deed, cancer cells are associated with dysregulation of many miRNA expressions, which occurs through a variety of mechanisms, such as genetic changes, epigenetic regulation, or altered expression of transcription factors.[135] On the other hand, miRNA expression also is altered in inflammatory conditions, and such alterations in miRNA expression appear to play roles not only in controlling chronic inflammation, but also in promoting cancer development.[136,137] Many of the changes in miRNA expressions observed in inflammatory tissues are derived from immune cells that may participate in hematopoietic tumorigenesis.[138] However, recent reports have shown that inflammation also induces changes in cancer-related miRNAs in epithelial cells, suggesting a direct link between alteration of miRNA expressions and inflammation-associated cancer development.[139,140]

miRNA expressions in epithelial cells can be altered during inflammation through various mechanisms such as NF-κB activation by Toll-like receptors or cytokine stimulation and STAT3 phosphorylation by IL-6 or other cytokines.[139-143] Among those, several miRNAs are identified as tumor-suppressor miRNAs. *miR-7* targets not only epidermal growth factor receptor (*Egfr*) but also latrophilin 2, brain abundant, membrane-attached signal protein 1, and musculoaponeurotic fibrosarcoma oncogene homolog, and thus is considered to be a tumor-suppressor miRNA.[142] In a mouse model of inflammation-associated cancer development, expression of *miR-7* was shown to be inhibited by activated macrophages in *Helicobacter*-infected gastritis mucosa and was shown to be involved in gastric cancer development, although it was increased in germ-free conditions.[142] Lethal-7 (*Let-7*), consisting of 12 members, targets the *RAS* family and *c-MYC*,[144,145] and genomic locations of *let-7* family members frequently are deleted in colon cancers and other solid cancers.[146] NF-κB activation enhances *Lin28B* transcription, which causes posttranscriptional inhibition of *let7* family member expression, and *let-7* directly inhibits IL-6 expression, a cytokine often produced in cancer cells. Thus, reduction of *let-7* expression by NF-κB activation appears to play a role in a positive feedback loop for NF-κB activation through an increase of IL-6 in cancer cells.[147]

miR-155, a possible oncogenic miRNA, is involved in blood cell maturation, immune responses, and autoimmune disorders, and high expression of *miR-155* is associated with the development of myeloproliferative disorders.[148] Recent studies have revealed a direct link between increase of *miR-155* and tumor formation and development in gastric and colon cancers.[148,149] *miR-155* expression is induced by NF-κB, interferon-β, and TLR stimulation,[150] and thus enhanced by *H pylori* and lipopolysaccharide (LPS) treatment.[151] Recently, Tilli et al[143] reported that TNF-α/LPS stimulation enhances *miR-155* expression in association with an increased mutation rate. They also showed that *miR-155* targets mitosis inhibitor protein kinase 1, which blocks cell-cycle progression, and therefore reasoned that reduction of mitosis inhibitor protein kinase by *miR-155* allowed cell division to continue even in the presence of DNA damage, leading to enhanced mutation induction. In another study, they also showed that *miR-155* promotes gene mutations by down-regulating the core mismatch repair proteins, hMSH2, hMSH6, and hMLH1.[152] Of particular interest are the recent reports showing that *miR-155* negatively regulates AID in B cells. Teng et al[153] showed that *miR-155* is up-regulated in B cells undergoing class-switch recombination, and regulates the germinal center reaction by modulating AID. Moreover, *miR-155* has been suggested to inhibit *MYC-IGH* translocation by reducing AID mRNA and protein in B cells.[154] Thus, although an inhibitory effect of *miR-155* on AID has not been examined in non-B cells, *miR-155* also may have a tumor-suppressor function in epithelial cells by inhibiting AID production.

A miRNA expression pattern distinct from normal colonic mucosa has been found in the colonic mucosa and in colitic tumor of patients with IBD as well as mice with colonic inflammation, including up-regulation of *miR-21* and *miR-3*.[155] *miR-21* is one of the most highly expressed miRNAs in colonic tissues of patients with ulcerative colitis,[155] and its expression is enhanced by LPS and IL-6 through STAT3 activation, targeting key regulators of cell proliferation and apoptosis such as phosphatase and tensin homolog and programmed cell death 4.[156] Olaru et al[157] recently showed that in colitic cancer development *miR-31* expression increases in a stepwise fashion from IBD to cancer, and that *miR-31* directly targets regulating factor inhibiting hypoxia-inducible factor 1, decreasing its repressor activity for hypoxia-inducible factor 1.

It is now evident that miRNAs exert various functions in inflammation-associated cancer development. However, alterations of miRNA expression observed in inflammatory tissues occur in both immune cells and epithelial cells. Accordingly, it is important to dissect miRNA changes in the 2 cell types because the patterns of the miRNA changes are different between immune cells and epithelial cells. Further elucidation of the changes of miRNA expression, particularly in epithelial cells, will facilitate our understanding of the role of tumor-related miRNAs in inflammation-associated cancer development.

Application to Cancer Prevention, Diagnostics, and Therapeutics

To prevent inflammation-associated cancer development, it is crucial to cure or control inflammation. Indeed, it has been shown repeatedly that long-term therapy with anti-inflammatory drugs resulted in fewer appearances of tumors.[158] The best way to control chronic inflammation is, of course, to eliminate causative infections. In other cases unrelated to infection such as IBD and PSC, one approach is to block the action of key regulators of inflammation. In this regard, NF-κB or STAT3, and their activators TNF-α or IL-6, respectively, may be good targets for suppressing the inflammatory response. However, because treatment usually needs to be continued for long periods to control chronic inflamma-

tion, agents without serious side effects with lower costs should be developed. For this purpose, many natural agents derived from vegetables, fruits, spices, and their components have been tested. Among them, curcumin, derived from yellow spice turmeric (Curcuma longa) has been used for centuries, and has been shown to suppress NF-κB- as well as STAT3-regulated inflammation,[159] and thus can be administered safely over the long term.[160] Indeed, a recent study showed that curcumin reduced TNF-α expression, prevented cancer-associated weight loss, and induced apoptosis of tumors in patients with colorectal cancer.[161] Resveratrol, a natural polyphenolic, nonflavonoid antioxidant found in grapes and other berries has been shown to have generalized inhibitory effects on inflammation-related molecules such as NF-κB, COX-2, and tyrosine kinases.[162] Recently, resveratrol was found to alter the expression of many tumor-related miRNAs.[163] Similar types of agents may have the potential to both prevent and treat cancers.[164]

In contrast to controlling inflammatory mediators, blocking genetic modulation appears to be difficult. One might consider inhibiting AID. However, because AID plays a critical role in immunoglobulin maturation in B cells, specific targeting for AID in the epithelial cells without affecting AID in B cells is critical. Control of epigenetic modulation can be considered from 2 aspects: suppression of methylation induction and reversal of induced methylation. Because induction of methylation is not essential in adult somatic cells, control of this process is a promising approach to prevent chronic inflammation-associated cancers. On the other hand, reversal of aberrant DNA methylation is an attractive idea to repair an epigenetic field defect, but targeting only aberrant DNA methylation without affecting physiological DNA methylation is currently very difficult.

H pylori eradication ameliorates chronic inflammation, and reduces the risk for gastric cancer. However, it is apparent that eradication cannot completely resolve chronic inflammation because some patients develop gastric cancer even after successful eradication.[165] Likewise, some patients with chronic hepatitis or cirrhosis as a result of HCV infection also develop HCC after obtaining a sustained virologic response.[166] As such, when inflammation is not appropriately controlled or even when inflammation is resolved after long-standing inflammation, accurate prediction for the risk of developing cancers in the inflammatory tissues becomes important. As was discussed, carcinogenesis is characterized by a stepwise accumulation of both genetic and epigenetic changes. Importantly, previous data suggested that the extent of those genetic and epigenetic modulations is paralleled with the duration or severity of inflammation,[15,167] and the degree of epigenetic field defect can be measured relatively easily and accurately. Thus, both qualitative and quantitative detection of these genetic and epigenetic changes in inflammatory tissues or tissues previously exposed to inflammation may provide a good risk marker for inflammation-associated cancer development. Indeed, epigenetic risk markers that can differentiate gastric mucosae of cancer patients from those of healthy individuals with odds ratios between 12.7 and 36.0 have been isolated,[168,169] and a prospective study is now being conducted.

Conclusions

Many cancers in digestive organs develop in the background of chronic inflammation. During chronic inflammation, a variety of mediators for inflammation such as cytokines, growth factors, eicosanoids, ROS, and NOS form complex networks not only for maintaining or reducing inflammation but also promoting cell growth, angiogenesis, and inhibiting apoptosis. These events eventually merge into and result in both genetic and epigenetic changes of the cellular genome, leading to inflammation-associated cancer development. In particular, AID plays a crucial role in inducing not only mutations, but also chromosomal aberrations during inflammation. Moreover, signals from macrophages with resulting mislocalization of DNMTs appear to be involved in the induction of epigenetic alterations.

Interestingly, epigenetic inactivation of *MLH1* leads to accumulation of genetic alterations.[170] At the same time, recent studies have shown that AID induces DNA demethylation through its deaminating activity on methylated cytosines.[131] Thus, genetic and epigenetic events are mutually related and work in concert in the development of inflammation-associated cancers.

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Conflicts of interest

The authors disclose no conflicts.

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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 *Sca*I-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*).

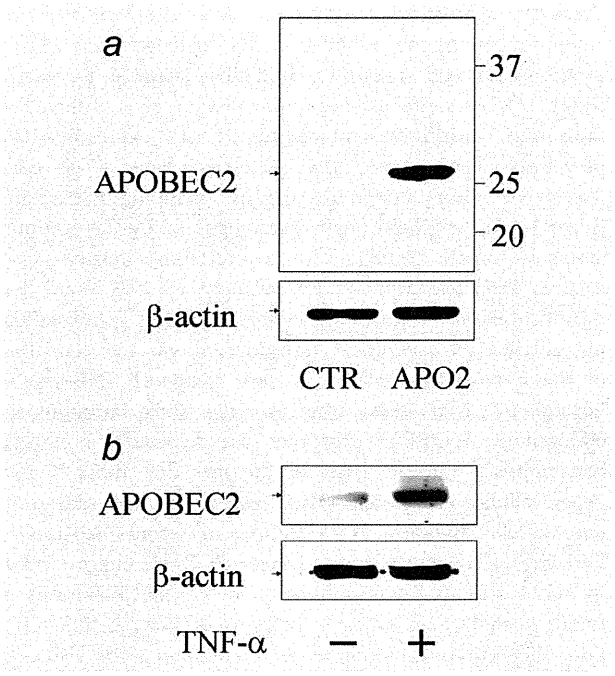

Figure 1. Detection of human APOBEC2 protein in hepatocytes by a specific anti-APOBEC2 antibody. (*a*) Huh7 cells were transfected with plasmid to induce the expression of human APOBEC2 (APO2) or control vector (CTR). After 48 hr, lysates of transfected cells were immunoblotted with anti-APOBEC2 antibody (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

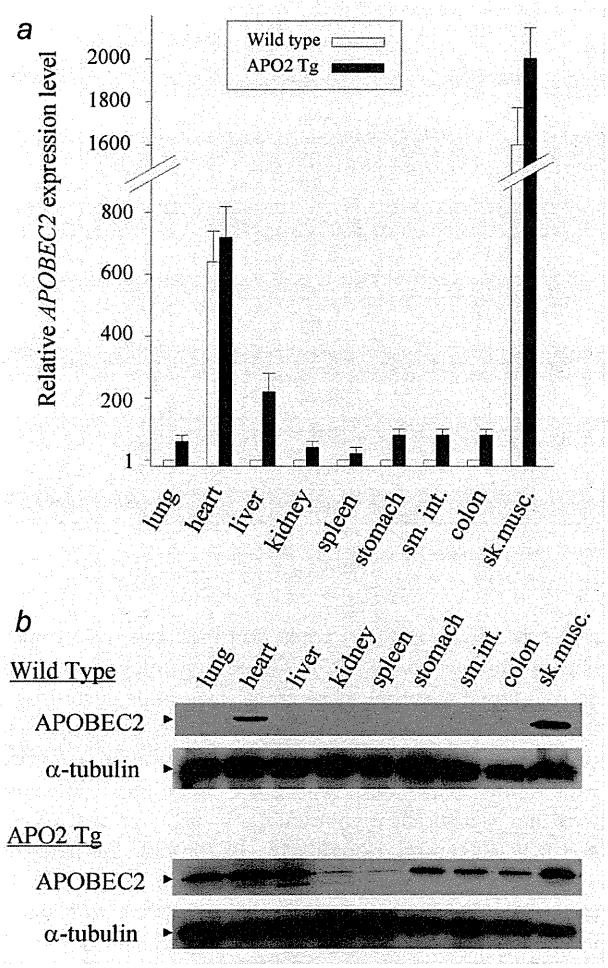

Figure 2. Expression analyses of APOBEC2 Tg mice. (*a*) Relative expression levels of *APOBEC2* transcripts calibrated by the amount of *18S rRNA* for indicated organs of adult APOBEC2 Tg mice (48-week-old) and their wild-type littermates. Data shown are mean results of quantitative real-time RT-PCR 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

				Nucleotide alterations		
Gene	Mice	Clone	Sequence reads	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

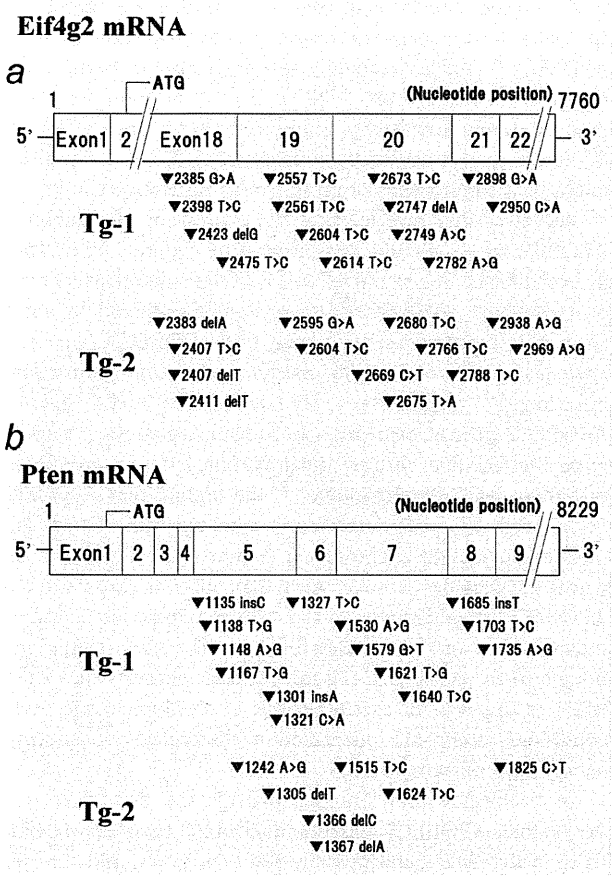

Figure 3. Distribution of nucleotide alterations in the Eif4g2 and Pten trascripts in the APOBEC2-expressing hepatocytes. The mRNA sequences between exon 18 and exon 21 of the Eif4g2 gene (*a*), and the mRNA sequences between exon 5 and exon 8 of the Pten gene (*b*) were determined in the nontumor liver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

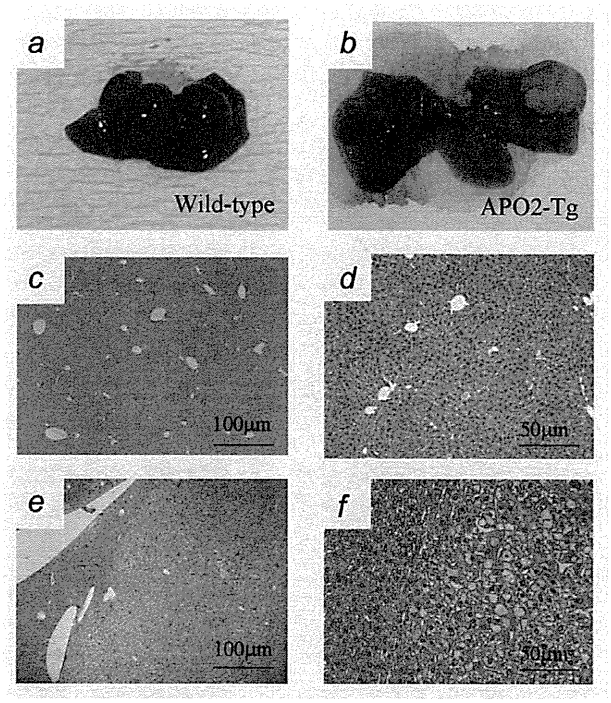

Figure 4. Tumors developed in the liver of APOBEC2 Tg mice. Macroscopic (*b*) and microscopic (haematoxylin and eosin) images (*e, f*) of the HCC that developed in a 72-week-old APOBEC2 Tg mouse and the non-cancerous liver of the same animal (*c,d*). Macroscopic image of the liver of a wild-type littermate 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

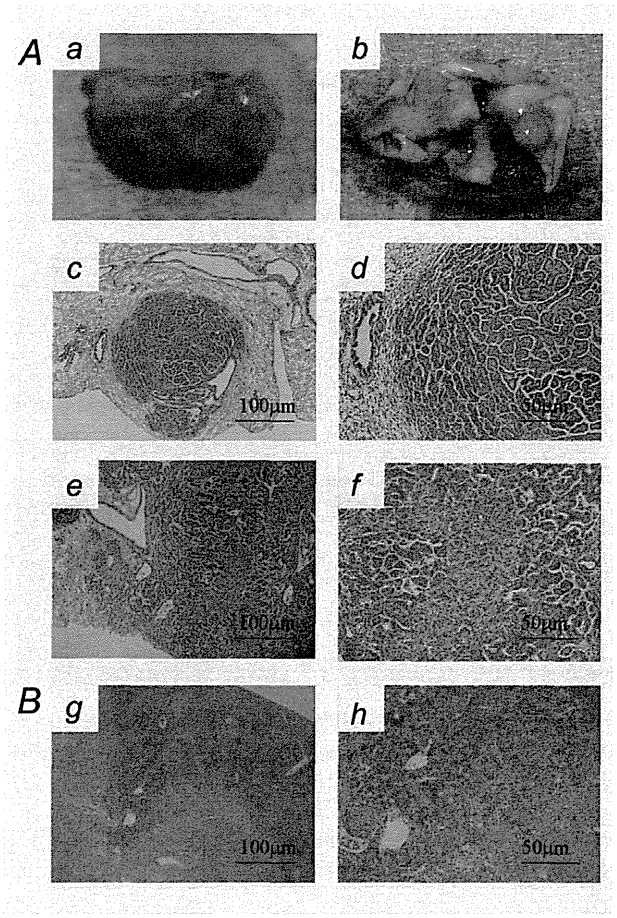

Figure 5. Lung tumors and lymphoma developed in APOBEC2 Tg mice. (*A*) Macroscopic view of a lung tumor that developed in a 72-week-old APOBEC2 Tg mouse (*b*). Microscopic view of a lung adenoma (*c,d*) and adenocarcinoma (*e,f*) that developed in a 72-week-old APOBEC2 Tg mouse. Macroscopic view of the 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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Effect of maintenance therapy with low-dose peginterferon for recurrent hepatitis C after living donor liver transplantation

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SUMMARY. Approximately 30% of patients who have recurrent hepatitis C after liver transplantation achieve sustained virological response (SVR) by taking a combination therapy of pegylated interferon and ribavirin. For the remaining non-SVR patients, an effective management treatment has not yet been established. In this study, efficacy of long-term peginterferon maintenance therapy for non-SVR patients was evaluated. Forty patients who had previously received the combination therapy for hepatitis C after living donor liver transplantation were classified into one of the following three groups: the SVR group (n = 11); the non-SVR-IFN group (n = 17), which received low-dose peginterferon maintenance therapy for non-SVR patients; and the non-SVR-Withdrawal group (n = 12), which discontinued the interferon treatment. We then compared histological changes among these three groups after 2 or more years follow-up. Activity grade of liver histology improved or remained stable in patients in the SVR and non-SVR-IFN groups, but deteriorated in half of the patients in the non-SVR-Withdrawal group. Fibrosis improved or remained stable in 10 of 11 SVR patients and in 13 of 17 non-SVR-IFN patients, but deteriorated in all non-SVR-Withdrawal patients. Mean changes in fibrosis stage between pretreatment and final liver biopsy were −0.18, +0.06 and +2.2 in the SVR, non-SVR-IFN and non-SVR-Withdrawal groups, respectively. Fibrosis stage deteriorated to F3 or F4 significantly more rapidly in the non-SVR-Withdrawal group than in the other two groups. In conclusion, continuing long-term maintenance therapy with peginterferon prevented histological progression of hepatitis C in patients who had undergone living donor liver transplantation.

Keywords: fibrosis, hepatitis C, liver transplantation, maintenance therapy, peginterferon, ribavirin.

INTRODUCTION

Cirrhosis and hepatocellular carcinoma caused by hepatitis C virus (HCV) infection is the leading indication for liver transplantation in Japan, the United States and western Europe. However, liver allograft infection with HCV following liver transplantation is universal, and almost all patients develop recurrent liver injury [1–6]. The progression of recurrent hepatitis C is often accelerated and, without appropriate antiviral therapy, 10–25% of patients develop cirrhosis within 5 years after transplantation, resulting in poorer prognosis for HCV-positive recipients than HCV-negative recipients [7].

To prevent the progression of hepatitis C after liver transplantation, a combined therapy of pegylated interferon plus ribavirin is commonly administered [8,9]. However, the efficacy of this combination therapy is limited: The mean sustained virological response (SVR) rate among patients with recurrent hepatitis C after liver transplantation was only 30% (range, 8–50%) [10]. Effective management of the remaining 70% of the patients who are unable to achieve SVR has not been established [11].

We recently reported the change in liver histology after combination therapy with interferon plus ribavirin in patients who have recurrent hepatitis C after living donor liver transplantations (LDLT). Among patients who did not achieve SVR, activity grade was not improved and fibrosis stage deteriorated. On the other hand, SVR was associated with reduced hepatic inflammation and suppression of liver fibrosis progression [12]. Because the histological progression of non-SVR patients occurred mainly after interferon

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; HCV, hepatitis C virus; LDLT, living donor liver transplantations; MMF, mycophenolate mofetil; PCR, polymerase chain reaction; SVR, sustained virological response.

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therapy was discontinued, we hypothesized that long-term, continuous interferon administration might be effective in slowing the progression of liver damage in these patients. Therefore, after our previous study, we prescribed a low-dose peginterferon maintenance therapy for non-SVR patients. Here, we evaluated the efficacy of this treatment by investigating long-term histological changes in these patients, as well as comparing them to the changes observed in SVR patients and non-SVR patients who did not receive maintenance treatment.

METHODS

Eighty patients who had previously received the combination therapy with interferon and ribavirin ($n = 40$) or peginterferon and ribavirin ($n = 40$) for recurrent hepatitis C after LDLT at Kyoto University between January 2001 and April 2007 were retrospectively analysed.

Patients

Between March 1999 and December 2006, 141 patients with HCV-related liver diseases underwent LDLT at Kyoto University. Of these, 100 patients had been followed up for more than 6 months after LDLT in our hospital. Antiviral therapy was given to 80 patients with recurrent hepatitis C between January 2001 and April 2007. The remaining 20 patients did not receive the antiviral therapy because of no histological recurrence of hepatitis C in the follow-up period. To evaluate the histological progression caused by hepatitis C, patients who were diagnosed as having other causes of liver injury, such as biliary complications, chronic rejection, and *de novo* autoimmune hepatitis (AIH), were excluded. Patients who discontinued the treatment within 3 months because of worsening of liver function caused by hepatitis C were also excluded, because the rapid progression of these patients is not comparable to the long-term progression and inclusion of these patients would have led to overestimation of the progression in the patients who discontinued treatment. Patients were also excluded if they did not have a liver biopsy more than 2 years after the initiation of treatment, because this prevented an analysis of long-term histological changes.

Treatment protocol and definition of responses to treatment

After liver transplantation, patients with recurrent HCV liver disease underwent treatment with interferon–α–2b (3 or 6 mega units thrice weekly) plus ribavirin (400–800 mg/day orally) for the first 6 months. This was followed by interferon monotherapy for 6 months [12]. This treatment protocol was employed between January 2001 through April 2004 inclusive. From May 2004 to April 2007, patients underwent combination antiviral therapy comprising peginterferon–α–2b (1.5 μg/kg body weight, weekly) and ribavirin (400–800 mg/day orally) [13]. Patients who became negative for serum HCV RNA within 12 months after initiating the treatment continued to receive the full (initial) dose for 8–22 months to achieve SVR; then, the treatment ended. Patients who were negative for serum HCV RNA for more than 6 months after completion of interferon therapy were defined as achieving SVR.

Patients who did not become negative for serum HCV RNA within 12 months of initiating the combination therapy, as well as patients who experienced a relapse after transient discontinuation of the treatment, continued to receive a low-dose peginterferon maintenance therapy (0.5–0.75 μg/kg of peginterferon-α-2b with or without ribavirin at 200 mg/day). Treatments occurred during the study period, May 2005–December 2009. During this time, the therapy was discontinued in patients with severe adverse events. Additionally, peginterferon treatments were discontinued when neutrophil and platelet counts fell below 500 and 30 000/μL, respectively, and ribavirin was discontinued when haemoglobin levels fell below 8 g/dL.

Histological assessment

Liver biopsies were performed when patients' alanine aminotransferase (ALT) levels were more than twice the upper limit of normal, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by two pathologists (H.H. and A.M.) with extensive experience in the pathology of liver transplantation. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores [14,15]. Grading was defined as A0 (no activity), A1 (mild activity), A2 (moderate activity) or A3 (severe activity); staging was defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) or F4 (cirrhosis) [14,15].

The following equations were used to analyse the histological changes:

(1) Changes in activity grade = grade at final biopsy − grade at pretreatment biopsy, and
(2) Changes in fibrosis stage = stage at final biopsy − stage at pretreatment biopsy.

Immunosuppression

Tacrolimus and low-dose steroid therapies were administered to induce immunosuppression [12,13,16]. The lower limit of the target for whole blood tacrolimus level was 10–15 ng/mL during the first 2 weeks, 10 ng/mL during weeks 2–8 and 5–8 ng/mL thereafter. Four patients received cyclosporine microemulsions, rather than tacrolimus, to induce immunosuppression (Table 1). Steroid therapy was initiated at a dose of 10 mg/kg before graft reperfusion and then tapered from 1 mg/kg per day on the first day to 0.3 mg/kg per day until the end of the first month, followed by 0.1 mg/kg per day until the end of the third month. After

Table 1 Baseline characteristics of 40 enrolled patients with recurrent hepatitis C after LDLT before interferon therapy

		Non-SVR		
	SVR (n = 11)	IFN (n = 17)	Withdrawal (n = 12)	P
Age (years)	55 (17–68)	57 (39–66)	58 (15–70)	0.724*
Males/Females	7/4	12/5	5/7	0.281†
Time since LDLT (months)	11.5 (4.2–39.1)	10.6 (1.1–51.2)	5.9 (1.8–85.3)	0.316*
HCV genotype 1/non-1	8/3	15/2	12/0	0.141†
HCV RNA (kIU/mL)	1120 (289–5000)	2810 (74–5000)	2320 (498–5000)	0.850*
White cell count (/μL)	4000 (2200–9000)	4600 (1300–6900)	4400 (1700–6900)	0.991*
Neutrophil count (/μL)	2220 (1235–4140)	2040 (793–4816)	2642 (836–4623)	0.884*
Haemoglobin (g/dL)	12.4 (11.6–17)	11.6 (9.2–15.5)	11.65 (8.9–15.2)	0.096*
Platelet count (10^4/μL)	11.7 (5.9–58.1)	11.3 (4.8–32.4)	14.9 (7.6–40)	0.529*
PT (INR)	1.00 (0.92–1.19)	1.04 (0.93–1.67)	1.07 (0.87–1.34)	0.561*
AST (IU/L)	106 (27–352)	78 (30–258)	107 (44–464)	0.539*
ALT (IU/L)	106 (38–395)	82 (37–275)	157.5 (40–354)	0.619*
ALP (IU/L)	492 (233–1954)	479 (234–828)	636 (306–2977)	0.221*
γ-GTP (IU/L)	293 (41–1447)	107 (29–457)	122.5 (23–1417)	0.147*
Bilirubin (mg/dL)	0.9 (0.4–1.8)	0.9 (0.4–2.6)	1.25 (0.3–10.4)	0.530*
Albumin(g/dL)	3.7 (3.3–4.7)	3.8 (2.7–4.5)	3.5 (2.9–4.4)	0.329*
METAVIR score				
A 0/1/2/3	0/8/3/0	0/8/8/1	0/7/5/0	0.594†
F 0/1/2/3/4	1/8/2/0/0	1/9/7/0/0	5/5/2/0/0	0.066†
Immunosuppression				
Tacrolimus	8	16	7	0.257†
Tacrolimus + MMF	2	0	3	
Tacrolimus + prednisolone	1	0	2	
Cyclosporine	0	1	0	
Cyclosporine + MMF	0	1	0	
Trough level for tacrolimus (ng/mL)	5.9 (3.4–8.7)	5.95 (3.3–10.9)	6.4 (3.8–9.1)	0.752*

PT, prothrombin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; MMF, mycophenolate mofetil; LDLT, living donor liver transplantations; SVR, sustained virological response. Qualitative variables are shown in number; and quantitative variables expressed as median (range). *Kruskal–Wallis test, †chi-square test.

that, steroid administration was terminated. Mycophenolate mofetil (MMF) was administered to patients who experienced refractory rejection or required reduction in tacrolimus or cyclosporine doses because of adverse events.

Virological assays

Hepatitis C virus genotype was determined using a genotyping system based on polymerase chain reaction (PCR) of the core region using genotype-specific PCR primers [17]. Serum HCV RNA load was evaluated once a month during treatment and 24 weeks after treatment, using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor; Roche Molecular Systems, Pleasanton, CA, USA).

Statistical analysis

Wilcoxon and Kruskal–Wallis tests, chi-square tests and t-tests were used to analyse the continuous variables, categorical variables and histological changes, respectively. The Kaplan–Meier method was used to estimate the rates of patients who showed a progression of fibrosis to stage F3 or F4 after the initiation of the interferon therapy; log-rank tests were used to compare these rates among groups. Significance was defined as $P < 0.05$.

RESULTS

Characteristics of patients

Hepatitis C virus RNA concentrations and histological evidence were used to diagnose 80 patients with recurrent hepatitis C after LDLT. These patients were given one of two combination therapies: interferon and ribavirin (n = 40) or peginterferon and ribavirin (n = 40) at Kyoto University between January 2001 and April 2007. Thirty-one of the 80 patients who received the combination therapy achieved SVR (Fig. 1). Among the remaining 49 non-SVR patients,

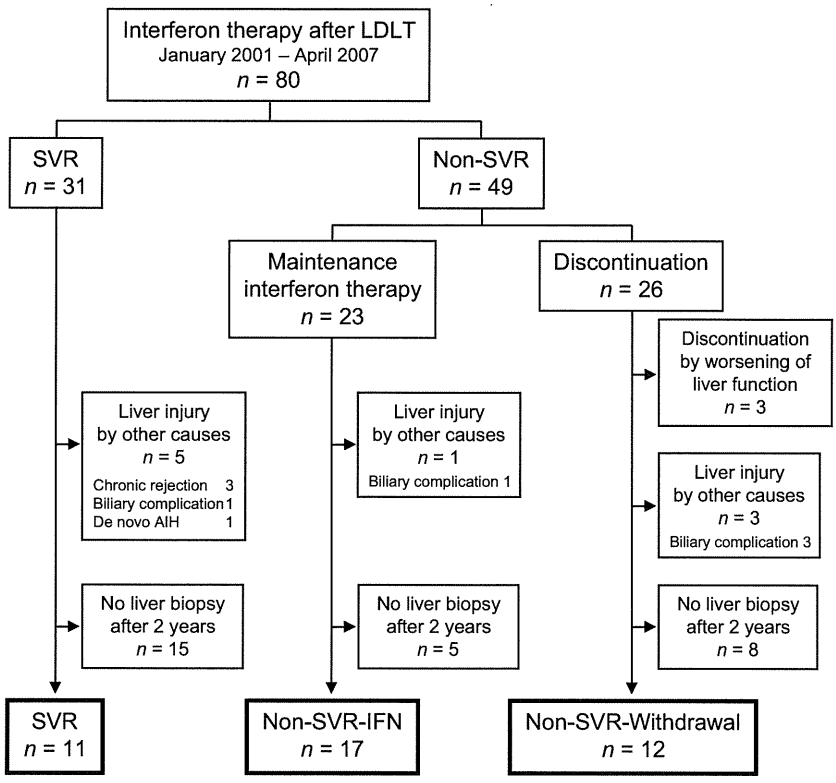

Fig. 1 Flow diagram showing the outcome of interferon therapy for patients with recurrent hepatitis C after living donor liver transplantation and indicating the classification of patients in this study.

23 (47%) received the low-dose peginterferon maintenance therapy, while 26 (53%) discontinued treatment within 12 months and did not receive low-dose peginterferon maintenance therapy as this was the patients' wish (n = 4), because of general fatigue (n = 4), recurrent hepatocellular carcinoma (n = 4), worsening of liver function (n = 3), biliary complications (n = 3), heart failure (n = 2), brain haemorrhage (n = 1), dementia (n = 1), sinusitis (n = 1), anaemia (n = 1), neutropenia (n = 1), and haemosputum (n = 1).

Of the 31 SVR patients, five were excluded because of chronic rejection (n = 3), biliary complications (n = 1) and *de novo* AIH (n = 1). Fifteen patients did not have liver biopsies more than 2 years after the initiation of the interferon therapy, mainly because liver function tests were normal. The remaining 11 patients were classified as the SVR group for analysis in this study. Among the 23 patients who received maintenance therapy, one patient with biliary complications and five patients who did not have liver biopsy more than 2 years after the initiation of therapy were excluded from the study. The remaining 17 patients were classified into the non-SVR-IFN group. Among the 26 patients who discontinued treatment within 12 months, three patients who initially experienced worsening of liver function were excluded because of the rapid progression of HCV; an additional three patients were excluded because of biliary complications. Eight patients were excluded because they had no liver biopsies taken more than 2 years after the initiation of the treatment. The remaining 12 patients were classified into the non-SVR-Withdrawal group. Cumulatively, we analysed the long-term histological changes of 40 patients: 11 in the SVR group (27.5% of the total), 17 in the non-SVR-IFN group (42.5% of the total) and 12 in the non-SVR-Withdrawal group (30% of the total).

There were no significant differences in the baseline characteristics among patients in the SVR, non-SVR-IFN, and non-SVR-Withdrawal groups (Table 1). The median age of patients at the beginning of therapy was 56.5 years (range, 15–70 years). The treatment started at a median of 9.5 months (range, 1.1–85.3 months) after LDLT. Thirty-five patients (88%) were infected with HCV genotype 1b. HCV genotypes of the remaining patients were 2a (n = 3), 2b (n = 1) and undetermined (n = 1). Median serum HCV RNA load was 2290 kIU/mL (range, 73.7–5000 kIU/mL); i.e. most patients had an extremely high viral load. Before the treatment, the necroinflammatory activity of all patients was A1 or greater, and 33 patients (83%) had a fibrosis score of F1 or greater. Among patients receiving tacrolimus for immunosuppression, the median serum trough level was 5.95 ng/mL (range, 3.3–10.9).

Effect of maintenance interferon therapy on liver histology

To evaluate the efficacy of long-term peginterferon therapy on histological changes, we compared scores between final biopsy samples (median, 44.0 months; range, 24.0–81.3 months) and those taken prior to treatment. Five patients in the non-SVR-IFN group discontinued maintenance

therapy between 26.5 and 53.1 months after the initiation of the treatment because of the adverse events. For these patients, the biopsies taken just before or within 3 months after discontinuation of the treatment were analysed as final biopsies. Despite the variation in time between pretreatment and final biopsy sample collection, there were no significant differences in the duration among the three groups ($P = 0.547$). Median duration from initiation of interferon therapy to final liver biopsy was 41.9 months (range, 24.0–81.3 months) in the SVR group, 41.7 months (range, 26.5–68.4 months) in the non-SVR-IFN group and 46.5 months (range, 30.4–79.6 months) in the non-SVR-Withdrawal group.

There were no significant differences in baseline activity grades or fibrosis stages of patients in the three treatment groups when they were first diagnosed with recurrent hepatitis C (Table 1). However, there were noticeable differences among the three groups by the end of treatment (Fig. 2a). The activity grade of all patients in the SVR and non-SVR-IFN groups improved or remained stable, whereas it deteriorated in 6 (50%) of 12 patients in the non-SVR-Withdrawal group. The fibrosis stage deteriorated in all patients in the non-SVR-Withdrawal group; nine of these patients (75%) deteriorated by more than one stage. In contrast, only four patients (24%) in the non-SVR-IFN group deteriorated, all by only a single stage. Furthermore, three patients actually improved. In the SVR group, fibrosis stage decreased or remained stable in 10 of 11 patients (91%).

In patients in the SVR and non-SVR-IFN groups, the mean activity grade was markedly reduced in the final biopsy, compared to the pretreatment biopsy (Fig. 2b). In contrast, patients in the non-SVR-Withdrawal group experienced an increase in activity grade. The differences between the non-SVR-Withdrawal group and both the SVR and the non-SVR-IFN groups were statistically significant ($P < 0.001$). The mean changes in fibrosis stage in the SVR and non-SVR-IFN groups were −0.18 and +0.06, respectively, suggesting that fibrosis did not change during the follow-up period. However, there was an obvious increase (+2.2) among patients in the non-SVR-Withdrawal group, indicating marked progression of fibrosis.

The Kaplan–Meier analysis allowed us to investigate whether patients in the three treatment groups experienced different progression rates to late-stage fibrosis (Fig. 2c). No patient in the SVR group and only 1 patient (6%) in the non-SVR-IFN group developed fibrosis stage F3 or F4, whereas nine patients (75%) in the non-SVR-Withdrawal group progressed to these stages. The rates of fibrosis progression were significantly higher in the non-SVR-Withdrawal group than in the non-SVR-IFN and SVR groups ($P = 0.0049$ and $P = 0.0086$, respectively). There was no significant difference between the SVR group and the non-SVR-IFN group ($P = 0.3980$). Five-year progression rates to F3 or F4 were 0% in the SVR group, 14% in the non-SVR-IFN group and 54% in the non-SVR-Withdrawal group.

Safety and tolerability of maintenance interferon therapy

Five of 17 patients (29%) who received low-dose maintenance peginterferon treatment discontinued interferon therapy because of biliary complications ($n = 2$), neutropenia ($n = 1$), anaemia ($n = 1$) and *de novo* AIH ($n = 1$), between 26.5 and 53.1 months after its initiation. The biliary complications were not related to interferon therapy. Patients with neutropenia and anaemia recovered after discontinuing interferon therapy and were able to resume therapy within months (3 and 10, respectively). Steroid therapy alleviated the *de novo* AIH, but the patients did not resume interferon therapy.

DISCUSSION

Studies have repeatedly shown the benefits of achieving SVR via interferon therapy after liver transplantation. For instance, the durability of the SVR is associated with improvements in hepatic inflammation and histological regression of fibrosis over the long-term [18–23]. In contrast, efficacy of interferon therapy for non-SVR patients after liver transplantation had not previously been investigated. Here, we have demonstrated that long-term peginterferon maintenance therapy suppresses histological progression of recurrent hepatitis C after LDLT.

Maintenance interferon therapy was recently shown to have no influence on either histological or clinical outcomes in patients with nontransplant hepatitis C [24]. This conclusion was drawn after observing that the rate of fibrosis progression was similar between treatment and control groups following a 3.5-year randomized controlled trial of low-dose peginterferon. As a large number of patients with advanced fibrosis were enrolled in the randomized controlled trial, it is difficult to compare with our study in which the number of patients studied is much smaller and patients with advanced fibrosis were not enrolled. In the current study after liver transplantation, however, we demonstrated that low-dose maintenance interferon therapy reduced necroinflammatory activity and fibrosis scores in non-SVR patients to levels similar to those in SVR patients. Furthermore, we found that non-SVR patients who discontinued treatment had significantly worse scores once no longer receiving therapy.

Although these results clearly suggest that low-dose peginterferon maintenance therapy is beneficial for non-SVR patients with recurrent hepatitis C after liver transplantation, the mechanism behind this positive response is unknown. Progression of hepatitis C and development of fibrosis after discontinuation of interferon treatment has been shown to proceed more rapidly in patients who have undergone liver transplantation [20,21]. Our results, indicating that activity grade and fibrosis stage markedly deteriorated in non-SVR patients who discontinued maintenance treatment, support these previous findings. Thus, such a

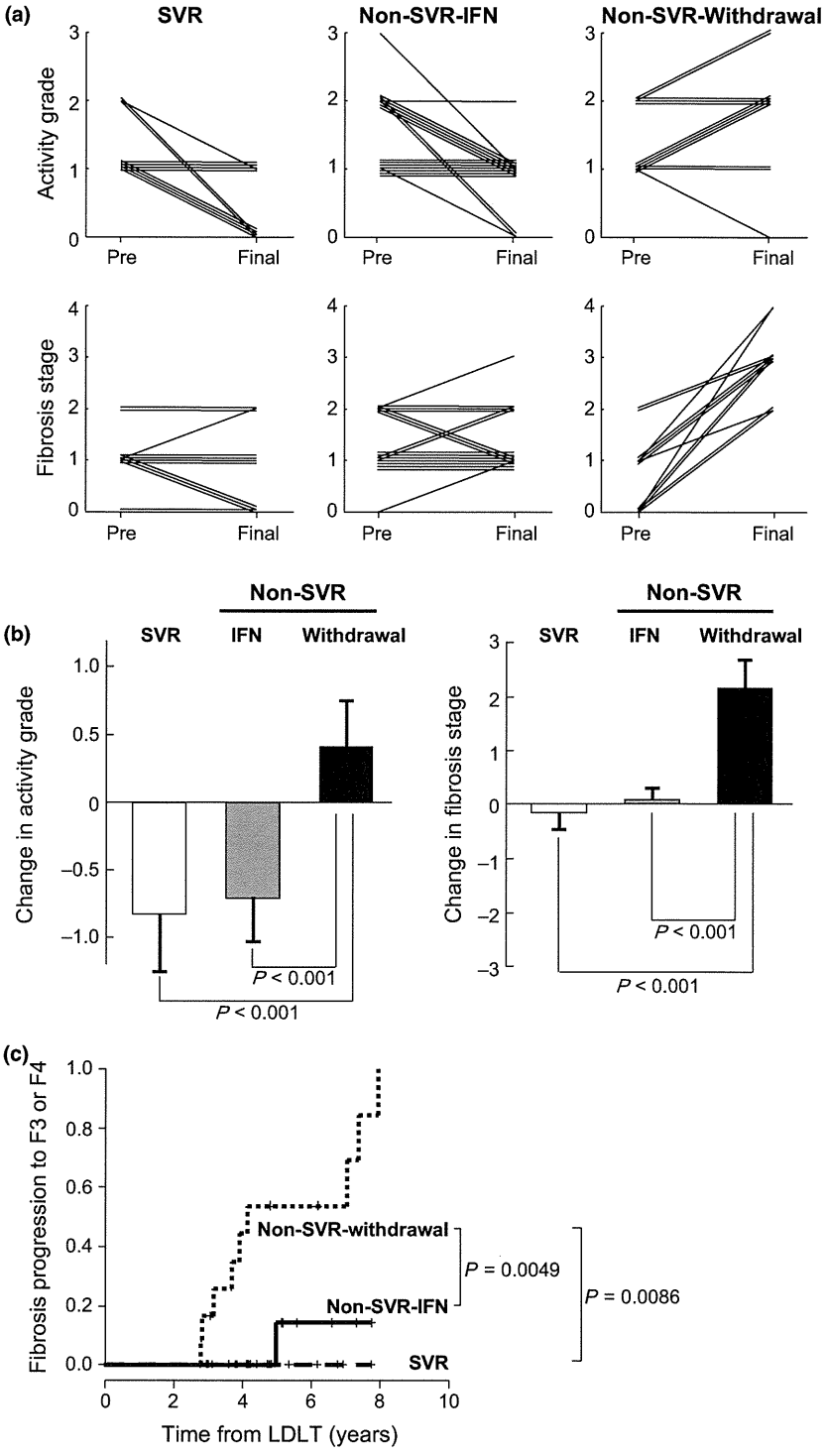

Fig. 2 Effect of maintenance interferon therapy on liver histology: (a) Changes in activity grade (upper) and fibrosis score (lower) of individual patients before interferon therapy (Pre) and at final biopsy (final). (b) Mean changes of liver activity grade (left) and fibrosis stage (right) between pretreatment liver biopsy and the final liver biopsy in each of the three treatment groups. The error bars represent 2 SEs. (c) Kaplan–Meier estimates of the progression rates among patients whose fibrosis advanced to F3 or F4. The dashed line indicates the sustained virological response (SVR) group, the solid line indicates the non-SVR-IFN group and the dotted line indicates the non-SVR-Withdrawal group.

rapid progression of recurrent hepatitis C in patients who discontinued interferon therapy may have highlighted the beneficial effect of the low-dose peginterferon maintenance therapy.

Another issue is the tolerability and safety of long-term peginterferon maintenance treatment. In this study, five patients (29%) discontinued the treatment during the peginterferon maintenance treatment, but only three did so for reasons directly related to the treatment. While two of these patients recovered simply by discontinuing the treatment, the third did require steroid pulse therapy to treat *de novo* AIH. Overall, however, the maintenance therapy did not result in the incidence of major adverse events, suggesting that it is both a tolerable and a safe treatment method.

Our work shows that long-term, low-dose peginterferon administration is an effective method for inhibiting the