacteria, or parasites,[3–7] and, moreover, that up to 25% of all cancers were associated with chronic inflammation irrespective of the presence or absence of infection.[5–7]

In inflammation-associated cancer development, in addition to infectious agents such as *Helicobacter pylori* and hepatitis C virus (HCV), many intrinsic mediators of inflammation including proinflammatory cytokines, eicosanoids, growth factors, and reactive oxygen species (ROS) and reactive nitrogen species exert important effects in cancer development through various mechanisms. These include enhancement of cell growth and mobility, induction of angiogenesis, and inhibition of apoptosis. However, a hallmark of cancer development is the stepwise accumulation of various genetic and epigenetic alterations of the genome. Indeed, recent genomewide analysis of human cancer tissues revealed that a single cancer cell generally possesses approximately 100 mutations in coding regions, 10–20 of which are known as driver genes that contribute to cancer development,[8–10] and, moreover, that there are many somatic gene rearrangements, including duplications, deletions, and inversions in human cancer genomes.[11,12] In addition to genetic alterations, recent studies also have shown that chronic inflammation enhances epigenetic changes as represented by DNA methylation.[13] It is estimated that several hundreds to thousands of genes are methylated in a cancer cell,[14] and that

Abbreviations used in this paper: A, adenine; AID, activation-induced cytidine deaminase; C, cytosine; CDKN, cyclin-dependent kinase inhibitor; COX-2, cyclooxygenase 2; DNMT, DNA methyltransferase; DSS, dextran sulfate sodium; EZH2, enhancer of zeste homolog 2; G, guanine; H, histone; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; Let-7, lethal-7; LPS, lipopolysaccharide; miRNA, microRNA; MAPK, mitogen-activated protein kinase; MLH1, mutL homolog 1; mRNA, messenger RNA; MSH, mutS homolog; NF-κB, activation of transcription factor nuclear factor κB; NOS, nitric oxide synthase; PSC, primary sclerosing cholangitis; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; T, thymine; TNF-α, tumor necrosis factor-α; U, uracil.

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Table 1. Inflammation-Associated Cancers in Digestive Organs

Inflammation-associated cancer	Underlying inflammation
Barrett's cancer	Reflux esophagitis
Gastric cancer	*H pylori*–induced chronic gastritis
Colitic cancer	Inflammatory bowel disease
	Celiac disease
HCC	HCV and hepatitis B virus chronic hepatitis
	Primary biliary cirrhosis
Cholangiocarcinoma	Primary sclerosing cholangitis
Pancreatic cancer	Chronic pancreatitis
	Hereditary pancreatitis
Lymphoma	*H pylori*–induced mucosa-associated lymphatic tissue lymphoma
	HCV-associated lymphoma
	Celiac disease–associated lymphoma

aberrant DNA methylation is present even in normal-appearing tissues, being involved in field cancerization.[13,15,16]

Digestive organs are inhabited by many microorganisms and are infiltrated by many immune cells in physiological and pathologic conditions, and thus they are more or less accompanied by certain levels of inflammation. Here, we review mechanisms of how inflammation is involved in cancer development in digestive organs, particularly focusing on the role of chronic inflammation in inducing genetic and epigenetic changes.

Cancers in Digestive Organs Associated With Inflammation

Many cancers arise in digestive organs. Indeed, gastric cancer remains the third leading cause of cancer death in men and the fifth leading cause in women, and colorectal cancer is the third most commonly diagnosed cancer in men and the second most commonly diagnosed in women worldwide.[17] In addition, hepatocellular carcinoma (HCC) is one of the most frequent malignancies and its incidence is increasing not only in an endemic area for the hepatitis virus but also in the United States and other Western countries.[18] Digestive organs cover a large part of the body surface in contact with the outer environment. Accordingly, they are inhabited not only by many microorganisms but also exposed to ingested food or chemical agents, and therefore infiltrated by many immune cells in pathologic as well as normal conditions, supporting the perpetuation of chronic inflammation. Therefore, it is reasonable that many cancers in digestive organs are associated with inflammation.

The best examples of inflammation-associated cancer in human beings are gastric cancer and HCC. Since the discovery of *H pylori* by Warren and Marshall[19] in 1982, it has been well established that *H pylori*–positive patients with chronic gastritis have a significantly higher risk for gastric cancer than *H pylori*–negative subjects,[20] and, moreover, careful investigations have shown more than 95% positivity for *H pylori* infection in gastric cancer patients.[21] On the other hand, hepatitis B virus and HCV infections account for approximately 60% and 33% of the total HCC cases in developing countries and 23% and 20% in developed countries, respectively,[6,22] and the majority of HCCs develop in patients who have chronic hepatitis or cirrhosis. Other inflammation-associated cancers in digestive organs are colitic cancers developed in patients with inflammatory bowel disease (IBD) or celiac disease,[23–25] primary sclerosing cholangitis (PSC)-associated cholangiocarcinoma,[26] primary biliary cirrhosis–associated HCC,[27] and Barrett's cancer developed in patients with reflux esophagitis.[28] In addition, the incidence of pancreatic cancer in patients with chronic pancreatitis is reported to be 4–8 times higher than in the general population,[29] and, more strikingly, the incidence of pancreatic cancer in patients with hereditary pancreatitis is 53 times higher than in the normal population,[30] indicating that chronic pancreatitis is a risk for pancreatic cancer.

In addition to cancers, inflammation is also a risk for developing various lymphomas in digestive organs. These include *H pylori*–induced mucosa-associated lymphatic tissue lymphoma or plasmacytoma,[31,32] HCV-related lymphoma,[33] and lymphoma related to celiac disease.[34]

Mechanisms for Inflammation-Associated Cancer Development

The inflammatory response is coordinated by a large range of mediators, which are released from immune cells, mesenchymal cells, and epithelial cells; these mediators exert various functions in maintaining or resolving inflammation, and at the same time are involved in cancer development. Among the mediators, cytokines play central roles in diversifying the inflammatory process, and interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 are known to be the major cytokines important for inflammation and cancer development.[35–37]

IL-1β and TNF-α act directly on epithelial cells to induce activation of transcription factor nuclear factor-κB (NF-κB), a key transcription factor mediating inflammation and cancer development.[36,37] NF-κB activation not only promotes growth or suppresses apoptosis of epithelial cells but also stimulates the production of growth factors and cytokines such as epidermal growth factor and IL-6, enhances cyclooxygenase (COX)-2 induction, and increases ROS production.[38] The induced COX-2 subsequently has many functions, including enhancement of cell growth and angiogenesis.[39] ROS modifies protein function.[40] IL-6 activates signal transducer and activator of transcription 3 (STAT3) and thereby enhances cell growth and stimulates growth factor production, including the Reg protein.[41] Interestingly, TNF-α and IL-6 often create a positive-feedback loop during cancer development.[42]

At the same time, these cytokines also activate mitogen-activated protein kinase (MAPK) cascades. For instance, TNF-α and IL-6 have been shown to activate the extracellular signal–regulated kinase/MAPK cascade, an impor-

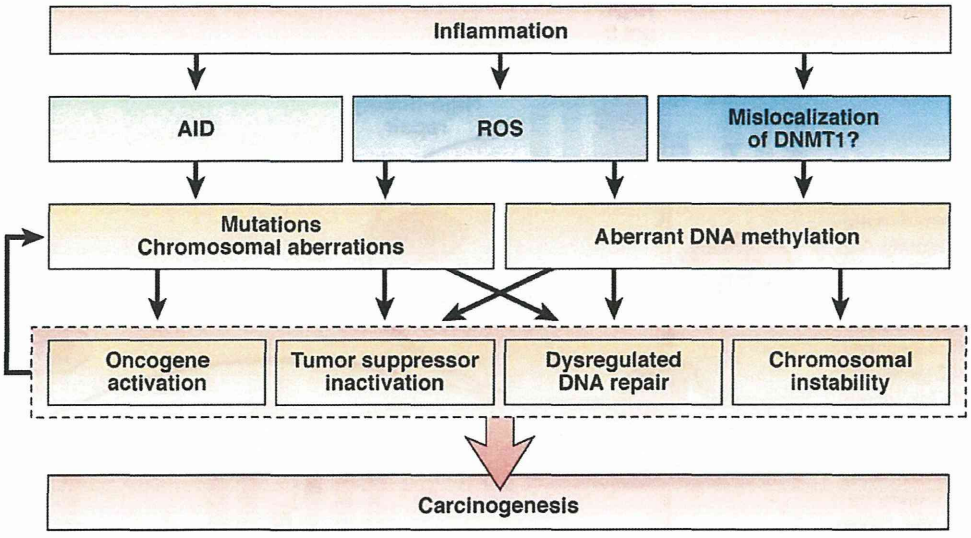

Figure 1. Molecular link between inflammation, genetic and epigenetic alterations, and carcinogenesis. Inflammation contributes to ROS production and transcriptional up-regulation of the DNA mutator enzyme, AID. These 2 factors were capable of inducing somatic mutations and chromosomal aberrations in tumor-related genes. On the other hand, inflammation results in mislocalization of DNMTs, inducing aberrant DNA methylation. The resulting genetic and epigenetic changes, including the activation of oncogenes, inactivation of tumor-suppressor genes, and dysregulation of DNA repair genes, could enhance genetic instability further, finally leading to carcinogenesis.

tant signaling pathway involved in many processes in carcinogenesis including cell proliferation, migration, and angiogenesis.[43,44] Similarly, IL1-β, TNF-α, and IL-6 all activate c-Jun N-terminal kinase (JNK). Although JNKs are attributed primarily to proapoptotic cell death or tumor suppression in response to inflammation or various stressors,[45] JNK activation, particularly JNK1, by proinflammatory cytokines has been reported to contribute to inflammation-associated cancer development through cell death–induced compensatory proliferation.[45–48] In this regard, an interesting thing to note is that *H pylori* directly activates extracellular signal–regulated kinase/MAPK and JNK in human gastric cells via a type IV secretion system–dependent mechanism.[49,50]

Thus, these mediators of inflammation form a complex of regulatory networks, and appear to work in concert to enhance cancer development. However, for normal cells eventually to be transformed and become cancer cells with clonal expansion, inflammation has to damage cellular DNA, either genetically or epigenetically, leading to permanent alteration within the genome.

Inflammation and Genetic Modulation

Cancer is a genetic disease resulting from stepwise accumulation of genetic and epigenetic alterations that drives the progressive transformation of normal cells into malignant derivatives.[51] Inactivation of tumor-suppressor genes and/or activation of oncogenes caused by somatic mutations, DNA copy number changes, or chromosomal aberrations are widely detectable in human cancer cells. Among them, the tumor suppressor *TP53* gene is one of the most frequent targets for genetic alterations in many human cancers.[52] An important point to note is that *TP53* mutations frequently are present also in noncancerous tissues with chronic inflammation before cancer development. Indeed, multiple genetic changes in the *TP53* gene have been detected in various inflammatory tissues such as IBD,[53,54] Barrett's esophagus,[55] and HCV-associated chronic hepatitis.[56] For example, by analyzing the individual crypt mutation burden across plaques of the dysplasia, it was shown that mutations in *TP53* genes could be identified in the majority of inflamed crypts of patients with ulcerative colitis.[57] Moreover, *TP53* mutations are detectable at the frequencies of 4–15 nucleotides of 10^4 nucleotides in the hepatocytes of the patients with chronic HCV infection.[56] Normal mutation rates cannot account for such abundant genetic changes that accumulate in inflamed epithelial cells, suggesting that certain molecular mechanisms underlie such a large number of genetic alterations. Therefore, to understand the mechanisms of inflammation-associated tumorigenesis, several possible intrinsic mutagens responsible for genetic aberrations in the inflammatory condition have been proposed. Among them, free radicals and intrinsic DNA mutator enzymes appear to be important candidates in the setting of chronic inflammation (Figure 1).

Free radicals refer to any molecular species with one or more unpaired electron(s), including ROS and reactive nitrogen species.[38] Interestingly, increases in *TP53* gene mutations at codons 247 and 248 are paralleled by an enhanced expression of nitric oxide synthase (NOS) in the inflamed lesions of the colonic tissues of patients with ulcerative colitis.[54] HCV infection also induces inducible NOS messenger RNA (mRNA) expression, thereby enhancing NO production, which in turn results in DNA breaks and enhanced mutation frequencies.[58] Moreover, an increased level of NO accelerated spontaneous tumor

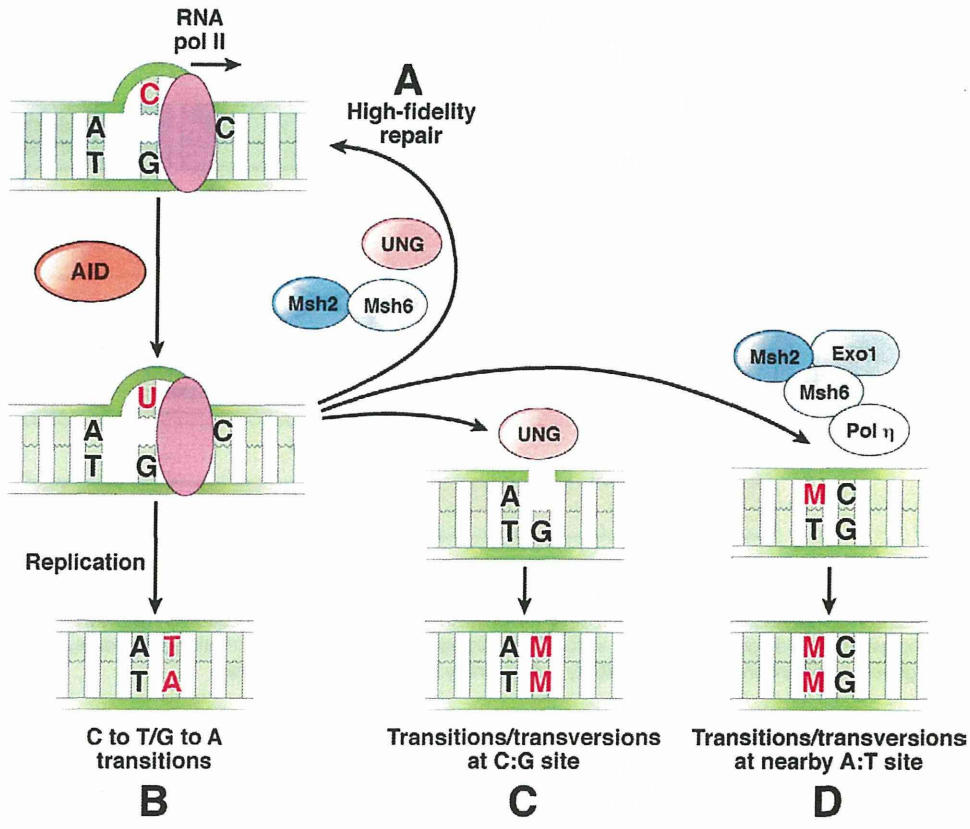

Figure 2. Mechanism of mutation induction by AID activity. AID deaminates cytosine (C), resulting in the generation of a uracil (U), and therefore can transform a DNA C:G pair into a U:G mismatch. (*A*) The AID-generated U:G mismatch can be recognized by uracil-DNA-glycosylase (UNG) or MSH2/MSH6 heterodimer and repaired correctly. (*B*) If DNA replication starts before recognition by the repair system, a U:G mismatch gives rise to C/G to T/A transition. Alternatively, (*C*) generation of an abasic site by UNG or (*D*) recognition of the U:G mismatch by the MSH2/MSH6 heterodimer induces any mutations in the AID-generated U:G mismatch or at a nearby A:T site, respectively, in an error-prone manner (indicated as M).

development, mostly lymphomas, in a *Trp53*-deficient mouse model infected with *Cryptosporidium parvum.*[59]

In the inflammatory condition, cellular ROS levels are increased substantially, and nucleic acids exposed to ROS generate various modified bases such as oxidatively altered purines and pyrimidines.[60] These modified nucleic acids could induce the putative DNA damage, including single- or double-stranded DNA breaks, DNA intrastrand adducts, and DNA protein cross-links.[61] In addition, ROS alters the mismatch repair function and allows mutations to accumulate in microsatellite sequences.[62] It has been well recognized that oncogene activation is capable of inducing genomic instability in precancerous lesions as well as cancer cells.[63] In this regard, ROS is also a putative mediator that links excessive activity of oncogene products and DNA damage. For example, oncogene *c-MYC* overexpression results in DNA damage before the S phase in association with ROS induction in normal human fibroblasts.[64] These findings suggested that the cumulative situation of ROS production, a condition of so-called *oxidative stress*, is involved in both the initiation and progression of inflammation-associated cancers through the induction of genetic instability.

Importantly, the typical mutation pattern induced by oxidative stress cannot account for a mutation signature observed in many human cancer tissues, particularly in inflammation-associated cancers. Among the oxidized nucleosides, one of the common products of free radical attack on DNA is 8-hydroxydeoxyguanine, which is considered to be a biomarker of oxidative stress.[65] The typical pattern of nucleotide alterations induced by 8-hydroxydeoxyguanine is guanine (G)/cytosine (C) to thymine (T)/adenine (A) transversions, which have been observed in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-RAS*) and *TP53* tumor-suppressor gene in lung and liver cancers.[66,67] However, recent genomewide analyses clearly showed that G/C to T/A transversions account for a minor proportion of the total mutations identified in human cancer cells, and instead C/G to T/A transitions are the most prevalent mutation pattern in various cancer tissues, including inflammation-associated cancers.[68] Thus, it appears reasonable to assume that there is an alternative mechanism that accounts for the most frequent mutational pattern, C/G to T/A transitions, detected in many human cancer tissues.

Recently, several human enzymes that are capable of inducing nucleotide alterations have been identified, providing a new avenue for understanding mutagenesis mechanisms.[69] Among them, activation-induced cytidine deaminase (AID) is a well-defined molecule involved in DNA mutations in the human genome. Through its enzymatic activity, AID can deaminate C on target DNA to produce a uracil (U), and therefore turns a DNA C:G pair into a U:G mismatch. When DNA replication starts before recognition by the repair system, a U:G mismatch gives rise to C/G to T/A transition. Alternatively, recognition of a U:G mismatch by uracil-DNA-glycosylase or mutS homolog 2 (MSH2)/mutS homolog 6 (MSH6) heterodimer

induces mutations in the U:G mismatch or at the nearby A:T site (Figure 2). As a result, AID can induce any type of mutations.[70] Under physiological conditions, AID contributes to generating antibody gene diversification in activated B lymphocytes by inducing somatic hypermutation and class switch recombination of immunoglobulin gene.[71] In sharp contrast to the favorable function of AID in the immune system, the role of AID in tumorigenesis through induction of genetic instability was first suggested in hematopoietic malignancies. A number of studies have shown that increased AID expression in various neoplasms of the B lymphocytic lineage was associated with unfavorable mutations and chromosomal translocations.[72,73] For instance, AID has been shown to be responsible for the chromosomal breaks in *c-MYC*, leading to a *c-MYC/IgH* translocation in B-cell lymphoma.[74] Moreover, AID induces *breakpoint cluster region–Abelson murine leukemia viral oncogene homolog 1* mutations leading to imatinib resistance in chronic myeloid leukemia cells.[75] Because the target of AID-mediated genotoxic effects was not restricted to immunoglobulin genes and a variety of other genes also received the AID-mediated mutations in B cells,[70] it was not surprising that aberrant up-regulation of AID induced genetic alterations in various tumor-related genes, leading to the transformation of hematopoietic cells.

As described, activation of NF-κB is induced in response to various inflammatory stimulations, and is deeply involved in multiple processes of cancer initiation and progression.[36] Interestingly, NF-κB is a major transcription factor for AID in B cells that is activated through cluster of differentiation 40-TNF receptor superfamily member 5 ligation by T cells (CD40),[76] suggesting that AID might link NF-κB activation and genetic instability in nonlymphoid cells in the setting of inflammation. In agreement with this hypothesis, AID expression is induced in response to proinflammatory cytokine stimulation via the NF-κB–dependent pathway in various epithelial cells (Figure 3). In hepatocytes, AID expression is induced by TNF-α through the I-κB kinase–dependent NF-κB signaling pathway.[77] Consistent with a previous finding that the HCV core protein triggers the activation of NF-κB in hepatocytes,[78] the HCV core protein itself also up-regulates endogenous AID in cultured hepatocytes.[77] NF-κB–mediated induction of AID expression is not limited to hepatocytes. In human gastric epithelial cells, AID expression is induced by TNF-α stimulation via activation of NF-κB, but is not detected in nonstimulated cells.[79] More interestingly, aberrant AID expression is induced by the infection of a pathogenic *H pylori* strain, the cytotoxin-associated gene pathogenicity island–positive strain that is capable of introducing bacterial virulence factors into the host cells through a type IV secretion system and activating NF-κB, indicating that both bacterial factors introduced into epithelial cells and the inflammatory mediators such as TNF-α and IL-1β induced by *H pylori* infection cooperatively promote aberrant AID expression in *H pylori*–infected gastric mucosal cells. Similar

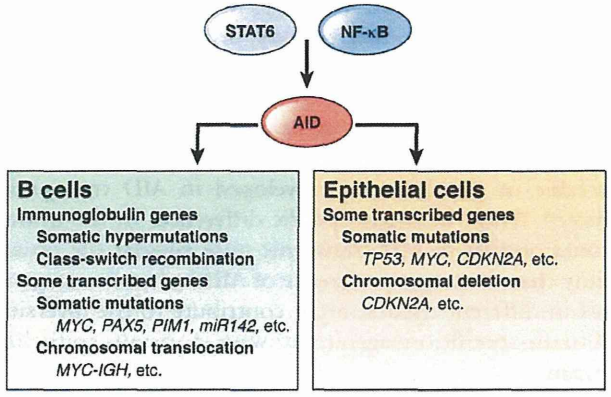

Figure 3. AID exerts both favorable and unfavorable effects. AID is a molecule that is indispensable for the diversification of immunoglobulin genes by inducing both somatic hypermutation and class-switch recombination in activated B lymphocytes. The genotoxic activity of AID, however, can be aimed to trigger the genetic alterations at both the nucleotide and chromosomal levels not only in B lymphocytes but also epithelial cells in the inflammatory conditions.

to hepatocytes and gastric mucosal cells, TNF-α stimulation resulted in up-regulation of endogenous AID in human colonic cells via the I-κB kinase–dependent NF-κB signaling pathway.[80] In addition, IL-4 and IL-13, which are involved in T helper 2 cell–type immune response in IBD, induced aberrant AID expression in a STAT6-dependent manner in human colonic epithelial cells.[80] Of note, IL-4 is known to induce AID also in B cells.[71]

Consistent with the in vitro analyses, aberrant AID expression is widely detectable in not only various inflammation-associated cancer tissues but also in a variety of inflamed epithelial tissues in which tumorigenic risk is high, including chronic hepatitis and cirrhosis caused by HCV infection,[56] chronic gastritis caused by *H pylori* infection,[79] IBD,[80] PSC,[81] and the columnar cell-lined Barrett's esophagus.[82]

The impact of AID expression in nonlymphoid epithelial cells was clarified using both in vivo and in vitro systems with aberrant AID expression. Constitutive and ubiquitous AID expression in transgenic mice induced lymphoma development via the accumulation of somatic mutations in various nonimmunoglobulin genes, including the proto-oncogene *c-Myc*.[83] More importantly, further phenotypic analyses revealed that AID transgenic mice also develop neoplasia in epithelial tissues, including lung, liver, and stomach, accompanied by the emergence of *Trp53* mutations, indicating that aberrant AID expression in epithelial cells can induce genetic instability, leading to cancer development.[83,84] It is widely recognized that the frequently mutated tumor-related genes differ among different cancers. For instance, nucleotide alterations in the *K-RAS* are detectable in almost all human pancreatic cancers,[85] whereas it is relatively low in other human tumors. Similarly, the *c-MYC* is a frequent target for genetic alterations in human lung cancers, whereas its nucleotide alterations are rare in hepatocellular carcinoma.[86] However, the mechanisms underlying the accumulation