Table 5. Prevalence of anti-HCV drug resistant mutations among the treatment-naïve patients.

Residue and Position	Drugs	Number of patients with mutated clones (%)	Frequency of the mutated clones (%)*
Resistant mutation to NS3/4A protease inhibitor			
T54S/A	Telaprevir Boceprevir	20/27 (74.1%)	0.49 (0.21–86.9)
V55A	Boceprevir	16/27 (59.3%)	0.4 (0.23–1.53)
Q80R/K	TMC435350	16/27 (59.3%)	0.36 (0.24–1.37)
V36A/M	Telaprevir Boceprevir	12/27 (44.4%)	0.47 (0.20–0.88)
V170A/T	Boceprevir	11/27 (40.7%)	0.52 (0.20–1.03)
A156T/V	Telaprevir	7/27 (25.9%)	0.35 (0.20–0.80)
R155K/T/Q	Telaprevir Boceprevir ITMN191/R7227 MK-7009 TMC435350 BI-201335	5/27 (18.5%)	0.42 (0.22–0.62)
A156S	Telaprevir Boceprevir	3/27 (11.15)	0.35 (0.24–0.83)
D168A/V/T/H	ITMN191/R7227 MK-7009 TMC435350 BI-201335	0/27 (0%)	
Resistant mutation to NS5B polymerase inhibitor			
V499A	BI-207127	20/27 (74.1%)	0.59 (0.20–99.1)
M423T/I/V	Filibuvir	12/27 (44.4%)	0.41 (0.21–1.48)
P495S/L/A/T	BI-207127	9/27 (33.3%)	0.37 (0.21–0.87)
P496A/S	BI-207127	1/27 (3.7%)	0.32
S282T	R7128	0/27 (0%)	

* Values are median (range).

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strated that IFN treatment resulted in no selective decrease of the viral clones comprising the previously defined mutational changes that were associated with a response to anti-viral therapy. Moreover, immediate virologic responders showed no common baseline nucleotide alterations that are efficiently eliminated in response to the administration of peg-IFNα2b plus RBV. Thus, our data suggest that an HCV sequence variation itself at a specific single nucleotide position does not directly reflect the virologic features regarding the sensitivity to IFN therapy in each viral clone, at least at the early stage of IFN administration. In contrast, several studies have provided evidence of the pre-existence of viral strains with an inherent resistance to IFN in patients who subsequently experienced a viral breakthrough or relapse [24,28]. Thus, there is room for further investigation to identify IFN-resistant clones by comparing the viral clones at baseline with those at the point of relapse using ultra-deep sequencing technology.

Notably, a distinct pattern of dynamic changes of HCV quasispecies was present between immediate responders and non-responders. Immediate responders showed a significant decrease of genetic complexity spanning all the viral genetic regions, resulting in a more homogeneous viral population after 1 week of peg-IFNα2b plus RBV administration. In contrast, non-responders showed no significant change in the genetic complexity in any of the HCV genomic regions. Our findings are consistent with the previous study showing that the early changes in HCV quasispecies determined by E1/E2 sequences provided prognostic information as early as the first 2 weeks after starting IFN therapy [28]. Moreover, the findings that there is no difference in the level of genetic complexity between early responders and non-responders at baseline and that almost none of the pre-existed HCV clones were eliminated in non-responder cases might suggest that the absence of sensitivity to IFN treatment in non-responders is due to host factors. Consistent with this hypothesis, recent studies revealed that host genetic variations at the IL28B gene are associated with a virologic response to peg-IFNα plus RBV combination therapy [29-32]. Alternatively, it is possible that a particular HCV protein of certain HCV mutants contributed to the strong inhibition of IFN-mediated anti-viral response in the liver of non-responders. Although dynamic changes in HVR1 sequences revealed that the minor viral clones were promptly eliminated in immediate virologic responders, the originally-inhabited major viral clones persisted 1 week after peg-IFNα2b plus RBV administration. Thus, further analyses are required to clarify how viral heterogeneity might be associated with the response to anti-viral therapy.

DAAs are promising drugs that could be more effective than peg-IFNα plus RBV therapy [33]. These DAAs include HCV NS3/4A protease and NS5B RNA-dependent RNA polymerase inhibitors, both of which have currently advanced to phase 1-3 trials. Increasing evidence, however, has clearly revealed that monotherapy with DAAs poses a high risk for the selection of resistant variants because of the high genetic heterogeneity of HCV [20]. Several studies reported the low prevalence of DAAs resistant mutants as the dominant clones in treatment-naïve cases [21,34-36]. For example, Kuntzen et al showed that drug-resistant mutations were detectable by conventional sequencing at individual frequencies between 0.3% and 2.8% in a treatment-naïve genotype 1 HCV-infected population [21]. In sharp contrast, ultra-deep sequencing identified that DAAs-resistant variants are common among treatment-naïve patients. Indeed, ultra-deep sequencing showed that 26 of 27 (96%) treatment-naive Japanese patients enrolled in this study possessed at least two clones resistant to DAAs, while 70.2% of the mutants presented as a very minor population (less than 1%) in each individual. It remains unclear whether these minor drug-resistant mutations have clinical significance, because the DAAs are not yet approved here in Japan. Recent *in vitro* findings, however, showed that minor but preexisting resistant mutants in HCV replicon cells were selected and expanded after DAAs therapy [37]. Lu et al revealed

that M414T mutants preexisting at a frequency of 0.22% and 0.18% in the treatment-naïve replicon population rapidly increased upon treatment with DAAs in a dose-dependent manner, reaching frequencies of 25% and 60% after 4 days of treatment. These findings suggest that those preexisting minor mutants might cause resistance against DAAs through the selection of dominant mutations. Thus, the significance of low-abundance variants in treatment-naïve patients requires further exploration.

The present study raises two limitations of ultra-deep parallel sequencing technology in the analyses of viral quasispecies. First, because the massive parallel ultra-deep sequencing platform is based on multitudinous short reads, it is difficult to separately evaluate the association between nucleotide sites mapped to different viral genome regions in a single viral clone. Indeed, it is difficult to clarify the potential mutational linkage between different viral genomic regions because of the short read length of the shotgun sequencing approach. Second, it is difficult to accurately analyze highly polymorphic regions such as the HVR by ultra-deep sequencing, because mutation findings strongly depend on mapping to the reference genome sequences. Thus, utilization of both conventional and ultra-deep sequencing technology might be necessary to fully clarify the significance and clinical relevance of the prominent HCV genomic heterogeneity.

In summary, using ultra-deep sequencing technology, we clearly demonstrated the extremely large genetic complexity in the genotype1b HCV derived from chronically infected patients. Although there was no significant difference in the level of viral complexity between immediate virologic responders and non-responders at baseline, immediate virologic responders, but not non-responders, showed a rapid reduction in the viral sequence variability at an early phase of peg-IFNα2b plus RBV administration. We also showed that drug-resistant mutants were widely present in treatment-naïve HCV-infected patients, indicating a putative risk for the expansion of resistant clones to DAAs. Further studies with a large number of patients are needed to fully elucidate the significance of viral heterogeneity in the clinical outcome of patients receiving anti-viral therapy.

Materials and Methods

Patients

The participants comprised 27 Japanese adult chronic hepatitis patients with genotype 1b HCV infection and the mean baseline level of serum HCV RNA determined by TaqMan RT-PCR (Applied Biosystems, Foster City, CA) was 6.9 log IU/ml. All patients received conventional peg-IFNα2b plus RBV combination therapy (Schering-Plough, Kenilworth, NJ) at Kyoto University and affiliated hospitals from February 2007 to December 2008. Indications for IFN-based combination therapy included high serum values of alanine aminotransferase and positivity for serum HCV RNA. Patients were treated with peg-IFNα2b (1.5 μg/kg) once per week, combined with daily oral RBV for 48 weeks [38]. The RBV dose was 600 mg/day in patients weighing less than 60 kg, 800 mg/day in those weighing at least 60 kg but less than 80 kg, and 1000 mg/day in those weighing 80 kg or more.

In this study, immediate virologic responders were defined as patients whose serum HCV RNA levels declined by more than 2 log IU/mL after 1 week of treatment with peg-IFNα2b plus RBV, while non-responders were defined as those whose serum HCV RNA levels declined less than 2 log IU/mL after peg-IFNα2b plus RBV administration. Of the original 27 patients, the serum before and 1 week after initiating treatment with peg-IFNα2b plus RBV of 16 cases was available for further analyses, and 8 of these cases were defined as immediate virologic responders and 8 cases were defined as non-responders. Among these non-responder cases, the serum HCV RNA levels in 6 of 8 (75.0%) patients changed by less than 1 log IU/mL after 1 week of treatment. The decline in HCV RNA levels in the remaining 2 cases was slightly over 1 log IU/mL (1.2 and 1.4 log IU/mL).

The ethics committee at Kyoto University approved the studies, and written informed consent for participation in this study was obtained from all patients.

Direct population Sanger sequencing

To define the representative reference sequences of full-length HCV in each clinical specimen, all samples were first subjected to direct population Sanger sequencing using Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) [39]. Serum samples were obtained before the start and at 1 week after initiation of peg-IFNα2b and RBV combination therapy. Total RNA was extracted from 140 μL of serum using a QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) and reverse-transcribed in a volume of 20 μL with the One step RNA PCR Kit AMV (Takara Bio, Ohtsu, Japan).

HCV genomes were amplified using Phusion High-Fidelity DNA polymerase (FINZYMES, Espoo, Finland). Oligonucleotide primers were designed to amplify the first-half (~5,000 bps) and the latter-half (~4,500 bps) of the genotype 1b HCV genome sequences (Table S3).

PCR products purified by the QIAquick Gel Extraction kit (Qiagen) were assayed for direct sequencing [40]. Nucleotide sequences of PCR products were determined using an ABI Prism Big Dye Terminator Ready Reaction Kit (Applied Biosystems). The serum of a healthy volunteer was used as a negative control.

Massive-parallel ultra-deep sequencing

Paired-end sequencing with multiplexed tags was carried out using the Illumina Genome Analyzer II. End-repair of DNA fragments, addition of adenine to the 3′ ends of DNA fragments, adaptor ligation, and PCR amplification by Illumina-paired end PCR primers were performed as described previously [41].

Briefly, the viral genome sequences were amplified with high-fidelity PCR and sheared by nebulization using 32 psi N2 for 8 min and the sheared fragments were purified and concentrated using QIAquick PCR purification Kit (Qiagen). The overhangs resulting from fragmentation were then converted into blunt ends using T4 DNA polymerase and Klenow enzymes, followed by the addition of terminal 3′ adenine-residues. Next, one of the adaptors containing six unique base pair (bp) tags, such as "ATCACG" and "CGATGT" (Multiplexing Sample Preparation Oligonucleotide Kit, Illumina), was ligated to each fragment using DNA ligase. Adaptor-ligated DNAs in the range of 200 to 350 bp were then size-selected by agarose gel electrophoresis. These libraries were amplified independently using a minimal PCR amplification step of 18 cycles with Phusion High-Fidelity DNA polymerase and then purified using a QIAquick PCR purification Kit for a downstream assay. Cluster generation and sequencing was performed for 64 cycles on the Illumina Genome Analyzer II following the manufacturer's instructions. Obtained images were analyzed and base-called using GA pipeline software version 1.4 with default settings provided by Illumina.

Genome Analyzer sequence data analysis

Using the high performance alignment software "NextGene" (SoftGenetics, State College, PA), the 64 base tags obtained from the Genome Analyzer II reads were aligned to the reference HCV RNA sequences of ~9200 bp that were determined by direct population Sanger sequencing in each clinical specimen. Entire reads were removed from the analysis when the median quality value score was below 20 and when containing more than 3 uncalled nucleotides. The low quality bases were trimmed from reads when more than 3 consecutive bases fell below a quality value score of 16. Based on the above criteria, reads with 90% or more bases matching a particular position of the reference sequence were aligned. Each position of the viral genome was assigned a coverage depth, representing the number of times the nucleotide position was sequenced.

Statistical analysis

Results are expressed as mean or median values and range (minimum and maximum). Pretreatment values were compared using the Mann–Whitney U-test. Categorical variables were analyzed by Fisher's exact test. P values of less than 0.05 were considered statistically significant. The viral quasispecies nature was evaluated by analyzing the genetic complexity based on the number of different sequences present in the population. Genetic complexity was determined by Shannon entropy values calculated as follows:

$$Sn = -\frac{\sum_{i=1}^{n} fi(\ln fi)}{N}$$

where n is the number of different species identified, fi is the observed frequency of the particular variant in the quasispecies, and N is the total number of clones analyzed [23,42]. Statistical comparisons of complexity between two groups were made using the Wilcoxon rank sum test or the Mann–Whitney U-test.

Supporting Information

Figure S1 Relationship between serum HCV RNA levels and the number of resistant mutant. No correlation was observed between serum HCV RNA levels (log IU/ml) and the number of resistant mutations against direct-acting antivirals in 27 cases in this study.
(TIF)

Table S1 Aligned reads, nucleotides, and mean coverage of each reference sequence in all patients.
(DOC)

Table S2 Mean genetic complexity in each viral genomic region of the 8 immediate virologic responders and 8 non-responders at pre-treatment and 1 week after IFN therapy.
(DOC)

Table S3 The oligonucleotide primers for PCR amplifying the whole HCV sequences.
(DOC)

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Author Contributions

Conceived and designed the experiments: AN HM. Performed the experiments: AN HM. Analyzed the data: AN HM NN TF FS KS TC YU. Contributed reagents/materials/analysis tools: AN HM YO YY TI TT. Wrote the paper: AN HM KT TC.

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Bile acid-induced expression of activation-induced cytidine deaminase during the development of Barrett's oesophageal adenocarcinoma

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Activation-induced cytidine deaminase (AID) induces somatic mutations in various host genes of non-lymphoid tissues, thereby contributing to carcinogenesis. We recently demonstrated that *Helicobacter pylori* infection and/or proinflammatory cytokine stimulation triggers aberrant AID expression in gastric epithelial cells, causing mutations in the tumour-suppressor *TP53* gene. The findings of the present study provide evidence of ectopic AID expression in Barrett's oesophagus and Barrett's oesophageal adenocarcinoma, a cancer that develops under chronic inflammatory conditions. Immunoreactivity for endogenous AID was observed in 24 of 28 (85.7%) specimens of the columnar cell-lined Barrett's oesophagus and in 20 of 22 (90.9%) of Barrett's adenocarcinoma, whereas weak or no AID protein expression was detectable in normal squamous epithelial cells of the oesophagus. We validated these results by analysing tissue specimens from another cohort comprising 16 cases with Barrett's oesophagus and four cases with Barrett's adenocarcinoma. *In vitro* treatment of human non-neoplastic oesophageal squamous-derived cells with sodium salt deoxycholic acid induced ectopic AID expression via the nuclear factor-kappaB activation pathway. These findings suggest that aberrant AID expression occurs in a substantial proportion of Barrett's epithelium, at least in part due to bile acid stimulation. Considering the genotoxic activity of AID, our current findings suggest that aberrant AID expression might enhance the susceptibility to genetic alterations in Barrett's columnar-lined epithelial cells, leading to cancer development.

Introduction

Chronic inflammation has a critical role in the development of many human cancers (1–4), such as hepatitis-associated hepatocellular carcinoma, colitis-associated colorectal cancers, cholangitis-related cholangiocarcinoma and *Helicobacter pylori* infection-related gastric cancers (3). Several studies have demonstrated various alterations in tumour-related genes in inflamed tissues before cancer onset (2,3,5). We previously demonstrated a substantial number of nucleotide alterations in the tumour-suppressor *TP53* gene in non-tumourous epithelial tissues of patients with chronic hepatitis (6) or *H. pylori*-related chronic gastritis (7). Although the molecular mechanisms underlying the transition from chronic inflammation to the accumulation of genetic mutations leading to tumourigenesis remain unknown, the discovery of nucleotide-editing enzymes that potentiate the genetic changes in DNA and/or RNA sequences (8,9) has provided a breakthrough in this research.

Among the human nucleotide-editing enzymes identified, only the activation-induced cytidine deaminase (AID) has been shown to induce mutations in human host DNA sequences (10). AID was originally identified as an inducer of somatic hypermutations, which diversifies the variable regions of immunoglobulin genes in activated B cells in germinal centres (11,12). Animal models with constitutive and ubiquitous expression of this nucleotide-editing enzyme, however, revealed that AID induces somatic mutations in various tumour-related genes, leading to the development of both lymphoid and non-lymphoid tumours (13,14). Moreover, we recently demonstrated that proinflammatory cytokine stimulation induces aberrant AID expression in various human epithelial cells, including colonic epithelium (15), hepatocytes (6,16), biliary cells (17) and gastric columnar cells (7). These findings indicate that inflammatory response-mediated aberrant AID expression may be a mechanism of the mutational accumulation required for malignant transformation in human epithelial cells (18,19).

Barrett's oesophagus is a metaplastic change from the normal stratified squamous epithelium of the lower oesophagus to a columnar-lined epithelium with intestinal-type differentiation (20). Barrett's oesophagus is clinically significant because it is associated with high risk of oesophageal adenocarcinoma (21). It is well recognized that chronic duodenogastro-oesophageal reflux and the resultant inflammatory response play a critical role in the development of Barrett's oesophagus and adenocarcinoma (21–23). Several previous studies demonstrated the involvement of *TP53* mutations at an early stage during malignant transformation of Barrett's oesophagus (24,25). In this regard, it is noteworthy that AID is aberrantly upregulated in response to inflammatory stimulation and AID activation induces the accumulation of multiple genetic alterations in *TP53* in the columnar cells of the stomach (7,26). Thus, because aberrant expression of AID in gastrointestinal columnar cells can be genotoxic during inflammation, we speculated that AID might also be involved in the pathogenesis of Barrett's oesophageal adenocarcinoma. Therefore, here, we investigated the expression of endogenous AID in human columnar epithelial cells lining Barrett's oesophagus and Barrett's oesophageal adenocarcinoma, a representative cancer that develops under chronic inflammatory conditions.

Abbreviations: AID, activation-induced cytidine deaminase; cDNA, complementary DNA; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; DCA, deoxycolic acid; NF-κB, nuclear factor-kappaB; RT–PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering RNA.

†These authors contributed equally to this work.

Materials and methods

Study population

The study group comprised patients who underwent endoscopic resection for Barrett's oesophageal adenocarcinoma or endoscopic biopsy for Barrett's oesophagus at Kyoto University Hospital or Saku Central Hospital between 2003 and 2005. Selection of patients enrolled in this study was based on the availability of a sufficient amount of tissue for analysis. The study group comprised 22 patients with Barrett's adenocarcinoma (Table I) and 6 patients with Barrett's oesophagus but no oesophageal adenocarcinoma [three men and three women, with a median age at the time of endoscopic treatment of 61 (range 38–81) years]. In addition to tumour tissues and the surrounding non-tumourous tissues of the columnar epithelium-lined Barrett's oesophagus, the normal squamous cells surrounding Barrett's epithelium were obtained from 16 cases with Barrett's adenocarcinoma. To validate the results of the initial analysis, we performed replication analyses using an independent another cohort from a different region of Japan (Shimane University). This second cohort included 16 patients with Barrett's oesophagus but no oesophageal adenocarcinoma [seven men and nine women, with a median age at the time of endoscopic treatment of 70 (range 45–80) years] and 4 patients with Barrett's oesophageal adenocarcinoma [three men and one woman, with a median age at the time of endoscopic treatment of 80 (range 65–85) years]. Barrett's oesophagus was defined by endoscopically recognizable columnar metaplasia

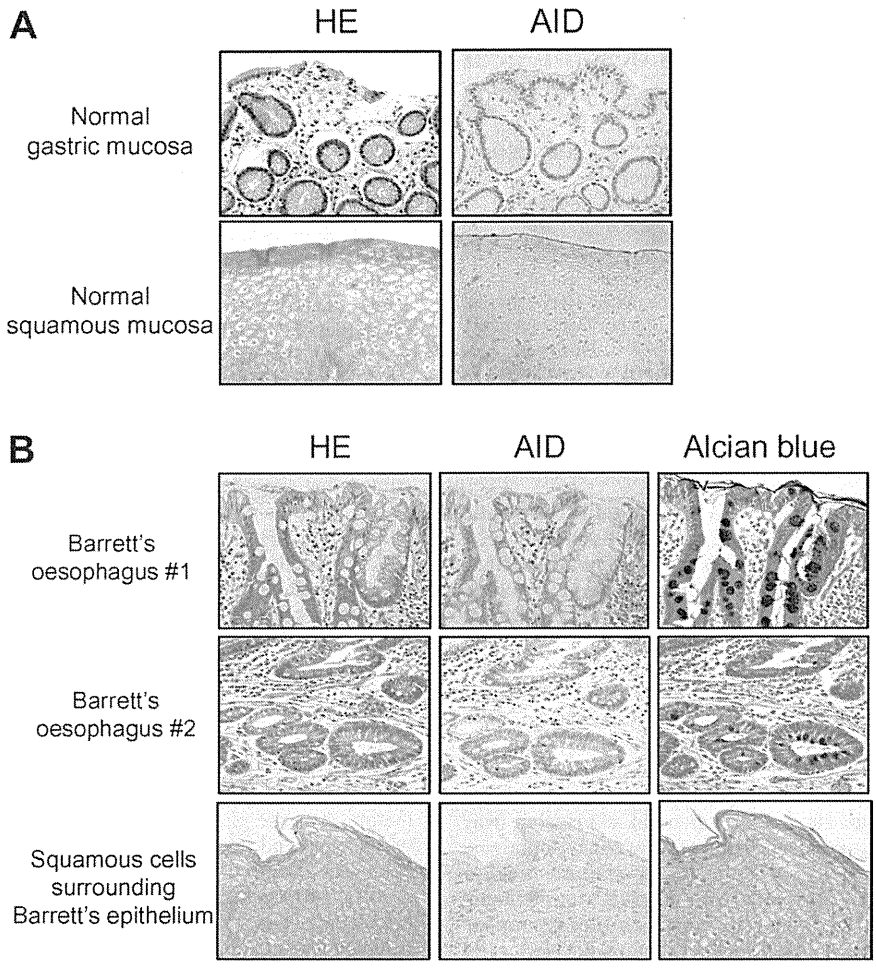

Fig. 1. Aberrant expression of AID protein in Barrett's oesophagus tissue specimens. Representative immunostaining for endogenous AID is shown. Immunohistochemistry was performed on normal columnar cell-lined gastric epithelium (A; upper panels), normal oesophageal squamous epithelium (A; lower panels), Barrett's oesophagus (cases #1 and #2, B; top and middle panels) and squamous cells surrounding Barrett's epithelium (B; bottom panels) (original magnification ×100).

Table I. Clinicopathological features of patients with Barrett's oesophageal adenocarcinoma

Age (years)	
Median age (range)	69 (30–81)
Gender	
Male	19
Female	3
Tumour size (cm)	
Median size (range)	2.0 (0.4–9.4)
Tumour differentiation	
Differentiated	17
Poorly	2
Unknown	3
TNM staging[a]	
0	2
I	13
II	3
III	0
IV	3
Unknown	1

[a]TNM staging is a cancer staging system that describes the extent of cancer in a patient's body.

of the oesophageal mucosa that was confirmed to have intestinal metaplasia by biopsy of the tubular oesophagus (27). Normal gastric mucosa and oesophageal squamous epithelium were obtained from biopsy samples of non-tumourous tissues from patients with benign submucosal tumours of the stomach or the oesophagus, none of which showed evidence of *H. pylori* infection and were used for AID immunostaining as a control. Written informed consent for the use of the resected tissues was obtained from all patients in accordance with the Declaration of Helsinki and the Kyoto University Graduate School and Faculty of Medicine Ethics Committee approved the study.

Immunohistochemistry

A polyclonal antibody against human AID was generated using purified recombinant AID protein as an immunogen (28). Immunohistochemical staining for endogenous AID protein was performed as described previously (7). For semi-quantitative analysis of the immunostaining results, the slides were scored for AID staining independently by two evaluators (S.M. and Y.M.). Visual assessment of the degree and intensity of the immunoreactivity were classified as no staining (−), no appreciable staining; weak positive staining (+), <50% of the gastrointestinal epithelial cells or tumour cells stained with AID and strong positive staining (++), >50% of the epithelial cells or tumour cells stained with AID.

Cell culture and transfection

Human non-neoplastic oesophageal squamous-derived Het-1A cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in Bronchial Epithelial Cell medium supplemented with the growth factors (BEGM Bulletkit, Lonza, Basel, Switzerland). To express the super-repressor form of the IκB-α protein, pcDNA3-IκB-α-del-N was made by inserting the complementary DNA (cDNA) fragment of human IκB-α into the BamHI–EcoRI sites of pcDNA3 (Invitrogen, Carlsbad, CA). The cDNA fragment for pcDNA3-IκB-α-del-N (amino acids 37-317) was synthesized by reverse transcription (RT)–polymerase chain reaction (PCR) with the oligonucleotide primers

Table II. Semiquantitation of AID immunoreactivity in normal columnar cells, normal squamous cells, Barrett's columnar cells and Barrett's adenocarcinoma

Cell types	Specimens analysed (*n*)	Specimens with AID immunoreactivity		
		(−)	(+)	(++)
Normal columnar cells	12	8	4	0
Normal squamous cells	10	4	6	0
Normal squamous cells surrounding Barrett's epithelium	16	7	9	0
Barrett's columnar cells	28	4	7	17
Barrett's adenocarcinoma	22	2	8	12

n = number of the patients; (++), strongly positive; (+), weakly positive; (−), negative.

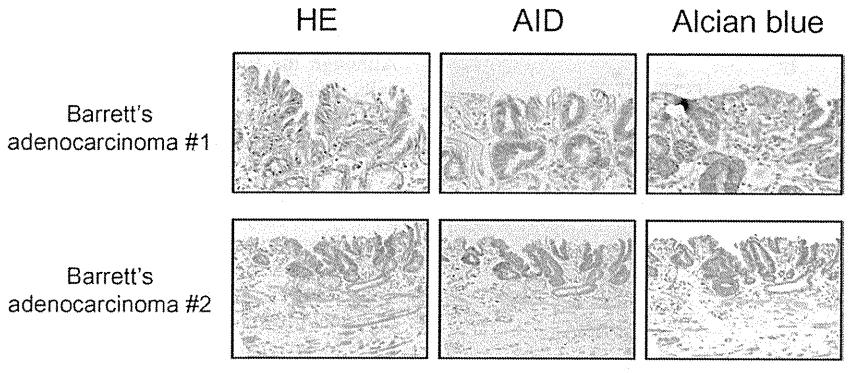

Fig. 2. AID protein production in tumour cells of Barrett's oesophageal adenocarcinoma. Representative moderate-to-strong AID immunostaining is shown in the tumour tissues of Barrett's oesophageal adenocarcinoma (original magnification ×100).

5′-CGCGGATCCATGAAAGACGAGGAGTACGA-3′ (forward) and 5′-CCGGAATTCTCATAACGTCAGACGCTGGC-3′ (reverse), as described previously (16). Lipofectamine 2000 reagent (Invitrogen) was used for transfection of the plasmids. The small interfering RNA (siRNA) duplex composed of 21 nucleotides against the endogenous non-catalytic subunit IκB kinase-γ [IKKγ; also known as NEMO, nuclear factor-kappaB (NF-κB) essential modulator] was obtained from Invitrogen (5′-UGCUCUUGAUGUGGUUGUCGUAUUC-3′, Stealth RNAi) and cells were treated with 0.07 nmol/ml siRNA for IKKγ/NEMO knockdown. A non-related control, Stealth RNAi Negative Control Duplex (Invitrogen), which lacks identity with known gene targets, was used as a control for non-sequence-specific effects. *Trans*IT-TKO reagent (Mirus, Madison, WI) was used for siRNA transfection. Sodium salt deoxycholic acid (DCA) and the NF-κB inhibitors MG132 and SN50 were purchased from Sigma (St Louis, MO), Calbiochem (Darmstadt, Germany) and Biomol International LP (Plymouth Meeting, PA), respectively.

Generation of stable cell lines

Stable cell lines derived from Het-1A cells were established by a lentiviral vector-mediated AID expression system (15,29). In brief, lentiviral stocks were produced in 293T cells in accordance with the manufacturer's protocol (Invitrogen). Virus-containing medium was collected 48 h post-transfection and filtered through a 0.45 μm filter. Het-1A cells were cultured in virus-containing medium in the presence of Neomycin (Sigma) until colonies of stably transfected clones grew. Genomic DNA was extracted from the cells derived from the three independent cultured colonies 8 weeks after viral transduction.

Subcloning and sequencing of tumour-related genes

The primer sets for amplification of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene were 5′-GAGGCCGATCCAGGTCAT-3′ (forward) and 5′-TTTACGGTAGTGGGGGAAGG-3′ (reverse). The primer sets for amplification of the *TP53* gene were 5′-CCCTTCCCAGAAAACCTACC-3′ (forward) and 5′-CCTCATTCAGCTCTCGGAAC-3′ (reverse). Amplification of the target sequences was performed using high-fidelity Phusion Taq polymerase (Finnzymes, Espoo, Finland), and the products were subcloned into a pGEM-T Easy vector (Promega, Madison, WI). The resulting plasmids were subjected to sequence analysis using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Quantitative real-time RT–PCR

Human AID gene expression was determined by quantitative real-time RT–PCR using a Light Cycler 480 and Fast Start Universal Probe Master (Roche, Mannheim, Germany) (7). The 6-carboxyfluorescein (FAM)-labelled probe specific for human *AID* was 5′-TCGGCGTGAGACCTACCTGTGC-TAC-3′. Standard curves for *AID* were generated for every target using a 10-fold serial dilution series of five independent transcripts derived from BL2-lymphoma cells that contain a high expression level of endogenous *AID*. To assess the quantity of isolated RNA as well as the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous messenger RNA levels of the housekeeping reference gene *18S ribosomal RNA* (*18S rRNA*) (15). For simplicity, ratios are represented as relative values compared with expression levels in a lysate from control cells. The PCR procedure was performed at least three times for each sample, and the results are expressed as box plots.

Statistical analysis

Statistical differences in AID expression levels were analysed using the Kruskal–Wallis *H*-test with Bonferroni correction. Statistical significance was evaluated using the χ^2 test for sequence analyses, and the χ^2 test with Bonferroni correction for immunostaining analyses. Values of $P < 0.05$ were considered statistically significant except when using the Bonferroni correction.

Results

Endogenous AID expression is upregulated in the columnar epithelial cells lining human Barrett's oesophagus and oesophageal adenocarcinoma cells

To clarify the expression and localization of AID protein in human oesophageal epithelium under physiological and pathological conditions, immunohistochemistry was performed using a specific antibody against human AID. Specificity of the antibody in immunostaining was confirmed by control staining performed on germinal centres of human mesenteric lymph nodes containing mostly activated B cells (6). Firstly, weak or no immunostaining for AID protein was observed

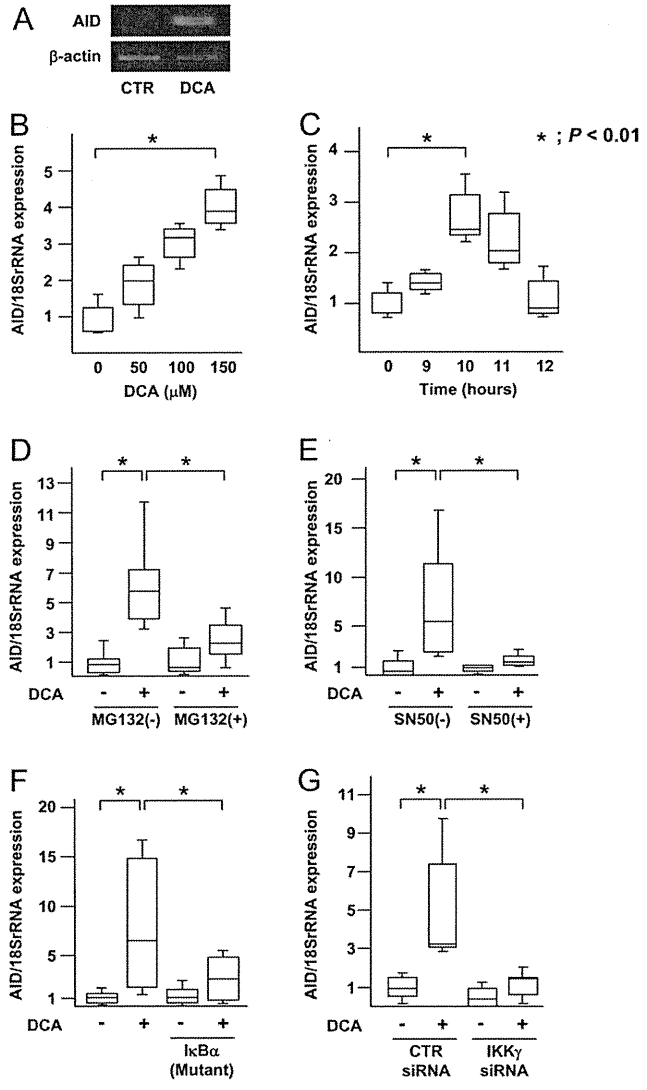

Fig. 3. DCA-mediated AID expression in human non-neoplastic oesophageal squamous epithelium-derived Het-1A cells. (A) RT–PCR for the expression of *AID* by Het-1A cells at 10 h after stimulation with 150 μM of DCA (upper panel). *β-actin* was quantified in each sample as an internal control (lower panel). (B) Het-1A cells were treated with various concentrations of DCA (0–150 μM) for 10 h. AID transcripts were measured by quantitative real-time RT–PCR. The expression levels were normalized to 18s ribosomal RNA (18s rRNA) as an endogenous control. The ratios are shown as relative value compared with the AID expression levels in non-stimulated Het-1A cells. (C) Time-dependent effects of DCA on AID gene expression. Het-1A cells were harvested and subjected to total RNA isolation immediately before (0) and 9, 10, 11 and 12 h after stimulation by DCA (100 μM). (D and E) Het-1A cells were treated with MG132 (0.15 μM) (D) or SN50 (250 ng/ml) (E) for 2 h and treated with DCA (150 μM) for 10 h. *AID* transcripts were analysed by quantitative real-time RT–PCR. (F) pcDNA3-IκB-α-del-N [IκBα (mutant)] was transfected into Het-1A cells and then treated with DCA (150 μM) for 10 h. Empty vector was used as a control. Total RNA was isolated from each cell and the expression levels of *AID* messenger RNA were measured by quantitative real-time RT–PCR. (G) siRNA-targeting IKKγ (IKKγ siRNA) or control siRNA (CTR siRNA) were transfected into Het-1A cells, followed by DCA stimulation for 10 h. Total RNA was isolated from each cell and the expression levels of *AID* messenger RNA were measured by quantitative real-time RT–PCR. $^*P < 0.01$.

in the columnar cells from the normal gastric mucosa that did not exhibit inflammatory changes, consistent with our previous findings (7) (Figure 1A and Table II). Similarly, weak or no AID expression was observed in normal squamous epithelial cells of individuals who lacked histological evidence of Barrett's oesophageal changes (Figure 1A and Table II). The normal squamous epithelial cells surrounding the columnar-lined Barrett's epithelial cells were weakly positive or negative for AID immunostaining (Figure 1B and Table II). No AID immunostaining was observed in any of the tissue specimens when non-immunized serum or phosphate-buffered saline was used instead of antibodies against AID (data not shown). In contrast, AID immunoreactivity was detected in 24 of 28 (85.7%) specimens and strong immunoreactivity for AID was detected in 17 of 28 (60.7%) specimens of the columnar epithelial cell-lined Barrett's oesophagus (Figure 1B and Table II). In the cancer tissues, AID immunoreactivity was detected in 20 of 22 (90.9%) patients, and strong expression of AID protein was present in 12 of 22 (54.5%) patients with Barrett's oesophageal adenocarcinoma (Figure 2 and Table II). The frequency of positivity for AID expression in Barrett's epithelial cells was significantly higher than that in normal squamous epithelial cells of patients without Barrett's oesophagus ($P = 0.021$, Table II). Although the frequency of positivity for AID expression in cancer cells tended to be higher than that in normal squamous epithelial cells of patients without Barrett's oesophagus, the difference was not significant [$P = 0.038$ (a level of $P < 0.025$ was considered significant), Table II]. We validated the AID immunostaining results by analysing the tissue specimens from another Japanese cohort comprising 10 subjects with normal squamous cells, 4 with Barrett's adenocarcinoma and 16 with Barrett's columnar cells but no oesophageal adenocarcinoma (Supplementary Table 1 is available at *Carcinogenesis* Online). Strong AID protein expression was observed in the columnar epithelial cells lining Barrett's oesophagus in 15 of 16 (94.0%) patients who did not develop oesophageal adenocarcinoma and in 4 of 4 (100%) patients with Barrett's adenocarcinoma (Supplementary Figure 1 is available at *Carcinogenesis* Online). The frequency of positivity for AID expression in Barrett's epithelial cells or in cancer cells was significantly higher than that in normal squamous epithelial cells ($P = 0.00025$, $P = 0.014$, respectively, Supplementary Table 1 is available at *Carcinogenesis* Online). These findings suggest that the inflammatory response related to the development of Barrett's oesophagus involves aberrant AID expression in the columnar epithelial cells lining Barrett's oesophagus. Regarding the effect of treatment with proton pump inhibitors on AID expression, we found no significant difference in the frequency of endogenous AID expression between patients with and without proton pump inhibitor medication (data not shown).

Aberrant AID expression is induced by DCA stimulation in both human oesophageal squamous and gastrointestinal columnar-lined cells

Bile reflux, which is usually associated with acid reflux, plays a critical role in the pathogenesis of Barrett's oesophagus and the development of Barrett's oesophageal cancer (21,30). Several studies have demonstrated that bile acid stimulation induces the activation of transcriptional factor NF-κB in gastrointestinal epithelial cells (31–34). We previously found that AID expression in gastric epithelial cells is regulated by the NF-κB pathway (7); therefore, we investigated whether bile acid stimulation could induce AID upregulation in oesophageal squamous cell epithelium *in vitro*. Accordingly, expression of AID messenger RNA transcripts was analysed by quantitative RT–PCR in human non-neoplastic oesophageal squamous epithelium-derived Het-1A cells in the absence or presence of DCA, a bile acid that has a putative role in the development of gastrointestinal cancer (35). Only small amounts of AID expression were detectable in the quiescent Het-1A cells without any stimulation. Endogenous AID expression, however, was induced in Het-1A cells in response to DCA treatment in a dose-dependent manner (Figure 3A and 3B). In addition, DCA induced a time-dependent transcriptional upregulation of AID messenger RNA in the cells, peaking at 10 h (Figure 3C). Next, we examined whether the DCA-mediated AID expression was achieved through the NF-κB signalling pathway. The cells were pretreated with the NF-κB inhibitory reagents MG132 and SN50,

Table III. Mutation frequency of *CDKN2A* and *TP53* genes in Het-1A cells with or without AID activation

	AID (+)			Control		
	Mean mutation frequency	(Mutated bases per total bases in the target gene sequenced)	Mutated clones/ total clones	Mean mutation frequency	(Mutated bases per total bases in the target gene sequenced)	Mutated clones/ total clones
CDKN2A	$2.70/10^4$	(6/22,231)	6/43	$0.41/10^4$	(1/24,299)	1/47
TP53	$1.52/10^4$	(5/32,956)	5/44	$0.74/10^4$	(3/40,446)	3/54

CDKN2A and *TP53* sequences were amplified from Het-1A cells with or without AID activation for 8 weeks, subcloned into the vector and then the cultured colonies carrying the amplified tumour-related gene fragments were randomly picked and subjected to sequence analyses. Data represent mean mutation frequency (left), number of mutated bases per number of total base pairs in *CDKN2A* or *TP53* genes sequenced (middle, in parentheses) and number of mutated clones per number of total clones examined (right).

followed by stimulation with DCA. Treatment with MG132 or SN50 significantly reduced the DCA-induced increase in AID transcripts ($P < 0.01$, Figure 3D and 3E). Moreover, the enhanced AID expression induced by DCA was significantly reduced by coproduction of the negative regulator of NF-κB, the dominant negative form of IκB-α kinase, in Het-1A cells ($P < 0.01$, Figure 3F). In addition, DCA significantly failed to elicit an increase in AID expression in cells in which the endogenous non-catalytic subunit IKKγ/NEMO was reduced by siRNA ($P < 0.01$, Figure 3G). Taken together, these findings indicate that the induction of AID expression in human oesophageal squamous cell epithelium by bile acid stimulation is achieved through the activation of NF-κB. We also tested whether DCA would be also involved in the aberrant AID expression in gastric columnar cell-lined epithelium. Quantitative RT–PCR analysis clearly showed that DCA stimulation in human gastric AGS cells substantially upregulated AID transcripts (Supplementary Figure 2 is available at *Carcinogenesis* Online). These findings suggest that aberrant AID expression in the oesophageal epithelium during the progression of Barrett's oesophagus is achieved by bile acid stimulation through the activation of NF-κB.

AID activation resulted in the accumulation of nucleotide alterations in tumour-related genes of the human oesophageal squamous epithelium-derived cells

We previously demonstrated that aberrant *AID* gene expression triggers the accumulation of genomic mutations in the *TP53* and *CDKN2A* genes of the cultured human gastric columnar epithelial cell lining (7,36). To clarify whether DCA-induced aberrant *AID* gene expression is genotoxic in oesophageal squamous epithelial cells, we investigated whether AID caused somatic mutations in the tumour-suppressor genes, *TP53* and *CDKN2A*, both of which have been reported to contain nucleotide alterations in human Barrett's oesophageal adenocarcinoma tissues (24,25,37,38). For this purpose, we established cultures of human non-neoplastic oesophageal squamous epithelium-derived Het-1A cells with constitutive AID expression using a lentiviral system (Supplementary Figure 3 is available at *Carcinogenesis* Online), followed by sequencing analyses performed on DNA samples extracted from the cells with or without AID activation for 8 weeks. Accordingly, over 40 cultured colonies carrying the amplified tumour-related gene fragments were randomly picked and subjected to sequence analyses. Less than one substitution per 1×10^4 nucleotides was detected in the tumour-related genes subcloned from control cells without AID activation (Table III). In contrast, more nucleotide alterations appeared in *CDKN2A* gene of the cells expressing *AID*, whereas four of the six mutations emerged in *CDKN2A* gene were identical silent mutations (Table III and Supplementary Table 2 is available at *Carcinogenesis* Online). The nucleotide alterations induced by *AID* gene activation were also observed in the *TP53* sequences, but the difference in the mutation frequency between the AID-expressing cells and control cells was not significant ($P = 0.32$, Table III and Supplementary Table 2 is available at *Carcinogenesis* Online). Although these findings suggest the possibility that aberrant *AID* gene expression might act as a DNA mutator for some tumour-suppressor genes in human oesophageal squamous epithelium cells, the evidence was insufficient to reach a clear conclusion.

Discussion

Barrett's oesophagus is the strongest known risk factor for the development of adenocarcinoma of the distal oesophagus (21). Various genetic alterations in relation to the dysregulation of cell growth and apoptosis occur during the development of Barrett's oesophageal cancers (24,25,39). For example, *TP53* is the most commonly mutated tumour-suppressor gene and is implicated in oesophageal adenocarcinoma developing in patients with Barrett's oesophagus (39). How somatic mutations accumulate through the process of carcinogenesis under the background of Barrett's oesophagus, however, remains unknown. In the present study, we demonstrated that a recently identified DNA-editing enzyme, AID, is upregulated in the columnar cell epithelium of Barrett's oesophagus, whereas normal gastric columnar cells and oesophageal squamous cells showed weak or no expression of AID protein. Moreover, strong AID expression was frequently observed in the tumour cells of Barrett's adenocarcinoma. Considering the genotoxic activity of AID, our present findings suggest the aberrant AID expression enhances the susceptibility to genetic alterations in Barrett's columnar-lined epithelial cells.

Increased exposure of the oesophagus to refluxed gastric and duodenal contents has a critical role in the development of Barrett's oesophagus and tumour development. The concentration of bile acids in the oesophageal refluxate correlates with the degree of oesophageal mucosal injury (22,40). More importantly, a secondary bile acid such as DCA is implicated in various cancers that develop in the gastrointestinal tract (41). In oesophageal adenocarcinoma, DCA is thought to contribute to carcinogenesis during reflux of the gastrointestinal contents (23,42,43). Several studies report that NF-κB is activated by bile acid components, resulting in the upregulation of a variety of genes involved in the development of metaplasia of Barrett's oesophagus and cancer development (31–33,39,44). We previously demonstrated that NF-κB enhances AID expression in hepatocytes and in colonic and gastric epithelial cells (7,15,16). In the present study, AID transcriptional upregulation was induced in response to bile acid stimulation in oesophageal epithelial cells via the NF-κB signalling pathway. Consistent with our findings, a recent paper demonstrated a non-linear dose–response to DCA for DNA damage and NF-κB activation in oesophageal cells (45). Thus, bile acid-mediated aberrant AID expression provides a novel link between gastroduodenal reflux and the increased susceptibility to carcinogenesis in Barrett's oesophagus.

Several clinical and molecular features differ markedly between squamous cell carcinoma and Barrett's adenocarcinoma of the oesophagus. In Barrett's oesophagus, the tissue undergoes a variety of genetic alterations and a prospective follow-up of lesions biopsied by endoscopy indicated that *TP53* gene mutations usually occur as an early event in the molecular pathogenesis of Barrett's oesophageal

adenocarcinoma (39). Previous studies also demonstrated the same *TP53* mutations in adenocarcinoma and adjacent non-cancerous Barrett's oesophageal epithelium, suggesting that the *TP53* mutation is an important step in the progression towards adenocarcinoma (25,46). Moreover, somatic mutations of the *CDKN2A* gene are frequently observed in human Barrett's oesophageal adenocarcinoma tissues (37,38). Our previous findings of constitutive AID expression in the columnar cell-lined gastric epithelium with nucleotide alterations in both the *TP53* and the *CDKN2A* genes (7,36) and submicroscopic deletions in the *CDKN2A* gene locus (36) support the hypothesis that AID expression contributes to the enhanced susceptibility of the columnar cell-lined epithelium in Barrett's oesophagus to tumourigenesis, potentially leading to adenocarcinoma. In the present study, we found that constitutive expression of AID in oesophageal squamous cells might also contribute to the accumulation of somatic mutations in both *CDKN2A* and *TP53* genes; however, the evidence was insufficient to conclude that AID was genotoxic in human oesophageal squamous cells.

In conclusion, the present findings indicate that aberrant AID expression occurs in a substantial proportion of human Barrett's epithelial and adenocarcinoma cells, suggesting that the genotoxicity of AID in columnar epithelial cells lining Barrett's oesophagus contributes to the accumulation of genetic alterations of tumour-related genes. Our findings also revealed that bile acid reflux plays a critical role in aberrant AID expression in the development of Barrett's oesophageal adenocarcinoma. A recent study suggested that Barrett's oesophagus is genetically heterogeneous where there are multiple independent clones (47), and a distinct clonal evolution from metaplasia to dysplasia was observed in the human stomach (48). Thus, further analyses are necessary to clarify whether aberrant AID expression in Barrett's oesophageal epithelium causes the genetic aberrations that contribute to expanding the clonal field of dysplasia, promoting the development of multiple independent subclones to enhance the risk of developing putative cancer cells.

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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.

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*).

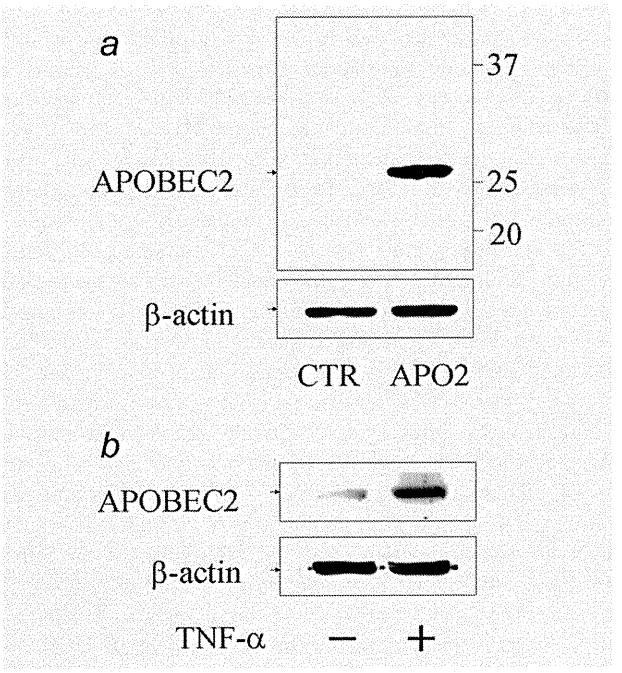

Figure 1. Detection of human APOBEC2 protein in hepatocytes by a specific anti-APOBEC2 antibody. (*a*) Huh7 cells were transfected with plasmid to induce the expression of human APOBEC2 (APO2) or control vector (CTR). After 48 hr, lysates of transfected cells were immunoblotted with anti-APOBEC2 antibody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

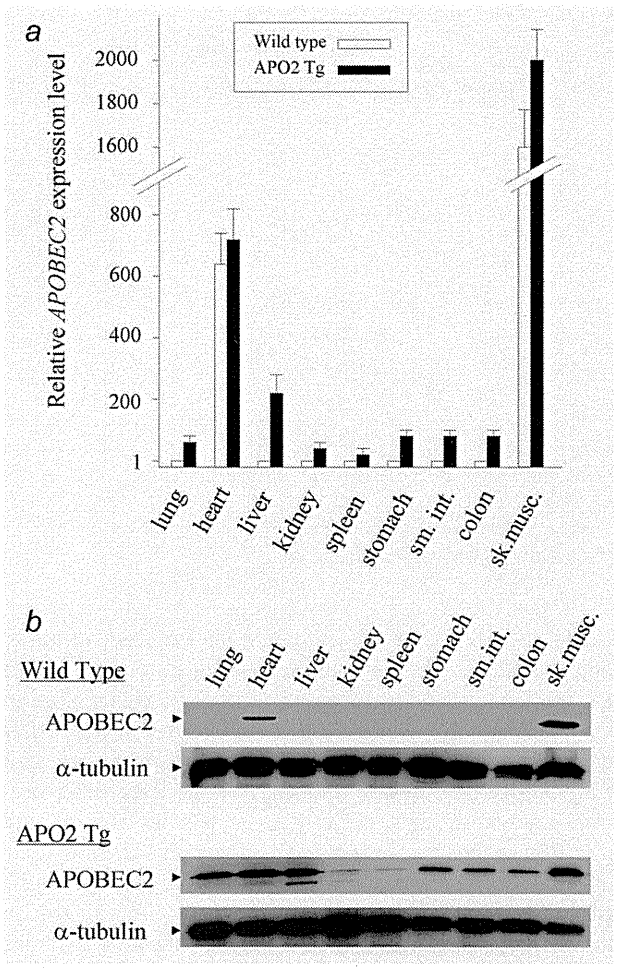

Figure 2. Expression analyses of APOBEC2 Tg mice. (*a*) Relative expression levels of *APOBEC2* transcripts calibrated by the amount of *18S rRNA* for indicated organs of adult APOBEC2 Tg mice (48-week-old) and their wild-type littermates. Data shown are mean results of quantitative real-time RT-PCR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

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

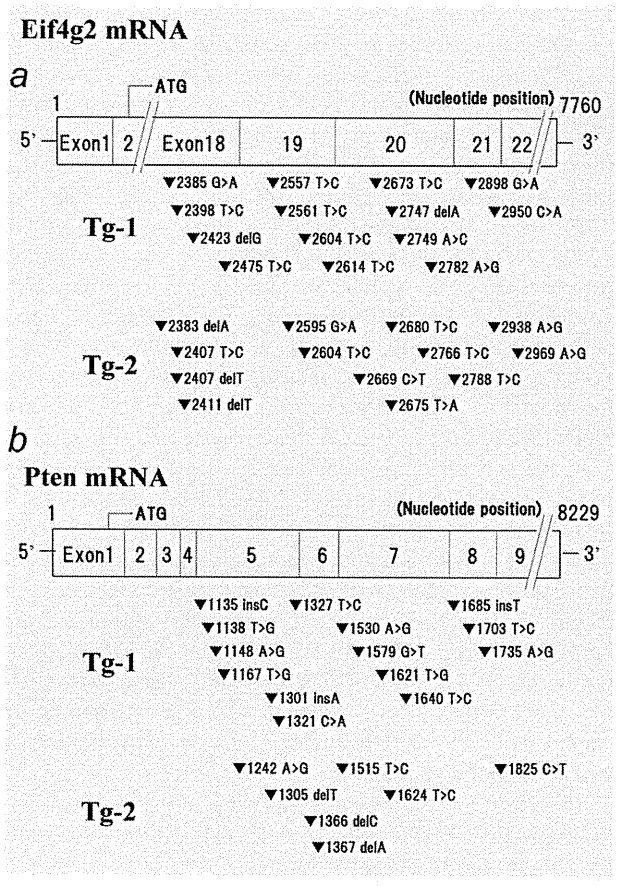

Figure 3. Distribution of nucleotide alterations in the Eif4g2 and Pten trascripts in the APOBEC2-expressing hepatocytes. The mRNA sequences between exon 18 and exon 21 of the Eif4g2 gene (*a*), and the mRNA sequences between exon 5 and exon 8 of the Pten gene (*b*) were determined in the nontumor liver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

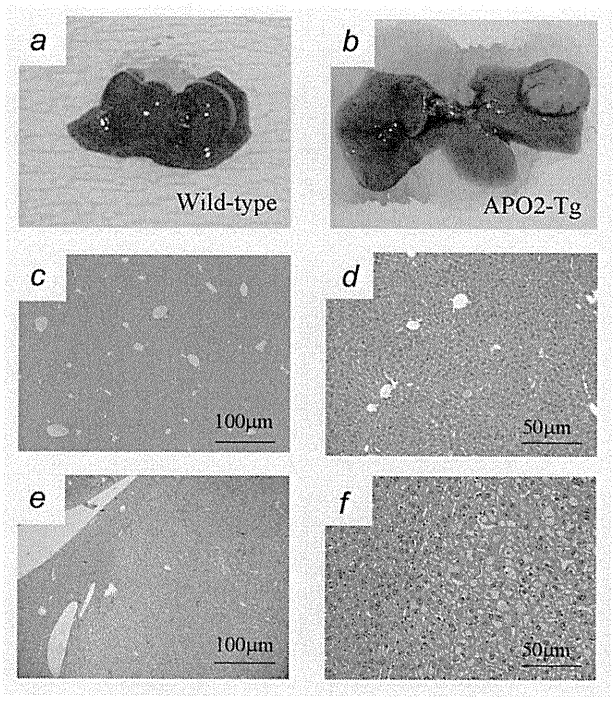

Figure 4. Tumors developed in the liver of APOBEC2 Tg mice. Macroscopic (*b*) and microscopic (haematoxylin and eosin) images (*e, f*) of the HCC that developed in a 72-week-old APOBEC2 Tg mouse and the non-cancerous liver of the same animal (*c,d*). Macroscopic image of the liver of a wild-type littermate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

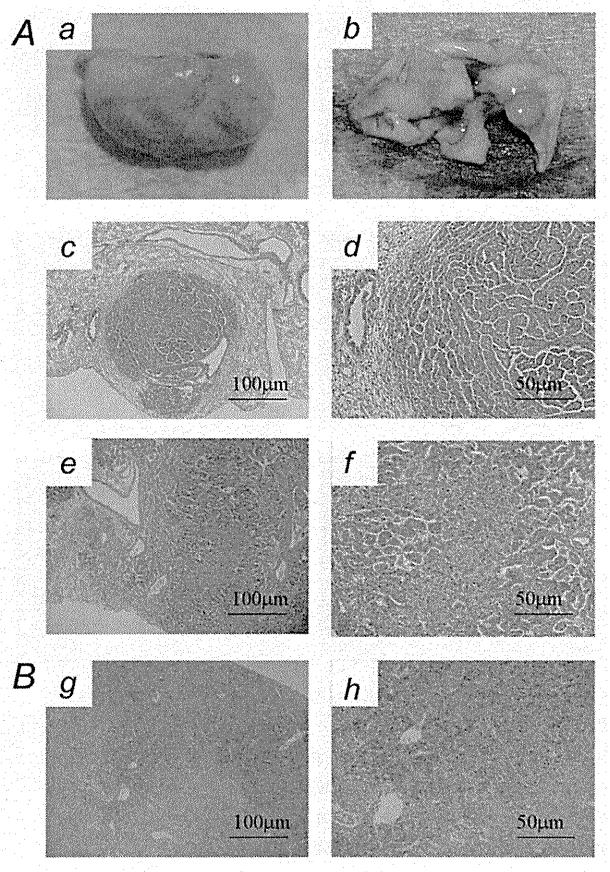

Figure 5. Lung tumors and lymphoma developed in APOBEC2 Tg mice. (*A*) Macroscopic view of a lung tumor that developed in a 72-week-old APOBEC2 Tg mouse (*b*). Microscopic view of a lung adenoma (*c,d*) and adenocarcinoma (*e,f*) that developed in a 72-week-old APOBEC2 Tg mouse. Macroscopic view of the 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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ORIGINAL ARTICLE

Targeting activation-induced cytidine deaminase prevents colon cancer development despite persistent colonic inflammation

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Inflammatory bowel disease (IBD) is an important etiologic factor in the development of colorectal cancer. However, the mechanism underlying carcinogenesis through chronic inflammation is still unknown. Activation-induced cytidine deaminase (AID) is induced by the inflammation and involved in various human carcinogenesis via its mutagenic activity. In the current study, we investigated whether the inflammation/AID axis plays an integral role in the development of colitis-associated cancers. Inflammation in the cecum was more severe than that in other colonic regions, and endogenous AID expression was enhanced most prominently in the inflamed cecal mucosa of interleukin (IL)-10$^{-/-}$ mice. Blockade of tumor necrosis factor (TNF)-α and IL-12 significantly suppressed AID expression. Although proinflammatory cytokine expression was comparable between IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice, sequencing analyses revealed a significantly lower incidence of somatic mutations in *Trp53* gene in the colonic mucosa of IL-10$^{-/-}$AID$^{-/-}$ than IL-10$^{-/-}$AID$^{+/+}$ mice. Colon cancers spontaneously developed in the cecum in 6 of 22 (27.2%) IL-10$^{-/-}$AID$^{+/+}$ mice. In contrast, none of the IL-10$^{-/-}$AID$^{-/-}$ mice developed cancers except only one case of neoplasia in the distal colon. These findings suggest that the proinflammatory cytokine-induced aberrant production of AID links colonic inflammation to an enhanced genetic susceptibility to oncogenic mutagenesis. Targeting AID could be a novel strategy to prevent colitis-associated colon carcinogenesis irrespective of ongoing colonic inflammation.

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Introduction

Chronic inflammation in epithelial tissues predisposes to the development of cancers (Mantovani *et al.*, 2008). For example, epidemiologic studies demonstrate that patients with chronic hepatitis caused by hepatitis virus infection and chronic gastritis caused by *Helicobacter pylori* infection leads to the development of hepatocellular carcinoma and gastric cancers, respectively (Chiba *et al.*, 2006; Ikeda *et al.*, 2007). Similarly, it is well recognized that the incidence of colorectal cancer is significantly higher in those with inflammatory bowel disease (IBD) than in the general population (Podolsky, 2002). Indeed, the cumulative risk of developing colorectal cancer for any patient with ulcerative colitis is estimated to be 1.6% at 10 years, 8.3% at 20 years and 18.4% at 30 years from disease onset (Eaden *et al.*, 2001). The mechanisms of colon carcinogenesis in chronically inflamed tissue remain unclear, but it is reasonable to assume that multistep gene alterations required for malignant transformation occur in the constitutively inflamed colonic mucosa. Consistent with this idea, genetic alterations such as the *tumor protein p53* (*TP53*) mutation appear to be an early event and are already present in colonic mucosa of patients with ulcerative colitis before cancer onset (Yin *et al.*, 1993; Kern *et al.*, 1994; Hussain *et al.*, 2000; Leedham *et al.*, 2009). Therefore, identifying the molecular pathway that links inflammation and genetic alterations in tumor-related genes is an important step to understand colitis-associated carcinogenesis.

Recently, we demonstrated that one of the human nucleotide-editing enzymes, activation-induced cytidine deaminase (AID), induces somatic mutations in several tumor-related genes, including *TP53*, in gastrointestinal epithelial cells (Endo *et al.*, 2007, 2008; Kou *et al.*, 2007; Matsumoto *et al.*, 2007; Komori *et al.*, 2008). AID was originally identified as an inducer of somatic mutations and class switch recombination of immunoglobulin genes, which diversifies the antibody production in B lymphocytes (Muramatsu *et al.*, 2000). Although AID expression is restricted to activated B cells under physiologic conditions, the inflammatory response can trigger aberrant AID expression in various epithelial organs. Stimulation of proinflammatory cytokines such