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

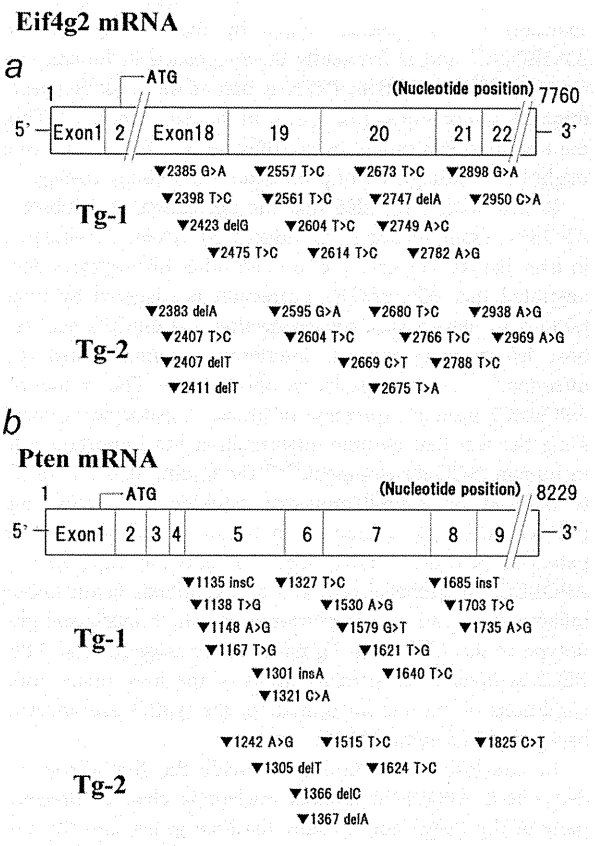

Figure 3. Distribution of nucleotide alterations in the Eif4g2 and Pten trascripts in the APOBEC2-expressing hepatocytes. The mRNA sequences between exon 18 and exon 21 of the Eif4g2 gene (*a*), and the mRNA sequences between exon 5 and exon 8 of the Pten gene (*b*) were determined in the nontumor liver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

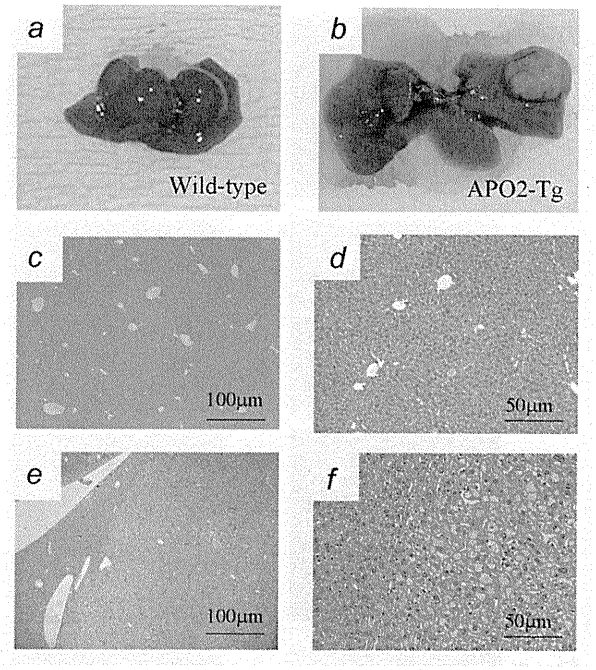

Figure 4. Tumors developed in the liver of APOBEC2 Tg mice. Macroscopic (*b*) and microscopic (haematoxylin and eosin) images (*e, f*) of the HCC that developed in a 72-week-old APOBEC2 Tg mouse and the non-cancerous liver of the same animal (*c,d*). Macroscopic image of the liver of a wild-type littermate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

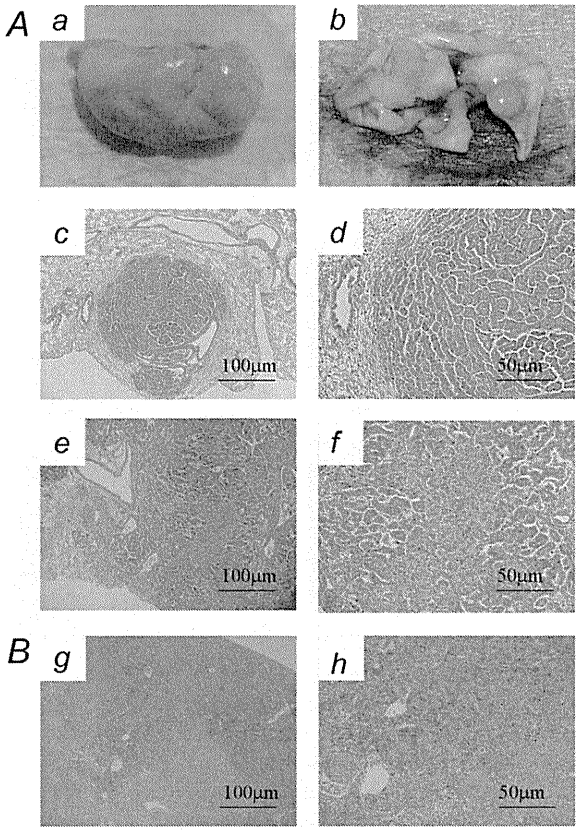

Figure 5. Lung tumors and lymphoma developed in APOBEC2 Tg mice. (*A*) Macroscopic view of a lung tumor that developed in a 72-week-old APOBEC2 Tg mouse (*b*). Microscopic view of a lung adenoma (*c,d*) and adenocarcinoma (*e,f*) that developed in a 72-week-old APOBEC2 Tg mouse. Macroscopic view of the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

References

1. Wedekind JE, Dance GS, Sowden MP, Smith HC. Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business. *Trends Genet* 2003;19:207–16.
2. Cascalho M. Advantages and disadvantages of cytidine deamination. *J Immunol* 2004; 172:6513–8.
3. Conticello SG, Thomas CJ, Petersen-Mahrt SK, Neuberger MS. Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Mol Biol Evol* 2005;22:367–77.
4. OhAinle M, Kerns JA, Malik HS, Emerman M. Adaptive evolution and antiviral activity of the conserved mammalian cytidine deaminase APOBEC3H. *J Virol* 2006;80:3853–62.
5. Pham P, Bransteitter R, Goodman MF. Reward versus risk: DNA cytidine deaminases triggering immunity and disease. *Biochemistry* 2005;44:2703–15.
6. Chen SH, Habib G, Yang CY, Gu ZW, Lee BR, Weng SA, Silberman SR, Cai SJ, Deslypere JP, Rosseneu M, *et al.* Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science* 1987;238:363–6.
7. Powell LM, Wallis SC, Pease RJ, Edwards YH, Knott TJ, Scott J. A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. *Cell* 1987; 50:831–40.

8. Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* 2003;424:99–103.
9. Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* 2003;424:94–8.
10. Harris RS, Bishop KN, Sheehy AM, Craig HM, Petersen-Mahrt SK, Watt IN, Neuberger MS, Malim MH. DNA deamination mediates innate immunity to retroviral infection. *Cell* 2003;113:803–9.
11. Shindo K, Takaori-Kondo A, Kobayashi M, Abudu A, Fukunaga K, Uchiyama T. The enzymatic activity of CEM15/Apobec-3G is essential for the regulation of the infectivity of HIV-1 virion but not a sole determinant of its antiviral activity. *J Biol Chem* 2003;278:44412–6.
12. Takaori-Kondo A. APOBEC family proteins: novel antiviral innate immunity. *Int J Hematol* 2006;83:213–6.
13. Noguchi C, Ishino H, Tsuge M, Fujimoto Y, Imamura M, Takahashi S, Chayama K. G to A hypermutation of hepatitis B virus. *Hepatology* 2005;41:626–33.
14. Suspene R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. *Proc Natl Acad Sci USA* 2005;102:8321–6.
15. Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, Takahashi S, Fujimoto Y, Ochi H, Abe H, Maekawa T, Yatsuji H, Shirakawa K, *et al.* Dual effect of APOBEC3G on Hepatitis B virus. *J Gen Virol* 2007;88:432–40.
16. Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, Honjo T. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* 1999;274:18470–6.
17. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000;102:553–63.
18. Liao W, Hong SH, Chan BH, Rudolph FB, Clark SC, Chan L. APOBEC-2, a cardiac- and skeletal muscle-specific member of the cytidine deaminase supergene family. *Biochem Biophys Res Commun* 1999;260:398–404.
19. Mikl MC, Watt IN, Lu M, Reik W, Davies SL, Neuberger MS, Rada C. Mice deficient in APOBEC2 and APOBEC3. *Mol Cell Biol* 2005;25:7270–7.
20. Yamanaka S, Balestra ME, Ferrell LD, Fan J, Arnold KS, Taylor S, Taylor JM, Innerarity TL. Apolipoprotein B mRNA-editing protein induces hepatocellular carcinoma and dysplasia in transgenic animals. *Proc Natl Acad Sci USA* 1995;92:8483–7.
21. Yamanaka S, Poksay KS, Arnold KS, Innerarity TL. A novel translational repressor mRNA is edited extensively in livers containing tumors caused by the transgene expression of the apoB mRNA-editing enzyme. *Genes Dev* 1997;11:321–33.
22. Morisawa T, Marusawa H, Ueda Y, Iwai A, Okazaki IM, Honjo T, Chiba T. Organ-specific profiles of genetic changes in cancers caused by activation-induced cytidine deaminase expression. *Int J Cancer* 2008;123:2735–40.
23. Okazaki IM, Hiai H, Kakazu N, Yamada S, Muramatsu M, Kinoshita K, Honjo T. Constitutive expression of AID leads to tumorigenesis. *J Exp Med* 2003;197:1173–81.
24. Matsumoto T, Marusawa H, Endo Y, Ueda Y, Matsumoto Y, Chiba T. Expression of APOBEC2 is transcriptionally regulated by NF-kappaB in human hepatocytes. *FEBS Lett* 2006;580:731–5.
25. Kou T, Marusawa H, Kinoshita K, Endo Y, Okazaki IM, Ueda Y, Kodama Y, Haga H, Ikai I, Chiba T. Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis. *Int J Cancer* 2007;120:469–76.
26. Iwai A, Marusawa H, Matsuzawa S, Fukushima T, Hijikata M, Reed JC, Shimotohno K, Chiba T. Siah-1L, a novel transcript variant belonging to the human Siah family of proteins, regulates beta-catenin activity in a p53-dependent manner. *Oncogene* 2004;23:7593–600.
27. Schroeder T, Just U. Notch signalling via RBP-J promotes myeloid differentiation. *Embo J* 2000;19:2558–68.
28. Endo Y, Marusawa H, Kinoshita K, Morisawa T, Sakurai T, Okazaki IM, Watashi K, Shimotohno K, Honjo T, Chiba T. Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappaB signaling. *Oncogene* 2007;26:5587–95.
29. Doi T, Kinoshita K, Ikegawa M, Muramatsu M, Honjo T. De novo protein synthesis is required for the activation-induced cytidine deaminase function in class-switch recombination. *Proc Natl Acad Sci USA* 2003;100:2634–8.
30. Zhao Y, Pan-Hammarstrom Q, Zhao Z, Hammarstrom L. Identification of the activation-induced cytidine deaminase gene from zebrafish: an evolutionary analysis. *Dev Comp Immunol* 2005;29:61–71.
31. Saunders HL, Magor BG. Cloning and expression of the AID gene in the channel catfish. *Dev Comp Immunol* 2004;28:657–63.
32. Prochnow C, Bransteitter R, Klein MG, Goodman MF, Chen XS. The APOBEC-2 crystal structure and functional implications for the deaminase AID. *Nature* 2007;445:447–51.
33. Buim ME, Soares FA, Sarkis AS, Nagai MA. The transcripts of SFRP1,CEP63 and EIF4G2 genes are frequently downregulated in transitional cell carcinomas of the bladder. *Oncology* 2005;69:445–54.
34. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. *Cell* 2008;133:403–14.
35. Tanaka Y, Marusawa H, Seno H, Matsumoto Y, Ueda Y, Kodama Y, Endo Y, Yamauchi J, Matsumoto T, Takaori-Kondo A, Ikai I, Chiba T. Anti-viral protein APOBEC3G is induced by interferon-alpha stimulation in human hepatocytes. *Biochem Biophys Res Commun* 2006;341:314–9.
36. Bonvin M, Achermann F, Greeve I, Stroka D, Keogh A, Inderbitzin D, Candinas D, Sommer P, Wain-Hobson S, Vartanian JP, Greeve J. Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. *Hepatology* 2006;43:1364–74.
37. Komohara Y, Yano H, Shichijo S, Shimotohno K, Itoh K, Yamada A. High expression of APOBEC3G in patients infected with hepatitis C virus. *J Mol Histol* 2006;37:327–32.
38. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002;31:339–46.
39. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003;362:1907–17.
40. Sato Y, Probst HC, Tatsumi R, Ikeuchi Y, Neuberger MS, Rada C. Deficiency in APOBEC2 leads to a shift in muscle fiber-type, diminished body mass and myopathy. *J Biol Chem* 2009;285:7111–18.

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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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Keywords: AID; colitis-associated cancer; colonic inflammation; IL-10$^{-/-}$ mouse

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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

as tumor necrosis factor (TNF)-α induces AID expression in hepatocytes, cholangiocytes and gastric epithelial cells (Endo *et al.*, 2007; Matsumoto *et al.*, 2007; Komori *et al.*, 2008). More importantly, aberrant AID expression in these epithelial cells results in the generation of nucleotide alterations in tumor-related genes and possible malignant transformation of the AID-expressing cells. Consistent with this hypothesis, animal models with constitutive and ubiquitous AID expression revealed that AID expression in epithelial tissues results in the accumulation of genetic mutations in various genes, leading to the development of liver, lung and gastric cancers (Morisawa *et al.*, 2008; Takai *et al.*, 2009). These findings strongly suggest that AID nucleotide-editing activity is intimately involved in the pathogenesis of inflammation-associated carcinogenesis (Chiba and Marusawa, 2009).

Chronic production of various proinflammatory cytokines is thought to be responsible for tumor development and progression in colitis-associated cancers (Lin and Karin, 2007). One example of the oncogenic effect of proinflammatory cytokines on colonic cells is provided by the colon carcinogenesis model, in which TNF-α blockade reverses azoxymethane and dextran sodium sulfate-induced colonic mucosal damage and attenuates subsequent colon cancer development (Popivanova *et al.*, 2008). What is noteworthy is that aberrant AID expression is induced in colonic epithelial cells in response to TNF-α via the IκB kinase-dependent nuclear factor (NF)-κB signaling pathways (Endo *et al.*, 2008). Moreover, we showed that enhanced expression of endogenous AID protein is detectable in the inflamed colonic mucosa of patients with ulcerative colitis or Crohn's disease (Endo *et al.*, 2008). These findings suggest that the inflammatory cytokine/AID axis may actually promote colon carcinogenesis by its genotoxic activity in the background of IBD. To clarify whether AID is a crucial mediator of the genetic alterations required for inflammation-mediated carcinogenesis, we investigated the impact of AID deficiency in the pathogenesis of colitis-associated colon cancer.

Results

AID gene expression in inflamed colonic mucosa of interleukin (IL)-10$^{-/-}$ mice

We first investigated endogenous AID expression in association with the degree of colonic inflammation in IL-10$^{-/-}$ mice, a representative model of human IBD. IL-10$^{-/-}$ mice were maintained under pathogen-free conditions and histological analysis was performed using paraffin-embedded tissue sections from the cecum, the proximal colon, the distal colon and the small intestine. IL-10$^{-/-}$ mice spontaneously developed intestinal inflammation after 8 weeks of age, whereas no inflammatory change was observed in the colons of the wild-type (WT) mice (Figure 1a). In 52-week-old IL-10$^{-/-}$ mice, the histological findings from the colonic tissue revealed epithelial hyperplasia, inflammatory cell infiltration and goblet cell loss (Figure 1a). Inflammatory lesions occurred at greater severity in the cecum compared with the proximal colon, the distal colon and the small intestine.

Colonic mucosal inflammation in IL-10$^{-/-}$ mice is mediated by proinflammatory cytokines as well as an excessive Th1 T-cell response associated with increased interferon (IFN)-γ and IL-12 secretion (Berg *et al.*, 1996; Davidson *et al.*, 1996). Consistent with previous studies, expression levels of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 were elevated in the inflamed cecal mucosa of the 52-week-old IL-10$^{-/-}$ mice (Figure 1b). Moreover, enhanced expression of Th1 cytokines, including IL-12 and IFN-γ, was observed in the 52-week-old IL-10$^{-/-}$ mice, but little expression of inflammatory cytokines was detected in 8-week-old IL-10$^{-/-}$ mice. In contrast, the expression levels of Th2 cytokines such as IL-4 and IL-13 did not differ between control and the IL-10$^{-/-}$ mice (Figure 1b).

We next examined whether aberrant AID expression appeared in association with colonic inflammation in IL-10$^{-/-}$ mice. Quantitative reverse transcription–PCR (RT–PCR) revealed a marked elevation of endogenous AID expression in the cecal epithelial cells of IL-10$^{-/-}$ mice >20 weeks of age, whereas only trace amounts of AID expression in younger IL-10$^{-/-}$ mice and in WT mice (Figure 1c, Supplementary Figure 1). In the 20- and 52-week-old IL-10$^{-/-}$ mice, AID was strongly expressed in the cecal mucosa compared with the epithelium of the proximal and distal colon (Figure 1d).

To determine whether the increased AID expression derives from epithelial cells or infiltrating B lymphocytes, we examined the expression pattern of endogenous AID in the inflamed cecal mucosa of IL-10$^{-/-}$ mice using *in situ* hybridization. The specificity of the *in situ* hybridization results was confirmed by control staining performed on an intestinal lymphoid follicle containing mostly activated B cells or the tissues derived from the transgenic mice with constitutive AID expression (Figure 1e, Supplementary Figure 2). No AID expression was detected in the normal cecal mucosa, liver and kidney of WT mice, or the murine B lymphoma cells with the small interfering RNA-mediated knockdown of endogenous AID transcripts (Figure 1e, Supplementary Figure 2). In contrast, high AID expression was observed mainly in the cytoplasm of both cecal epithelium and lymphocytes in inflamed intestinal tissues (Figure 1e, Supplementary Figure 3).

These findings suggest that persistent inflammation in the cecum of the older IL-10$^{-/-}$ mice is closely associated with the enhanced production of various inflammatory cytokines, leading to the induction of aberrant AID expression in inflamed colonic mucosa.

Inhibition of TNF-α and IL-12 suppressed AID expression with the decrease of colonic inflammation in IL-10$^{-/-}$ mice

To clarify the role of TNF-α and IL-12 in the pathophysiology of colonic inflammation in association with aberrant AID expression in the IL-10$^{-/-}$ mice, the biologic activity of TNF-α and IL-12 was inhibited using the TNF antagonist etanercept and neutralizing

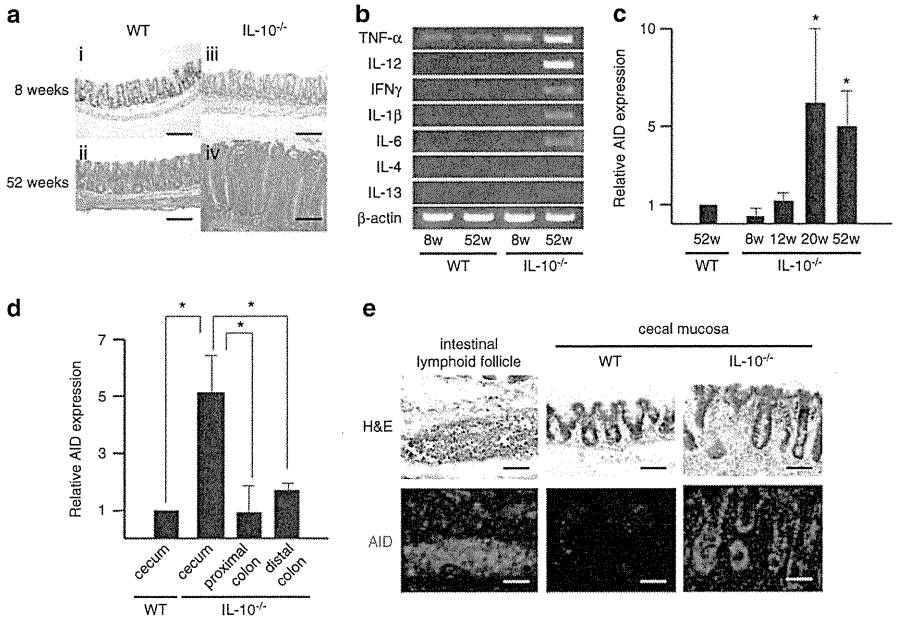

Figure 1 Endogenous AID expression in inflamed colonic mucosa of IL-10$^{-/-}$ mice. (**a**) Microscopic (hematoxylin and eosin (H&E) stain) images of the cecum of IL-10$^{-/-}$ mice and its WT littermate. The images of 8-week-old mice (i, iii) and 52-week-old mice (ii, iv) are shown. Scale bars are 200 μm. (**b**) Representative results of RT–PCR for the expression of various proinflammatory cytokines in the cecal mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from cecal mucosa of 8- and 52-week-old IL-10$^{-/-}$ mice and their WT littermates. RT–PCR was performed using oligonucleotides specific for murine TNF-α, IL-12, IFN-γ, IL-1β, IL-6, IL-4, IL-13, and β-actin. (**c**) Time course changes of AID expression in the cecal mucosa of IL-10$^{-/-}$ mice. Total RNA was isolated from mucosa at the cecum of 8-, 12-, 20- and 52-week-old IL-10$^{-/-}$ mice and WT littermates of 52-week-old IL-10$^{-/-}$ mice. Quantitative RT–PCR was performed using oligonucleotides specific for murine AID. *$P<0.05$ versus WT mice. (**d**) AID expression in various regions of colonic mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from mucosa at the cecum, and the proximal and distal colon of 52-week-old IL-10$^{-/-}$ mice and WT littermates. Quantitative RT–PCR was performed using oligonucleotides specific for murine AID. *$P<0.05$. (**e**) Representative images of AID expression in inflamed cecal mucosa determined by *in situ* hybridization assay. The images show the intestinal lymphoid follicle (left panels), the cecal mucosa of WT (middle panels) and IL-10$^{-/-}$ mice (right panels) stained with H&E (upper panels) or hybridized with the probe specific for the murine AID transcript (lower panels). Scale bars are 100 μm.

IL-12p40 monoclonal antibody (mAb), respectively (Liu *et al.*, 2006; Watanabe *et al.*, 2006; Popivanova *et al.*, 2008). First, we confirmed that the expression of both TNF-α and IL-12 was significantly upregulated in the cecum compared with the proximal and distal colon ($P<0.05$, Figure 2a). In agreement with established findings that TNF-α augments the expression of various cytokines and chemokines (Marra *et al.*, 1993; Popivanova *et al.*, 2008), etanercept treatment resulted in the suppression of a variety of proinflammatory cytokines and chemokines such as TNF-α, IL-12, IFN-γ, IL-1β, IL-6, and monocyte chemoattractant protein-1 (Figure 2b, Supplementary Figure 4). In contrast, there was no apparent difference in the expression levels of IL-5, a Th2 cytokine, between the etanercept and control groups (Supplementary Figure 4). Histopathological findings revealed that the cecal inflammation was substantially reduced in mice treated with etanercept compared with the control mice (Figure 2c, left and middle panels). Similar to etanercept, IL-12p40 mAb treatment effectively suppressed cecal inflammation in association with reduced levels of proinflammatory cytokines and chemokines other than IL-5 compared with the control mice (Figure 2b, Supplementary Figure 4).

NF-κB p65 is strongly activated in the inflamed colonic mucosa of IL-10$^{-/-}$ mice (Neurath *et al.*, 1996; Inoue *et al.*, 2009). To determine the effect of inhibition of the TNF-α- or IL-12-mediated signaling pathways on NF-κB activity, immunohistochemical staining of the cecal tissue from IL-10$^{-/-}$ mice was performed using the phospho-NF-κB p65 antibody. In the vehicle-treated IL-10$^{-/-}$ mice, phospho-NF-κB p65 was strongly positive in the nucleus of the epithelial cells, whereas NF-κB activation was markedly suppressed in the cecal mucosa of mice treated with the etanercept or IL-12p40 mAb (Figure 2c, right panels).

Endogenous AID expression is induced in response to TNF-α or IL-12 treatment and AID transcription is regulated via IκB kinase-dependent NF-κB signaling pathways in human colonic cells (Endo *et al.*, 2008). Thus, we investigated AID expression levels when TNF-α or IL-12 activity was inhibited in the colonic tissue of IL-10$^{-/-}$ mice. Quantitative RT–PCR analyses showed that endogenous AID expression was markedly decreased in the cecal mucosa in both the etanercept and IL-12p40 mAb groups when compared with the control group (Figure 2d). These findings suggest that TNF-α and IL-12 play a critical role in not only intestinal

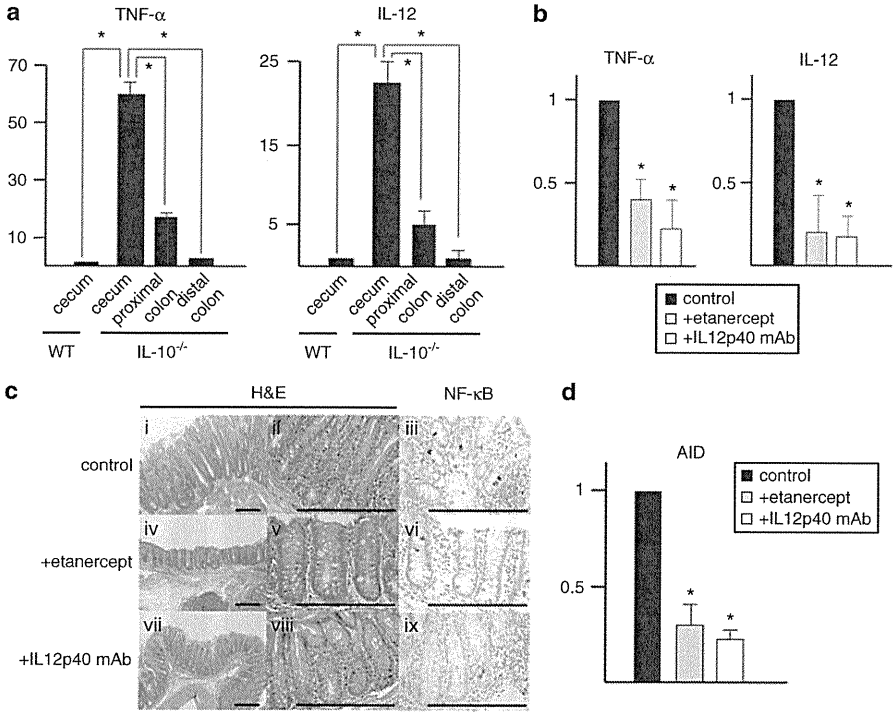

Figure 2 Effects of TNF-α and IL-12 blockade on various cytokine expression, NF-κB activation and AID expression in the colonic mucosa of IL-10$^{-/-}$ mice. (**a**) TNF-α and IL-12 expression in various regions in colonic mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from mucosa at the cecum, and the proximal and distal colon of 52-week-old IL-10$^{-/-}$ mice and the cecum of the WT littermates. Quantitative RT–PCR was performed using oligonucleotides specific for murine TNF-α and IL-12. *$P<0.05$. (**b**) Changes of various cytokine expression under the inhibition of the biologic activity of TNF-α and IL-12. Total RNA was isolated from mucosa at the cecum of IL-10$^{-/-}$ mice treated with phosphate-buffered saline (PBS), etanercept and IL-12p40 mAb. Quantitative RT–PCR was performed using oligonucleotides specific for murine TNF-α and IL-12. Relative values of these cytokines are shown. *$P<0.05$ versus control mice. (**c**) Changes of histopathological image and NF-κB activation induced by administration of etanercept and IL-12p40 mAb. Microscopic images (hematoxylin and eosin (H&E) stain) of the cecum of IL-10$^{-/-}$ mice treated with PBS (i, ii), etanercept (iv, v) and IL-12p40 mAb (vii, viii). Immunohistochemical staining for phospho-NF-κB p65 was performed using each sample including control group (iii), etanercept-treated group (vi) and IL-12p40 mAb-treated group (ix). Scale bars are 500 μm. (**d**) Results of quantitative RT–PCR for AID expression in the cecum of IL-10$^{-/-}$ mice treated with PBS, etanercept and IL-12p40 mAb. *$P<0.05$ versus control mice.

inflammation but also the aberrant AID expression in the cecal mucosa of the IL-10$^{-/-}$ mice.

AID deficiency did not affect the expression levels of proinflammatory cytokines in colonic mucosa

To gain insight into the role of AID expression in the inflamed colonic mucosa, we evaluated the effect of AID deficiency on the inflammatory response by crossing AID$^{-/-}$ mice with IL-10$^{-/-}$ mice (Figure 3a), and the cecal mucosa isolated from IL-10$^{-/-}$AID$^{-/-}$ mice were subjected to further analysis. Histological findings revealed that mucosal inflammatory changes in IL-10$^{-/-}$AID$^{-/-}$ mice, including pronounced inflammatory infiltration and marked epithelial hyperplasia, were comparable with those of the IL-10$^{-/-}$AID$^{+/+}$ littermates (Figure 3b). In agreement with a previous study (Fagarasan *et al*., 2002), hyperplasia of isolated lymphoid follicles developed in the cecum of 52-week-old AID$^{-/-}$ mice as well as in IL-10$^{-/-}$AID$^{-/-}$ mice (data not shown). RT–PCR analyses revealed only trace amounts of cytokine expression in WT or AID$^{-/-}$ mice (Figure 3c). In contrast, enhanced expression levels of various cytokines such as TNF-α, IL-12, IFN-γ, IL-1β and IL-6 observed in the IL-10$^{-/-}$AID$^{-/-}$ mice were comparable with those in the IL-10$^{-/-}$AID$^{+/+}$ mice (Figure 3c). Quantitative RT–PCR analyses also showed markedly enhanced expression levels of monocyte chemoattractant protein-1 in the cecum of IL-10$^{-/-}$AID$^{-/-}$ mice to almost the same levels as that in IL-10$^{-/-}$AID$^{+/+}$ mice (Figure 3d). Thus, the production levels of inflammatory cytokines and chemokines in the cecal mucosa did not differ between IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice.

Deficiency of endogenous AID resulted in the reduced frequencies of nucleotide alterations in the Trp53 *gene in IL-10$^{-/-}$ mice*

To clarify whether the AID upregulation induced by chronic colitis is genotoxic in colonic mucosa, we determined the nucleotide sequences of the *Trp53*, *Apc*, *Ctnnb1* and *Kras* genes, all of which are thought to be involved in human colorectal carcinogenesis (Fearon and Vogelstein, 1990). We first confirmed that the incidence of nucleotide alterations was <1.00

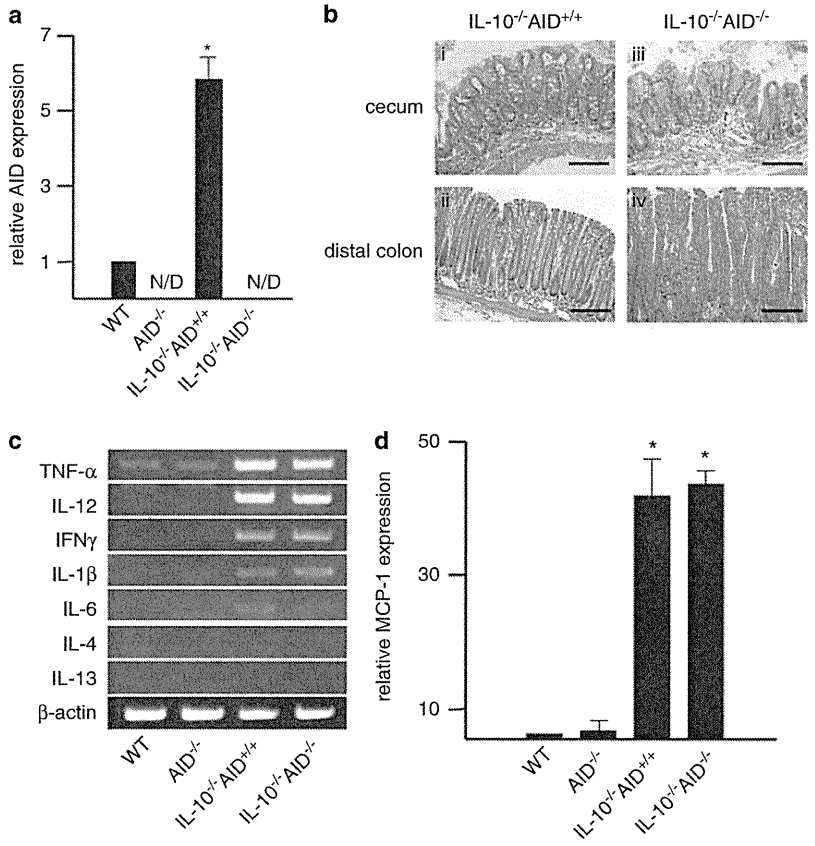

Figure 3 Effects of AID deficiency in the colonic mucosa of IL-10$^{-/-}$ mice. (**a**) Results of quantitative RT–PCR for AID expression. Total RNA was extracted from the mucosa at the cecum of 52-week-old WT, AID$^{-/-}$, IL-10$^{-/-}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. Quantitative RT–PCR was performed using oligonucleotides specific for murine AID. *$P<0.05$ versus WT mice. N/D, not detected. (**b**) Microscopic images (hematoxylin and eosin (H&E) stain) of the large intestine of 52-week-old IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$AID$^{-/-}$ mice. Upper panels (i, iii) are the images of the cecum and lower panels (ii, iv) are the images of the distal colon. Scale bars are 200 μm. (**c**) Representative results of semiquantitative RT–PCR for the expression of various proinflammatory cytokines in the cecum of 52-week-old WT, AID$^{-/-}$, IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. RT–PCR analyses were performed using specific primers for murine TNF-α, IL-12, IFN-γ, IL-1β, IL-6, IL-4, IL-13 and β-actin. (**d**) Results of quantitative RT–PCR for monocyte chemoattractant protein-1 (MCP-1) expression in the cecum of each genotype. *$P<0.05$ versus WT mice.

substitution per 10^4 nucleotides in these four tumor-related genes obtained from over 40 randomly picked clones from the normal cecal epithelial cells of WT mice, determined by conventional sequencing analyses with high-fidelity PCR amplification (data not shown). We then determined the sequences of the *Trp53, Apc, Ctnnb1* and *Kras* genes in 50 randomly selected clones amplified from the cecal epithelial cells of three IL-10$^{-/-}$AID$^{+/+}$ as well as three IL-10$^{-/-}$AID$^{-/-}$ mice (representative data are shown in Table 1). Nucleotide alterations had accumulated in the *Trp53* gene in the inflamed cecal mucosa of the IL-10$^{-/-}$AID$^{+/+}$ mice with a frequency of 2.19 substitutions per 10^4 nucleotides (Table 1). Although all the nucleotide changes determined in the *Trp53* gene were different in each clone, 9 of 12 (75%) alterations were accumulated in the regions corresponding to the DNA-binding motif of the human *TP53* gene (Figure 4). Among the nucleotide changes observed in the cecal epithelial cells of IL-10$^{-/-}$AID$^{+/+}$

Table 1 Gene mutation frequencies in inflamed cecal mucosa of the IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$ AID$^{-/-}$ mice

Gene	*Genotype*	*Nucleotide alterations*		P-*value**
		Number[a]	*Frequency (/10^4)*	
Trp53	AID (+/+)	12/54 787	2.19	<0.05
	AID (−/−)	5/70 380	0.71	
Apc	AID (+/+)	2/33 717	0.59	0.43
	AID (−/−)	1/42 762	0.23	
Ctnnb1	AID (+/+)	5/38 988	1.28	0.51
	AID (−/−)	2/36 037	0.55	
Kras	AID (+/+)	4/36 603	1.09	0.84
	AID (−/−)	3/31 955	0.94	

Abbreviations: AID, activation-induced cytidine deaminase; IL-10, interleukin-10.
Representative results of mutation frequencies in *Trp53, Apc, Ctnnb1* and *Kras* genes in inflamed cecal mucosa of IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice are shown.
[a]Number of mutated nucleotides/number of total nucleotides examined.
**P*-value is calculated using the χ^2 test.

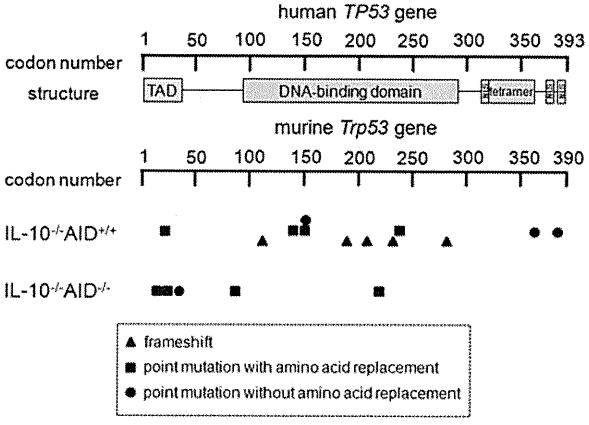

Figure 4 Distribution of murine *Trp53* mutations found in the cecal mucosa of IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. Murine p53 codon numbers are shown with equivalent human p53 domain structure with transactivation (TAD), DNA-binding and tetramerization (tetramer) domains and nuclear localization signal (NLS).

mice, 7 (58.3%) of 12 genetic changes were single-base substitutions and 4 of these 7 alterations in the *Trp53* coding sequences resulted in amino-acid replacements with potential functional consequences. In contrast, the mutation frequency of the *Trp53* gene in the cecal epithelial cells of IL-10$^{-/-}$AID$^{-/-}$ mice (0.71 substitutions per 10^4 nucleotides) was significantly lower than that of IL-10$^{-/-}$AID$^{+/+}$ mice ($P<0.05$, Table 1). On the other hand, the *Apc*, *Ctnnb1* and *Kras* genes did not have remarkable numbers of nucleotide alterations in the inflamed cecal mucosa of IL-10$^{-/-}$AID$^{+/+}$ mice, and thus the incidence of nucleotide changes in the *Apc*, *Ctnnb1* and *Kras* genes was not significantly different between IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice (Table 1). These findings suggest that the *Trp53* gene is a specific target gene in chronically inflamed cecal mucosa in IL-10$^{-/-}$ mice, and the accumulation of genetic changes in the *Trp53* gene of the inflamed colonic mucosa was due to AID activity.

The incidence of colon cancer was reduced in IL-10$^{-/-}$ mice in the absence of endogenous AID

The findings that AID deficiency in IL-10$^{-/-}$ mice had no significant impact on the levels of colonic inflammation but reduced the frequencies of somatic mutations in the tumor-suppressor *Trp53* gene led us to speculate that the knockout of endogenous AID might reduce the incidence of colonic cancer development irrespective of ongoing colonic inflammation. Thus, we compared the neoplastic phenotype of the IL-10$^{-/-}$AID$^{+/+}$ mice with that of IL-10$^{-/-}$AID$^{-/-}$ mice. The frequency and spectrum of colonic tumors that developed in IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$AID$^{-/-}$ mice are summarized in Table 2. Dysplastic changes in the mucosa of the large intestine were equally observed in most of these mice. These dysplastic lesions more frequently developed in the cecum than in the proximal and distal colon. Interestingly, invasive adenocarcinomas were detected

Table 2 Incidence of colonic tumors observed in IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice

	IL-10$^{-/-}$AID$^{+/+}$ (n=22)	*IL-10$^{-/-}$AID$^{-/-}$ (n=23)*
Mean age (weeks)	54.5	51.2
Male/female	13/9	13/10
Tumor formation		
Adenoma	20 (90.9%)	21 (91.3%)
Cecum[a]	20	20
Proximal colon[a]	1	1
Distal colon[a]	13	16
Adenocarcinoma	6* (27.2%)	1* (4.3%)
Cecum[a]	6	0
Proximal colon[a]	0	0
Distal colon[a]	0	1

Abbreviations: AID, activation-induced cytidine deaminase; IL-10, interleukin-10.
[a]Number of animals that developed adenoma or adenocarcinoma is shown.
*$P<0.05$ (P-value is calculated using Fisher's test).
Values in parentheses show percentages of animals that developed adenoma or adenocarcinoma.

in 6 of 22 IL-10$^{-/-}$AID$^{+/+}$ mice, and all the tumors characteristically developed from the dysplastic mucosa in the cecum (Table 2). Histopathological analysis of colonic tumors revealed moderate to poorly differentiated adenocarcinomas and invasive tumor cells beyond the submucosa with strong β-catenin expression (Figure 5a, Supplementary Figure 5). In contrast, IL-10$^{-/-}$AID$^{-/-}$ mouse developed no tumors in the inflamed colonic mucosa except only one tumor in the distal colon (Table 2). The colonic tumor that developed in the IL-10$^{-/-}$AID$^{-/-}$ mouse showed a trabecular pattern of growth within the submucosa, consistent with the morphologic appearance of well-differentiated adenocarcinoma (Figure 5b). These findings suggest that the upregulation of endogenous AID in the cecal mucosa driven by the inflammatory response contributes to the development of colonic cancers.

Discussion

The causal association between colonic inflammation and carcinogenesis is now well recognized (Eaden *et al.*, 2001; Podolsky, 2002). A recent genetic-linkage analysis of patients with IBD revealed that loss-of function mutations in genes encoding the IL-10 receptor proteins are associated with severe, early-onset enterocolitis, a finding that underscores the pivotal role of IL-10 in mediating the signals that control inflammation in the human gut (Glocker *et al.*, 2009). Consistent with the clinical finding, a mouse model with targeted disruption of the *IL-10* gene invariably develops enterocolitis that eventually progresses to colon cancer under conventional housing conditions; this mouse model is thus extremely useful as a disease model of human IBD (Kuhn *et al.*, 1993; Berg *et al.*, 1996; Sturlan *et al.*, 2001). In the present study, expression of AID was most prominent in the inflamed cecal mucosa of IL-10$^{-/-}$ mice. Moreover, we demonstrated that a deficiency of

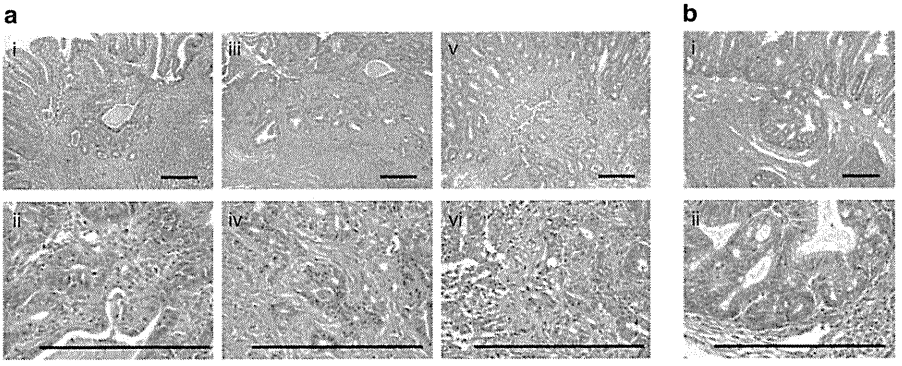

Figure 5 Colonic adenocarcinomas developed in IL-10$^{-/-}$ mice. (**a**) Microscopic images (hematoxylin and eosin (H&E) stain) of adenocarcinomas developed in the cecum of the IL-10$^{-/-}$AID$^{+/+}$ mice (i–vi). Scale bars are 500 μm. (**b**) Microscopic images (H&E stain) of adenocarcinoma developed in the distal colon of the IL-10$^{-/-}$AID$^{-/-}$ mouse (i, ii). Scale bars are 500 μm.

endogenous AID reduces the incidence of both the accumulation of somatic mutations in the *Trp53* gene and the development of colon cancer in inflamed colonic mucosa. *In vitro*, we previously demonstrated that aberrant AID expression is induced in response to proinflammatory cytokine stimulation, and colonic epithelial cells underlying chronic inflammation acquire the genetic mutations achieved by AID genotoxic activity (Endo *et al.*, 2008). Together, these findings suggest that inappropriate AID expression plays a pivotal role in the development of colorectal cancers via the accumulation of genetic alterations in the colonic mucosa of IBD.

We revealed here that endogenous AID is upregulated in inflamed colonic mucosa of elder IL-10$^{-/-}$ mice and the degree of AID expression paralleled extent of colonic inflammation. This observation is consistent with the findings that AID protein expression was detected in the colonic epithelium of inflammatory lesions from patients with IBD (Endo *et al.*, 2008). Colonic mucosal inflammation is usually mediated by either an excessive Th1 T-cell response associated with increased IFN-γ and IL-12 secretion or an excessive Th2 T-cell response associated with increased IL-4, IL-5 and IL-13 secretion (Fuss *et al.*, 1996). We previously found that the proinflammatory cytokine TNF-α, the Th2 cytokines IL-4 and IL-13 and Th1 cytokine IL-12 enhanced aberrant AID expression in cultured colonic epithelial cells (Endo *et al.*, 2008). On the other hand, TNF-α expression is elevated in colonic tissues of IL-10$^{-/-}$ mice (Berg *et al.*, 1996), and colitis in IL-10$^{-/-}$ mice is predominantly mediated by Th1-type T cells with increased production of IL-12 (Berg *et al.*, 1996; Davidson *et al.*, 1996). Consistent with these previous findings, in the present study, blockade of the activity of TNF-α or IL-12 suppressed AID expression in association with reduced production of various proinflammatory cytokines in the inflamed colonic mucosa of IL-10$^{-/-}$ mice. Thus, it is reasonable to assume that cytokine signalings, especially those mediated by TNF-α and IL-12, contribute to aberrant AID expression in the colonic cells of IL-10$^{-/-}$ mice.

A causal relationship between colonic inflammation and the accumulation of *TP53* tumor-suppressor gene mutations has been reported in human IBD (Yin *et al.*, 1993; Kern *et al.*, 1994; Hussain *et al.*, 2000; Leedham *et al.*, 2009). Alterations in the *TP53* gene, a late event in the pathogenesis of sporadic colorectal cancers, occur in dysplastic lesions with a background of ulcerative colitis (Yin *et al.*, 1993; Holzmann *et al.*, 1998) and are likely to proceed to dysplasia (Lashner *et al.*, 1999). Thus, the increased *TP53* mutation load in inflamed colonic epithelium of patients with IBD suggests that *TP53* mutations in noncancerous colon tissue of IBD patients specifically confer susceptibility to the development of colorectal cancers in an inflammatory microenvironment (Hussain *et al.*, 2000). In the present study, we found that high frequencies of nucleotide alterations had accumulated in *Trp53* gene mutation in inflamed mucosa of IL-10$^{-/-}$AID$^{+/+}$ mice. In addition, we demonstrated that a deficiency of endogenous AID in inflamed colonic mucosa resulted in a significantly reduced occurrence of somatic mutations in the *Trp53* genes, whereas no significant accumulation of somatic mutations appeared in the *Apc*, *Ctnnb1* and *Kras* genes in the inflamed colonic mucosa of IL-10$^{-/-}$AID$^{+/+}$ mice compared with IL-10$^{-/-}$AID$^{-/-}$ mice. It is unclear why the *Trp53* gene was more sensitive to AID-mediated genotoxic activity than the *Apc*, *Ctnnb1* and *Kras* genes in colonic epithelial cells of IL-10$^{-/-}$ mice. The present findings, however, are consistent with a previous observation that target gene selection for AID-induced somatic mutations varies among tissues and target cells (Morisawa *et al.*, 2008), and AID expression in cultured human colonic epithelial cells preferentially targets the *TP53* gene *in vitro* (Endo *et al.*, 2008). On the other hand, alterations in the *APC* and *KRAS* genes are also detected in dysplastic lesions and cancer tissues that develop in human IBD (Redston *et al.*, 1995). Therefore, we assume that the mutations in *APC*, *CTNNB1*, and *KRAS* genes were also present, but that their frequencies were below the detection limits of the present study. Further comprehensive sequencing analyses are required to determine how the AID-mediated genotoxic effects

achieve the target gene selection and whether IL-10$^{-/-}$ mice and human IBD share a similar process of mutational accumulation in tumor-related genes.

It is noteworthy that AID deficiency resulted in the reduced incidence of colitis-associated colon cancer development. AID deficiency caused the development of hyperplasia of isolated lymphoid follicles associated with an expansion of anaerobic flora in the small intestine (Fagarasan *et al.*, 2002; Suzuki *et al.*, 2004). We found no significant differences in the production levels of inflammatory cytokines in the colonic mucosa between the IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. This observation might be consistent with the previous findings that activated B cells were not the primary mediator of inflammatory response in the colon of IL-10$^{-/-}$ mice, as evidenced by their ability to transfer colitis to immunodeficient RAG2$^{-/-}$ mice (Davidson *et al.*, 1996). In contrast to the similar levels of colonic inflammatory activity, the incidence of colon cancers was significantly lower in IL-10$^{-/-}$AID$^{-/-}$ mice compared with the IL-10$^{-/-}$AID$^{+/+}$ mice harboring endogenous AID. It may be emphasized that expression levels of endogenous AID in the cecal mucosa was significantly higher than those of the remaining sites of the colon, and all the colon cancers that developed in IL-10$^{-/-}$AID$^{+/+}$ mice were located at the cecum, whereas none of the IL-10$^{-/-}$AID$^{-/-}$ mice developed cancers in their cecum. Only one IL-10$^{-/-}$AID$^{-/-}$ mouse developed a tumor in the distal colon. Histological examination indicated that this tumor had the morphologic appearance of well-differentiated adenocarcinoma located within the submucosa, whereas all the cancers developed in IL-10$^{-/-}$AID$^{+/+}$ mice invaded the muscularis propria or adventitia with the characteristics of moderate to poorly differentiated adenocarcinomas. Based on the above discussion, ectopic AID expression in the inflamed colonic mucosa is an indispensable factor for the development of colon cancers in IL-10$^{-/-}$ mice.

Recent studies revealed that AID is involved in regulating DNA methylation in certain systems (Rai *et al.*, 2008; Bhutani *et al.*, 2010; Guo *et al.*, 2011). Moreover, infiltrating leukocytes, including B cells, might modulate tumor cell properties via the production of certain chemokines or cytokines (Ammirante *et al.*, 2010). Therefore, further studies are necessary to examine the incidence of inflammation-associated cancers in mice in which AID is specifically deficient in the epithelial cells, and to clarify whether AID has a role in inflammation-associated tumorigenesis through the epigenetic modification of tumor-related genes.

In conclusion, we demonstrated that the proinflammatory cytokine TNF-α and the Th1 cytokine IL-12 are responsible for aberrant AID expression in the colonic mucosa of IL-10$^{-/-}$ mice with chronic inflammation. Aberrant AID expression in the inflamed colon is associated with the accumulation of somatic mutations in tumor-suppressor *Trp53* gene, and AID deficiency resulted in a reduced incidence of colitis-associated colon cancers. These findings may lead to a novel strategy for preventing carcinogenesis by targeting AID irrespective of the ongoing colonic inflammation in patients with IBD.

Materials and methods

Animal experiments

The generation of AID$^{-/-}$ mice was described previously (Fagarasan *et al.*, 2001). IL-10$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME, USA) and AID$^{-/-}$ mice were crossed on a C57BL/6 background to generate IL-10$^{-/-}$AID$^{-/-}$ mice. All mice were maintained in a specific pathogen-free facility at Kyoto University Faculty of Medicine. Cecal and colonic epithelium was purified as follows: cecum and colon were cut into 2.0 cm long and incubated with 2 mM EDTA in Hank's balanced salt solution without calcium and magnesium for 10 min at room temperature. Then, the tissues were tumbled for 6 min and the mucosa was selectively stripped from the submucosa. The stripped mucosa was washed with phosphate-buffered saline three times and the supernatant containing floating cells and debris were discarded. The obtained epithelial tissue samples and nonepithelial tissue samples were frozen in liquid nitrogen for nucleotide extraction. In some experiments, IL-10$^{-/-}$ mice were intraperitoneally injected with TNF antagonist etanercept and neutralizing antibody to murine IL-12p40 (Watanabe *et al.*, 2006). Accordingly, 40-week-old IL-10$^{-/-}$ mice were injected with etanercept at a dose of 3 mg/kg body weight over 5 days and killed on day 12. Other 40-week-old IL-10$^{-/-}$ mice were injected with IL-12p40 mAb at a dose of 0.5 mg/body weight on days 1 and 8, and killed at day 12. All animal experiments were approved by the ethics committee for animal experiments and performed under the Guidelines for Animal Experiments of Kyoto University.

Histopathological and immunohistochemical analyses

The entire colon was removed and washed with phosphate buffered saline. The cecum, the proximal colon and the distal colon were dissected transversely and fixed in 4% (w/v) formaldehyde. The fixed tissue was embedded in paraffin and sectioned at 3 μm in a random manner. In particular, two types of histological preparations were sectioned from the tissues of the cecum. These samples were stained with hematoxylin and eosin and analyzed histologically in a blind fashion by three readers. Immunohistochemical staining was performed according to a previously described protocol (Toda *et al.*, 1999). The polyclonal antibodies for phospho-NF-κB p65 (Ser276) and β-catenin were purchased from Cell Signaling Technology (Danvers, MA, USA) and BD Biosciences (Franklin Lakes, NJ, USA), respectively.

In situ *hybridization*

A digoxigenin-labeled RNA probe specific for murine AID was transcribed with digoxigenin-11-UTP according to the manufacturer's instructions (Roche, Basel, Switzerland) from a 1.7-kb complementary DNA (cDNA) amplified using the following primers: 5′-ATGGACAGCCTTCTGGTGATGAA-3′ and 5′-CTTGTTCCCAAGGTCGCAAGGAAAGG-3′. Similarly, an RNA probe for murine villin1 was transcribed from a 1.6-kb cDNA amplified using the following primers: 5′-TGAATGCC CAACTCAAAGGCTCTCTC-3′ and 5′-ACCTCAAAAGCC TTGGTGTTATCAGC-3′. *In situ* hybridization was performed as described previously (Nakatani *et al.*, 2004). The alkaline phosphatase chromogen reaction was performed using Fast Red (Roche) as the substrate at room temperature for

48 h. The sections were then washed with distilled water and mounted on glass slides in mounting medium.

Semiquantitative and quantitative RT–PCR
Total RNA was extracted from the tissues using QuickGene RNA Tissue Kit (Fuji, Tokyo, Japan). cDNA was synthesized using Transcriptor First-Strand cDNA Synthesis Kit (Roche). PCR amplification was performed using Takara Ex Taq DNA polymerase (Takara, Tokyo, Japan). The oligonucleotide primers for the semiquantitative RT–PCR are shown in Supplementary Table 1. Gene expression was quantified by quantitative real-time RT–PCR using LightCycler 480 System II (Roche). The oligonucleotide primers for the quantitative RT–PCR are shown in Supplementary Table 2. To assess the quantity of isolated RNA as well as the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene *18S rRNA* (Matsumoto *et al.*, 2007). For simplicity, ratios are represented as relative values compared with expression levels in lysate from control specimens.

Subcloning and sequencing analyses of tumor-related genes
The oligonucleotide primers for the amplification of the murine *Trp53*, *Apc*, *Ctnnb1* and *Kras* genes are shown in Supplementary Table 3. Amplification of targeted sequences was performed using high-fidelity Phusion Taq Polymerase (Finnzymes, Espoo, Finland), and the products were subcloned using pGEM-T Easy Vector Systems (Promega, Madison, WI, USA). The resulting plasmids were subjected to sequence analysis using Applied Biosystems 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

Statistical analysis
Statistical analysis was performed using the Mann–Whitney *U* test, χ^2 test and Fisher's test. Differences were considered to be statistically significant if *P*-values were <0.05.

Abbreviations

AID, activation-induced cytidine deaminase; cDNA, complementary DNA; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; NF, nuclear factor; RT–PCR, reverse transcription–PCR; TNF, tumor necrosis factor; *TP53*, *tumor protein p53*; WT, wild type.

Conflict of interest

The authors declare no conflict of interest.

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References

Ammirante M, Luo JL, Grivennikov S, Nedospasov S, Karin M. (2010). B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature* **464**: 302–305.

Berg DJ, Davidson N, Kuhn R, Muller W, Menon S, Holland G *et al.* (1996). Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* **98**: 1010–1020.

Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM. (2010). Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature* **463**: 1042–1047.

Chiba T, Marusawa H. (2009). A novel mechanism for inflammation-associated carcinogenesis; an important role of activation-induced cytidine deaminase (AID) in mutation induction. *J Mol Med* **87**: 1023–1027.

Chiba T, Seno H, Marusawa H, Wakatsuki Y, Okazaki K. (2006). Host factors are important in determining clinical outcomes of Helicobacter pylori infection. *J Gastroenterol* **41**: 1–9.

Davidson NJ, Leach MW, Fort MM, Thompson-Snipes L, Kuhn R, Muller W *et al.* (1996). T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *J Exp Med* **184**: 241–251.

Eaden JA, Abrams KR, Mayberry JF. (2001). The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* **48**: 526–535.

Endo Y, Marusawa H, Kinoshita K, Morisawa T, Sakurai T, Okazaki IM *et al.* (2007). Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappaB signaling. *Oncogene* **26**: 5587–5595.

Endo Y, Marusawa H, Kou T, Nakase H, Fujii S, Fujimori T *et al.* (2008). Activation-induced cytidine deaminase links between inflammation and the development of colitis-associated colorectal cancers. *Gastroenterology* **135**: 889–898, 898 e1–3.

Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. (2001). In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* **413**: 639–643.

Fagarasan S, Muramatsu M, Suzuki K, Nagaoka H, Hiai H, Honjo T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. *Science* **298**: 1424–1427.

Fearon ER, Vogelstein B. (1990). A genetic model for colorectal tumorigenesis. *Cell* **61**: 759–767.

Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA *et al.* (1996). Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* **157**: 1261–1270.

Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schaffer AA, Noyan F *et al.* (2009). Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* **361**: 2033–2045.

Guo JU, Su Y, Zhong C, Ming GL, Song H. (2011). Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell* **145**: 423–434.

Holzmann K, Klump B, Borchard F, Hsieh CJ, Kuhn A, Gaco V *et al.* (1998). Comparative analysis of histology, DNA content, p53 and Ki-ras mutations in colectomy specimens with long-standing ulcerative colitis. *Int J Cancer* **76**: 1–6.

Hussain SP, Amstad P, Raja K, Ambs S, Nagashima M, Bennett WP *et al.* (2000). Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. *Cancer Res* **60**: 3333–3337.

Ikeda K, Marusawa H, Osaki Y, Nakamura T, Kitajima N, Yamashita Y *et al.* (2007). Antibody to hepatitis B core antigen

and risk for hepatitis C-related hepatocellular carcinoma: a prospective study. *Ann Intern Med* **146**: 649–656.
Inoue S, Nakase H, Matsuura M, Mikami S, Ueno S, Uza N *et al.* (2009). The effect of proteasome inhibitor MG132 on experimental inflammatory bowel disease. *Clin Exp Immunol* **156**: 172–182.
Kern SE, Redston M, Seymour AB, Caldas C, Powell SM, Kornacki S *et al.* (1994). Molecular genetic profiles of colitis-associated neoplasms. *Gastroenterology* **107**: 420–428.
Komori J, Marusawa H, Machimoto T, Endo Y, Kinoshita K, Kou T *et al.* (2008). Activation-induced cytidine deaminase links bile duct inflammation to human cholangiocarcinoma. *Hepatology* **47**: 888–896.
Kou T, Marusawa H, Kinoshita K, Endo Y, Okazaki IM, Ueda Y *et al.* (2007). Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis. *Int J Cancer* **120**: 469–476.
Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**: 263–274.
Lashner BA, Shapiro BD, Husain A, Goldblum JR. (1999). Evaluation of the usefulness of testing for p53 mutations in colorectal cancer surveillance for ulcerative colitis. *Am J Gastroenterol* **94**: 456–462.
Leedham SJ, Graham TA, Oukrif D, McDonald SA, Rodriguez-Justo M, Harrison RF *et al.* (2009). Clonality, founder mutations, and field cancerization in human ulcerative colitis-associated neoplasia. *Gastroenterology* **136**: 542–550 e6.
Lin WW, Karin M. (2007). A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest* **117**: 1175–1183.
Liu R, Bal HS, Desta T, Behl Y, Graves DT. (2006). Tumor necrosis factor-alpha mediates diabetes-enhanced apoptosis of matrix-producing cells and impairs diabetic healing. *Am J Pathol* **168**: 757–764.
Mantovani A, Allavena P, Sica A, Balkwill F. (2008). Cancer-related inflammation. *Nature* **454**: 436–444.
Marra F, Valente AJ, Pinzani M, Abboud HE. (1993). Cultured human liver fat-storing cells produce monocyte chemotactic protein-1. Regulation by proinflammatory cytokines. *J Clin Invest* **92**: 1674–1680.
Matsumoto Y, Marusawa H, Kinoshita K, Endo Y, Kou T, Morisawa T *et al.* (2007). Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nat Med* **13**: 470–476.
Morisawa T, Marusawa H, Ueda Y, Iwai A, Okazaki IM, Honjo T *et al.* (2008). Organ-specific profiles of genetic changes in cancers caused by activation-induced cytidine deaminase expression. *Int J Cancer* **123**: 2735–2740.
Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**: 553–563.
Nakatani T, Mizuhara E, Minaki Y, Sakamoto Y, Ono Y. (2004). Helt, a novel basic-helix-loop-helix transcriptional repressor expressed in the developing central nervous system. *J Biol Chem* **279**: 16356–16367.
Neurath MF, Pettersson S, Meyer zum Buschenfelde KH, Strober W. (1996). Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat Med* **2**: 998–1004.
Podolsky DK. (2002). Inflammatory bowel disease. *N Engl J Med* **347**: 417–429.
Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S *et al.* (2008). Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* **118**: 560–570.
Rai K, Huggins IJ, James SR, Karpf AR, Jones DA, Cairns BR. (2008). DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45. *Cell* **135**: 1201–1212.
Redston MS, Papadopoulos N, Caldas C, Kinzler KW, Kern SE. (1995). Common occurrence of APC and K-ras gene mutations in the spectrum of colitis-associated neoplasias. *Gastroenterology* **108**: 383–392.
Sturlan S, Oberhuber G, Beinhauer BG, Tichy B, Kappel S, Wang J *et al.* (2001). Interleukin-10-deficient mice and inflammatory bowel disease associated cancer development. *Carcinogenesis* **22**: 665–671.
Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T *et al.* (2004). Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci USA* **101**: 1981–1986.
Takai A, Toyoshima T, Uemura M, Kitawaki Y, Marusawa H, Hiai H *et al.* (2009). A novel mouse model of hepatocarcinogenesis triggered by AID causing deleterious p53 mutations. *Oncogene* **28**: 469–478.
Toda Y, Kono K, Abiru H, Kokuryo K, Endo M, Yaegashi H *et al.* (1999). Application of tyramide signal amplification system to immunohistochemistry: a potent method to localize antigens that are not detectable by ordinary method. *Pathol Int* **49**: 479–483.
Watanabe T, Kitani A, Murray PJ, Wakatsuki Y, Fuss IJ, Strober W. (2006). Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis. *Immunity* **25**: 473–485.
Yin J, Harpaz N, Tong Y, Huang Y, Laurin J, Greenwald BD *et al.* (1993). p53 point mutations in dysplastic and cancerous ulcerative colitis lesions. *Gastroenterology* **104**: 1633–1639.

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The forkhead box M1 transcription factor as a candidate of target for anti-cancer immunotherapy

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The present study attempted to identify a target antigen for immunotherapy for cholangiocarcinoma. Forkhead box M1 (FOXM1) was selected as a candidate antigen based on the data of previous cDNA microarray analysis of clinical samples of cholangiocarcinoma. The level of *FOXM1* mRNA was more than 4 times higher in cancer cells in comparison to adjacent normal epithelial cells, in all of 24 samples of cholangiocarcinoma tissues. An immunohistochemical analysis also detected FOXM1 protein in the cancer cells but not in the normal cells. Twenty-three human FOXM1-derived peptides predicted to bind to HLA-A2 were analyzed to determine their ability to induce HLA-A2-restricted T cells in HLA-A2 transgenic mice. $FOXM1_{362-370}$ (YLVPIQFPV), $FOXM1_{373-382}$ (SLVLQPSVKV), and $FOXM1_{640-649}$ (GLMDLSTTPL) peptides primed HLA-A2-restricted cytotoxic T lymphocytes (CTLs) in the HLA-A2 transgenic mice. Human CTL lines reactive to these 3 peptides could also be established from HLA-A2-positive healthy donors and cancer patients. Natural processing of the 3 epitopes from FOXM1 protein was confirmed by specific killing of HLA-A2-positive FOXM1-transfectants by peptide-induced CTLs. FOXM1 is expressed in various types of cancers and it is also functionally involved in oncogenic transformation and the survival of cancer cells. Therefore, FOXM1 may be a suitable target for immunotherapy against various cancers including cholangiocarcinoma.

Key words: FOXM1, cDNA microarray, CTL epitopes, tumor-associated antigen, CTLs, HLA-A2

Abbreviations: BM-DC: bone marrow-derived dendritic cell; CTL: cytotoxic T lymphocyte; DC: dendritic cell; ELISPOT: enzyme-linked immunospot; FOXM1: forkhead box M1; HA: hemagglutinin; HLA: human leukocyte histocompatibility antigen; HRP: horseradish peroxidase; NSCLC: non-small cell lung carcinoma; PBMC: peripheral blood mononuclear cell; TAA: tumor-associated antigen; Tgm: transgenic mouse

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Cholangiocarcinoma is a devastating malignancy, which is difficult to diagnose and treat. Unfortunately, only a few patients are considered suitable for surgery, and chemotherapy and radiotherapy have not yielded substantial improvements in the survival rate. The overall survival rate is poor, with less than 5% of patients surviving upto 5 years. There has been no significant change in this rate over the past 30 years.[1] Recent studies have identified some therapeutic molecular targets in cholangiocarcinoma in preclinical studies, thus supporting the therapeutic potential of selected targeting strategies against cholangiocarcinoma.[2] The results were encouraging, although they were obtained from *in vitro* studies and tumor cell xenograft models. Therefore, novel therapeutic strategies for cholangiocarcinoma are urgently needed.

In cancer immunotherapy, effective induction of cytotoxic T lymphocytes (CTLs) by tumor-associated antigen (TAA) has shown promising results.[3] The utilization of CTLs elicited by TAA is an ideal therapeutic approach, if they specifically attack tumor cells expressing the antigen and reveal little or no adverse effects on normal cells. The development of cDNA microarray technologies, coupled with genome information, has provided comprehensive profiles of the gene expression of malignant cells, which have been compared to those of normal cells.[4,5] TAAs should be expressed excessively and preferentially by the tumor cells but not by the

normal tissues, and gene expression profiling with cDNA microarray technologies is an effective approach for the identification of new TAAs.[6–8] Previous studies have identified several molecular targets useful for the diagnosis and immunotherapy by analyzing genome-wide gene expression profiles of various cancerous tissues with cDNA microarrays.[9–14] The present study identified a candidate TAA, forkhead box M1 (FOXM1), based on the results of previous cDNA microarray analysis of the tissues from 25 patients with intrahepatic cholangiocarcinoma.[15] Recently, an elevated expression of *FOXM1* has been reported in a variety of human cancers,[12,15–20] including lung cancer,[21] which is the most common causes of cancer-related deaths in Western countries.[22] Among normal adult human organs, FOXM1 is expressed only in the testis, thymus, small intestine and colon.[23]

The identification of immunodominant epitopes of TAA has highlighted the utilization of these epitopes as promising therapeutic tools for cancer immunotherapy. However, the identification of CTL epitopes remains difficult, because many TAA-derived peptides must be screened for their immunogenicity by using a large number of peripheral blood mononuclear cells (PBMCs) isolated from the patients with cancer. HLA transgenic mice (Tgm) provides versatile animal models for the preclinical evaluation of peptide-based immunotherapy.[24] Previous investigations have used HLA-A2 Tgm for the identification of HLA-A2-restricted antigenic epitopes.[11] The present study identified FOXM1-derived HLA-A2-restricted epitopes by using HLA-A2 Tgm. CTL lines reactive to these epitopes were also established from cancer patients and healthy donors. The data presented herein suggest that FOXM1 may therefore be a suitable target for immunotherapy of various cancers, including cholangiocarcinoma.

Tumor Immunology

Material and Methods

cDNA microarray analysis

Intrahepatic cholangiocarcinoma tissue specimens were obtained from 25 patients who underwent hepatectomy at Kyoto University Hospital with written informed consents. All tumors were histologically diagnosed to be cholangiocarcinoma by pathologists. The profiling of gene expression by cDNA microarray analysis was carried out, as described previously.[15]

Mice, peptides and cell lines

HLA-A2 transgenic mice described previously were kindly provided by Dr. F.A. Lemonnier (Department SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, France).[11] Peptides (purity > 90%) were purchased from Anygen (Gwangju, South Korea). Three human cholangiocarcinoma (TFK, HuCCT-1, MEC), two human hepatocellular carcinoma (HepG2 and SK-Hep1), and the 23 human lung cancer cell lines including 7 adenocarcinomas (A427, A549, LC319, PC-3, PC-9, PC-14 and NCI-H1666), two bronchiolo-alveolar carcinomas (NCI-H1373, NCI-H1781), 7 squamous cell carcinomas (EBC-1, LU61, NCI-H520, NCI-H1703, NCI-H2170, RERF-LC-AI and SK-MES-1), 2 adenosquamous carcinomas (NCI-H226 and NCI-H647), 1 large-cell carcinoma (LX1), and 4 small-cell lung cancers (DMS114, DMS273, SBC-3 and SBC-5) were used. Human SAEC cells were used as controls. A transporter associated with antigen processing (TAP)-deficient and *HLA-A*0201*-positive cell line, T2, and a colon cancer cell line, Caco-2, were obtained from the RIKEN Bioresource Center (Tsukuba, Japan). These cells were maintained *in vitro* in RPMI 1640 medium or DMEM supplemented with 10% heat inactivated fetal calf serum, except for Caco-2 cells that were cultured in medium supplemented with 20% heat inactivated fetal calf serum. SAEC cells were grown in optimized medium (SAGM) purchased from Cambrex Bio Science (Walkersville, MD).

Blood and tissue samples

The clinical research using PBMCs from the donors was approved by the Institutional Review Board of Kumamoto University. Blood samples were obtained from healthy donors and from patients with either cholangiocarcinoma or lung cancers who provided their written informed consent. The expression of HLA-A2 was examined by flowcytometry with an anti-HLA-A2 monoclonal antibody, BB7.2 (One Lambda, Canoga Park, CA), to select HLA-A2-positive donors. Cancerous tissues were excised from patients during surgery, after obtaining written informed consent.

RT-PCR and Northern blot analysis

Total RNA was extracted from the cell lines and the tissues using the RNeasy kit (Qiagen, Dusseldorf, Germany) according to the manufacturers' instructions. Total RNA of human normal tissues was purchased from Clontech (Palo Alto, CA) and RT-PCR was done as described previously.[25] The primer sequences were: *FOXM1*, 5′-CACCCCAGTGCCAACCGC TACTTG-3′ and 5′-AAAGAGGAGCTATCCCCTCCTCAG-3′ (that can detect three splicing variants *FOXM1a*, *FOXM1b* and *FOXM1c*) or 5′-CCCTGACAACATCAACTGGTC-3′ and 5′-GTCCACCTTCGCTTTTATTGAGT-3′ (that cannot detect the variants); *GAPDH*, 5′-CCCATCACCATCTTCCAG-GAGC-3′ and 5′-CCAGTGAGCTTCCCGTTCAGC-3′; *ACTB*, 5′-GAGGTGATAGCATTGCTTTCG-3′ and 5′-CAAGTCAGTGTACAGGTAAGC-3′. A Northern blot analysis was done as described previously.[18] Gels with 20 μg of the total RNA of the human normal tissue (Clontech) per lane were blotted onto nylon membranes. The membranes were hybridized with ^{32}P-labeled *FOXM1*-specific cDNA as a probe. The blots were then autoradiographed with intensifying screens at −80°C for 120 hr.

Immunohistochemical analysis

An immunohistochemical analysis was done as described previously.[25] Sections of paraffin-embedded cholangiocarcinoma and lung cancer specimens were stained with a mouse monoclonal anti-human FOXM1 antibody (clone 3A9,

Abnova, Taipei, Taiwan) by standard procedure. Staining signals were visualized using the DakoCytomation EnVision + System-HRP (DakoCytomation, Carpinteria, CA).

Generation of FOXM1 transfectant

A cDNA encoding for FOXM1 tagged with Influenza virus Hemagglutinin (HA) was cloned into a pENTR vector (Invitrogen, Carlsbad, CA) and then transferred into a self-inactivating lentivirus expression vector, CSII-EF-RfA,[26] by LR recombination reaction (Invitrogen). Lentivirous-mediated gene transfer was performed as described previously.[27] The expression of the transgene-derived FOXM1 protein was confirmed by Western blot analysis, using horseradish peroxidase (HRP)-conjugated rat monoclonal anti-HA antibody (clone 3F10, Roche, Mannheim, Germany). β-actin was also detected by using mouse monoclonal anti-β-actin antibody (clone AC-15, Sigma, St. Louis, MO) and HRP-conjugated rabbit anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) antibody. The signals were developed and visualized using a chemiluminescence system (ECL, Amersham Biosciences).

Identification of HLA-A2-restricted CTL epitopes of FOXM1 using HLA-A2 transgenic mice

The immunization of mice with peptide-loaded bone marrow-derived DCs (BM-DCs) was done as previously described.[12,15] $CD4^-$ spleen cells were isolated from the immunized mice, and the frequency of cells producing IFN-γ among 5×10^4 cells upon stimulation with syngeneic 1×10^4/well BM-DCs, pulsed with and without each peptide, was assayed by an enzyme-linked immunospot (ELISPOT) assay as previously descibed.[28]

HLA-peptide-binding assay

HLA-A*0201-positive and TAP-deficient T2 cells, pre-incubated overnight at 26°C, were cultured in the presence of 50 μM FOXM1 peptides and 5 μg/ml β_2-microglobulin (Sigma, St Louis, MO) in serum-free medium at 26°C again for 1.5 hr and subsequently at 37°C for 18 hr. The level of expression of cell surface HLA-A2 was analyzed by flow cytometry using anti-HLA-A2 monoclonal antibody, BB7.2. HLA-A24-restricted human Cytomegalovirus-derived peptide (QYDPVAALF) as a negative control and SARS-A2-S-7 peptide (NLNESLIDL), which is SARS coronavirus-derived CTL epitope peptide with high binding affinity to HLA-A2 (work in preparation), as a positive control were also used. The fluorescence index (FI) was calculated from the mean fluorescence intensity (MFI) of HLA-A2 expressed on T2 cells determined by flow cytometry, using the formula; FI = (MFI [T2 cells with FOXM1 peptide]/MFI [T2 cells with CMV-peptide]) − 1.

Induction of FOXM1-reactive human CTLs

PBMCs were isolated from heparinized blood of HLA-A2-positive Japanese cancer patients or healthy donors by Ficoll-Conray density gradient centrifugation. Peripheral monocyte-derived dendritic cells (DCs) were generated as previously described.[29] $CD8^+$ T cells were isolated, using anti-CD8 microbeads (Miltenyl Biotec, Bergisch Gladbach, Germany) from PBMCs of the same donors, and thereafter, peptide-

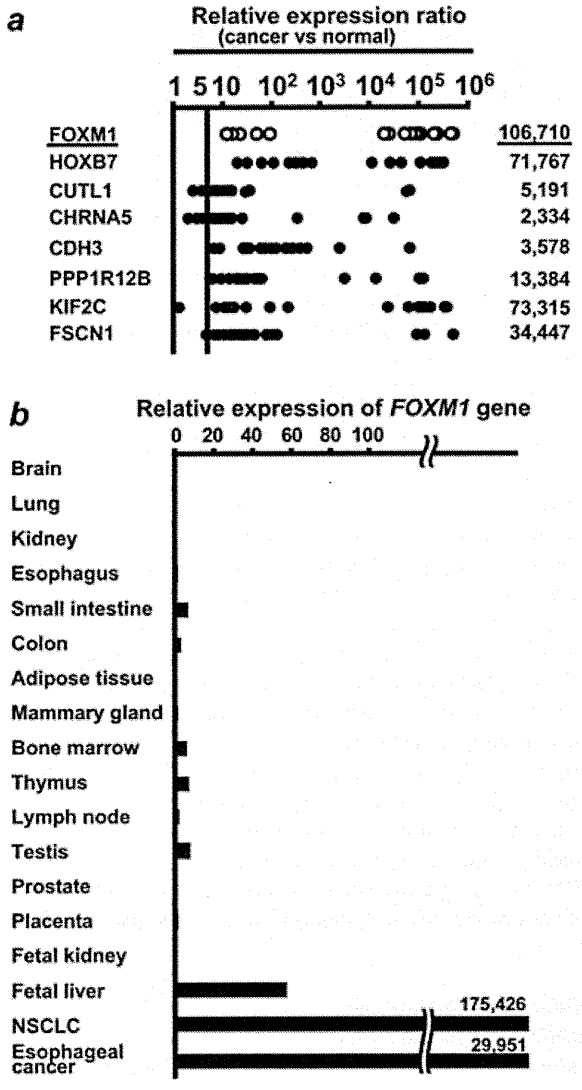

Figure 1. cDNA microarray analysis revealed a markedly enhanced expression of *FOXM1* mRNA in intrahepatic cholangiocarcinoma tissues. (*a*) A list of up-regulated genes in intrahepatic cholangiocarcinoma tissue. These genes exhibited greater than 4-fold increased expression in cancer cells in comparison to their normal counterparts in more than 15 out of 25 intrahepatic cholangiocarcinoma patients. The expression of *FOXM1* mRNA in intrahepatic cholangiocarcinoma tissue specimens was markedly enhanced in 24 of 25 patients with intrahepatic cholangiocarcinoma. (*b*) The expression levels of *FOXM1* in the normal tissues were far lower than those in non-small cell lung carcinoma (NSCLC) or esophageal cancer tissues based on the cDNA microarray analysis.

Table 1. Human FOXM1-derived peptides predicted to bind to HLA-A2 (*A*0201*)

No.	Position	Sequence[1]	Predicted binding score	
			BIMAS[2]	SYFPEITHI[3]
1	42–50	NQAEASKEV	29	15
2	241–249	YMAMIQFAI	201	21
3	256–264	RMTLKDIYT	30	12
4	288–296	NLSLHDMFV	383	20
5	290–299	SLHDMFVRET	53	23
6	355–363	LLPRVSSYL	200	22
7	355–364	LLPRVSSYLV	118	23
8	362–370	YLVPIQFPV	1856	24
9	366–375	IQFPVNQSLV	44	15
10	373–382	SLVLQPSVKV	70	26
11	374–382	LVLQPSVKV	38	23
12	409–418	LLAEEGIAPL	342	32
13	429–438	LLFGEGFSPL	255	25
14	545–553	LLFSEGPST	47	20
15	571–579	SQLSYSQEV	26	17
16	616–625	KVGGIDFSPV	40	17
17	640–649	GLMDLSTTPL	324	23
18	660–669	RLLSSEPLDL	79	23
19	661–669	LLSSEPLDL	36	23
20	702–711	SLTEGLVLDT	70	22
21	711–719	TMNDSLSKI	71	24
22	719–728	ILLDISFPGL	1047	27
23	720–728	LLDISFPGL	28	24

[1]Underlined amino acids are not conserved between human and mouse FOXM1. [2]http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform. [3]http://www.syfpeithi.de/home.htm.

reactive $CD8^+$ CTLs were generated as described.[30] Six days after the last stimulation, antigen-specific responses of the CTLs were analyzed by IFN-γ ELISPOT assay as described above and also by 4 h-^{51}Cr release assay as described previously.[31] Briefly, ^{51}Cr labeled target cells (5×10^3) suspended in 100 μl of assay medium were seeded into wells of 96-well plates. Various numbers of effector cells suspended in 100 μl of assay medium were added into the wells and were incubated for 4 hr, and the supernatant was collected from each well to measure the ^{51}Cr radioactivity.

Statistical analysis

The statistical significance of the differential findings among the experimental groups was determined by Student's *t*-test. Avalue of $p < 0.05$ was considered to be statistically significant.

Results

Markedly enhanced expression of FOXM1 in intrahepatic cholangiocarcinoma tissues

A laser microdissection-based cDNA microarray analysis of 25 cases of intrahepatic cholangiocarcinoma has been reported previously.[15] These data were used to search for genes showing a high relative expression ratio between cancer cells and normal epithelial cells of intrahepatic bile duct. This process identified 8 genes with greater that 4-fold increased expression in cancer cells in more than 15 out of 25 tissue samples (Fig. 1*a*). Among the 8 up-regulated genes, the expression of *FOXM1* exhibited the greatest increase in the expression in cancer cells in comparison to the normal cells (Fig. 1*a*). Therefore, FOXM1 was evaluated as a candidate antigen for immunotherapy.

The expression of *FOXM1* was analyzed in 16 kinds of normal tissues including fetal kidney and liver and also non-small cell lung cancer and esophageal cancer, using cDNA microarray (Fig. 1*b*). *FOXM1* was expressed in the testis, bone marrow, small intestine, thymus, and fetal liver among normal tissues. However, the levels of expression in these normal tissues were far lower than those in non-small cell lung carcinoma and esophageal cancer tissues (Fig. 1*b*).

cDNA microarray analysis of expression of FOXM1 in various cancer tissues

The previously reported data of cDNA microarray analysis of various kinds of cancerous tissues[12–21] were used to analyze the expression of *FOXM1* in 21 different types of cancer tissues. Supporting Information Table 1 shows the expression of *FOXM1* to be elevated in various cancer tissues. A particularly enhanced expression was recognized in bladder cancer, lung cancer (both small cell carcinoma and non-small cell carcinoma) and pancreatic cancer.

Expression of FOXM1 in various cancer cell lines and tissues

Further investigation of FOXM1 was conducted not only in cholangiocarcinoma but also in lung cancer, because lung cancer is the most common cause of cancer death in Western countries.[22]

The expression of the *FOXM1* gene in cholangiocarcinoma, hepatocellular carcinoma and lung cancer cell lines (adenocarcinoma, bronchiolo-alveolar carcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell carcinoma and small cell carcinoma), and lung cancer tissues was analyzed by RT-PCR.

The *FOXM1* gene contains 10 exons, 2 of which are alternatively expressed, giving rise to 3 differentially expressed mRNA isoforms.[23] Cholangiocarcinoma cell lines, TFK, HuCCT-1, and MEC, and hepatocellular carcinoma cell lines, HepG2 and SK-Hep1 strongly expressed *FOXM1b* and *FOXM1c* (*FOXM1b* < *FOXM1c*) mRNA (Fig. 2).

FOXM1 was expressed in all the lung cancer cell lines examined (Supporting Information Fig. 1). In addition, all of the tissue samples of lung adenocarcinoma, squamous cell carcinoma, and small cell carcinoma also expressed *FOXM1* mRNA, whereas normal lung epithelium did not (Supporting Information Fig. 1). *FOXM1* was strongly expressed in all cancer cell lines examined, except for Caco-2, which is a

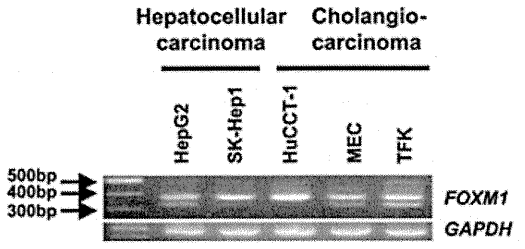

Figure 2. The expression of *FOXM1* mRNA in cancer cell lines. RT-PCR analysis was done to detect *FOXM1* mRNA expression in cholangiocarcinoma and hepatocellular carcinoma cell lines. The same cDNA samples were tested for *GAPDH* for control.

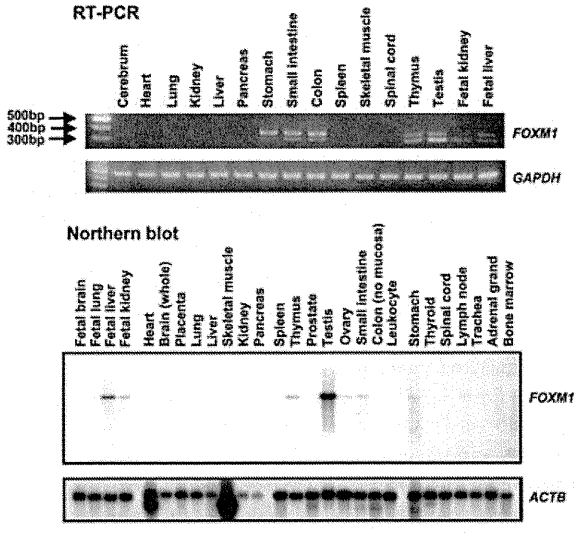

Figure 3. *FOXM1* expression in various normal tissues analyzed by RT-PCR and Northern blot analysis. The same Northern blot membrane was stripped and rehybridized with a β-actin probe to prove the RNA integrity and to assess the loading of equal amounts of RNA.

colon cancer cell line expressing a trace amount of *FOXM1* mRNA (data not shown).

Among the normal tissue specimens, *FOXM1* mRNA was expressed in the testis, fetal liver and thymus (*FOXM1b* > *FOXM1c*), stomach, small intestine and colon (*FOXM1b* < *FOXM1c*) (Fig. 3). A Northern blot analysis revealed relatively strong expression of *FOXM1* mRNA in testis and weak expression in the fetal liver, thymus and gastrointestinal tracts (Fig. 3). These RT-PCR and Northern blot analyses were consistent with the results of the cDNA microarray analysis (Fig. 1*b*).

Tissue samples of cholangiocarcinoma and lung cancer were analyzed immunohistochemically to determine the expression of FOXM1 protein in cancer cells (Fig. 4). An

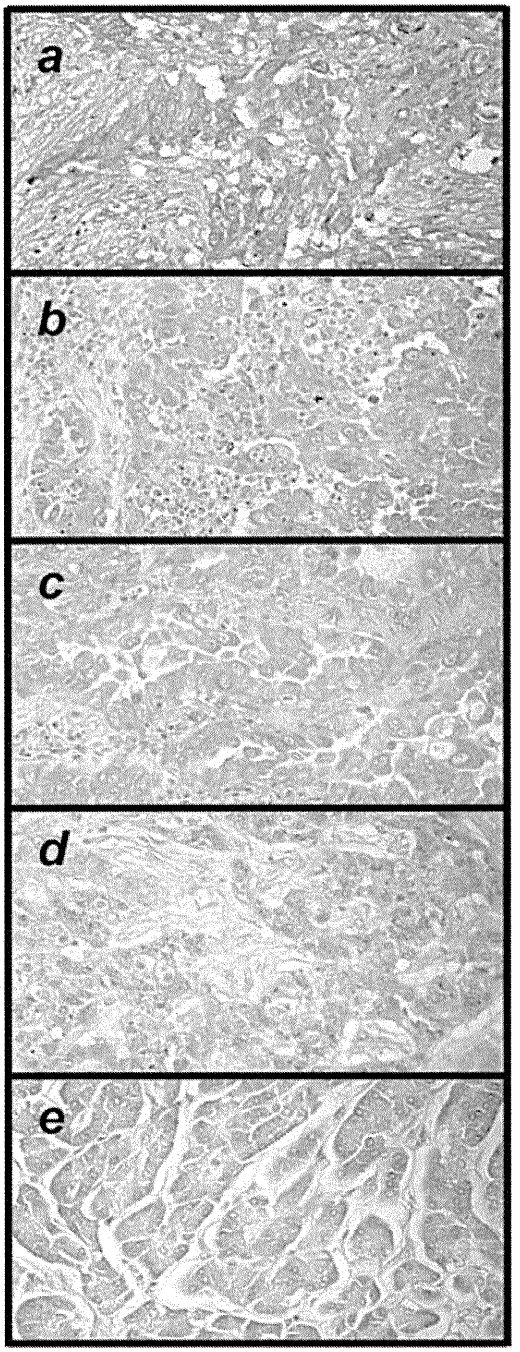

Figure 4. Immunohistochemical staining of FOXM1 protein in the intrahepatic cholangiocarcinoma (*a*), lung adenocarcinoma (*b, c*), cholangiocarcinoma (*d*), non-cancerous lesion (pancreas) of *d* (*e*). The sections were stained with monoclonal anti-FOXM1 antibody. Objective magnification was 200×.

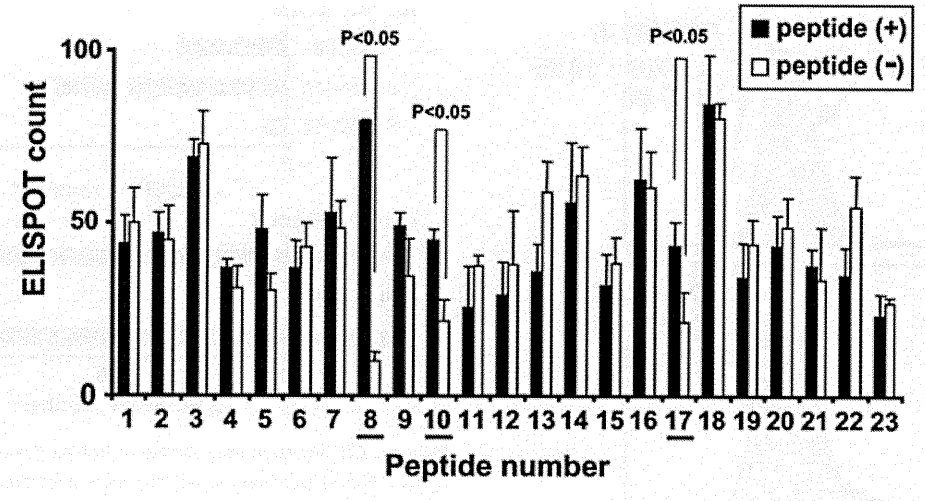

Figure 5. The identification of HLA-A2-restricted CTL epitopes of FOXM1 by using HLA-A2 Tgm. HLA-A2 Tgm were primed by injection of BM-DCs (5×10^5) pulsed with the mixture of FOXM1-derived peptides carrying HLA-A2 (*A*0201*) binding motif into the peritoneal cavity of the Tgm twice with a 1 week interval. Seven days after the 2nd vaccination, spleens were isolated and $CD4^-$ spleen cells (5×10^6/well) were stimulated with syngeneic irradiated BM-DCs (5×10^5/well) pulsed with each peptide for 6 days. Cultured $CD4^-$ spleen cells were subjected to ELISPOT assay to count IFN-γ-producing cells/5×10^4 $CD4^-$ spleen cells upon stimulation with 1×10^4 BM-DCs with (black) or without (white) peptide loading. The columns and bars indicate mean of triplicate assays and SE, respectively. The data are each representative of 3 independent experiments. The underlines indicate the peptides that induced peptide-specific responses ($p < 0.05$).

increased expression of FOXM1 protein was observed in the cancerous tissues, but not in the adjacent normal tissues.

Identification of HLA-A2-restricted FOXM1 epitopes by using HLA-A2.1 Tgm

Human FOXM1-derived peptides, carrying binding motifs for HLA-A2 (*HLA-A*0201*) were searched using the BIMAS software package (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD, http://www-bimas.cit.nih.gov/) and SYFPEITHI (Biomedical Informatics, http://www.syfpeithi.de/). We selected 23 peptides predicted to bind to HLA-A2 from the amino acid sequence conserved among human FOXM1a, FOXM1b and FOXM1c isoforms. The scores of the selected peptides calculated based on BIMAS and SYFPEITHI algorithms predicting HLA-A2-binding affinity are shown in Table 1.

Further investigations were conducted to identify HLA-A2-restricted epitopes from the selected 23 peptides, using HLA-A2 Tgm. The 23 peptides were loaded onto BM-DCs of HLA-A2 Tgm and injected intraperitoneally twice into the mice with a 7-day interval. Spleen cells were isolated from the mice 7 days after the second injection and cultured with BM-DCs pulsed with the peptides. Subsequently, the cultured cells were subjected to an ELISPOT assay to analyze production of IFN-γ upon stimulation with the same peptides. A series of repeated experiments revealed a reproducible response to $FOXM1_{362\text{-}370}$, $FOXM1_{373\text{-}382}$ and $FOXM1_{640\text{-}649}$ (designated as FOXM1-8, FOXM1-10 and FOXM1-17, respectively; Table 1: Fig. 5). On the other hand, the peptide-reactive CTL lines did not respond to the peptides pulsed onto non-transgenic C57BL/6 mice-derived BM-DCs, thus verifying that the responses to these peptides were restricted by HLA-A2.

The amino acid sequences of FOXM1-8 and FOXM1-10 are conserved between human and mouse, whereas that of FOXM1-17 is not conserved; human GLM*D*LSTTPL *vs.* mouse GLM *E*LNTTPL. To determine whether the immunization of the mice with the FOXM1 peptides caused autoimmunity, a histological analysis was done on various organs isolated from HLA-A2 Tgm immunized with FOXM1-8 or FOXM1-10 peptides. We observe no pathological changes, such as lymphocyte infiltration or tissue destruction and repair in the brain, heart, lung, liver, small intestine, kidney, or testis of the immunized Tgm (data not shown). These results suggest that the CTL response elicited by immunization with FOXM1-8 or FOXM1-10 peptide may not cause damage to normal tissues.

Induction of FOXM1-reactive human CTLs from HLA-A2-positive donors

To know whether or not the identified 3 FOXM1-derived peptides actually bind to HLA-A2, we analyzed the binding of the peptides to HLA-A2 by using TAP-deficient and HLA-A2-positive T2 cells. As shown in Figure 6, stabilization of cell surface HLA-A2 by addition of FOXM1 peptides was observed, thus confirming the actual binding of the peptides to HLA-A2.