progression of liver damage for recurrent hepatitis C after liver transplantation. Unfortunately, this was not a randomized control study, and only a small number of patients were eligible for research. Therefore, we recommend further work to more fully explore the effects of this treatment and to improve the outcomes for patients who do not achieve SVR.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Decrease in alpha-fetoprotein levels predicts reduced incidence of hepatocellular carcinoma in patients with hepatitis C virus infection receiving interferon therapy: a single center study

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Abstract
Background Increasing evidence suggests the efficacy of interferon therapy for hepatitis C in reducing the risk of hepatocellular carcinoma (HCC). The aim of this study was to identify predictive markers for the risk of HCC incidence in chronic hepatitis C patients receiving interferon therapy.
Methods A total of 382 patients were treated with standard interferon or pegylated interferon in combination with ribavirin for chronic hepatitis C in a single center and evaluated for variables predictive of HCC incidence.
Results Incidence rates of HCC after interferon therapy were 6.6% at 5 years and 13.4% at 8 years. Non-sustained virological response (non-SVR) to antiviral therapy was an independent predictor for incidence of HCC in the total study population. Among 197 non-SVR patients, independent predictive factors were an average alpha-fetoprotein (AFP) integration value ≥10 ng/mL and male gender. Even in patients whose AFP levels before interferon therapy were ≥10 ng/mL, reduction of average AFP integration value to <10 ng/mL by treatment was strongly associated with a reduced incidence of HCC. This was significant compared to patients with average AFP integration values of ≥10 ng/mL ($P = 0.009$).
Conclusions Achieving sustained virological response (SVR) by interferon therapy reduces the incidence of HCC in hepatitis C patients treated with interferon. Among non-SVR patients, a decrease in the AFP integration value by interferon therapy closely correlates with reduced risk of HCC incidence after treatment.

Keywords Alpha-fetoprotein · Hepatocellular carcinoma · Hepatitis C · Interferon

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Introduction

Hepatitis C virus (HCV) infection is a predominant cause of liver cirrhosis and hepatocellular carcinoma (HCC) in many countries, including Japan, the United States, and countries of Western Europe [1–5]. The annual incidence of HCC in patients with HCV-related cirrhosis ranged from 1 to 8% [6–9]. Even in the absence of liver cirrhosis, patients with chronic hepatitis caused by HCV infection are at a high risk of developing HCC. Indeed, a large-scale Japanese cohort study showed that the annual incidence of HCC is 0.5% among patients with stage F0 or F1 fibrosis and 2.0, 5.3, and 7.9% among those with F2, F3, and F4 fibrosis, respectively [9]. Periodic surveillance is recommended to detect HCC as early as possible in patients with HCV-related chronic liver disease; however, this may not be cost-effective. For patients with chronic hepatitis C, more effective detection and prevention of HCC is being sought by two important routes: (1) the attempt to discover noninvasive predictive markers and (2) development of treatment strategies to reduce the risk of HCC. There have been several attempts to discover non-invasive markers capable of predicting the risk of HCC incidence in patients with chronic hepatitis C [6, 10]. For example, a cohort

derived from the Hepatitis C Antiviral Long-term Treatment Against Cirrhosis (HALT-C) Trial identified older age, African American race, lower platelet count, higher alkaline phosphatase, and esophageal varices as risk factors for HCC [11].

There have also been a number of studies to evaluate the effect of anti-viral treatment of chronic hepatitis C on the incidence of HCC [12–19]. The results were summarized in a meta-analysis, which concluded that the effect of interferon on risk of HCC is mainly apparent in patients achieving a sustained virological response (SVR) to interferon therapy [13]. In addition, a number of studies have suggested the incidence of HCC is reduced in treated patients compared to historical controls [12, 15, 16, 19]. However, the recent HALT-C randomized control trial revealed that long-term pegylated interferon therapy does not reduce the incidence of HCC among patients with advanced hepatitis C who do not achieve SVRs. Reduction in the risk of HCC by maintenance therapy was shown only in patients with cirrhosis [14, 17]. These controversial results suggest that interferon therapy reduces the risk of HCC only in a group of patients with HCV-related chronic liver disease. Thus, it is important to evaluate the risk of HCC development in hepatitis C patients receiving interferon therapy and it will be clinically useful to discover markers distinguishing high- and low-risk groups.

Serum alpha-fetoprotein (AFP) has been widely used as a diagnostic marker of HCC [20–22]. However, elevation of serum AFP levels is often found in non-neoplastic liver diseases without evidence of HCC, including acute liver injury and chronic viral hepatitis [23–27], especially among patients with advanced chronic hepatitis C [28]. An increase of AFP after liver damage is interpreted as a sign of dedifferentiated hepatic regeneration [27]. There have been some reports that AFP is a significant predictor of HCC in patients with chronic hepatitis C [4, 5, 29]. In addition, it has recently been shown that AFP levels decrease in response to interferon administration in patients with chronic hepatitis C [30, 31], and that long-term interferon therapy for aged patients with chronic HCV infection is effective in decreasing serum AFP levels and preventing hepatocarcinogenesis [32, 33]. However, little is known about the relationship between changes in serum AFP level over time during interferon therapy and the development of HCC.

The aim of this large single center study was to identify predictive markers for the risk of HCC development in patients receiving interferon therapy for chronic hepatitis C. For this purpose, patients treated with standard or pegylated interferon, in combination with ribavirin, for chronic hepatitis C were enrolled and subjected to scheduled periodic surveillance for HCC and a number of potential predictive markers, including AFP and alanine aminotransferase (ALT) integration values, at a single center.

Materials and methods

Patients

Between January 2002 and April 2010, 528 patients with chronic hepatitis C received combination therapy with standard interferon and ribavirin ($n = 84$) or pegylated interferon and ribavirin ($n = 444$) at Osaka Red Cross Hospital. Eligibility criteria for treatment were positivity for serum HCV RNA and histological evidence of chronic hepatitis C ($n = 427/444$; 80.9%), or positivity for serum HCV RNA, liver enzyme levels greater than the normal upper limit, and an ultrasound image demonstrating chronic liver damage ($n = 101/444$; 19.1%). Exclusion criteria for treatment were as follows: neutrophil count <750 cells/μL, platelet count <50,000 cells/μL, hemoglobin level ≤9.0 g/dL, and renal insufficiency (serum creatinine levels >2 mg/dL).

Of 528 patients who received interferon therapy for chronic hepatitis C, 146 were excluded from this study for the following reasons: follow-up <24 weeks after the termination of the interferon therapy ($n = 122$), previously treated for HCC ($n = 22$), or occurrence of HCC during or within 24 weeks after treatment ($n = 2$). Therefore, 382 patients were enrolled for the study and were retrospectively analyzed.

To detect early-stage HCC, ultrasonography, dynamic contrast enhanced computed tomography (CT), dynamic contrast enhanced magnetic resonance imaging (MRI), and/or measurement of tumor markers (including AFP) were performed for all patients at least every 6 months. HCC was diagnosed radiologically as liver tumors displaying arterial hypervascularity and venous or delayed phase washout by dynamic contrast enhanced CT or MRI.

The study protocol was approved by the Ethics Committee at Osaka Red Cross Hospital and performed in compliance with the Helsinki Declaration.

Treatment protocol and definition of responses to treatment

The basic treatment protocol for patients with chronic hepatitis C consisted of 6 mega units of interferon-α-2b 3 times a week or 1.5 μg/kg of pegylated interferon α-2b once a week, combined with ribavirin at an oral dosage of 600–1000 mg/day. Duration of the treatment was 48–72 weeks for those with HCV genotype 1 and serum HCV RNA titer of >5 log IU/mL, and 24 weeks for all other patients.

Patients who were negative for serum HCV RNA for >6 months after completion of interferon therapy were defined as showing an SVR. Patients whose serum ALT levels decreased to the normal range and remained normal for >6 months after the termination of interferon therapy were defined as showing a sustained biochemical response (SBR).

Patients who did not achieve SVR received ursodeoxycholic acid and/or glycyrrhizin containing preparation (Stronger Neo-Minophagen C), when serum ALT levels were higher than the upper limit of normal.

Virological assays

HCV genotype was determined by polymerase chain reaction (PCR) amplification of the core region of the HCV genome using genotype-specific PCR primers [34]. Serum HCV RNA load was evaluated once a month during and 24 weeks after treatment using a PCR assay (Cobas Amplicor HCV Monitor, Roche Molecular Systems, Pleasanton, CA, USA).

Measurement of AFP and calculation of average integration value

AFP was measured in serum samples obtained from each patient at intervals of 1–3 months. The median number of examinations was 15 (range 1–70) in each patient. Serum AFP levels were determined by enzyme-linked immunosorbent assay, which was performed using a commercially available kit (ELISA-AFP, International Reagents, Kobe, Japan). Integration values of AFP and ALT were calculated as described in previous reports [35]. For example, the integration value of AFP was calculated as follows, $(y_0 + y_1) \times x_1/2 + (y_1 + y_2) \times x_2/2 + (y_2 + y_3) \times x_3/2 + (y_3 + y_4) \times x_4/2 + (y_4 + y_5) \times x_5/2 + (y_5 + y_6) \times x_6/2$, i.e., the area of each trapezoid representing an AFP value was measured the sum of the resulting values used to calculate the integration value (Fig. 1). The average integration value was obtained by dividing the integration value by the observation period from initiation of the treatment.

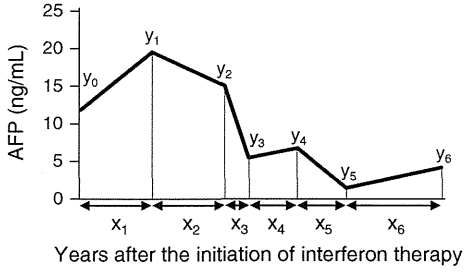

Fig. 1 Example plot of data used for calculation of average integration value of alpha-fetoprotein (AFP)

Statistical analysis

The Kaplan–Meier method was used to estimate the rates of development of HCC in patients after interferon therapy. Log-rank tests were used to evaluate the effects of predictive factors on incidence of HCC. Significance was defined as $P < 0.05$. Multivariate Cox regression analysis using the stepwise method was used to evaluate the association between HCC incidence and patient characteristics, and to estimate hazard ratio (HR) with a 95% confidence interval (CI). A P value of 0.1 was used for variable selection and was regarded as statistically significant. SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

Results

Characteristics of patients and incidence of HCC

This study included 382 patients treated for chronic hepatitis C with standard interferon or pegylated interferon in combination with ribavirin. Baseline clinical and virological characteristics of patients included in the study are summarized in Table 1. The median age of the patients at the outset of therapy was 59.0 years (range 18–81 years) and the median follow-up period was 4.1 years (range 0.1–8.4 years). The majority of patients were infected with HCV genotype 1b ($n = 229$; 60%), and median serum HCV RNA load was 6.1 log IU/mL (range 2.3–7.3 log IU/mL). Baseline (before interferon therapy) median serum AFP level was 6.9 ng/mL (range 1.6–478.3 ng/mL).

During follow-up, 23 patients (4.9%) developed HCC. The cumulative incidences of HCC, which was estimated using the Kaplan–Meier method, were 3.1, 6.6, and 13.4% at 3, 5, and 8 years, respectively (Fig. 2).

Predictive factors for incidence of HCC in all patients

Predictive factors for incidence of HCC in all 382 patients were analyzed using log-rank tests (Table 2). Univariate analysis showed that age ≥70 years ($P = 0.040$), non-SVR ($P < 0.0001$), non-SBR ($P = 0.027$), average ALT integration value ≥40 IU/L ($P = 0.001$), baseline AFP ≥10 ng/mL ($P = 0.005$), average AFP integration value ≥10 ng/mL ($P < 0.0001$), and baseline platelet count <150,000 platelets/μL ($P = 0.001$) were all significantly associated with the incidence of HCC. After multivariate analysis, the only variable remaining in the model was non-SVR (HR 8.413, 95% CI 1.068–66.300, $P = 0.043$).

Table 1 Characteristics of 382 patients with hepatitis C treated with interferon therapy in this study

Age (years)	59.0 (18–81)
[a]Males/females	192/190
Observation period (years)	4.1 (0.1–8.4)
[a]IFN + RBV/PEG-IFN + RBV	69/313
HCV genotype 1/2/unclassified	229/57/96
HCV RNA (log IU/mL)	6.1 (2.3–7.3)
White blood cell count (/μL)	4950 (2050–9970)
Hemoglobin (g/dL)	14.0 (10.3–18.8)
Platelet (10^4/μL)	15.0 (5.3–36.4)
AST (IU/L)	56 (17–244)
ALT (IU/L)	67 (16–416)
Bilirubin (mg/dL)	0.8 (0.3–2.4)
AFP (ng/mL)	6.9 (1.6–478.3)

Qualitative variables ([a]) are shown in number, and quantitative variables expressed as median (range)

IFN interferon, *RBV* ribavirin, *PEG-IFN* pegylated interferon, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AFP* alpha-fetoprotein

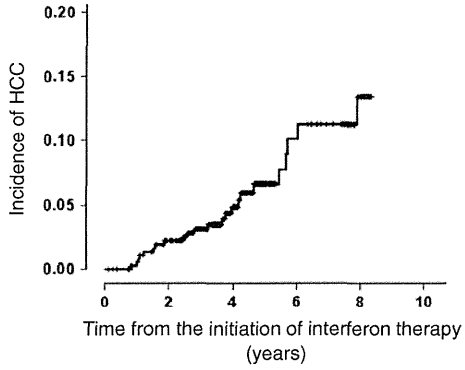

Fig. 2 Incidence of hepatocellular carcinoma (HCC) in 382 patients with hepatitis C who received interferon therapy, estimated using the Kaplan–Meier method

Further, although patients with average AFP integration values ≥10 ng/mL also appeared to have an increased risk of HCC, the difference did not reach statistical significance in the multivariate analysis ($P = 0.050$) (Table 3).

Predictive factors for incidence of HCC in non-SVR patients

Because non-SVR was the only predictive factor across the entire study cohort, to clarify predictive factors for incidence of HCC within this group, the same variables were further analyzed in non-SVR patients alone. By univariate analysis, average AFP integration value ≥10 ng/mL ($P = 0.019$) and baseline platelet count <150,000 ($P = 0.0022$) (Table 2) were again identified as significant predictive factors for incidence of HCC. In addition, male gender was significantly associated with incidence of HCC in non-SVR patients ($P = 0.022$). Multivariate analysis, however, indicated that only two variables were independently associated with incidence of HCC in non-SVR patients: average AFP integration value ≥10 ng/mL (HR 4.039, 95% CI 1.570–10.392, $P = 0.004$), and male gender

Table 2 Univariate analysis of predictive factors for incidence of hepatocellular carcinoma in all 382 and 197 non-SVR patients

Factors	All ($n = 382$)			Non-SVR ($n = 197$)		
	No.	Incidence of HCC ($n = 23$)		No.	Incidence of HCC ($n = 22$)	
		No. (%)	*P* value[a]		No. (%)	*P* value[a]
Age (years)						
<70	359	19 (5)	0.040	182	18 (10)	0.089
≥70	23	4 (17)		15	4 (27)	
Sex						
Female	190	8 (4)	0.125	111	8 (7)	0.022
Male	192	15 (8)		86	14 (16)	
HCV genotype						
1	229	12 (5)	0.452	137	12 (9)	0.796
Non-1	57	1 (2)		10	1 (10)	
Virological response						
SVR	185	1 (1)	<0.0001			
Non-SVR	197	22 (11)				
Biochemical response						
SBR	282	12 (4)	0.027	102	11 (11)	0.857
Non-SBR	86	11 (13)		81	11 (14)	
ALT before IFN therapy						
<40	79	2 (3)	0.274	39	2 (5)	0.319
≥40	301	21 (7)		158	20 (13)	
ALT integration value						
<40	238	6 (3)	0.001	79	5 (6)	0.153
≥40	142	17 (12)		118	17 (14)	
AFP before IFN therapy						
<10	230	7 (3)	0.005	102	7 (7)	0.124
≥10	116	14 (12)		75	13 (17)	
AFP integration value						
<10	258	8 (3)	<0.0001	115	8 (6)	0.019
≥10	63	12 (19)		53	11 (21)	
Platelet before IFN therapy						
<150,000	187	20 (11)	0.001	121	19 (16)	0.022
≥150,000	194	3 (2)		76	3 (4)	

[a] Log-rank test

SVR sustained virological response, *SBR* sustained biochemical response, *ALT* alanine aminotransferase, *IFN* interferon, *AFP* alpha-fetoprotein

Table 3 Multivariate analysis of the predictive factors for incidence of hepatocellular carcinoma in all 382 patients

Factors	Hazard ratio	95% CI	*P* value
Virological response			
SVR	1		
Non-SVR	8.413	1.068–66.300	0.043
AFP integration value			
<10	1		
≥10	2.580	0.999–6.659	0.050

SVR sustained virological response, *IFN* interferon, *AFP* alpha-fetoprotein

Table 4 Multivariate analysis of predictive factors for incidence of hepatocellular carcinoma in 197 non-SVR patients

Factors	Hazard ratio	95% CI	*P* value
AFP integration value			
<10	1		
≥10	4.039	1.570–10.392	0.004
Sex			
Female	1		
Male	3.636	1.383–9.563	0.009

AFP alpha-fetoprotein

(HR 3.636, 95% CI 1.383–9.563, $P = 0.009$) (Table 4). There was no significant difference in other variables including those identified as predictive factors in the entire study population (i.e., age, non-SBR, ALT integration value, AFP before interferon therapy) (Table 2).

AFP integration value as a predictive factor for HCC

Further analysis focused on the AFP integration value as this was the strongest predictive factor for incidence of HCC in non-SVR patients. Of the 382 patients, both baseline and AFP integration values were available for 321. These were divided into four groups: (1) AFP "low–low," (2) AFP "low–high," (3) AFP "high–low," and (4) AFP "high–high," for baseline AFP-average AFP integration values, respectively, where "high" is ≥10 ng/mL and "low" is <10 ng/mL. As shown in Fig. 3a, of the 321 patients, 211 (65.7%) showed baseline AFP levels <10 ng/mL. Of these 211, 207 (98%), were in the AFP low–low group, and only four in the AFP low–high groups. Baseline characteristics, including age, gender, serum HCV-RNA, aspartate aminotransferase (AST), ALT, bilirubin, white blood cell, hemoglobin, platelet, observation periods, and number of times of AFP measurement, were not different between AFP high–low group and high–high group. However, AFP-low group, which is a combination of the low–high and low–low groups, showed significantly lower AST level ($P < 0.00001$), lower ALT level ($P < 0.00001$), higher platelet count ($P < 0.00001$), shorter observation period ($P = 0.01448$), and fewer number of times of AFP examination ($P = 0.00035$), compared to both AFP high–high and AFP high–low group. Six patients (2.8%) with baseline AFP levels <10 ng/mL developed HCC in the follow-up period and none of these patients were among the four low–high group patients. Even in patients with high baseline AFP levels, incidence of HCC was only 3.9% among the AFP high–low group (2 of 51 patients). In contrast, 20.3% of patients in the AFP high–high group developed HCC during the follow-up period.

The incidence rate of HCC in three patient groups, "AFP-low" (a combination of the "low–high" and "low–low" groups),"high–low," and "high–high", was estimated using the Kaplan–Meier method and compared using log-rank tests (Fig. 3b). The rate of HCC incidence was significantly higher in the AFP high–high group compared to both the AFP high–low group and patients with low baseline AFP levels ($P = 0.009$ and 0.001, respectively). There was no significant difference between patients with low baseline AFP levels and the AFP high–low group. The 7-year incidence rate of HCC was 32.3% in the AFP high–high group, compared to only 6.6% in the AFP high–low group, and 8.1% in all patients with low pre-treatment levels.

Discussion

It is well recognized that the most effective strategy for the prevention of HCC development in patients with chronic hepatitis C is likely to be the complete elimination of the HCV infection accompanied by the resultant normalization of liver function [7, 12, 13, 15, 16, 19]. Indeed, we confirmed here that non-SVR is the most significant predictive factor for incidence of HCC in patients receiving interferon therapy for chronic hepatitis C. However, it should be noted that the risk of HCC, even in non-SVR patients, differs between individuals. In the current study, we identified AFP integration value and male gender as independent risk factors for incidence of HCC in non-SVR patients. The incidence of HCC was significantly reduced in individuals with average AFP integration values < 10 ng/mL after interferon therapy, which suggests that the decrease of AFP by interferon therapy lowers the risk of developing HCC. Indeed, even where patients had high baseline AFP levels, incidence of HCC was reduced when the AFP integration value decreased after interferon therapy. Thus, our current findings identify AFP integration value as a useful predictive marker of HCC development in non-SVR patients.

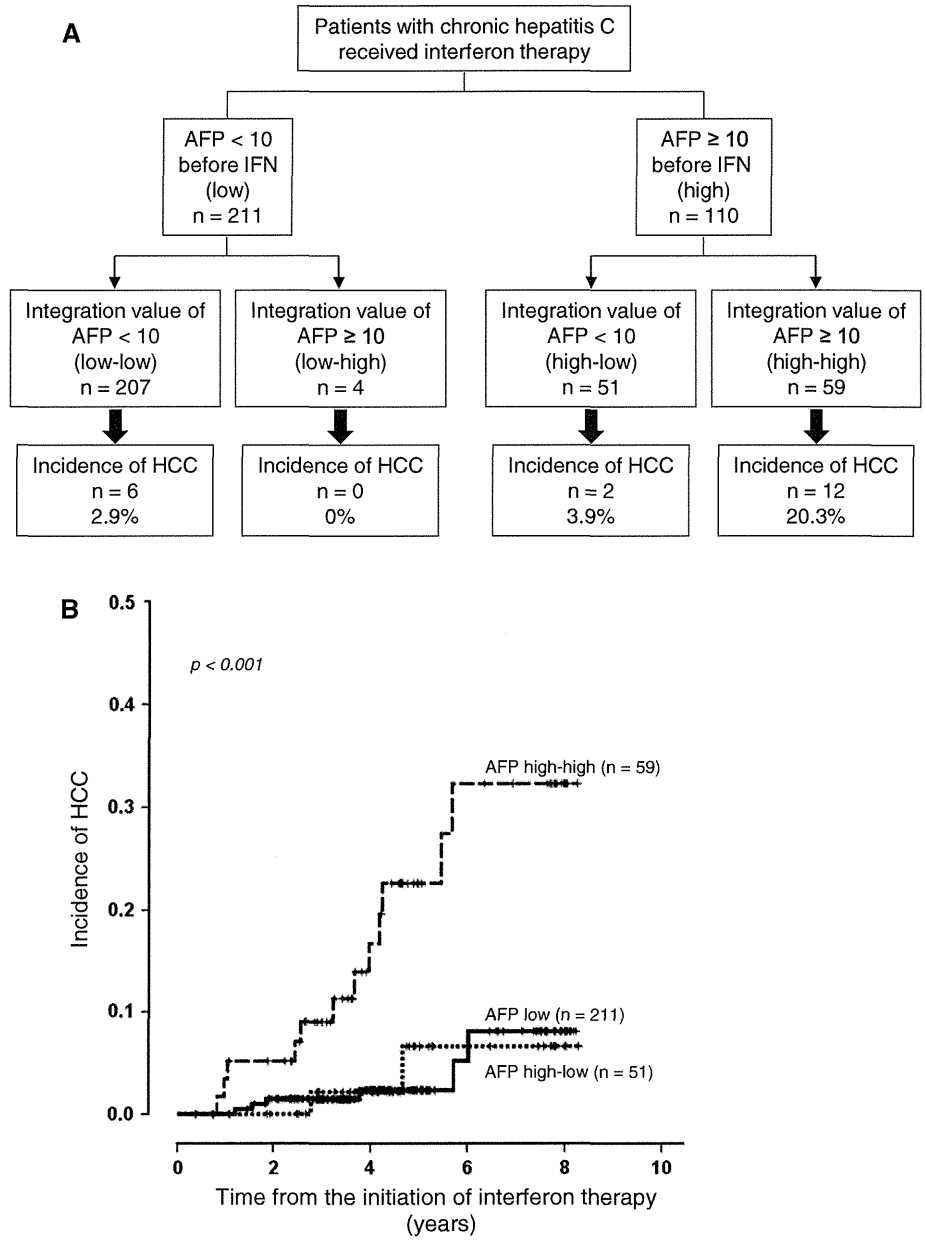

Fig. 3 AFP integration value as a predictive factor for HCC. **a** *Flow diagram* showing the number of patients (*n*) classified by baseline alpha-fetoprotein (AFP) levels before interferon (IFN) therapy and average AFP integration value, and the incidence of hepatocellular carcinoma (HCC) of each group. **b** Kaplan–Meier estimates of the incidence of HCC. *Solid line* AFP-low group (AFP levels before interferon therapy <10 ng/mL); *dotted line* AFP high–low group (baseline AFP levels ≥10 ng/mL, average AFP integration value <10 ng/mL); *dashed line* AFP high–high group (both baseline and average AFP integration values ≥10 ng/mL)

Data from several previous studies suggest that the continuous normalization of alanine aminotransferase (ALT) levels by interferon therapy can reduce the risk of HCC development [36–39]. In addition, one recent study suggested that the ALT integration value is a predictive factor for HCC [35]. In contrast to published data (22), our multivariate analysis did not identify the ALT integration value as a significant predictive factor for HCC incidence, although it was identified as significant by univariate analysis in all 382 patients. Since the previous study did not evaluate AFP levels as a factor for prediction of HCC [35], our results indicate that the AFP integration value is superior to that of ALT as a predictive factor for incidence of HCC. We do not know the reason for this result, but it is speculated that significance of AFP as a marker of hepatic regeneration resulted in the more accurate prediction of hepatocarcinogenesis by integration value of AFP than that of ALT.

As AFP is a diagnostic marker for the existence of HCC, high integration value of AFP in the present study might be a result of HCC development. However, we concluded that the high AFP integration values in patients who developed HCC were not caused by a result of existence of HCC, because of the following two reasons. First, the last AFP values before detection of HCC were not the highest level in the follow-up periods in 19 of 23 patients who developed

HCC, suggesting that the AFP was not produced by the developing HCC in these patients. Second, to exclude the influence of the remaining four patients whose last AFP levels were the highest in the follow-up periods, we analyzed the same statistical analysis by using average AFP integration values excluded the last two examinations of AFP before the detection of HCC. The results of the analysis also showed average integration value of AFP as a significant predictive factor for incidence of HCC.

Male gender was also identified as an independent risk factor for HCC in non-SVR patients in this study. Several reports have shown that men are at a higher risk of developing HCC than women [6, 10, 33, 40, 41]. The male gender also appears to be a risk factor for more severe disease and a greater risk of developing cirrhosis in chronic hepatitis C [42]. Although the association of male gender with the risk of HCC is as yet unexplained, hormonal or genetic factors may lead to increased risk for HCC and cirrhosis in men as previously discussed [10].

In conclusion, a decrease in the AFP integration value predicts reduced incidence of HCC in patients with hepatitis C receiving interferon therapy. Further prospective studies with a larger number of patients are required to validate the significance of these findings.

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Conflict of interest The authors declare that they have no conflict of interest.

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exhibit linear clearance, suggesting that larger individuals clear antibodies more rapidly than smaller individuals without compensatory increases in FcRn-mediated salvage. The effects of other demographic factors, such as age, gender, and renal or hepatic function on the pharmacokinetics of antibodies, are controversial and rarely reported.

An additional clearance mechanism is the development of an immune response against the therapeutic antibody (eg, anti-infliximab or anti-adalimumab antibodies). This affects the pharmacokinetics by increasing clearance, and/or impairing binding. Antiglobulin responses are classed as neutralizing or non-neutralizing, depending on their effect on the activity of the antibody. All therapeutic antibodies approved to date have shown some immunogenicity, even in immunosuppressed patients, although relatively short halve-lives for some chimeric antibodies relative to their FcRn-binding affinity may be related to an enhanced immunogenic response in comparison with human antibodies. On the other hand, the route of administration can sometimes affect immunogenicity, with the intravenous route of administration usually being the least immunogenic. Generally, the intramuscular and subcutaneous routes are more immunogenic. Testing immunogenicity of infliximab and adalimumab in the particular disease of interest is paramount also to understand differential therapeutic effects.

Finally, concomitant administration of other agents that may affect antibody clearance by competing for binding sites, reducing receptor density, or affecting immunogenicity must be considered as potentially affecting clearance. In particular, the role of cotreatment with corticosteroids or immunosuppressive drugs should be further clarified. Beside their impact on therapeutic antibodies immunogenicity, an effect on non–immune-mediated clearance has also been suggested.

Although based on available evidence, the magnitude and the relevance of the correlation between trough levels of anti-TNF therapeutic antibodies and clinical response in inflammatory bowel disease remains unclear, particularly for adalimumab, nonresponders or patients losing response very often have low trough levels. All these considerations point to the urgent need to perform a fine and precise pharmacokinetic profiling, and characterize the pharmacokinetics–pharmacodynamics relationship very early in the development of an antibody therapy, and for any new therapeutic indication. This is key for more successful drug development and to provide greater benefit to patients.

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LARGE-SCALE IDENTIFICATION OF EFFECTOR GENES THAT MEDIATE THE TYPE I INTERFERON ANTIVIRAL RESPONSE

Schoggins JW, Wilson SJ, Panis M, et al. **A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 2011;472:481–485.**

Type I interferons (IFNs) are multifaceted cytokines with a central role in the host innate defense against viral infection. Upon viral infection, the host elicits a type I IFN response, mediated essentially by the expression of hundreds of IFN-stimulated genes (ISGs; Annu Rev Immunol 2005;23:307–336). Although it is assumed that these ISGs function together and are required for establishment of the antiviral state, few have been characterized regarding their antiviral potential, target specificity, and mechanisms of action.

To address these issues, in their recently published paper in the *Nature*, researchers at the Rockefeller University in New York conducted a large-scale, fluorescence-activated, cell sorting-based screen of antiviral ISGs. Based on previously published microarray gene expression data, the authors selected 389 human ISGs as candidate effector genes for screening. Each selected ISG was inserted into a bicistronic lentiviral vector co-expressing the red fluorescent protein TagRFP. The generated lentiviral ISG stocks were used for the transduction of target cells. ISG-TagRFP–expressing target cells were then challenged with a panel of green fluorescent protein-expressing viruses, and viral replication was monitored by quantification of the green fluorescent protein-positive cells in the RFP-positive population. This high-throughput screening strategy allowed for sensitive, quantitative, and systematic evaluation of the antiviral effect of individual ISGs against several medically important viruses, including hepatitis C virus (HCV), HIV, yellow fever virus, West Nile virus, Venezuelan equine encephalitis virus, and chikungunya virus.

Through extensive screening and subsequent validation experiments, they demonstrated that each virus tested was susceptible to inhibition by a unique set of ISGs, with the overall ISG inhibition profile overlapping among viruses. Antiviral ISG hits included broad-acting effectors and specific effectors: The former ISGs including *IRF1, RIG-I, MDA5,* and *IFITM3* showed broad effects on multiple viruses, whereas the latter ISGs, including *DDX60, IFI44L, IFI6,* and *MOV10,* had specific effects on limited viral species. Based on the magnitude of their antiviral effect, the ISGs were categorized as strong inhibitors that broadly act on IFN-mediated or other signaling pathways and modest inhibitors that may have more specific effector functions. Gene ontology analysis classified these validated ISG hits into 3 main molecular functions—nucleic acid binding, hydrolase activity, and helicase activity—and 3 main biologic processes—signal transduction, transcrip-

tion initiation, and small molecule transport. A long-standing hypothesis is that the cooperative action of ISGs is prerequisite for an effective type I IFN response (J Leukoc Biol 2001;69:912-920; Virology 1999;258:435-440). In support of this hypothesis, the authors demonstrated that combinational expression of ISGs generally enhanced their antiviral effects. Surprisingly, they also showed that the expression of several ISGs, including *ADAR, FAM46C, LY6E,* and *MCOLN2,* enhanced viral replication, although how these ISGs do so and why they are induced by IFN signaling remain to be elucidated. Finally, to dissect the antiviral mechanisms of ISGs, they examined the stage of the viral life cycle at which the validated ISGs exert their antiviral functions. The assays using HCV pseudoparticles and subgenomic replicons expressing reporter gene revealed that translational block is the primary and common antiviral mechanism of these effector ISGs, highlighting the surprising host strategy that multiple effectors with diverse molecular functions cooperatively suppress HCV by targeting a single HCV life-cycle stage.

Together, the identification and characterization of novel antiviral effectors presented in this study unraveled the heretofore unsuspected diversity of ISG-mediated IFN effector mechanisms.

Comments. The innate immune response represents the first line of defense against viral assault. Type I IFNs, a family of cytokines with pleiotropic functions, are central players in antiviral innate immunity (Annu Rev Immunol 2005;23:307-336). Since the first description of IFN as a soluble factor produced by influenza virus-infected chick embryo cells that confers resistance to subsequent virus infection (Proc R Soc Lond B 1957;147:258-267), several outstanding studies have contributed to our understanding of many important aspects of IFN system (Immunity 2006;25:343-348). Notably, there is now substantial knowledge about the virus-sensing machineries that lead to type I IFN induction and IFN receptors and their downstream pathway, the so-called JAK-Stat signaling pathway (Nat Immunol 2006;7:131-137; Immunity 2006; 25:361-372).

Activation of the JAK-Stat pathway through IFN receptors induces the expression of numerous ISGs. Most of the well-characterized examples of ISGs are 2′-5′ oligoadenylate synthases, double-stranded RNA-dependent protein kinase R (PKR), and myxovirus resistance proteins. The 2′-5′ oligoadenylate synthases, activated by viral dsRNA, produce 2′-5′ oligoadenylates, which in turn activate the latent nuclease RNase L, resulting in the degradation of viral RNA transcripts as well as host RNAs (Annu Rev Biochem 1998;67:227-264). PKR, a member of the eukaryotic initiation factor 2α kinase family, is another ISG. Activation of PKR by dsRNA results in eukaryotic initiation factor 2α phosphorylation, leading to the translational block of viral and cellular mRNA (Cell 1990; 62:379-390). Myxovirus resistance proteins, large IFN-inducible GTPases of the dynamin family, have antiviral activity against influenza and vesicular stomatitis virus (Cell 1990;62:51-61; Ciba Found Symp 1993;176:233-243). Their similarity with dynamin suggests that they interfere with viral assembly and trafficking in the cell. Furthermore, in association with HCV, ISG56 was recently demonstrated to suppress HCV RNA translation through direct interaction with eIF3, which blocks ribosome recruitment to the viral RNA (J Virol 2004;78: 11591-11604).

Although these examples clearly suggest that ISGs represent essential effector components of IFN signaling to establish an "antiviral state" and indeed hundreds of ISGs have been identified since their discovery >25 years ago (Proc Natl Acad Sci U S A 1979;76:1824-1828; Proc Natl Acad Sci U S A 1984;81:6733-6737; J Leukoc Biol 2001; 69:912-920), the majority of ISGs remain to be characterized with respect to their antiviral activity. Thus, "antiviral state" is a generic term and the larger picture of how the IFN system exerts an antiviral response through the induction of numerous ISGs has long been an open question for most researchers in this field.

These novel findings presented by Schoggins et al provide a long-sought answer for this challenging theme. Overcoming the technical barriers that hampered the systematic overexpression of hundreds of genes, they developed an elegant, cell-based screening system by which they identified multiple novel antiviral ISGs. The major findings of this study are as follows. Each ISG has a diverse range of antiviral potential and virus target specificity. ISGs exert their antiviral effect in a combinatorial fashion, and translational inhibition is a common mechanism of ISG-mediated antiviral action. These findings, together with the fact that IFN therapy is currently the first-choice therapy for HCV eradication and is also used for the treatment of several other viral infections, have potentially important implications for the development of new antiviral therapies. The side effects of IFN therapy, which frequently limit its clinical use, may be due to undesirable global ISG expression. Thus, selective utilization of ISG sets optimized for target viruses might be a more effective and safer therapeutic option.

This work is very exciting and expands our knowledge of downstream IFN effector mechanisms. However, some issues remain to be resolved in future studies. First, the effects of viral evasion mechanisms are dismissed in this study. Most viruses have evolved unique strategies to interfere with various aspects of the IFN system (Nat Rev Immunol 2002;2:675-687). For example, HCV is equipped with multiple evasion mechanisms: NS3/4A serine protease blockade of type I IFN production by the cleavage of IPS-1, a key signaling molecule of the IFN-inducing pathway (Trends Immunol 2006;27:1-4), disruption of JAK-Stat signaling by NS5A, and inhibition of PKR by NS5A and E2 proteins (Nat Rev Immunol 2002;2:675-687). The overexpression platform used in this study may not reflect the events that actually happen during viral infection in vivo. Second, further investigation of the proviral ISGs presented in this study is needed. It is of great interest that several ISGs exhibit proviral activity and the data suggest

that the effect of IFN is more complex than previously thought. What are the physiologic functions of proviral ISGs and why does the IFN system induce them? A loss-of-function study might be useful to answer these questions. Finally, the screening assays were performed on only positive-sense RNA viruses (HCV, yellow fever virus, Venezuelan equine encephalitis virus, West Nile virus, and chikungunya virus) and 1 retrovirus (HIV-1), but not on DNA viruses. Yet the host also obviously activates the type I IFN system against DNA viruses, such as cytomegalovirus and herpes simplex virus, utilizing Toll-like receptor 9 and a not-yet identified cytoplasmic DNA sensor molecule(s) (Nat Immunol 2006;7:131-137; Curr Opin Immunol 2010;22:41-47; Biochem Pharmacol 2010;80:1955-1972). Given that DNA viruses have their own viral life cycle and evasion strategies distinct from those of viruses tested here, there might be other antiviral ISG profiles with mechanisms of action that are entirely unique to DNA viruses. Elucidation of these issues, together with further dissection of the antiviral mechanisms of the validated ISGs, would provide additional insight into the type I IFN antiviral response.

In conclusion, this is the first report of a comprehensive evaluation of antiviral potentials of ISGs. A newly developed, cell-based screening assay identified multiple novel antiviral effectors in the type I IFN system that were previously unanalyzable. Given that current IFN therapy has undesirable side effects, this work opens the door to designing new therapeutic strategies based on antiviral ISGs.

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DIAGNOSTIC ENDOSCOPIC RETROGRADE PANCREATOGRAPHY FOR AUTOIMMUNE PANCREATITIS: ONE SIZE DOES NOT FIT ALL

Sugumar A, Levy MJ, Kamisawa T, et al. **Endoscopic retrograde pancreatography criteria to diagnose autoimmune pancreatitis: an international multicentre study. Gut 2011;60:666-670.**

Autoimmune pancreatitis (AIP) is being increasingly recognized worldwide. Although the exact etiology and pathogenesis of AIP remain unclear, we do have some insights into the disease process. It has been proposed that AIP is the pancreatic manifestation of an immunoglobulin (Ig)G4-associated systemic fibro-inflammatory disorder that can also affect the bile duct, kidneys, retroperitoneum, orbits, lymph nodes, and salivary glands (Pancreatology 2006;6:132-137). Histologically, AIP is characterized by an inflammatory process, with infiltration of the pancreas by a lymphoplasmactic infiltrate rich in IgG4-positive cells. The inflammatory process can be focal or diffuse. Additionally, IgG4-positive plasma cells and other inflammatory cells have been described as infiltrating other organs in patients with AIP (J Gastroenterol 2003;38:982-984), thus supporting the notion that this process may be systemic. Clinically, AIP presents most commonly as obstructive jaundice; this presentation can often be confused with pancreatic adenocarcinoma. Less commonly, AIP can present with abdominal pain or acute pancreatitis. AIP is treated with a course of corticosteroids, and the response to treatment is often dramatic. A reliable and accurate diagnosis of the disease, particularly differentiation from pancreatic adenocarcinoma, which AIP can mimic, continues to present a clinical challenge (Clin Gastroenterol Hepatol 2006;4:1010-1016; Am J Gastroenterol 2003;98:2694-2699). Histology, considered to be the gold standard for diagnosis, requires a biopsy that is often not easily obtainable in the pancreas. Fine-needle aspiration of the pancreas, although readily available, does not yield a definitive diagnosis of AIP (Clin Gastroenterol Hepatol 2006;4:1010-1016). A core biopsy may be diagnostic, with the caveat that, with patchy distribution of disease or in the presence of a strong desmoplastic reaction, even a core specimen may not yield a diagnosis. Thus, in the appropriate clinical setting, a negative biopsy does not exclude pancreatic cancer or AIP. A surgical biopsy, although certainly not feasible in all cases, may thus be required for diagnosis. Investigators in Asia and the United States have developed several diagnostic classification systems based on clinical, imaging, laboratory, and pathologic criteria, and response to treatment. According to the Japanese Pancreas Society, a diagnosis of AIP can be made when a patient exhibits ≥ 1 imaging feature and either 1 serologic or histologic feature. The Mayo HISORt criteria are more commonly used in the United States, and include ≥ 1 of the following: diagnostic histology, characteristic imaging, elevated serum IgG4 levels, involvement of other organ systems, and response to treatment with glucocorticoids. Although there seems to be an emerging consensus toward uniformity in the criteria, some major differences remain, 1 of which is the use of endoscopic retrograde cholangiopancreatography (ERCP)for ductal imaging; the Asian criteria mandate endoscopic retrograde pancreatography (ERP) for ductal imaging (J Gastroenterol 2006;41:626-631), whereas the Mayo HISORt criteria do not (J Gastroenterol 2007;42[Suppl 18]:39-41).

To advance our understanding of AIP, an international group of experts has formed the Autoimmune Pancreatitis International Cooperative Study group. Given the discrepancy in the use of ERP to diagnose AIP, 1 of the first goals of this collaboration was to determine the performance characteristics of ERP for the diagnosis of AIP.

Sugumar et al performed an international, multicenter study in 2 phases (Gut 2011;60:666-670). A total of 21 physicians from 4 centers in Asia, the United Kingdom,

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Review Article

Inflammation-mediated genomic instability: roles of activation-induced cytidine deaminase in carcinogenesis

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Chronic inflammation is a strong risk factor for the development of cancer. Many previous studies have demonstrated that a transcriptional factor, nuclear factor (NF)-κB, plays an important role in the association between inflammation and cancer development, particularly tumor promotion and tumor progression. Although it is well recognized that cancer develops via stepwise accumulation of genetic aberrations, the mechanisms underlying the generation of these genetic alterations in normal epithelial cells under inflammatory conditions are not known. We recently demonstrated that pathogenic bacterial or viral factors and the subsequent inflammatory reactions lead to the aberrant expression of a DNA mutator enzyme, activation-induced cytidine deaminase (AID), in various epithelial cells via NF-κB activation, which causes the accumulation of genetic alterations in tumor-related genes. AID activation is widely observed in gastrointestinal tissues with cancer-associated inflammation, such as chronic viral hepatitis, *Helicobacter pylori*-related gastritis, Barrett's esophagus and inflammatory bowel disease. Furthermore, a deficiency of endogenous AID expression reduces both accumulation of somatic mutations in tumor-related genes and tumor incidence in a mouse model of inflammation-associated cancer development. These findings strongly suggest that AID plays an integral role in inflammation-associated carcinogenesis and is therefore a potential target molecule for the prevention and treatment of cancers. (*Cancer Sci* 2012; 103: 1201–1206)

Cancer cells have various genetic alterations, including somatic mutations, chromosomal rearrangements, copy number alterations and epigenetic changes.[1] Recent genome technology innovations, such as ultra-deep sequencing and comprehensive genome hybridization analyses, have allowed investigators to better understand the landscape of genetic alterations in cancer tissues.[2] Using these technologies, comprehensive genome analyses of various cancer tissues have clarified that cancer cells have numerous nucleotide alterations, including "passenger mutations, which might not be involved in cancer development, and 'driver mutations", which directly contribute to carcinogenesis.[3] In contrast, chronic inflammation plays important roles in the development of various human cancers, and tumor cells are considered to be generated from stepwise accumulation of genetic alterations in various genes during the process of inflammation-associated carcinogenesis. In some diseases, including hereditary non-polyposis colorectal cancer, genetic abnormalities in the DNA repair system result in accumulation of genetic alterations in various genes, leading to colorectal carcinogenesis.[4,5] However, in most sporadic cancers the molecular mechanisms for acquiring genetic alterations under inflammatory conditions are unknown.

Nuclear Factor (NF)-κB is a Key Molecule in Inflammation-Associated Carcinogenesis

Many epidemiological studies have clearly demonstrated that chronic inflammation predisposes to tumor formation in various organs, including hepatocellular carcinoma (HCC) caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, gastric cancer caused by *Helicobacter pylori* infection, colorectal cancer caused by inflammatory bowel diseases (IBD), bile duct cancer caused by primary sclerosing cholangitis and esophageal cancer caused by Barrett's esophagus.[6–11] Accordingly, over 25% of human cancer cases are thought to be associated with chronic inflammation.[12]

Chronic inflammation is characterized by a continued active inflammatory response and tissue destruction, followed by irreversible tissue remodeling. Various cytokines, chemokines and transcription factors contribute to pathogenesis of chronic inflammation, and are also involved in the carcinogenesis process.[13] One of the most well known molecules is the transcription factor NF-κB, which is heavily involved in the processes of both tumor promotion and tumor progression and is considered a key regulator of cancer-associated inflammation. NF-κB is activated by various proinflammatory cytokines and microbial products, and regulates various cytokines and chemokines related to the determination of cell fate by binding their gene promoter.[14] Although NF-κB is physiologically associated with immune and inflammatory cell function, activated NF-κB proteins are detected in epithelial cells under many chronic inflammatory conditions related to carcinogenesis, such as *H. pylori*-related chronic gastritis, HCV-associated chronic liver disease and IBD.[15–18] NF-κB activation in inflamed epithelial cells, premalignant cells and malignant cells is involved not only in regulating cell survival, proliferation and growth, but also in the epithelial-to-mesenchymal transition.[19] In addition to these crucial processes that could contribute to tumorigenesis, a novel molecule, activation-induced cytidine deaminase (AID), a nucleotide-editing enzyme that is directly involved in DNA instability, has been recently identified to be regulated by NF-κB[20] (Fig. 1).

AID Triggers Both Somatic Mutations and DNA Double-Strand Breaks

Several molecules that possess the capacity to induce mutations in target DNA or RNA have been identified. Most of these molecules are members of the apolipoprotein B

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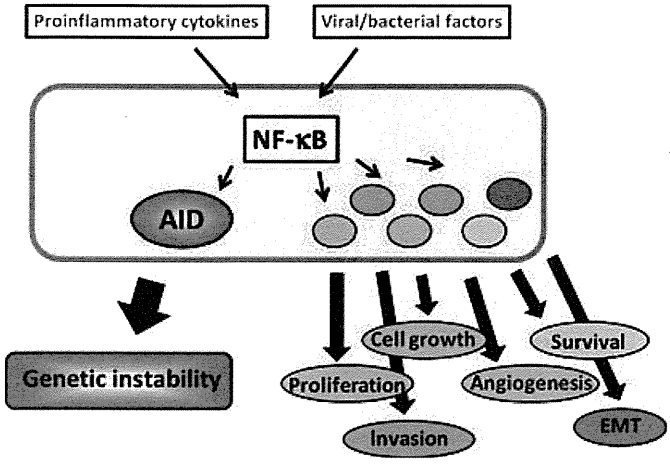

Fig. 1. Link between inflammation and carcinogenesis. Nuclear factor (NF)-κB is activated by various proinflammatory cytokines and microbial products and regulates various cytokines and chemokines related to the determination of cell fate. NF-κB activation in epithelial cells and malignant cells is involved in generating genetic instability via aberrant activation-induced cytidine deaminase (AID) activation, as well as cell growth, survival, proliferation, angiogenesis and epithelial-to-mesenchymal transition (EMT).

mRNA-editing enzyme, catalytic polypeptide-like (APOBEC) family.[21] Among the APOBEC family members, AID is the only molecule that can induce genetic alterations in human DNA sequences.[22] AID is a key molecule for generating immune diversity and is essential for inducing both somatic hypermutation, which occurs in variable regions of the immunoglobulin (Ig) genes, and class-switch recombination, which occurs in switch regions of the Ig genes (Fig. 2A).[23,24] It is considered that AID acts on single-strand DNA during the transcriptional stage, resulting in the conversion of cytosine (C) to uracil (U). The generated U:G mismatches can usually be repaired to C:G by the high-fidelity repair system. However, if the U:G mismatch is not repaired before the onset of DNA replication and is replicated over, it give rise to C:G to T:A transitions.[25] In contrast, nicks in the near sites of both strand sequences of switch regions are generated by the repair process of AID-induced U:G mismatches, resulting in DNA double-strand breaks that are required for class-switch recombination[26] (Fig. 3).

Constitutive AID Expression Contributes to Tumorigenesis

Under physiological conditions, AID has a strong preference for targeting the Ig genes in activated B cells, but it also mutates a number of non-Ig genes, including Bcl6, Pax5, mirR142, Pim1 and c-myc.[27,28] Strikingly, approximately 25% of all genes expressed in germinal center B cells are mutated by AID activity.[29] Additionally, a number of studies demonstrated that high AID expression is frequently observed in various types of human B-cell lymphomas and leukemias, and AID expression is associated with various gene mutations and chromosomal translocations.[30–34] These findings call attention to the concept that while the nucleotide-editing function of AID is physiologically essential for immune diversity in activated B cells, AID could be involved in the malignant transformation of lymphocytes via the accumulation of genetic alterations in tumor-related genes. This hypothesis was confirmed by generating a transgenic (Tg) mouse model with constitutive and ubiquitous AID expression.[35] All AID-Tg mice developed T cell lymphomas via the accumulation of mutations in non-Ig genes, such as T cell receptor and *c-myc*. Notably, the AID-Tg mice not only developed malignant lymphomas but also various epithelial tumors, including liver, lung and gastric cancers.[36] Thus, the cancer phenotypes of AID-Tg mice further suggest that epithelial cells with constitutive AID expression are transformed into malignant cells via the accumulation of genetic alterations in tumor-related genes (Fig. 2B).

Role of AID During Inflammation-Associated Human Carcinogenesis

Under physiological conditions, expression of the AID gene *Aicda* is strictly regulated by many factors, because AID has a dangerous ability to induce genomic instability.[37] Four regions with transcription factor-binding motifs have been identified in and around the *Aicda* locus.[38] Among these regions, the transcription enhancer activity of region 4, the area approximately 8 kb upstream of the transcription start site, has a primary role in the induction of AID by environmental stimuli.[39] The major transcription factors that mediate the signals initiated by CD40L, interleukin (IL)-4 and transforming growth factor-β stimuli are identified as NF-κB, STAT6 and Smad3/4, respectively. Furthermore, NF-κB-binding sites are also found immediately upstream of the transcription start site in region 1.[40,41] The NF-κB site in region 4 is considered to have a main role against CD40 stimulation in activated B cells, while the site in region 1 is responsible for the cell's response to viral infection and tumor necrosis factor (TNF)-α (Fig. 4).[39] These findings indicate that NF-κB plays a key role in the regulation of AID expression and suggest that activation of NF-κB in epithelial cells under many inflammatory conditions might induce AID, leading to genetic instability.

Liver carcinogenesis related to chronic HCV infection. Epidemiological studies have demonstrated that most HCC arise in the setting of chronic liver disease with features of chronic hepatitis or liver cirrhosis. HCV infection is one of the leading causes of chronic liver damage and induces the development of HCC at an annual rate of 0.5–1.0% in chronic hepatitis and 5–8% in liver cirrhosis.[6] Recent studies demonstrated that a number of genetic alterations, including the tumor-suppressor *TP53* gene, are present not only in human HCC but also in the surrounding hepatitis or liver cirrhosis.[42] Strikingly, aberrant AID expression is observed in both HCC cells and the surrounding non-cancerous hepatocytes, while no AID expression is observed in normal liver tissues.[42] Previous studies have revealed that high TNF-α expression levels are found in the liver of patients with chronic viral hepatitis, which activates the NF-κB classical pathway, and the HCV core protein has the potential to induce NF-κB activation in human hepatocytes.[43,44] Consistent with the regulation of AID by NF-κB in activated B cells, AID expression is induced in response to TNF-α stimulation in cultured human hepatocytes through the NF-κB pathway.[45] Likewise, AID is expressed in cultured human hepatocytes expressing HCV core protein via the NF-κB pathway. Furthermore, aberrant expression of AID in hepatocytes results in the accumulation of genetic alterations of various genes, including the *TP53* gene.[45] These findings provide the concept that both the response to chronic inflammation and HCV infection itself induce the aberrant expression of AID in hepatocytes, contributing to the genomic instability required for hepatocarcinogenesis.

Gastric carcinogenesis related to *H. pylori* infection. *Helicobacter pylori* causes chronic gastric inflammation and is defined as a class one carcinogen for human gastric cancer.[46] Patients with a previous history of *H. pylori*-related gastric cancer are at a higher risk of subsequent development of gastric cancers.[47] Although eradication of *H. pylori* reduces the development of metachronous gastric cancer, *H. pylori*-infected

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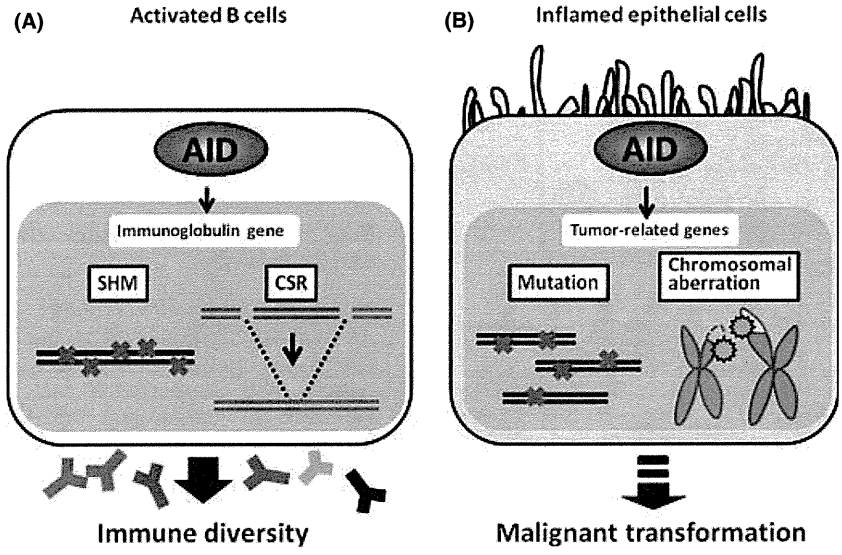

Fig. 2. Activation-induced cytidine deaminase (AID) function under physiological and inflammatory conditions. (A) Under physiological conditions, AID is expressed only in activated B cells and is a key molecule for generating immune diversity by inducing somatic hypermutation (SHM) and class-switch recombination (CSR) in immunoglobulin genes. (B) Under inflammatory conditions, AID is aberrantly expressed in epithelial cells. AID can induce somatic mutations and chromosomal aberrations in tumor-related genes, contributing to malignant transformation.

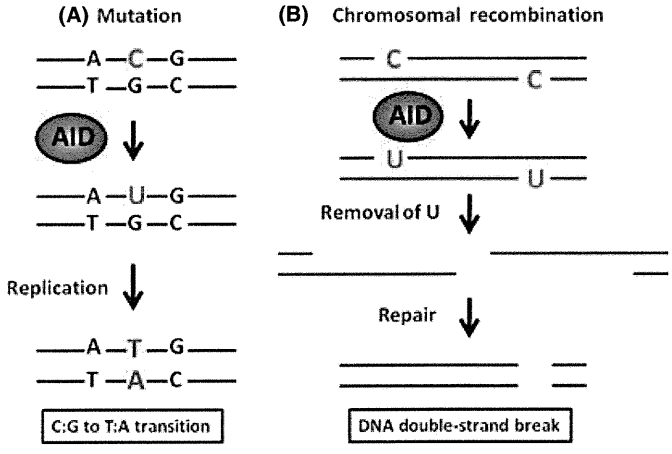

Fig. 3. Molecular mechanisms of the induction of mutation and chromosomal recombination by activation-induced cytidine deaminase (AID). (A) AID acts on single-strand DNA during the transcriptional stage. AID binds to the target DNA and deaminates deoxycytidine (dC) to deoxyuracil (U), creating a U–G mismatch. The general DNA replication without repair pathways can convert U to T and G to A, generating C:G to T:A mutations. (B) DNA double-strand breaks are generated through the repair process of AID-induced U:G mismatches through the production of abasic sites. Class-switch recombination (CSR) occurs by recombination of double-strand breaks.

mucosa might already have a number of genetic alterations, leading to multicentric cancer development.[48,49] Interestingly, AID protein is aberrantly expressed in a substantial proportion of *H. pylori*-associated human gastric epithelium and gastric cancer tissues, although no AID expression is observed in normal gastric mucosa or in gastric tissues after *H. pylori* eradication.[50]

Helicobacter pylori can be subclassified into "*cag*" pathogenicity island (*cag*PAI)-positive and *cag*PAI-negative strains based on the presence or absence of *cag*PAI, a 40-kb genome fragment containing 31 genes.[51] The *cag*PAI-positive isolates are more virulent strains that produce severe pathological infection in humans and are deeply associated with an increased risk for gastric cancer.[52] Previous studies showed that infection with *cag*PAI-positive *H. pylori* is associated with increased expression of NF-κB in gastric epithelial cells both *in vitro* and *in vivo*.[53,54] Intriguingly, infection with *cag*PAI-positive *H. pylori* ectopically induces a high expression of AID in human gastric epithelial cell lines, while cag*PAI*-negative *H. pylori* has no effect on AID expression. Similar to cag*PAI*-positive *H. pylori* infection, TNF-α stimulation increases the expression of endogenous AID protein in gastric epithelial cells through the NF-κB pathway. Furthermore, aberrant AID expression in gastric epithelial cells causes a number of somatic mutations in tumor-related genes, including the *TP53* gene, and knockdown of endogenous AID significantly reduces the number of *TP53* mutations observed in *H. pylori*-infected cells.[50]

In contrast, it is reasonable to assume that AID is capable of inducing chromosomal aberrations in epithelial cells because of its ability to trigger DNA double-strand breaks. Consistent with this hypothesis, AID expression in gastric epithelial cells causes chromosomal aberrations, mainly submicroscopic deletions, at various chromosomal loci.[55] Among these deleted loci, the recurrently deleted chromosomal regions harbored the tumor-suppressor genes cyclin-dependent kinase inhibitor (*CDKN*)*2A/CDKN2B*. Oral infection of wild-type mice with *H. pylori* reduces the copy number of the *Cdkn2b-Cdkn2a* locus, whereas no such changes are observed in the gastric mucosa of *H. pylori*-infected AID-deficient mice.[55] Taken together, these findings suggest that both *H. pylori* infection and the resulting inflammatory responses induce aberrant AID expression in gastric epithelial cells via NF-κB activation, leading to accumulation of genetic alterations, including somatic mutations and submicroscopic deletions in tumor-related genes during *H. pylori*-related gastric carcinogenesis.

Colitis-associated carcinogenesis. Inflammatory bowel diseases is an important etiological risk for the development of colorectal cancer.[56] It is well known that the risk of colitis-associated colorectal cancer increases according to the number of years after disease onset.[57] The cumulative risk of developing colorectal cancer in patients with ulcerative colitis (UC) is estimated to be 1.6% at 10 years, 8.3% at 20 years and 18.4% at 30 years from disease onset.[9] Recent studies have shown that patients with Crohn's disease are also at high risk of developing colon cancer.[58] Importantly, colitis-associated

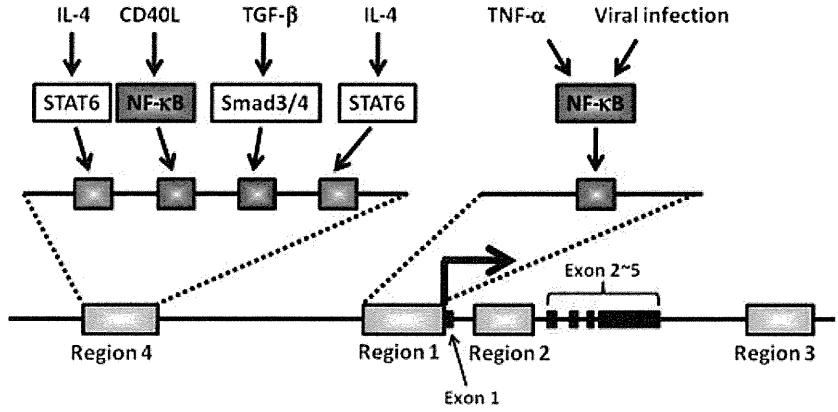

Fig. 4. Transcriptional regulation of activation-induced cytidine deaminase (AID) expression induced by environmental stimuli. The transcriptional regulatory elements for AID are localized to four regions. Nuclear factor (NF)-κB-binding sites are present in region 4 and region 1. The site in region 4 has a main role against CD40 stimulation in activated B cells, while the site in region 1 might be responsible for the cell's response to viral infection and tumor necrosis factor (TNF)-α.

colorectal cancer has several distinct characteristics compared with sporadic colorectal cancers. In most sporadic colorectal cancers, adenomatous polyps first develop through inactivation of the *APC* gene, and the subsequent acquisition of other gene abnormalities, such as *KRAS*, *TP53* and *DCC* genes, contribute to cancer development.[59] In contrast, mutations in the *TP53* gene are already present in dysplastic lesions with a background of UC, suggesting that colonic inflammation induces the accumulation of mutations in the *TP53* gene.[60,61] Interestingly, AID protein is expressed in colonic epithelial cells of patients with IBD, while AID is not detected in normal colonic mucosa.[62]

Colonic mucosal inflammation is usually mediated by either an excessive T helper cell (Th) 1 T-cell response associated with increased interferon-γ and IL-12 secretion, or an excessive Th2 T-cell response related to increased IL-4, IL-5 and IL-13 secretion.[63] Although the concentration of the Th2 cell-driven cytokine IL-4 varies in UC colon tissue, UC is considered to have a Th2 profile.[64] In addition to the finding that the proinflammatory cytokine TNF-α induces AID expression through the NF-κB pathway, Th2 cytokines IL-4 and IL-13 enhance the aberrant expression of AID via STAT6 activation in cultured colonic epithelial cells.[62] Moreover, constitutive AID expression in colonic epithelial cells generates a number of mutations of the *TP53* gene.

Recent studies have revealed that IL-10 has a pivotal role in mediating the signals that control inflammation in the human gut.[65] Moreover, a mouse model with target disruption of the IL-10 gene spontaneously develops intestinal inflammation followed by colon cancer, and thus this mouse model has been used to represent human IBD.[66,67] We found that AID expression is observed in the inflamed cecal mucosa of IL-10-deficient mice, and mice deficient in both IL-10 and AID do not develop colon cancer in the cecum, whereas IL-10-deficient mice develop spontaneous colon cancers.[68] Furthermore, somatic mutation rates of *Trp53* in the cecal mucosa of mice deficient in both IL-10 and AID are significantly lower than those in the mucosa of mice deficient in IL-10 only, regardless of the same inflammation conditions. Therefore, it is conceivable that aberrant AID expression in the inflamed colonic mucosa plays an integral role in the development of colitis-associated cancers via accumulation of genetic abberations.

Barrett's esophageal carcinogenesis related to bile acid exposure. Barrett's esophagus is a well known high-risk factor for the development of esophageal adenocarcinoma.[11] Barrett's esophagus is a metaplastic change from the normal stratified squamous epithelium of the lower esophagus to a columnar-lined epithelium with intestinal-type differentiation due to chronic duodenogastro-esophageal reflux and resultant inflammation.[69] Several studies have demonstrated that NF-κB is activated by bile acid components, resulting in the upregulation of various genes involved in the development of metaplasia of Barrett's esophagus and cancer formation.[70] Interestingly, AID is aberrantly expressed in the columnar cell-lined Barrett's esophagus and Barrett's adenocarcinoma, whereas weak or no expression of AID is observed in normal squamous epithelial cells of the esophagus.[71] *In vitro* experiments using human non-neoplastic esophageal squamous-derived cells showed that exposure to deoxycholic acid induces endogenous AID upregulation via NF-κB activation. Moreover, constitutive AID expression in esophageal squamous cells causes the accumulation of mutations in *TP53*.[71] Taken together, these findings suggest that bile acid reflux-mediated aberrant AID expression in Barrett's columnar-lined epithelium enhances the susceptibility to genetic alterations, contributing to the development of Barrett's adenocarcinoma.

Conclusion

Recent epidemiological studies have revealed that removing the causes of chronic inflammation, such as eradication of HCV or *H. pylori*, reduced cancer development, suggesting that regulation of inflammation contributes to suppressing cancer development.[48] To date, we have demonstrated that aberrant AID expression in various epithelial cells induces the accumulation of genetic alterations, and a deficiency of endogenous AID reduces the accumulation of somatic mutations in various genes in inflamed tissues, resulting in reduced incidence of inflammation-associated cancer development.[68] These findings might provide a novel strategy for cancer prevention by targeting AID-related pathways. Recently, several studies have shown that deamination activity of AID promote active DNA demethylation via the base excision repair pathway, suggesting that AID is involved in the epigenetic regulation of various genes.[72,73] Thus, it is hoped that further elucidation of the link between AID and the DNA methylation system will lead to a better understanding of the mechanisms of inflammation-associated carcinogenesis.

Disclosure Statement

The authors declare no potential conflict of interest.

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Original Article

Detection of highly prevalent hepatitis B virus co-infection with HIV in Indonesia

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Aim: The prevalence of hepatitis B virus (HBV) co-infection with HIV is increasing worldwide because of shared transmission routes. This study aimed to assess the prevalence of HBV and HIV co-infection in Indonesia, and its molecular and clinical characteristics.

Methods: A total of 118 serum samples from HIV-infected patients (age 33.3 ± 8.9 years, 99 male, 19 female) collected in 2009 were serologically examined. HBV DNA was assessed by polymerase chain reaction (PCR) analysis targeting the S region.

Results: Overall, 15.3% (18/118) of the patients were hepatitis B surface antigen (HBsAg) positive, whereas 27.1% (32/118) were HBsAg negative but HBV DNA positive, and were considered to have occult HBV infection. HBsAg antibodies and/or HBV core antibodies were detected in 45.6% (31/68) of HBV DNA negative patients.

Conclusion: HBV co-infection, including occult HBV infection, was common in Indonesian HIV patients. Hepatic damage by the interaction of host immunity and HBV is still a remaining issue in these immunosuppressive patients, and further study will be needed.

Key words: co-infection, hepatitis B virus, HIV, Indonesia

INTRODUCTION

THERE ARE AN estimated 400 million people worldwide with chronic hepatitis B virus (HBV) infection and an estimated 40 million people are infected with HIV. Co-infection with HBV and HIV is common because of shared blood-borne transmission routes, particularly injection drug use (IDU), with estimates for the prevalence of co-infection ranging 4–23%.[1–3] In areas of highly endemic HBV infection, co-infection with HBV was reported in 50% of HIV patients.[4]

In this setting, the mortality rate was higher in patients with HBV and HIV co-infection than for HIV or HBV infection alone.[5] Since the introduction of highly active antiretroviral therapy (HAART) in many countries, including Indonesia, the mortality rate attributed to AIDS has declined. Liver disease, however, has emerged as a significant cause of morbidity and mortality among HIV-infected individuals[6–9] because HIV promotes HBV replication and the progression of hepatic damage.[10] These changes cause prolonged elevations in alanine aminotransferase (ALT) levels,[11] shorten the time to the onset of cirrhosis[12] and increase the risk of developing hepatocellular carcinoma. The risk of death attributable to liver diseases in patients with HBV and HIV co-infection is 14-times higher than that of patients infected with either virus alone.[12,13] The prevalence of HBV-related liver diseases is expected to increase in HIV-infected patients because HAART has now been introduced in HBV endemic areas and the incidence of AIDS-related death is decreasing.

Occult HBV infection is defined as the presence of HBV DNA in serum and/or liver tissue of individuals with HBV core antibodies (anti-HBc) without hepatitis B surface antigen (HBsAg).[14] The prevalence of occult HBV infection varies greatly in HIV-infected patients and its clinical significance is unclear.[15] Occult HBV infection is possibly caused by changes in antigenicity,

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but it may also occur following suppression of viral replication, gene expression and virus secretion.[16]

Indonesia is experiencing one of the fastest increases in the incidence of HIV in Asia,[17] with a concentrated HIV epidemic in populations characterized by high-risk behaviors. In HBV endemic countries, such as Indonesia (5–20%), transmission mostly occurs perinatally or in early childhood. Therefore, HBV infection usually precedes HIV infection by several decades.[2] The main route of HIV transmission is IDU, followed by sexual contact.[17–19] The aims in this study were to assess the prevalence of HBV, including occult HBV infection, in HIV-infected patients in Surabaya, Indonesia, and determine its molecular and clinical characteristics, to understand the interaction between these two chronic viral infections.

METHODS

Study population

SERUM SAMPLES WERE collected from 118 HIV-infected individuals (mean age, 33.3 ± 8.9 years; 99 male, 19 female) with unknown HBsAg status who routinely visited a private clinic in Surabaya, the second biggest city in Indonesia, in 2009. Most of the patients were male (83.9%). The main routes of HIV transmission were IDU (63.8%; 74/118), sexual contact (35.6%; 42/118) and unknown exposure (1.7%; 2/118). Ninety-four percent of patients (109/118) were on HAART with activity against AIDS (lamivudine, zidovudine, and nevirapine or efavirenz). The study protocol was reviewed and approved by the Ethics Committees of Kobe University in Japan and Airlangga University in Indonesia. Informed consent was obtained from all patients. Demographic, clinical and laboratory data were also retrieved from the patient database maintained at the clinic.

Serological assays

Serological tests for HBV markers were performed on all 118 serum samples from HIV-infected patients. Commercially available kits were used to assess HBsAg by reverse passive hemagglutination (Mycell II HBsAg; Institute of Immunology, Tokyo, Japan), HBsAg antibodies (anti-HBs) and anti-HBc by passive hemagglutination (Mycell II anti-HBs and Mycell anti-rHBc; Institute of Immunology). An enzyme immunoassay (EIA) (Espline HBsAg; Fuji Rebio, Tokyo, Japan) and an enzyme-linked immunosorbent assay (ELISA) (Surase B-96; General Biologicals, Hsin Chu, Taiwan) were used to confirm HBsAg positivity in samples positive for HBV DNA on polymerase chain reaction (PCR).

HBV amplification and sequencing

After assaying HBV serological status, DNA was extracted from 200 μL of serum that had been stored at −80°C using a DNA extractor kit (QIAamp DNA Blood Mini Kit; QIAGEN, Tokyo, Japan) from all 118 HIV-infected individuals. The presence of HBV DNA was assayed by PCR with primer pairs for the precore/core (nt. 1611–2072) and S (nt. 18–557 and/or 414–989) regions. PCR amplification was performed as previously described.[20] The amplified fragments were directly sequenced using a Big Dye Deoxy Terminator cycle sequencing kit with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The HBV genotypes/subgenotypes were determined using the phylogenetic tree of 13 strains in the S (nt. 84–465) region. If the genotypes/subgenotypes could not be determined using these methods, the amplified fragments (nt. 406–646) of the small S region detected by real-time PCR were genotyped. Reference sequences were retrieved from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database. Alignments were performed using CLUSTAL X software, and phylogenetic trees were constructed by the neighbor-joining method. To confirm the reliability of phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. These analyses were carried out using Molecular Evolutionary Genetics Analysis (MEGA) software (available at www.megasoftware.net).

Detection of HBV mutations and measurement of the HBV viral load

Hepatitis B virus nucleotide sequences encoding the *a* determinant region of HBsAg were translated to amino acid sequences (124–147) and aligned with reference sequences. Lamivudine-resistance mutations, within the amino acid sequence 125–213 of HBV polymerase, were assessed as previously described.[21] Viral load was assessed by real-time PCR using an ABI 7500 real-time PCR system (Applied Biosystems). HBV DNA was amplified with a primer and probe set, as described previously.[22]

Statistical analysis

Statistical analysis was performed using the χ^2-test or Fisher's exact test for categorical variables. The indepen-