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

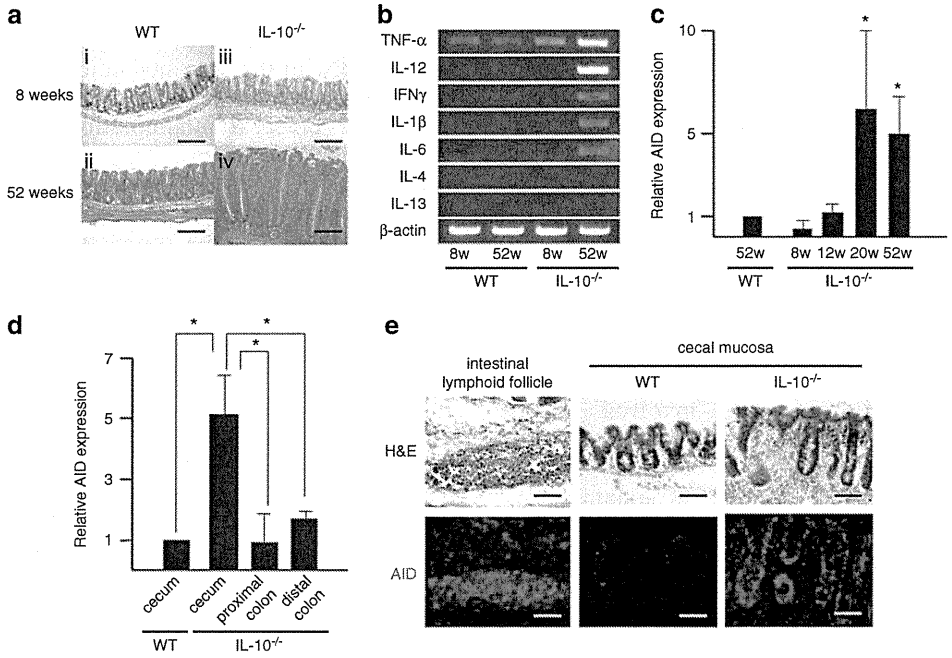

Figure 1 Endogenous AID expression in inflamed colonic mucosa of IL-10$^{-/-}$ mice. (**a**) Microscopic (hematoxylin and eosin (H&E) stain) images of the cecum of IL-10$^{-/-}$ mice and its WT littermate. The images of 8-week-old mice (i, iii) and 52-week-old mice (ii, iv) are shown. Scale bars are 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

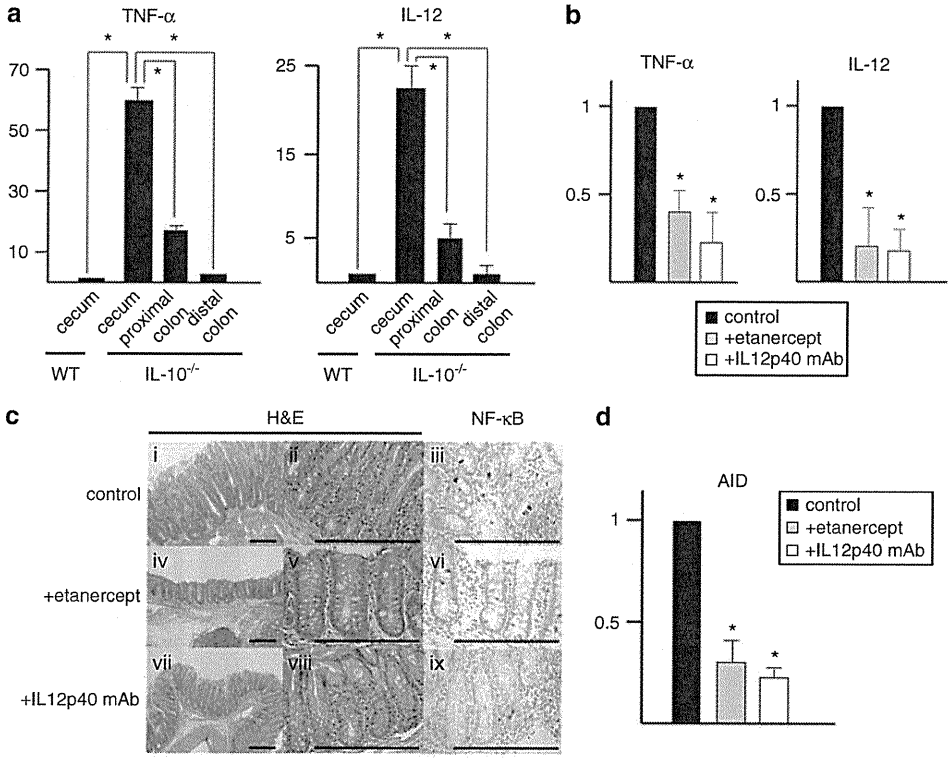

Figure 2 Effects of TNF-α and IL-12 blockade on various cytokine expression, NF-κB activation and AID expression in the colonic mucosa of IL-10$^{-/-}$ mice. (**a**) TNF-α and IL-12 expression in various regions in colonic mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from mucosa at the cecum, 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

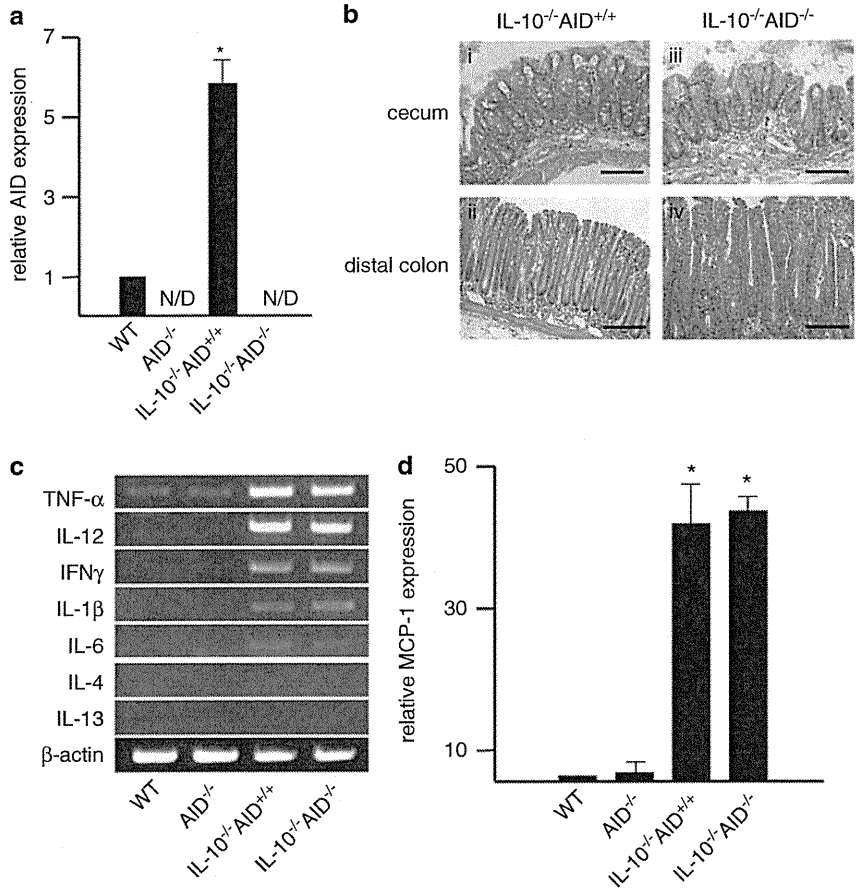

Figure 3 Effects of AID deficiency in the colonic mucosa of IL-10$^{-/-}$ mice. (**a**) Results of quantitative RT–PCR for AID expression. Total RNA was extracted from the mucosa at the cecum of 52-week-old WT, AID$^{-/-}$, IL-10$^{-/-}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. Quantitative RT–PCR was performed 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.
Number of mutated nucleotides/number of total nucleotides examined.
*P-value is calculated using the χ^2 test.

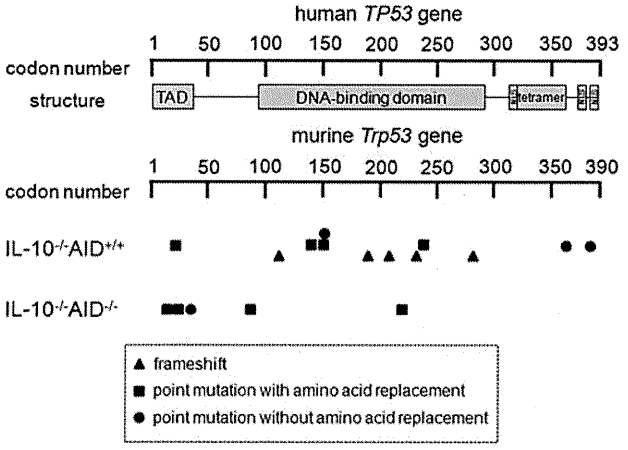

Figure 4 Distribution of murine *Trp53* mutations found in the cecal mucosa of IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. Murine p53 codon numbers are shown with equivalent human p53 domain structure with transactivation (TAD), DNA-binding and tetramerization (tetramer) domains and nuclear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

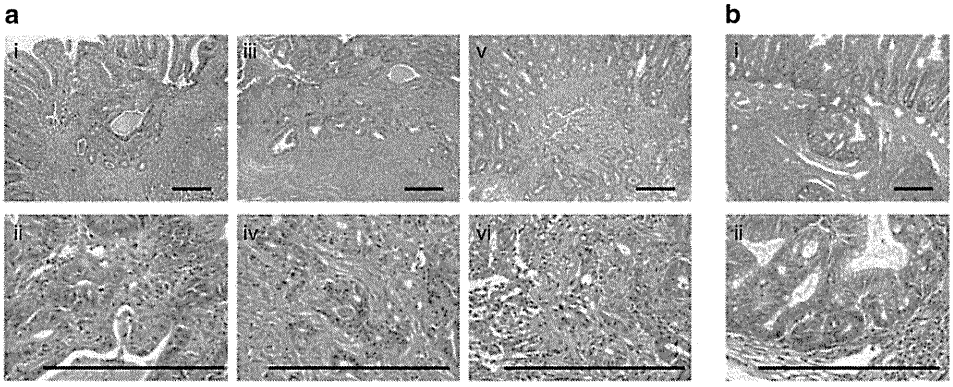

Figure 5 Colonic adenocarcinomas developed in IL-10$^{-/-}$ mice. (**a**) Microscopic images (hematoxylin and eosin (H&E) stain) of adenocarcinomas developed in the cecum of the IL-10$^{-/-}$AID$^{+/+}$ mice (i–vi). Scale bars are 500 μm. (**b**) Microscopic images (H&E stain) of adenocarcinoma developed 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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The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased in Kyushu area

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: The incidence of hepatocellular carcinoma (HCC) in Japan has still been increasing. The aim of the present study was to analyze the epidemiological trend of HCC in the western area of Japan, Kyushu.

Material/Methods: A total of 10,010 patients with HCC diagnosed between 1996 and 2008 in the Liver Cancer study group of Kyushu (LCSK), were recruited for this study. Cohorts of patients with HCC were categorized into five year intervals. The etiology of HCC was categorized to four groups as follows; B: HBsAg positive, HCV-RNA negative, C: HCV-RNA positive, HBsAg negative, B+C: both of HBsAg and HCV-RNA positive, non-BC: both of HBsAg and HCV-RNA negative.

Results: B was 14.8% (1,485 of 10,010), whereas 68.1% (6,819 of 10,010) had C, and 1.4% (140 of 10,010) had HCC associated with both viruses. The remaining 1,566 patients (15.6%) did not associate with both viruses. Cohorts of patients with HCC were divided into six-year intervals (1996–2001 and 2002–2007). The ratio of C cases decreased from 73.1% in 1996–2001 to 64.9% in 2002-2007. On the other hand, B and -nonBC cases increased significantly from 13.9% and 11.3% in 1996-2001 to 16.2% and 17.6% in 2002-2007, respectively.

Conclusions: The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased after 2001 in Kyushu area. This change was due to the increase in the number and proportion of the HCC not only nonBC patients but also B patients.

key words: hepatitis virus • hepatocellular carcinoma • Japan

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Background

The three leading causes of death in Japan are malignancy neoplasms, cardiovascular diseases, and cerebrovascular diseases. Since 1981, malignant neoplasms have been the leading cause of death in Japan. For the last 30 years, liver cancer has been the third leading cause of death from malignant neoplasms in men. In women, liver cancer has ranked fifth during the past decade [1]. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancers[2] and the age-adjusted HCC mortality rate has increased in recent decades in Japan [3]. Similarly, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia [4,5]. HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, or nonalcoholic fatty liver disease. Of the hepatitis viruses which cause HCC, HCV is predominant in Japan [6–9].

Although the age-adjusted incidence of HCC has increased in Japan, sequential changes in etiology of HCC patients between 2001 and 2008 are not fully understood [10]. To clarify factors affecting epidemiological changes in Japanese HCC patients, especially the recent trend of HCC, we analyzed the epidemiological trend of HCC in the western area of Japan, Kyushu area.

Material and Methods

Patients

A total of 10,010 patients with HCC diagnosed between 1996 and 2008 in the Liver Cancer study group of Kyushu (LCSK), were recruited for this study. The diagnosis of HCC was based on AFP levels and imaging techniques including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG), and/or tumor biopsy. The diagnostic criteria for HCC were either a confirmative tumor biopsy or elevated AFP (>20 ng/mL) and neovascularization in HAG and/or CT.

Etiology of HCC

A diagnosis of chronic HCV infection was based on the presence of HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg). The etiology of HCC was categorized to four groups as follows; **B:** HBsAg positive, HCV-RNA negative, **C:** HCV-RNA positive, HBsAg negative, **B+C:** both of HBsAg and HCV-RNA positive, **nonBC:** both of HBsAg and HCV-RNA negative.

Statistical analysis

The data were analyzed by the Mann-Whitney test for the continuous ordinal data, the x2 test with Yates' correction and the Fisher exact test for the association between two qualitative variables. The standard deviation was calculated based on the binomial model for the response proportion. P<0.05 was considered statistically significant.

Results

Clinical features of the studied patients

A total of 10,010 patients with HCC were diagnosed at our study group from 1996 to 2008. Table 1 show that the proportion of patients diagnosed with B was 14.8% (1,485 of 10,010), whereas 68.1% (6,819 of 10,010) had C, and an additional 1.4% (140 of 10,010) had HCC associated with both viruses. The remaining 1,566 patients (15.6%) did not associate with both viruses. In analysis of patients in HCC by category, the median age of patients at diagnosis of B was 57 years old significant younger than other types HCC(**C: 69, nonBC: 70,.B+C 65 years old**).

As shown in Figures 1 and 2, the number and ratio of B cases remained unchanged from1996 to 2001 and thereafter increased and plateaued, whereas C rapidly increased from 1996 to 2000 and thereafter decreased and plateaued. In addition, the number and ratio of the **nonBC** cases has increased continued gradually and continued in this study period.

Change of etiology in patients with HCC during the period 1996–2007 with 6-years intervals

Cohorts of patients with HCC were divided into six-year intervals (1996–2001 and 2002–2007). Table 2 show that the incident rate of C decreased significantly from 73.1% in 1996-2001 to 64.9% in 2002-2007 (1996–2001 vs. 2002–2007, p=<0.001). On the other hand, the incident rate of B and **-nonBC** increased significantly from 13.9% and 11.3% in 1996-2001 to 16.2% and 17.6% in 2002-2007, respectively. Not only the incident rate but also number of B and -nonBC became larger in same 6 years periods.

Table 3 shows that male/female ratio of C and **-nonBC** decreased significantly from 2.2 and 4.0 in 1996-2001 to 1.8 and 2.7 in 2002-2007, respectively (p<0.001). The ratio became clearly smaller, indicates an increase in female patients with C and **nonBC.** On the other hand, the male/female ratio of B patients did not significantly change during the period. The median age at diagnosis of **B, C,** and **nonBC** in six-year intervals were significant increase from 56 to 58, from 67 to 71 and from 68 to 71 years of age during the period.

Discussion

Our study was the twenty-three major liver center-based study designed to examine the sequential change in the background of HCC patients during the past 13 years, 1996–2008. More than 80% of our patients had chronic HBV or HCV infections. During this observation period, the number and proportion of HCC-C reached a peak in 2000 and thereafter decreased and became stabilized. Previous studies from Japan reported that the proportion of the HCC patients with HCV infection had been increased and reached a plateau in the period of 1981–2001 [1,3,10–12]. However, in our study, the number and proportion of the HCC patients with HCV infection cases decreased in 2001–2008. The reason may be explained as follows; interferon therapy for chronic hepatitis C may have been associated with a decreased incidence of HCC [13–17]. Oral supplementation with a oral branched-chain amino acids has been useful in the prevention HCC [18]. Finally, the chronically HCV-infected

Table 1. The characteristic of HCC patients during the period of 1996–2008.

Age (y.o.)	B		C		nonB		B+C		Total
	Male	Female	Male	Female	Male	Female	Male	Female	
0–	1	0	0	1	0	0	0	0	2
10–	4	1	0	0	0	2	0	0	7
20–	6	2	1	0	1	1	0	0	11
30–	31	5	4	0	11	3	2	0	56
40–	204	22	130	12	32	15	12	0	427
50–	507	66	728	145	167	32	31	6	1,682
60–	287	118	1836	741	411	102	35	13	3,543
70–	140	64	1775	947	483	133	22	14	3,578
80–	9	18	271	214	97	65	1	4	679
90–	0	0	9	5	9	2	0	0	58
Total	1,189	296	4,754	2,065	1,211	355	103	37	10,010
	1,485 (4.8%)		6,819 (68.1%)		1,566 (15.6%)		140 (1.4%)		
Median	57	63	67	70	68	70	61	68	67
	57		69		70		65		
Mean	56	64	68	71	69	71	62	68	67
	58		68		68		63		
Range	1–87	14–89	27–94	0–93	28–96	17–90	36–82	55–82	0–96
	1–89		0–94		17–96		36–82		

Age: B vs. C $p \leq 0.001$; B vs. B+C $p \leq 0.001$; B vs. nonBC $p \leq 0.001$; C vs. BC $p \leq 0.001$; C vs. nonBC $p = 0.043$; BC vs. nonB+C $p \leq 0.001$. IQR – interquartile range; SD – standard deviation.

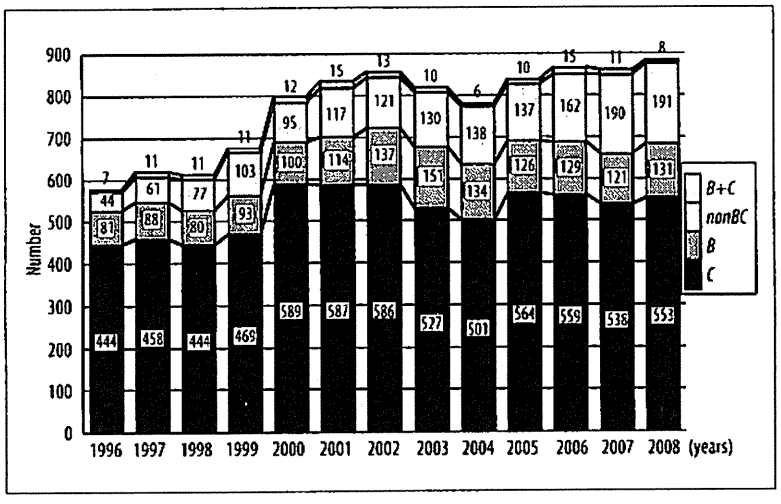

Figure 1. Sequential changes in the number of HCC patients categorized by etiology during the period 1996–2008.

population is aging in Japan. Yoshizawa et al. reported that age-specific prevalence for the presence of HCVAb among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the age, reaching the highest proportion of 7% in individuals who were more than 70 years old [10,19]. In this study, the median age of the HCC patients with HCV infection steadily increased from 67 to 71 years of age during the studied period. In a word, HCV infected people become older with years in Japan and they were regarded as a high risk for HCC.

The prevalence rate of HBV in Kyushu area has been reported to be higher than other area in Japan [1]. In Kyushu area, 95% of patients with chronic HBV infection had HBV genotype C except for Okinawa [20]. HBV genotype C is thought to be associated with higher incidence of HCC

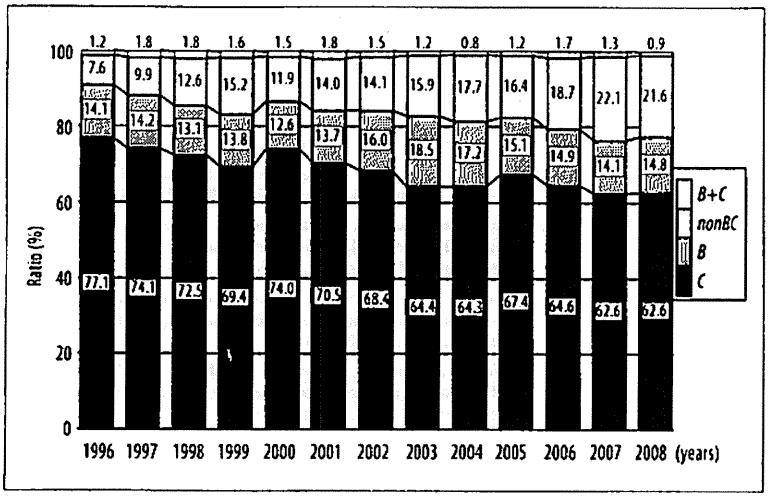

Figure 2. Sequential changes in the ratio of HCC patients categorized by etiology during the period 1996–2008.

Table 2. Change of etiology in patients with HCC during the period 1996–2007 with 6-years intervals.

Period		1996–2001	2002–2007	P value
Number		3,023	4,173	
Sex	Male	2,162	2,849	
	Female	861	1,324	
Ratio (male/female)		2.5	2.2	0.003
Age (y.o.) (IQR)		66 (14)	69 (12)	<0.001
Hepatitis virus (%)	B	13.9	16.2	
	C	73.1	64.9	
	B+C	1.7	1.3	
	nonBC	11.3	17.6	0.001

QR – interquartile range.

Table 3. The median age and male/female ratio of HCC patients during the period of 1996–2007.

Period		1996–2001	2002–2007	P value
B				
Age (y.o.) (IQR)		56 (14)	58 (15)	0.001
Sex	Male	331	519	
	Female	88	157	
Ratio (male/female)		3.8	3.3	0.391
C				
Age (y.o.) (IQR)		67 (9)	71 (11)	<0.001
Sex	Male	1,524	1,753	
	Female	687	955	
Ratio (male/female)		2.2	1.8	0.002
nonBC				
Age (y.o.) (IQR)		68 (12)	71 (13)	<0.001
Sex	Male	273	534	
	Female	69	201	
Ratio (male/female)		4.0	2.7	0.012

QR – interquartile range.

compared with other HBV genotypes [21]. In the present study, the incident rate of HCC patients with HBV infection became larger in this study period. To explain this change, we must consider from two viewpoints. The one is that the number of patients with HCC caused by HCV infection decreased, the other is that the proportion of chronic HBV infected patients who have reached the age of developing HCC is relatively high as described below.

Nationwide health survey for HBsAg in the over 40 years of age population had been done between 2002 and 2006 in Japan. This survey reports indicated that the average HBsAg prevalence was 1.2% in the total Japanese population patients with chronic HBV infection [10] and the age-specific prevalence of HBsAg was higher in the group aged between 50 (1.4%) and 55 years (1.5%). In the HCC patients with HBV genotype C, the mean age was 55 years in Japan [20]. This overlap between age-specific prevalence and hepatocellular carcinogenic age would be associated with the increase of HCC patients with HBV infection. Nucleoside analogue reverse transcriptase inhibitor (NARTI) therapy effectively reduces the incidence of HCC in chronic hepatitis B patients [22,23]. However, Interferon therapy for chronic hepatitis C started from 1992, whereas NARTI therapy for HBV started from 2000 in Japan [24,25]. Hence, HBV associated HCC will probably decrease in Japan during the next 10 to 20 years.

The survey of HCC patients associated with nonBC infection in Japan was conducted by Inuyama Hepatitis Research Group from 1995 to 2003. The ratio of HCC patients with nonBC accounted 9.3% [1]. In the present study, the ratio of HCC patients with nonBC was 14.1%. Furthermore, the number and the proportion of HCC patients with nonBC have been gradually increasing in the periods. The current two studies account for the increase in number and proportion of HCC patients with nonBC. First, Lai et al. reported

that type 2 diabetes increases the risk of developing HCC in those who are HCV negative or have a high level of total cholesterol [26]. Second, Nakano et al. reported that epidemiological studies on diabetes mellitus revealed that the number of patients with diabetes mellitus is gradually increasing in Japan along with development of car society and westernization of food intake. Since prevalence of diabetes mellitus increases with aging, proportion of individuals with diabetes mellitus aged over 60 has exceeded two-third of estimated total number of patients (7.40 million in 2002) in Japan where aging of society is rapidly progressing [27]. In a word, the number of type 2 diabetes people is increasing in Japan and they were regarded as a high risk for HCC. Then, the number and the proportion of HCC patients with nonBC have been increased recent twelve years in Japan.

It is known that 2 to 4 decades of chronic HCV infection are required to develop cirrhosis and subsequent HCC [28–31]. The number of HCC cases has increased in Japan, because individuals infected with HCV during the past have grown old and have reached the cancer-bearing age. The prevalence of HCV infection in young Japanese individuals is low and the incidence of HCVAb is very low because of preventative actions against HCV infection such as the screening of blood products for HCV and the use of sterile medical equipment [32]. Additionally, we showed that the number and proportion of patients with HCC-C cases decreased, whereas the number and ratio of HCC-nonBC steadily increased during the studied period. These findings may be expected that the incidence of HCC patients with nonBC in Japan may continue to increase even after the consequence of the HCV epidemic level off, a country that is far advanced with regard to HCC patients with HCV infection, in the near future.

Conclusions

In summary, HCC patients had increased from 1996 to 2000 and this increase was originated from HCC patients with HCV infection. The number and proportion of HCC patients with HCV infection reached a peak in 2000 and thereafter decreased and became stabilized. The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased after 2001 in Kyushu area. This change was due to the increase in the number and proportion of the HCC not only nonBC patients but also B patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Alpha-fetoprotein above normal levels as a risk factor for the development of hepatocellular carcinoma in patients infected with hepatitis C virus

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Abstract
Background Noninvasive risk factors are required for predicting the development of hepatocellular carcinoma (HCC) not only in patients with cirrhosis but also in those with chronic hepatitis who are infected with hepatitis C virus (HCV).
Methods A total of 707 patients with chronic HCV infection without other risks were evaluated for the predictive value of noninvasive risk factors for HCC, including age, sex, viral load, genotype, fibrosis stage, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count, and alpha-fetoprotein (AFP) at entry to the study, as well as interferon (IFN) therapy they received.
Results The ten-year cumulative incidence rates of HCC for patients with fibrosis stages F0/F1, F2, F3, and F4 were 2.5, 12.8, 19.3, and 55.9%, respectively. Multivariate analysis identified age ≥57 years [hazard ratio (HR) 2.026, $P = 0.004$], fibrosis stage F4 (HR 3.957, $P < 0.001$), and AFP 6–20 ng/mL (HR 1.942, $P = 0.030$) and ≥20 ng/mL (HR 3.884, $P < 0.001$), as well as the response to IFN [relative risk (RR) 0.099, $P < 0.001$], as independent risk factors for the development of HCC. The ten-year cumulative incidence rates of HCC in the patients with AFP levels of <6, 6–20, and ≥20 ng/mL at entry were 6.0, 24.6, and 47.3%, respectively.
Conclusions Not only high (>20 ng/mL), but also even slightly elevated (6–20 ng/mL) AFP levels, could serve as a risk factor for HCC to complement the fibrosis stage. In contrast, AFP levels <6 ng/mL indicate a low risk of HCC development in patients infected with HCV, irrespective of the fibrosis stage.

Keywords Alpha-fetoprotein · Hepatitis C virus · Hepatocellular carcinoma

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Introduction

Worldwide, an estimated 170 million people are persistently infected with hepatitis C virus (HCV) [1, 2], and they are at high risk of developing hepatocellular carcinoma (HCC) [1, 3–5]. Several factors have been identified that increase the risk of HCC, including, age, male gender, and alcohol intake, as well as cirrhosis and the duration of infection [3, 5]. Of these factors, the stage of liver fibrosis parallels the risk for HCV-associated HCC. The annual incidence of HCC in patients with HCV-related cirrhosis ranges from 1 to 7% [6, 7]. Although liver biopsy is the gold standard for the assessment of hepatic fibrosis [8, 9], it is too invasive a procedure to be acceptable as a routine test [10, 11]. In place of liver biopsy, the platelet count is used to estimate the degree of fibrosis [12–14], and low platelet counts have been shown to be a risk factor for the development of HCC in cirrhotic patients [13, 15, 16]. In this study, we tried to identify noninvasive markers for predicting the development of HCC in a large cohort of

patients with chronic HCV infection during a long observation period.

Patients and methods

Study design

Between January 1992 and December 2003, 832 patients were identified who were positive for both anti-HCV, by a second or third-generation enzyme-linked immunosorbent assay (ELISA), and for HCV RNA by polymerase chain reaction (PCR). These patients underwent liver biopsy guided by ultrasonography (US) at the National Nagasaki Medical Center. Of the 832 patients, 125 (15.0%) were excluded according to the following criteria: (1) positive for hepatitis B surface antigen (HBsAg) ($n = 12$); (2) heavy habitual drinking defined as an average daily consumption of >100 g ethanol ($n = 26$); (3) presence of autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension ($n = 8$); (4) positive antinuclear antibody (defined as a titer of >320×) without a diagnosis of AIH ($n = 8$); or (5) a short follow-up period (<180 days) ($n = 71$). The remaining 707 patients were analyzed retrospectively for the incidence of HCC. Their medical histories had been recorded, with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of US-guided liver biopsy at regular intervals. Complete blood cell counts and biochemical tests were performed, using automated procedures, at the clinical pathology laboratories of the National Nagasaki Medical Center. Iinformed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a-priori approval by the institution's human research committee.

Staging of hepatic fibrosis

Liver biopsy was taken by fine-needle aspiration (18G or 16G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al. [17].

HCV RNA, HCV core antigen, and HCV genotypes

HCV RNA was determined by reverse transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the lumispot EIKEN HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified as low or high with the cutoff at 1,000 fmol/L [18, 19]. Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan) [20, 21].

Interferon therapy

During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (PEG)-IFN monotherapy, combination therapy with IFN and ribavirin, or PEG-IFN and ribavirin. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA by the end of treatment that persisted for longer than 6 months thereafter, while failure in meeting these criteria was judged as non-SVR. There was no relapse of viremia after 6 months among SVR patients.

Diagnosis of hepatocellular carcinoma

Patients were followed up with hematological and biochemical tests at intervals of 1–12 months. Liver imaging was performed by US at 6- to 12-month intervals in most patients at fibrosis stages F0–F2, while computed tomography (CT), magnetic resonance imaging (MRI), or US was performed at 3- to 6-month intervals in patients at fibrosis stages F3 and F4. HCC was diagnosed by typical vascular patterns on CT, MRI, or angiography, or by fine-needle biopsy of space-occupying lesions detected in the liver.

Statistical analysis

Continuous variables [platelet counts, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha-fetoprotein (AFP), HCV core antigen] were dichotomized with respect to the median value or clinically meaningful values in a multivariate analysis. To estimate the cumulative risk of developing HCC, the Kaplan–Meier method and the log-rank test were used. Cox proportional hazards regression analysis was performed to evaluate risk factors for HCC. Analysis was performed by Bonferroni's correction and data analysis was performed with SPSS ver. 11.0 (SPSS, Chicago, IL, USA).

Results

Characteristics at enrollment

Table 1 lists the characteristics of the 707 patients at enrollment. The median age was 57.0 years; 120 (17.0%)

Table 1 Demographic, clinical, and virological characteristics of 707 patients persistently infected with hepatitis C virus (HCV)

Age (years)	57.0 (19–79)
Male	351 (49.6%)
Observation period (years)	8.2 ± 4.4[a]
Interferon therapy	373 (52.8%)
Habitual alcohol intake	135 (19.1%)
Fibrosis stage	
F0/F1	273 (38.6%)
F2	193 (27.3%)
F3	121 (17.1%)
F4	120 (17.0%)
Platelet count ($\times 10^3/mm^3$)	156 (30–391)
Albumin (g/dL)	4.2 (2.7–5.3)
Total bilirubin (mg/dL)	0.7 (0.1–2.5)
Aspartate aminotransferase (AST; IU/L)	53 (11–422)
Alanine aminotransferase (ALT; IU/L)	82 (1–1,057)
Alpha-fetoprotein (AFP; ng/mL)	6 (1–510)
HCV core antigen	
≥1,000 fmol/L	539 (76.2%)
HCV genotype	
1b	510 (72.1%)
2a/2b	195 (27.6%)
Unknown	2 (0.3%)

Values are medians with ranges in parentheses, or means with SD in parentheses

[a] Mean ± SD

patients were diagnosed histologically with liver cirrhosis (fibrosis stage: F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The average follow-up period was 8.2 years. The patients were classified into three categories by the level of AFP; 350 patients (49.5%) had AFP levels of <6 ng/mL, 254 (35.9%) had levels between 6 and 20 ng/mL, and the remaining 103 (14.6%) had levels of ≥20 ng/mL.

IFN therapy and IFN response

Of the 120 patients with cirrhosis (fibrosis stage F4), 46 (38.3%) received IFN while the remaining 74 (61.7%) did not. The proportions of IFN-treated patients showing an SVR were 40.8% (56/137) in patients with F1; 37.6% (44/117) in those with F2; 32.8% (24/73) in those with F3; and 32.6% (15/46) in those with F4.

Risk factors for HCC

Cox regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, and biochemical as well as virological parameters. The following factors were identified as showing an increased risk for HCC by the univariate analysis: age; IFN therapy; fibrosis stage; platelet count; albumin; AST, ALT, and AFP levels; and HCV genotype (Table 2). Multivariate analysis was performed on these factors (Table 3), and the following were identified as independent risk factors: fibrosis stage (F4), AFP (6–20 and ≥20 ng/mL), age (≥57 years), and IFN therapy (SVR).

Development of HCC

During the follow-up period, HCC developed in 110 (15.6%) patients. Of the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Among the patients with HCC, only eight (7.2%) had AFP <6 ng/mL at the time of diagnosis of HCC. Figure 1 shows Kaplan–Meier estimates of the cumulative risk of HCC with respect to fibrosis stage at entry. The 10-year cumulative incidence rates of HCC for stages F0/F1, F2, F3, and F4 were 2.5, 12.8, 19.3, and 55.9%, respectively.

There were significant differences in cumulative incidence rates among the three groups of patients with different AFP levels. The 10-year cumulative risk of HCC was 6.0% in the 350 patients with AFP <6 ng/mL at the study entry, 24.6% in the 254 patients with AFP 6–20 ng/mL, and 47.3% in the 103 patients with AFP ≥20 ng/mL ($P < 0.001$) (Fig. 2). Of the 350 patients with AFP <6 ng/mL, 21 eventually developed HCC during the observation period. Fourteen of these 21 patients were ≥57 years old and 10 had fibrosis stage F3 or F4. In remarkable contrast, HCC ultimately developed in 84.5% of the patients with AFP ≥20 ng/mL.

The 10-year cumulative incidence rates of HCC were 3.1% in patients with SVR to IFN, 14.6% in patients with non-SVR, and 29.5% in the patients without IFN therapy (Fig. 3). Of the 139 patients with SVR, three (2.2%) eventually developed HCC during the observation period. These three patients had advanced fibrosis stages at the study entry (1 with F3 and 2 with F4). Figure 4 shows the cumulative incidence of HCC in the patients with different AFP levels, stratified by the fibrosis stage. In the patients with fibrosis stage F4, there were significant differences in HCC incidence between those with AFP levels of <6 and those with levels of ≥20 ng/mL.

Figure 5 shows the proportions of patients with different AFP levels stratified by the fibrosis stage. The proportion of patients with AFP <6 ng/mL decreased with the advance of fibrosis stage, and conversely, the proportion of patients with AFP ≥20 ng/mL increased with the advance of fibrosis stage. There was a strong correlation between AFP levels and the fibrosis stage.

Table 2 Factors increasing the risk for hepatocellular carcinoma (HCC), determined by univariate analysis

Features	Hazard ratio	*P* value
Age		
<57 years	1	
≥57 years	3.889	<0.001
Sex		
Female	1	
Male	1.146	0.475
Alcohol intake		
None	1	
Habitual	1.012	0.962
Interferon therapy		
None	1	
Non-SVR	0.523	0.002
SVR	0.063	<0.001
Fibrosis stage		
F0/F1	1	
F2	1.863	0.096
F3	3.985	<0.001
F4	13.045	<0.001
Platelet count		
$\geq 150 \times 10^3/mm^3$	1	
$<150 \times 10^3/mm^3$	4.644	<0.001
Albumin		
≥4.2 g/dL	1	
<4.2 g/dL	2.952	<0.001
Total bilirubin		
<0.7 mg/dL	1	
≥0.7 mg/dL	1.438	0.065
AST		
<53 IU/L	1	
≥53 IU/L	2.501	<0.001
ALT		
<82 IU/L	1	
≥82 IU/L	1.514	0.035
AFP		
<6 ng/mL	1	
6–20 ng/mL	4.628	<0.001
≥20 ng/mL	10.335	<0.001
HCV core antigen		
<1,000 fmol/L	1	
≥1,000 fmol/L	1.112	0.645
HCV genotype		
2a/2b	1	
1b	1.730	0.027

SVR sustained virological response

Table 3 Factors increasing the risk for HCC, determined by multivariate analysis

Features	Hazard ratio (95% CI)	*P* value
Fibrosis stage		
F0/F1	1	
F2	1.030 (0.471–2.253)	0.942
F3	1.682 (0.632–3.713)	0.198
F4	3.957 (1.861–8.411)	<0.001
AFP		
<6 ng/mL	1	
6–20 ng/mL	1.942 (1.066–3.538)	0.030
≥20 ng/mL	3.884 (2.014–7.433)	<0.001
Age		
<57 years	1	
≥57 years	2.026 (1.261–3.255)	0.004
Interferon therapy		
None	1	
Non-SVR	0.704 (0.453–1.094)	0.119
SVR	0.099 (0.029–0.334)	<0.001

CI confidence interval

Discussion

In the present study, four variables were identified as risk factors for HCC in patients with chronic HCV infection: fibrosis stage, AFP level, age, and IFN therapy. Previous reviews have analyzed risk factors for the development of HCC [3, 22–25]. Yoshida et al. [6] have reported that the annual incidence increases with the stage of liver fibrosis, from 0.5% in patients with stage F0 or F1 to 7.9% in patients with stage F4 (cirrhosis). In our study, the cumulative incidence of HCC increased along with the advance of fibrosis stage. AFP is used as a serological marker of HCC, and is employed in combination with US for screening HCC [3]. Several reports have shown an elevated AFP level as a risk factor for the development of HCC among patients infected with HCV [16, 25–32]. Most of the studied patients had cirrhosis that was not definitely diagnosed by clinical symptoms and ultrasonographic findings. There have been few studies on patients with chronic hepatitis C, in addition to those with cirrhosis [27]. Thus, it has been unclear whether elevated AFP levels are a risk factor for the development of HCC in patients infected with HCV. Against this background, we were prompted to analyze the utility of AFP as a risk factor for the development of HCC in patients who had been histologically diagnosed by US-guided liver biopsy. In the present study,

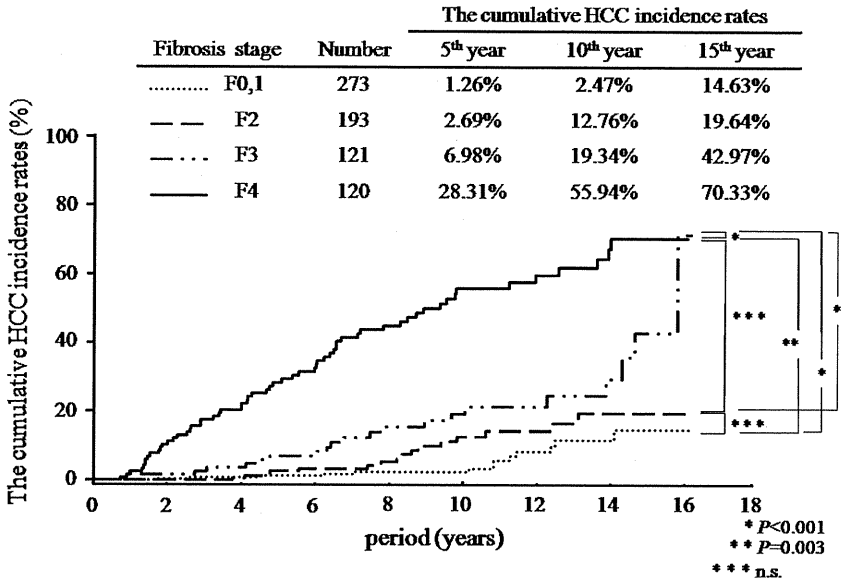

Fig. 1 Cumulative incidence of hepatocellular carcinoma (*HCC*) according to the fibrosis stage

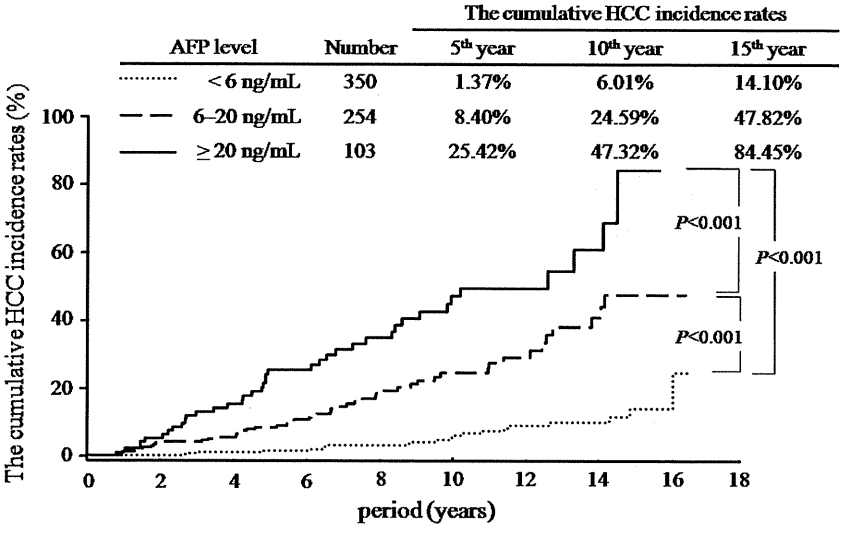

Fig. 2 Cumulative incidence of HCC according to alpha-fetoprotein (*AFP*) levels

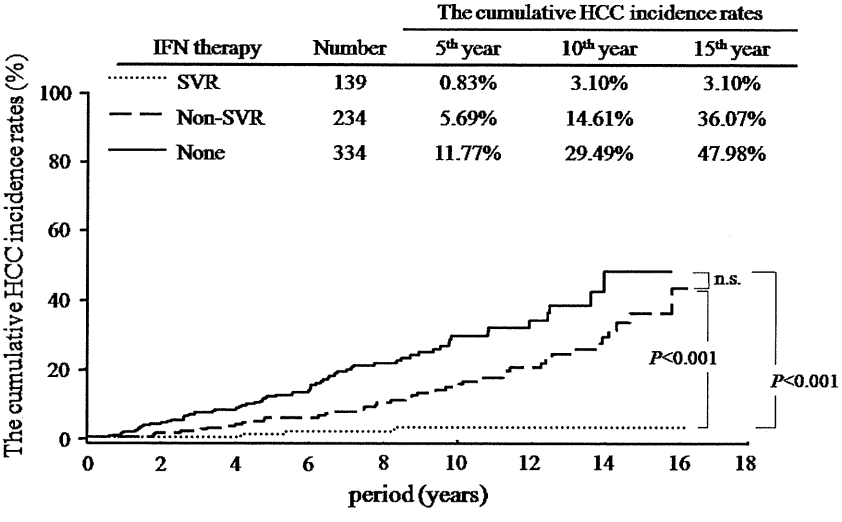

Fig. 3 Cumulative incidence of HCC according to interferon (*IFN*) therapy. *SVR* Sustained virological response

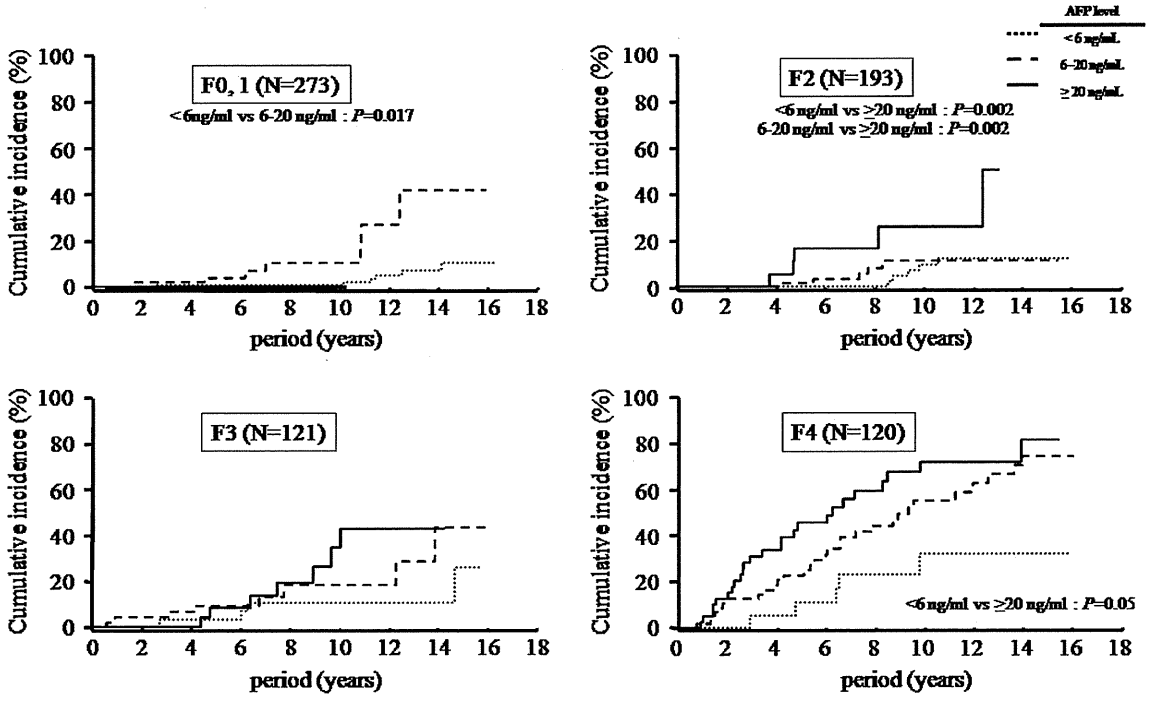

Fig. 4 Cumulative incidence of HCC according to AFP levels, stratified by the fibrosis stage

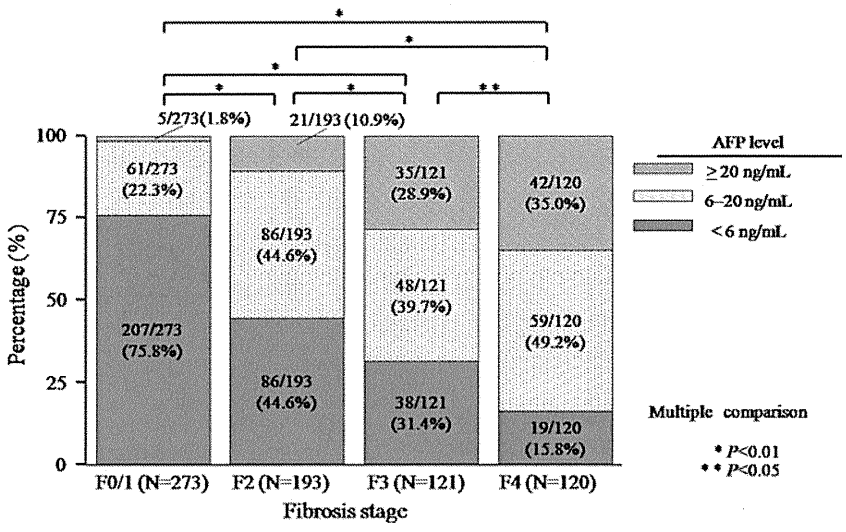

Fig. 5 Proportions of patients with three different AFP levels (<6 ng/mL, 6–20 ng/mL, and ≥20 ng/mL) at different fibrosis stages

among patients infected with HCV, including not only those with cirrhosis but also those with chronic hepatitis, we found AFP levels to be a dependable risk factor for HCC, in addition to the fibrosis stage. Of particular note, not only the patients with high AFP levels (≥20 ng/mL) but also those with even slightly elevated AFP levels (between 6 and 20 ng/mL) had increased risks for the development of HCC. In the patients in this study, the median AFP level was 6 ng/mL. It deviated slightly from serum levels of AFP in healthy adults that have been reported to range from 0.1 to 5.8 ng/mL [33]. Hence, we performed analyses by setting various AFP cutoff levels for evaluating their performance as risk factors. However, there were no significant differences in the analysis with the use of AFP cutoff levels exceeding 7 ng/mL. On the basis of these observations, an AFP cutoff level of 6 ng/mL was adopted in this study. In previous reports, AFP levels were associated with advanced fibrosis stage in patients infected with HCV in the absence of HCC [34–38]. In the present study, AFP levels were elevated in parallel with advanced fibrosis stages and correlated well with the fibrosis stage. As the patients with even slightly elevated AFP levels, between 6 and 20 ng/mL, had moderately advanced liver fibrosis stages, these AFP levels could