Table 3 Multivariate analysis of factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Long-term survival			Recurrence-free survival		
Variables	Hazard ratio (95% CI)	*P*-value	Variables	Hazard ratio (95% CI)	*P*-value
Percutaneous ablation			Percutaneous ablation		
AFP-L3 (%)			HBsAg		
<15	1		Negative	1	
≥15	2.098 (1.169–3.765)	0.013	Positive	2.823 (1.090–7.310)	0.033
Tumor size (cm)			DCP		
<3	1		<40 (mAU/ml)	1	
≥3	1.998 (1.123–3.553)	0.018	≥40 (mAU/ml)	2.767 (1.267–6.046)	0.011
			AFP-L3		
			<15 (%)	1	
			≥15 (%)	3.463 (1.437–8.347)	0.006
Hepatectomy			Hepatectomy		
Tumor size (cm)			Tumor number		
<3	1		Single	1	
≥3	6.162 (1.457–26.064)	0.013	Multiple	4.654 (0.936–23.149)	0.060
Tumor number					
Single	1				
Multiple	3.170 (1.442–6.921)	0.004			

Hazard ratio and *P*-value were calculated using Cox's stepwise proportional hazard model

CI confidence interval, *AFP* alpha-fetoprotein, *HBsAg* hepatitis B surface antigen, *DCP* des-gamma-carboxy prothrombin

patients with early stage HCC who underwent percutaneous ablation and patients who underwent hepatectomy (Fig. 1). No significant difference was observed between groups with or without AFP-L3 elevation ($P = 0.53$) in patients who underwent hepatectomy. In contrast, a close-to-significant ($P = 0.054$) difference was observed between the groups of patients with and without AFP-L3 elevation who underwent percutaneous ablative therapy.

In summary, the results of the retrospective and prospective studies demonstrated that AFP-L3 status was a statistically significant prognostic factor of long-term survival and recurrence-free survival in patients who underwent percutaneous ablative therapy, but did not affect prognosis in patients who underwent hepatectomy.

Discussion

AFP-L3, a fucosylated species of AFP, is the product of alpha 1-6 fucosyltransferase (FUT8) in the presence of GDP-fucose. Our previous result revealed that FUT8 levels in HCC tissue were higher than those in the surrounding non-cancerous tissues and that FUT8 levels of HCC tissue increased in accordance with tumor dedifferentiation [21]. Several reports have shown the relationship between AFP-L3 status and histologic grade in HCC. Miyaaki et al. [16] showed that the frequency of poorly differentiated HCC was significantly higher in AFP-L3-positive patients than in AFP-L3-negative patients. Oka et al. [14] reported that AFP-L3-positive HCC was characterized by portal vein invasion and poorer differentiation, and that tumors in AFP-L3-positive HCC were advanced, even if they were small and the patient had a low serum AFP concentration. These results indicate the relationship between increased AFP-L3 level and increased degree of malignant behavior of HCC tissue.

Recurrence after treatment is an important factor affecting prognosis. Vascular invasion is an established adverse prognostic indicator of recurrence of HCC [22, 23]. Yamashita et al. [24] suggested that portal vein invasion is associated with AFP-L3 positivity, and that there is a strong possibility of intrahepatic invasion when there is positive conversion of this marker. Hayashi et al. [13] reported the relationship between AFP-L3 status and pattern of recurrence in patients with HCC. In their report, intrahepatic metastasis was significantly more common in AFP-L3-positive patients than in negative patients, although the recurrence rate of multicentric tumors did not differ significantly between the two groups with or without AFP-L3 elevation. From this point of view, hepatectomy—especially anatomical resection, which can remove venous tumor thrombi together with the primary lesion—is more suitable than local ablative therapies for the treatment of AFP-L3-positive patients.

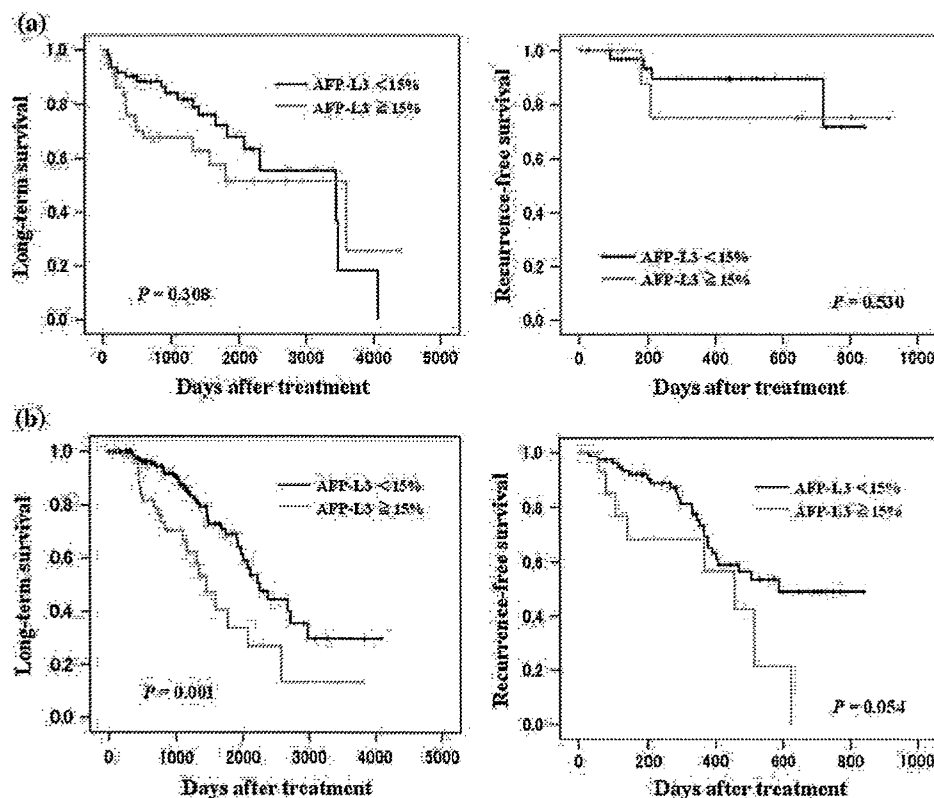

Fig. 1 Comparison of long-term survival rates and recurrence-free survival rates between patients with and without AFP-L3 elevation who underwent hepatectomy (**a**) and who underwent percutaneous ablation (**b**)

In our study, the pathological diagnosis was made by individual pathologists at each hospital. At Niigata University Hospital, 58 HCC patients underwent hepatectomy, of whom 23 had an elevated serum AFP-L3 level (≥15%) and the remaining 35 were negative for AFP-L3 (<15%). Among the 23 patients with AFP-L3 elevation, only two (8.7%) were diagnosed as having well-differentiated HCC on the basis of the resected specimens, 14 (60.9%) had moderately differentiated HCC, and seven (30.4%) had poorly differentiated HCC. In contrast, among the 35 patients who were negative for AFP-L3,.7 (20.0%) were diagnosed as having well-differentiated HCC, 24 (68.6%) had moderately differentiated HCC, and only four (11.4%) had poorly differentiated HCC. Although no statistically significant differences were observed by Fisher's exact test, the group showing AFP-L3 elevation tended to have a poorer histopathological grading ($P = 0.141$). Only eight out of 331 patients who underwent percutaneous ablative therapy were diagnosed as having HCC on the basis of histological findings in four hospitals. Therefore, we were unable to investigate whether poorly differentiated tumors were more frequent in the groups who underwent percutaneous ablative therapy and hepatectomy. Portal vein invasion was investigated similarly in 58 patients, and was found to be present in six of 23 AFP-L3-positive patients and six of 35 AFP-L3-negative patients. No significant difference was observed between AFP-L3 and portal vein invasion in this limited investigation.

We demonstrated here in a multicenter retrospective study that AFP-L3 status was a significant prognostic factor affecting the long-term survival of patients who underwent percutaneous ablative therapy. In addition, to evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we performed a multicenter prospective study to identify the prognostic factors for recurrence-free survival in patients with early stage HCC. Although this evaluation was conducted over a short period of time, we confirmed that AFP-L3 status was a significant prognostic predictor of recurrence-free survival in patients who underwent percutaneous ablative therapy, but it did not affect the prognosis of patients who underwent hepatectomy.

A number of studies have shown that AFP-L3 status is an independent prognostic factor in patients with HCC [12, 13, 15]. We previously reported that AFP-L3-positive (>15%) patients had a lower survival rate than negative (<15%) patients in subgroups with a low serum AFP concentration. Moreover, the statistically significant differences were more distinct in the subgroups with lower AFP concentrations [20]. However, the patients in these studies had received various treatments such as hepatectomy, RFA, and transcatheter arterial embolization, and

there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities. Tateishi et al. [15] demonstrated that pre-treatment AFP-L3 positivity (>15%) was a significant predictor of HCC recurrence in patients who underwent curative ablation, and that AFP-L3 positivity after ablation was the strongest predictor of HCC recurrence by multivariate analysis. Although their study was performed in only one center and did not evaluate long-term survival, their results are compatible with ours.

Treatment of HCC patients with cirrhosis faces a dilemma in that minimization of damage to noncancerous liver tissue improves long-term survival, but incomplete treatment of subsequent HCC recurrences results in a poor prognosis. Accordingly, if a useful indicator of choice of therapeutic modality were to be available before the initial therapy, there would be several advantages in not only the treatment, but also the follow-up, of patients with HCC.

In conclusion, present results revealed that AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Although this study was not a randomized control trial, AFP-L3 might be a promising scale to improve the prognostic estimate and appraisal of therapeutic outcome in patients with HCC.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Regular surveillance by imaging for early detection and better prognosis of hepatocellular carcinoma in patients infected with hepatitis C virus

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Abstract

Purpose This study evaluated the usefulness of regular check-ups by ultrasonography and contrast-enhanced imaging for early detection of hepatocellular carcinoma (HCC) in a retrospective analysis.

Patients and methods From April 2001 to March 2007, 240 consecutive patients with HCC who were infected with hepatitis C virus (HCV) were divided into three groups. Patients diagnosed with HCC by repeated imaging constituted Group A (surveillance group). Group B comprised patients in whom HCC was detected during scheduled doctor visits for liver disease or other diseases such as diabetes. Group C comprised non-screened patients.

Results The prevalence of solitary tumors decreased from Group A through Group B to Group C (66, 48 and 24%, respectively, $P < 0.001$). The proportion of patients in stages I and II decreased from 83% (103/124) in Group A to 53% (42/79) in Group B and 24% (9/37) in Group C ($P < 0.001$). The proportion of patients who were treated with curative procedures, such as resection or ablation, was highest at 80% (99/124) in Group A, and lower at 53% (42/79) in Group B and 27% (10/37) in Group C ($P < 0.001$). The cumulative survival rate was better in Group A than B ($P < 0.05$), and in Group B than C ($P < 0.001$). Periodical medical check-ups without imaging did not necessarily detect early-stage disease, even when HCC-related markers including des-γ-carboxy prothrombin were tested.

Conclusions Regular surveillance with ultrasonography and contrast-enhanced imaging is useful for detecting early-stage HCC and increase chances for curative treatments in patients with HCV-related chronic liver disease.

Keywords Hepatocellular carcinoma · Early detection · Curative procedures · Survival rates · Surveillance

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1], and patients with HCC continue to suffer an unsatisfactory prognosis. Surveillance for HCC should aim at decreasing mortality, and early detection is vital for therapeutic success. Serum levels of alpha-fetoprotein (AFP) and ultrasonography are widely accepted screening tests for early diagnosis of HCC [2–11].

However, serological markers including des-γ-carboxy prothrombin (DCP) and glycosylated AFP have shown limited success in detecting HCC in early stages [12–14]. Recent advances in imaging technologies allow the detection of early HCC, as reported in the guideline of the American Association for the Study of Liver Diseases [14]. Patients need to be surveyed for HCC, taking into consideration the incidence of HCC and cost-effectiveness. The discovery of hepatitis B virus (HBV) and hepatitis C virus (HCV), responsible for the majority of HCC cases [15], has enabled providers to identify the population at risk for HCC.

In Japan, HBV and HCV infections are associated with HCC in 15 and 80% of the cases, respectively [16, 17]. This retrospective study focused on HCV-associated HCC in Japan, and compared the efficacy of three methods for diagnosing HCC diagnosis. As the results show, regular repeated imaging was useful for early detection of HCC in patients infected with HCV.

Patients and methods

Patients

From April 2001 to March 2007, 338 consecutive patients were diagnosed with HCC in our institution. Among them, 240 patients infected with HCV were enrolled in this study. We retrospectively examined the procedure of diagnosis from clinical records and classified patients into one of three groups according to the method for diagnosing HCC. A total of 124 patients were diagnosed with HCC by regular imaging procedures such as ultrasonography, and they were categorized into the surveillance group (Group A). Hepatic damages such as rough surface pattern of the liver and dullness on the liver edge, as well as the detection of obvious varices on the first ultrasonography, led them to receive repeated imaging procedures. In 82% (102/124) of Group A patients, the interval between the latest imaging and diagnosis of HCC was within 6 months. The average interval between the latest imaging and diagnosis of HCC was 4.3 months [median, 3.6 months (range 2–11 months)]. They also received tests for HCC-related markers at least every 3 months. Group B comprised 79 patients who had been diagnosed with HCC during scheduled doctor visits for HCV-related liver disease or other diseases such as diabetes. These patients were not enrolled in a surveillance program at the time, and had not undergone any imaging procedures for at least 1 year before the diagnosis of HCC, while they received tests for HCC-related markers at least every 3 months. Among them, 26 patients received imaging due to elevated levels of HCC-related markers, such as AFP and DCP. In the remaining 53 patients in Group B, imaging was performed incidentally; they had not received imaging over the previous 1 year. The 37 patients who had not been screened for HCC were classified into Group C. They were diagnosed with HCC when symptoms developed (32 patients) or incidentally during a diagnostic workout for unrelated medical conditions such as traffic accident (5 patients). The study conformed to the ethical guidelines of the declaration of Helsinki, and was approved by the Institutional Review Board.

Surveillance strategy

Figure 1 outlines the surveillance program. Briefly, detection of any mass on ultrasonography instigated repeated imagings if the nodule diameter was up to 1 cm, or a dynamic study if the diameter exceeded 1 cm. HCC nodules are characterized by an intense contrast uptake during the arterial phase of dynamic computed tomography (CT) or magnetic resonance imaging (MRI), with the contrast washed away during the delayed or venous phase [12–14]. In the present study, the specific pattern of arterial uptake followed by venous washout was considered to represent HCC, since the value of "washout" in the venous phase has been recognized recently. If the vascular pattern on CT or MRI was not specific for HCC in a nodule with a diameter >1.5 cm, angiographically assisted CT or biopsy was undertaken to establish the diagnosis. Patients with nodules <1.5 cm in diameter who did not reveal HCC by angiographically assisted CT or biopsy underwent repeated surveillance procedures, and subsequent enlargement of the nodule during follow-ups indicated shifting to a dynamic study.

Diagnosis of cirrhosis

The diagnosis of chronic liver disease was made at the time of HCC detection by the following procedures.

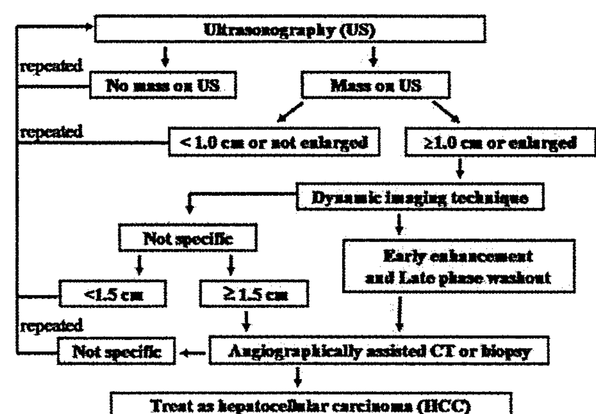

Fig. 1 Flow chart for the surveillance program including repeated imaging procedures

Histological findings were obtained in surgical specimens from 85 patients, and cirrhosis was diagnosed in 61 and chronic hepatitis or liver fibrosis in the remaining 24. Gastrointestinal varices in an additional 24 patients were considered diagnostic of cirrhosis. The remaining 131 patients were diagnosed to have cirrhosis according to the histologic scoring system [18].

Staging

Cancer stage was assessed by ultrasonography and dynamic CT or MRI. A total of 193 patients (80%) underwent angiography and/or angiographically assisted CT to obtain further details prior to resection, ablation or transarterial chemoembolization. In those patients, staging was also assessed by imaging on angiography and/or angiographically assisted CT. All patients underwent a chest X-ray, while additional investigations to detect metastases were performed only when extrahepatic involvement was suspected. Staging was not assessed by histologic findings on surgically resected specimens, even when they were available. Staging was determined according to the Liver Cancer Study Group of Japan classifications [19]. Staging was made also by the Milan criteria [20].

Treatment selection

Hepatic resection was indicated particularly to the patients with localized HCC who had maintained hepatic reserve capacity. When resection was contraindicated or refused by patients, the most appropriate treatment was selected according to the tumor status and liver function preserved [21]. Percutaneous ablation by ethanol injection [22] or radiofrequency ablation (RFA) [23] was considered in patients who had 1–3 nodules <3 cm in diameter, and were without vascular invasion or extrahepatic metastases. Transarterial chemoembolization [24] was offered to patients with either a paucifocal nodule not treatable by percutaneous ablation or multiple tumors not accompanied by thrombosis in main portal veins or extrahepatic metastasis. For the patients in Child-Pugh class C, transarterial chemoembolization was not recommended. In this study, resection and ablation were considered curative procedures based on their high efficacy.

Statistical analysis

The following 11 parameters were analyzed: age, sex, AFP, DCP, prothrombin activity, serum albumin level, total bilirubin level, liver state, tumor stage, HCC treatment and survival. Efficacy of the imaging program was evaluated by comparing clinical manifestation and prognosis among patients in the three groups. Differences in the distributions of tumor stage, tumor markers, and HCC treatment were evaluated by chi-squared test or Student's *t* test. Survival was calculated from the time of treatment start in patients who received it, and from the time of cancer diagnosis in patients without treatment. Data were censored at the time of death or the last follow-up visit. Survival was calculated according to the Kaplan–Meier method, and survival curves were compared by log-rank test. *P* values less than 0.05 were considered statistically significant.

Results

Background characteristics

There is no difference between Groups A and B in background of the patients except the programs with or without imaging. Table 1 details the background characteristics of all patients. Although the prevalence of cirrhosis was similar among the three groups, patients in Group C had poorer hepatic reserve with respect to albumin and total bilirubin levels ($P < 0.001$). The prevalence of non-cirrhotic liver in patients under 74 years was 26% (42/161), and 42% (33/79) in patients over 75 years. These differences were statistically significant ($P < 0.01$).

Features of HCC

The majority of HCC nodules were diagnosed by dynamic study including angiographically assisted CT, while HCC nodules in only 4 (1.7%) were confirmed by fine needle biopsy. Table 2 compares characteristics of HCC among the three groups. The frequency of solitary tumors was 66% (82/124) in Group A, 48% (38/79) in Group B, and 24% (9/37) in Group C, with a significant difference among three groups ($P < 0.001$). Nodules measuring less than 2 cm were detected in 64% (80/124) of patients in Group A, 25% (20/79) of those in Group B, and only 5% (2/37) of those in Group C ($P < 0.001$). The frequency of non-advanced tumor state decreased from 88% (109/124) in Group A, to 52% (41/79) in Group B, and to 27% (10/37) in Group C ($P < 0.001$). Cut-off values were set at 200 ng/ml and 40 mAU/ml, respectively, on AFP and DCP. In Group A, 47% (58/124) of the cases were negative for both, 46% (57/124) were positive for either, and 7% (9/124) were positive for both. In Group B, 11% (9/79) of the patients were negative for both, while 65% (51/79) were positive for ether, and 24% (19/79) were positive for both. In Group C, 11% (4/37) of the patients were negative for both, 57% (21/37) were positive for either, and 32% (12/37) were positive for both. These differences were statistically significant ($P < 0.001$). Thus, most patients in Groups B and C were positive for

Table 1 Background characteristics of patients

	Group A (surveillance) (n = 124)	Group B (scheduled doctor visits) (n = 79)	Group C (non-screened) (n = 37)	P value
Age at diagnosis of HCC (years)				
Median (range)	69.7 (49–89)	72.8 (49–87)	69.6 (50–87)	<0.05[b]
Gender				
Men	79 (64%)	52 (66%)	28 (76%)	NS
Women	45 (36%)	27 (34%)	9 (24%)	
History of blood transfusion	28 (22%)	19 (24%)	6 (16%)	NS
Excessive alcohol intake[a]	25 (20%)	20 (25%)	15 (49%)	NS
Liver state				
Not cirrhotic	34 (27%)	31 (39%)	10 (27%)	NS
Cirrhosis	90 (73%)	48 (61%)	27 (73%)	
Prothrombin activity (%)				
Median (range)	86 (48–125)	88 (57–135)	83 (39–124)	NS
Albumin (g/dl)				
Median (range)	3.6 (2.1–4.6)	3.8 (2.8–5.1)	3.4 (2.5–4.5)	<0.001[c]
Total bilirubin (mg/dl)				
Median (range)	0.9 (0.3–2.7)	0.8 (0.2–6.8)	1.4 (0.3–6.8)	<0.001[c]

NS not significant

[a] Excessive alcohol intake was defined as consumption of more 86 g alcohol/day

[b] Significant difference between Group B and Group A or Group C

[c] Significant difference between Group C and Group A or Group B

Table 2 Characteristics of the HCC nodule

	Group A (surveillance) (n = 124)	Group B (scheduled doctor visits) (n = 79)	Group C (non-screened) (n = 37)	P value
Solitary	82 (66%)	38 (48%)	9 (24%)	<0.001[b]
Size of main nodule				
–2 cm	80 (64%)	20 (25%)	2 (5%)	<0.001[b]
2.1–3 cm	31 (25%)	14 (18%)	6 (16%)	
3.1–5 cm	12 (10%)	33 (42%)	8 (22%)	
5.1 cm–	1 (1%)	12 (15%)	21 (57%)	
Vascular thrombus	4 (3%)	9 (11%)	10 (27%)	<0.001[b]
Distant metastases	1 (1%)	1 (1%)	5 (14%)	<0.001[c]
Tumor marker[a]				
Both negative	58 (47%)	9 (11%)	4 (11%)	<0.001[d]
Either positive	57 (46%)	51 (65%)	21 (57%)	
Both positive	9 (7%)	19 (24%)	12 (32%)	
Within the Milan criteria	109 (88%)	41 (52%)	10 (27%)	<0.001[b]

[a] HCC related tumor marker: AFP, DCP. Arbitrary cutoff values of 200 ng/ml and 40 mAU/ml were used for AFP and DCP, respectively

[b] Significant difference among all three groups

[c] Significant difference between Group C and Group A or Group B

[d] Significant difference between Group A and Group B or Group C

either or both AFP and DCP. Most patients in Group C who were in early tumor stages were diagnosed with HCC incidentally.

Figure 2a shows the distribution of tumor stages according to the Liver Cancer Study Group of Japan [19]. Proportions of patients in stages I and II were highest in the

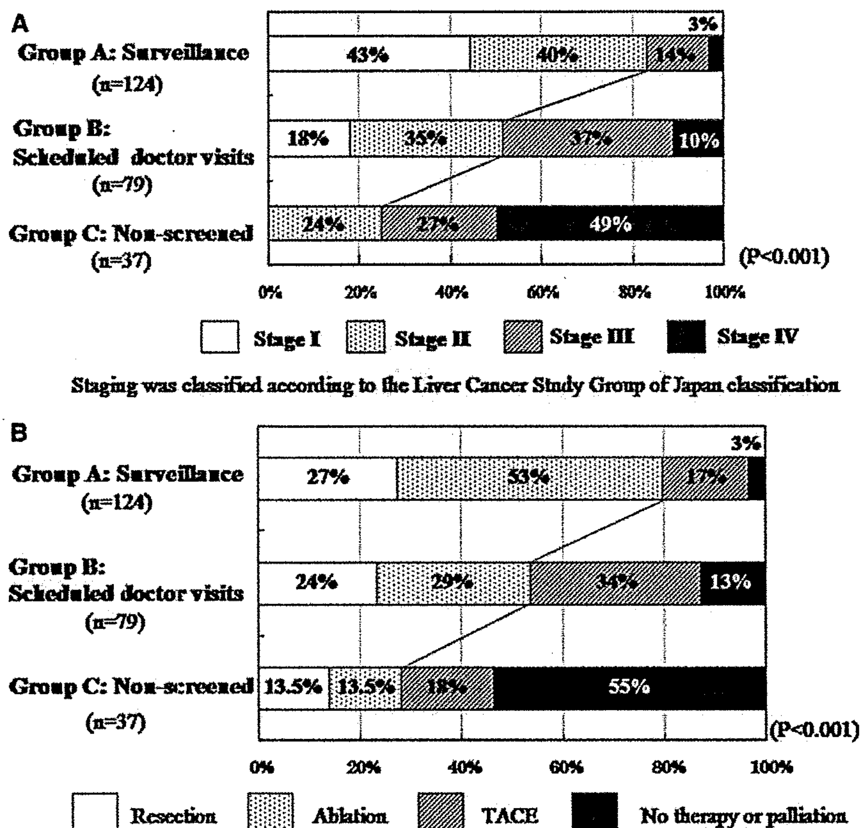

Fig. 2 **a** distribution of tumor stage according to the Liver Cancer Study Group of Japan [19]. **b** Distribution of treatment selected based on tumor stage and hepatic reserve

surveillance group (Group A); they decreased progressively through Group B to Group C ($P < 0.001$). The incidence of vascular thrombosis increased from 3% (4/124) in Group A to 11% (9/124) in Group B, and to 27% (10/37) in Group C ($P < 0.001$). Distant metastases were more frequent in Group C [14% (5/37)] than in Groups A and B [1% (1/124) and 1% (1/79), respectively] ($P < 0.001$). In Group A, the proportions of stages I and II was comparable between the patients with an interval between the latest imaging and diagnosis of HCC within 6 months and those with that of longer than 6 months [84% (86/102) vs. 77% (17/22)].

Treatment selection

Figure 2b shows the distribution of treatments selected based on the tumor stage and hepatic reserve. The proportion of patients treated with curative procedures, such as resection and ablation, was highest in Group A, and was lower in Groups B than C ($P < 0.001$). In Group C, the majority of patients received systemic chemotherapy or conservative care in hospice (palliation); most patients treated with curative procedures were diagnosed incidentally.

Survival

The median follow-up period was 35 months (range 3–94 months). During follow-ups, 148 patients died. Causes of death were cancer-related in 110 cases, liver failure in 6 (unrelated to treatment), gastrointestinal bleeding in 8, and others in the remaining 24. The distribution was similar between Groups A and B, while cancer-related causes were most prevalent (96%) in Group C. Figure 3a compares overall survival rates among the three groups. The cumulative survival rate was higher in group A than B ($P < 0.05$), and higher in group B than C ($P < 0.001$). Although survival rates of patients treated by curative procedures, such as resection and ablation, tended to be higher than the overall survival rate, there were no significant differences in the survival rates among patients in the three groups (Fig. 3b).

Discussion

For achieving better outcomes in patients with HCC, it is necessary to increase their eligibility for curative treatment. In the present study, 83% of patients under regular

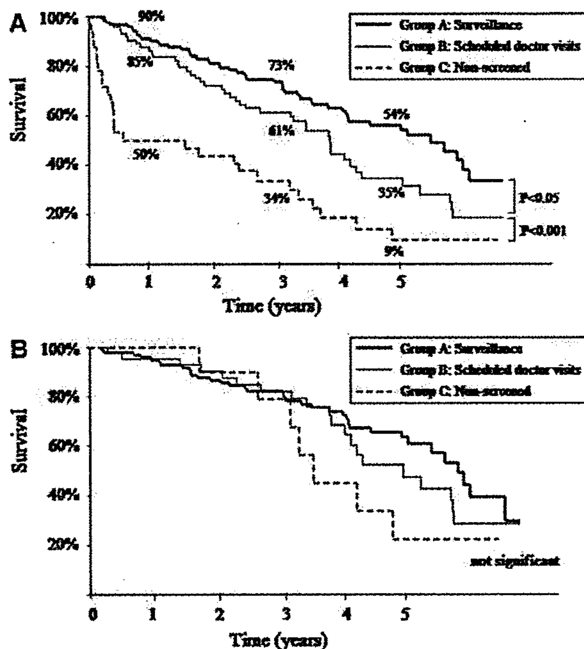

Fig. 3 **a** Survival rates in the three groups with different surveillance procedures. **b** Survival rates of the patients in three groups who had received curative treatments, such as resection and ablation

surveillance (Group A) were diagnosed with HCC at stage I or II, and the majority of them were indicated to curative treatments including surgical resection and RFA. As the results, patients in the surveillance group had a significantly better prognosis than those in the other groups without regular imaging screening (Group B) or none at all (Group C). Other reasons for the difference in prognosis among the three groups may include the following. Since the severity of underlying liver disease is a critical factor influencing the efficacy of surveillance programs, surveillance is reported to have few effects on improving the prognosis of patients with advanced cirrhosis [4, 10]. Although prevalence of cirrhosis was no different among the three groups, hepatic reserve was poorer in Group C than Group A or Group B. The dismal prognosis of patients in Group C, therefore, was attributed to either or both advanced tumor stage and poorer hepatic function. Indeed, analysis of only the patients who had received curative treatments, such as resection or ablation, revealed no significant differences in the survival among the three groups. However, the proportion of patients who had received curative treatment differed among the three groups with distinct diagnostic procedures.

Performance of surveillance would depend on the treatment selected and its efficacy. The 5-year survival of patients with a solitary HCC < 5 cm or up to 3 nodules <3 cm (Milan criteria [20]) exceeds 70% after transplantation, and that after resection surpasses 50% [12–14]. In general, transplantation offers the best long-term survival, and should be considered. In Japan, however, it is quite difficult for HCC patients to receive liver transplantation due to the shortage of donors [16], and liver resection is regarded as safe with less than 1% mortality [25]. Due to these background considerations, transplantation was not performed in the present study.

Should patients within the Milan criteria have undergone transplantation, differences in the outcome between Group A and Group B would have been reduced. In actuality, differences in the proportion of patients within the Milan criteria were lower that those in the distribution of stage I or II between them. The 5-year survival after resection was accomplished by 61% of patients with stage-II HCC and 73% of those with stage-I HCC; the staging was in accord with the definition of the Liver Cancer Study Group of Japan [16]. Thus, survival after resection in patients in Group A was comparable to that reported in transplanted patients within the Milan criteria. Indeed, the 5-year survival of patients in Group A who received curative treatments reached 63%. At present, the lack of sufficient liver donation is a worldwide problem in performing liver transplantation. Our results may indicate that surveillance by regular imaging can gain an excellent outcome where and when transplantation is hardly feasible, especially in patients with small HCC that can be treated by RFA or surgical resection.

With respect to HCC-related serological markers, most patients in Group A were negative for either AFP or DCP when they were diagnosed with HCC, in remarkable contrast to the majority of patients in Group B or C who were positive for either or both markers. In Group B, one-third of patients were tested for tumor markers during their scheduled doctor visits. However, the distribution of tumor stages was comparable between the patients with and without tumor-marker testing. Although yearly office visits would be helpful in early detection of HCC, periodical medical check-ups without screening by imaging may not necessarily detect early-stage disease, even if HCC-related markers such as AFP and DCP are tested for. This is the first report of poor performance of tumor markers including DCP in detecting early-stage HCC, and it suggests that various imaging procedures help detect HCC at a stage before levels of tumor markers elevate. Our results support the AASLD guideline that AFP alone should not be used for HCC screening when ultrasonography is not available [14]. On the other hand, it should be noted that 17% of patients in Group A in this study were diagnosed with HCC in stage III or IV, and 86% (18/21) of them were positive for either AFP or DCP. We therefore propose that HCC surveillance by regular imaging should be complemented with intermittent tests for tumor-markers, insofar as their elevated levels may reflect invisible nodules. As an

extension to this, repeated imaging with intermittent measurements of two different HCC-related tumor markers are included in the algorithm of the HCC surveillance program; it is described in Evidence-Based Clinical Practice Guidelines for HCC supported by the Japanese Ministry of Health, Labor and Welfare [26].

In a cirrhotic liver, small lesions detected by ultrasonography are likely to represent HCC. Even lesions not typical of cancer might transform into bona fide HCC during subsequent follow-ups. Generally, the incidence of HCC increases with the nodule size. In the present study, lesions >1 cm in diameter were examined by dynamic study, together with follow-ups by imaging at 3–6 month intervals, even when the appearance was atypical of HCC. Lesions >1.5 cm should be evaluated by dynamic study, preferably in combination with angiographically assisted CT or biopsy. Since the incidence of hypervascularity and moderately or poorly differentiated histology increases in HCC >1.5 cm [27–30], a 1.5-cm threshold in diameter may improve early diagnosis of HCC.

The AASLD guidelines recommend at-risk patients be screened by ultrasonography at 6–12-month intervals [14]. In our study, patients in Group B who had not undergone imaging for at least one year before the diagnosis often presented with advanced disease. A surveillance interval <12 months is therefore desirable. Although most patients in Group A were diagnosed with HCC within 6 months after the latest imaging, the proportion of stage I or II was similar between patients with the interval between the latest imaging and diagnosis of HCC below and above 6 months. However, optimal frequency of imaging was not determined in the present study. Further studies are required to determine the optimal screening interval.

Surveillance with imaging is feasible only in populations at risk for HCC, because radiological procedures are highly labor-intensive in comparison with serological testing. Major causes of cirrhosis in patients with HCC include HBV, HCV, alcoholic liver disease, exposure to aflatoxin, and possibly nonalcoholic steatohepatitis (NASH). Persistent infection with HBV or HCV is the most common cause of chronic liver disease including HCC, and increases the risk of HCC by approximately 20-fold. Heavy alcohol use and aflatoxin ingestion are environmental carcinogenic factors, and act synergistically with other risk factors [12–15]. In evaluating risks for HCC, geographic variations in incidence has to be taken into account. A recent study suggested an increased risk of HCC among patients with metabolic diseases such as diabetes or NASH [31–35]. However, the rate of HCC development in patients with NASH-related cirrhosis was significantly lower than that in those with HCV-related cirrhosis [33]. Thus, it remains uncertain how to assign surveillance programs to patients with metabolic disease.

In conclusion, surveillance programs including regular ultrasonography are useful for identifying HCC in early stages. HCC detected early is frequently indicated to curative treatments, such as resection and RFA, and is associated with better survival. Recently, several studies demonstrated that elderly patients infected with HCV developed HCC despite low-grade fibrosis stages [36, 37]. Elderly patients with HCV would be at high risk for the development of HCC, even though they do not show progression to cirrhosis. In the present study, most patients over 75 years were non-cirrhotic. Management of HCC should include early detection programs in all patients with HCV-related chronic liver disease including elderly patients in Japan.

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Absence of viral interference and different susceptibility to interferon between hepatitis B virus and hepatitis C virus in human hepatocyte chimeric mice☆

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***Background/Aims*: Both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate in the liver and show resistance against innate immunity and interferon (IFN) treatment. Whether there is interference between these two viruses is still controversial. We investigated the interference between these two viruses and the mode of resistance against IFN.**

***Methods*: We performed infection experiments with either or both of the two hepatitis viruses in human hepatocyte chimeric mice. Huh7 cell lines with stable production of HBV were also established and transfected with HCV JFH1 clone. Mice and cell lines were treated with IFN. The viral levels in mice sera and culture supernatants and messenger RNA levels of IFN-stimulated genes were measured.**

***Results*: No apparent interference between the two viruses was seen *in vivo*. Only a small (0.3 log) reduction in serum HBV and a rapid reduction in HCV were observed after IFN treatment, regardless of infection with the other virus. In *in vitro* studies, no interference between the two viruses was observed. The effect of IFN on each virus was not affected by the presence of the other virus. IFN-induced reductions of viruses in culture supernatants were similar to those in *in vivo* study.**

***Conclusions*: No interference between the two hepatitis viruses exists in the liver in the absence of hepatitis. The mechanisms of IFN resistance of the two viruses target different areas of the IFN system.**
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Keywords: **Superinfection; JFH-1; IFN-stimulated genes**

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Abbreviations: GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; OAS, 2′,5′-oligoadenylate synthetase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

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1. Introduction

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 million people are infected with HBV, and more than 170 million people are infected with HCV [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and lead to death due to liver failure and hepatocellular carcinoma [3]. To date, interferon (IFN) remains one of the most important drugs available for the treatment of both types of hepatitis viral infections. Although it is assumed that IFN suppresses viral replication through the effect of IFN-induced gene products such as mixovirus resistance protein A (MxA), RNA-dependent protein kinase (PKR), and 2′,5′-oligoadenylate synthetase (OAS) [4], the precise mechanism of action of these proteins on both hepatitis viruses are unknown.

Coinfection with both viruses leads to a rapid and severe progression of chronic liver disease [5], with a higher risk of hepatocellular carcinoma [6]. Currently, there is a debate about whether or not there is interference between the two hepatitis viruses, with some favoring such interference [7] and others arguing against such a concept [8]. A number of mechanisms can cause interference between viruses. A major mechanism of interference is induction of IFN by one virus to prevent replication of the second virus; however, viruses develop their own strategies to resist the effect of IFN. In clinical practice, practitioners often perceive that reduction of HBV in serum by IFN therapy is poorer compared with HCV. HCV levels in sera of IFN-treated patients decrease relatively rapidly, and a proportion of patients eventually show complete eradication of the virus. Furthermore, the recent use of pegylated IFN (PEG-IFN) in combination with ribavirin has improved the eradication rate [9]. Eradication of HBV by IFN, however, is usually difficult, even when using IFN combined with ribavirin [10].

The mechanisms developed by viruses to resist host innate immunity, including IFN signaling, are well established in some viruses. Such mechanisms involve interruption of IFN signaling by interacting molecules that transduce the signal from the IFN receptor through the Janus kinase (Jak) signal transducer and activator of transcription (STAT) pathway [4]. Viral proteins of paramixoviruses, for example, inhibit IFN signaling [11]. Several studies have also examined the mechanisms by which HCV resists the host immune system. These include degradation of Cardif adaptor protein by NS3A/4 protease [12]. Generally, expression of HCV protein is associated with inhibition of STAT1 function independent of STAT tyrosine phosphorylation [13]. Additionally, expression of the HCV core protein in cultured cells is associated with increased expression levels of the suppressor of cytokine signaling 3 (SOCS-3) [14]. The NS5A and E2 proteins are both inhibitors of PKR [15]. These strong actions of HCV against innate immunity are consistent with the high chronicity rate of the virus. IFN, however, effectively reduces HCV replicon in Huh7 cells [16], suggesting that the virus has little potential to disturb the actions of IFN.

In contrast to HCV, the mechanisms of IFN resistance by HBV are poorly understood. To date, only a few studies have reported the molecular mechanisms of HBV resistance against the actions of IFN. The HBV-related resistance to IFN, for example, involves upregulation of protein phosphatase 2A (PP2A) as the primary event, which subsequently leads to inhibition of protein arginine methyltransferase 1 (PRMT1) and reduced STAT1 methylation [17]. In addition to these molecular mechanisms, microarray analyses of serial liver biopsies of experimentally infected chimpanzees showed striking differences in the early immune responses to HBV and HCV. HCV, for example, induced early changes in the expression of many intrahepatic genes, including genes involved in type 1 IFN response [18], whereas HBV did not induce any detectable changes in the expression of intrahepatic genes in the first weeks of infection [19].

HBV–HCV double infection is a good model to use for assessment of the mechanism of IFN resistance by these two viruses because one can test the effect of IFN on one virus in the presence of the other virus. Recently, Bellecave et al. [20] established a novel *in vitro* model system in Huh7 cells that allowed the analysis of both viruses in a replicating context and reported the absence of direct viral interference. To this end, we used human hepatocyte chimeric mice and cell culture systems in the present study. The results showed that the presence of HBV does not affect the actions of IFN on HCV and vice versa. These results suggest the lack of interference between the two viruses in liver cells and indicate that the reported interference between the two viruses might be via inflammation including death of infected cells by cytotoxic T cells, cytokines including IFN-α and IFN-β, and interleukins produced by hepatocytes and infiltrating T cells.

2. Materials and methods

2.1. Transfection of Huh7 cells with HBV DNA and HCV RNA

Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37 °C and under 5% CO_2. Cloning of HBV DNA and the plasmid construction were performed as described previously [21]. For production of stably transfected cell lines, Huh7 cells were seeded onto 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pTRE-HB-wt [21] was transfected by the calcium phosphate precipitation method. Twenty-four hours after transfection, the cells were split and cultured in Hygromycin B-DMEM selection medium (300 μg/ml; Invitrogen Japan K.K., Osaka, Japan), while 50 colonies were isolated and cultured for identification of virus-producing cell lines. Clones positive

for both hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were selected and further analyzed for production of HBV particles. Finally, three cell lines that produced more than 10^5 copies per milliliter of HBV DNA in supernatant were selected and used for further experiments.

For transfection with HCV RNA, we used pJFH1, which contains the complementary DNA of full-length genotype 2a HCV clone JFH1 downstream of the T7 promoter [22]. *In vitro* synthesis of HCV RNA and electroporation into Huh7 cells were performed as described previously [22,23]. Briefly, cells were treated with trypsin, washed twice with ice-cold RNase-free phosphate-buffered saline, and resuspended in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) at a final concentration of 7.5×10^6 cells per milliliter. Then, 10 μg of HCV RNA to be electroporated was mixed with 0.4 mL of cell suspension and subjected to an electric pulse (950 μF and 260 V) using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish.

2.2. Generation of human hepatocyte chimeric mice

Generation of the urokinase-type plasminogen activator $(uPA)^{+/+}$ and severe combined immunodeficiency $(SCID)^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [21,23,24]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and euthanasia were performed under ether anesthesia. The concentration of serum human serum albumin, which correlates with the repopulation index [24], was measured in mice as described previously [21]. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.

2.3. Human serum samples

Human serum samples containing high titers of either HBV DNA (5.3×10^6 copies per milliliter) or genotype 1b HCV (2.2×10^6 copies per milliliter) were obtained from patients with chronic hepatitis with a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use. Chimeric mice were injected intravenously with 50 μL of either HBV- or HCV-positive human serum. Some mice were injected with HBV-positive human serum at 6 weeks after injection of HCV-positive human serum.

2.4. Analysis of HBV and HCV

HBsAg and HBeAg in culture supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Japan, Osaka, Japan). DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL H_2O [21,25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μL RNase-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20-μL reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY, USA).

2.5. RNA extraction and measurement of mRNAs of interferon-induced genes by quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One nanogram of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, OAS, and PKR. Amplification and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). Results were normalized to the transcript levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

2.6. Statistical analysis

Changes in HBV DNA and HCV RNA in mice sera were compared by Mann–Whitney test and unpaired *t* test. Differences in HBV DNA and HCV core antigen in mice sera and culture supernatants were analyzed by one-way analysis of variance followed by Scheffe's test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Infection of chimeric mouse with HBV and HCV and susceptibility to interferon

To investigate the interference between HBV and HCV and to examine the effect of IFN on both of these two viruses *in vivo*, we used six human chimeric mice. Each of six mice was inoculated intravenously with 50 μL of serum samples obtained from either HBV- or HCV-positive patients. The median HBV DNA level in HBV-positive serum-inoculated mice was 1.4×10^8 copies per milliliter (range: 5.3×10^6–3.6×10^9 copies per milliliter) at 6 weeks after inoculation (Fig. 1A), similar to our recent observation [21]. Similarly, the median HCV RNA level in HCV-positive human serum-inoculated mice was 1.0×10^7 copies per milliliter (range: 1.2×10^6–0.8×10^7 copies per milliliter) at 4 weeks after inoculation (Fig. 1B), as reported recently by our group [23]. Six weeks after inoculation, three of six HBV- or HCV-infected mice were treated daily with 7000 IU/g per day of intramuscular IFN-α for 2 weeks. Treatment resulted in a decrease of only 0.3 log in mice serum HBV DNA level compared to that in mice without treatment (Fig. 1A). In contrast, the same therapy resulted in a rapid decrease in HCV RNA to undetectable levels, as confirmed by quantitative polymerase chain reaction (PCR; Fig 1B).

To investigate the direct interference of the two viruses, we performed double-infection experiments. Ten chimeric mice were first inoculated intravenously with 50 μL of HCV-positive human serum samples. Six weeks after HCV infection when the mice developed HCV viremia, 50 μL of HBV-positive human serum samples were inoculated intravenously in 5 of 10 HCV-infected mice. All five mice became positive for both HBV and HCV at 2 weeks after HBV infection. No significant decrease in HCV RNA levels was observed in these superinfected mice before or after the development of HBV viremia (Fig. 2A). After HBV infection, there was no apparent decrease in HCV titer (Fig. 2B). Moreover, HBV DNA level in HBV–HCV-coinfected mice was comparable with that of only HBV-infected mice (Fig. 2B). These results sug-

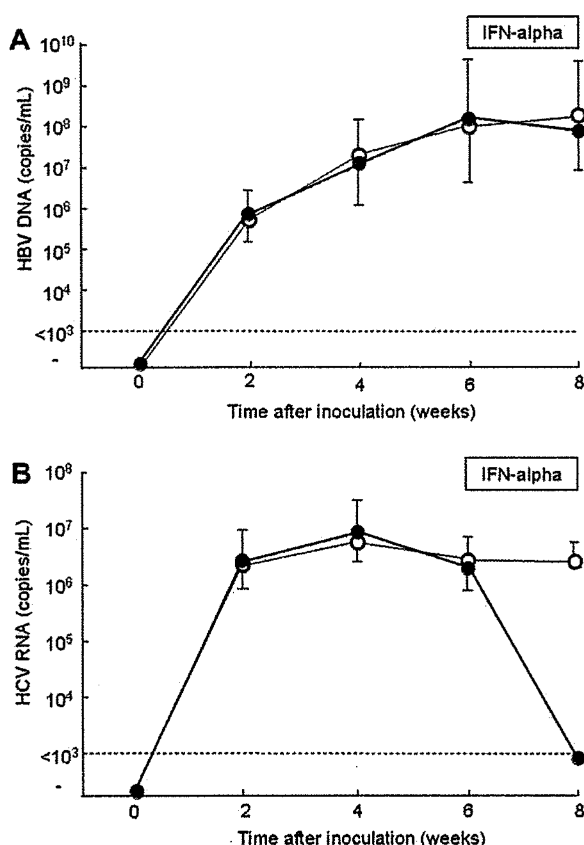

Fig. 1. Changes in serum virus titers in mice inoculated with hepatitis B virus (HBV) – positive or hepatitis C virus (HCV) – positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples. Six weeks after inoculation, three of six mice were treated daily with (closed circles) or without (open circles) 7000 IU/g per day of interferon-alpha intramuscularly for 2 weeks. Mice serum samples were extracted every 2 weeks after inoculation. Data are mean plus or minus standard deviation (*n* = 3). The horizontal dashed line represents the detection limit (10^3 copies per milliliter).

gest no interference between the two viruses in mice, which lack immunocytes known to cause hepatitis.

To further investigate if infection with either of the two hepatitis viruses alters the effect of IFN against the other virus, three HBV–HCV-coinfected mice were treated with IFN-α (Fig. 3A). Such treatment resulted in a rapid decrease in HCV RNA in all mice to undetectable levels as confirmed by quantitative PCR (Fig. 3B). In contrast, no significant decrease in HBV DNA titers was observed in these mice (Fig. 3B). These results are similar to the reduction of HCV RNA and HBV DNA in mice that were infected with either of these hepatitis viruses. These results indicate that HCV is more susceptible to IFN-α than HBV and that each virus does not alter the effect of IFN on the other virus. Because the effect of IFN on HCV was not disturbed by HBV, we assumed that HBV has no effect on the signal from IFN receptor to IFN-stimulated genes. It is possible, however, that HBV and HCV replicated in different cells in these mice. Because it was impossible to detect HCV protein and RNA in HCV-infected mouse liver by histologic examination, we performed *in vitro* experiments.

3.2. Production of both HBV- and HCV-producing cells and the effect of interferon

To investigate the effect of IFN on HBV and HCV *in vitro*, we created cell lines that produce both HBV and HCV. First, we established stable HBV-producing Huh7 cell lines. Three cell lines (Clone-39, -42, and -53) that produced HBsAg, HBeAg, and HBV DNA into the supernatant were selected (Table 1). These cell lines continuously produced HBV for more than 3 months (data not shown). Next, JFH1 RNA was transfected into these HBV-producing cell lines to produce both HBV DNA and HCV proteins into the supernatant. HBV DNA levels in the supernatants of these cell lines decreased in Clone-39, increased in Clone-42, and did not change in Clone-53 after JFH1 transfection (Fig. 4A). In contrast, HCV core antigen levels in the supernatants were higher in two of the three cell lines (Clone-39 and -42) than in Huh7 cells, and the level was not different in the remaining cell line (Clone-53) (Fig. 4B). These results indicate that the production of each of the two viruses does not disturb the replication of the other virus.

3.3. Effects of interferon on HBV and HCV in vitro

The effects of IFN on virus production in both HBV- and HCV-producing cell lines was examined by adding different amounts of IFN-α (0, 50, and 500 IU/mL) into the culture. The mRNA levels of intracellular IFN-stimulated genes such as MxA, OAS, and PKR increased in a dose-dependent manner in all three cell lines as well as in parental Huh7 cells (Fig. 5A). Following the addition of IFN, no apparent reduction of HBV was noted in the supernatant of HBV–HCV-cotransfected cell lines (Fig. 5B). In contrast, the levels of HCV core antigen in the supernatant decreased in all three cell lines treated with IFN, and the decrease was dose-dependent (Fig. 5C).

4. Discussion

Although IFN treatment for chronic HCV infection has improved with the advent of PEG-IFN, the rate of viral eradication remains unsatisfactory [9]. The mechanism responsible for failure of IFN to eradicate the virus completely must be clarified. To study the mechanism of viral resistance against IFN, analysis of viral interference may give us some hints because one of the major mechanisms of interference is through the action of IFN.

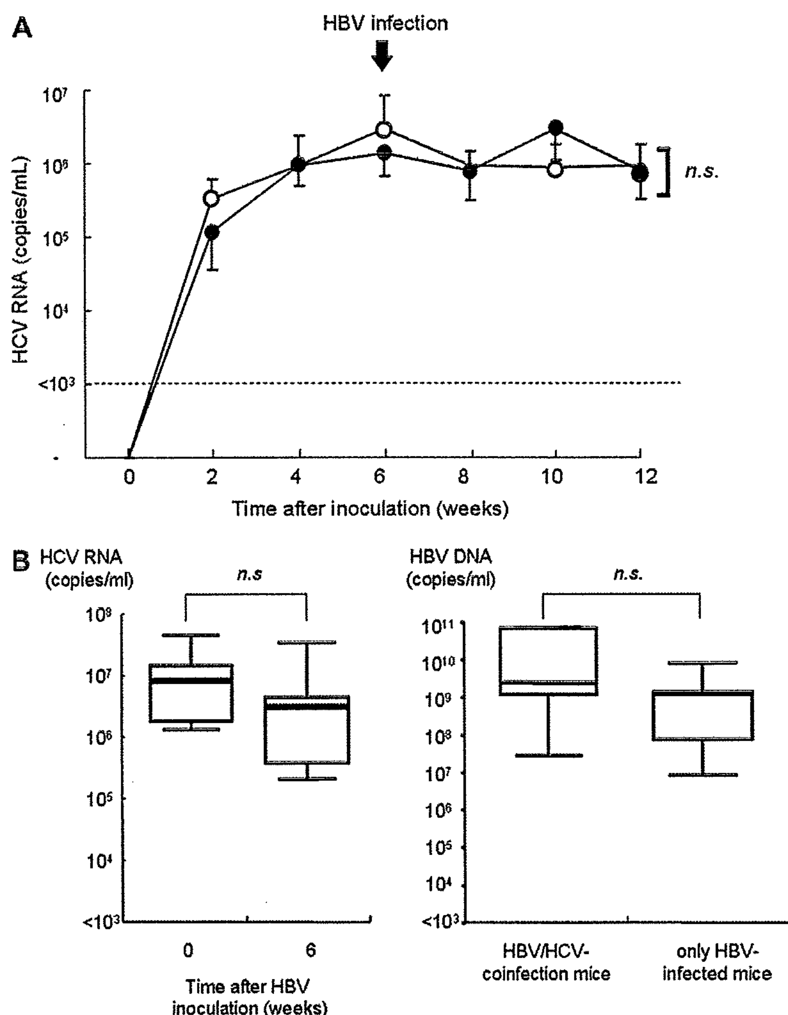

Fig. 2. Comparison of hepatitis C virus (HCV) and hepatitis B virus (HBV) titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining five mice (open circles) did not receive HBV inoculation. Data are mean plus or minus standard deviation ($n = 3$). (B) Serum HCV RNA titers in five mice infected with HCV before and at 6 weeks after HBV superinfection (left panel). Serum HBV DNA titers in five mice coinfected with HBV and HCV were compared with those of five mice with HBV infection only (Fig. 1) at 12 weeks after HCV inoculation (right panel). In these box-and-whisker plots, lines within the boxes represent the median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Accumulation of mononuclear cells is usually seen in the livers of infected individuals, in association with the state of inflammation. It is thus difficult to examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte chimeric mouse model is ideal for investigating such interaction. We expected either reduction of HCV after inoculation of HBV in HCV-infected mice or failure to develop HBV viremia or low-level HBV viremia in these mice due to viral interference; however, no reduction in HCV titers occurred in these mice, and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

Wieland et al. reported that HBV did not induce any genes during entry or expansion in HBV-infected chimpanzee livers and suggested that HBV was a stealthy virus early in the infection [19]. Because no reduction in HCV was noted during and after the development of high-level HBV viremia, we assume that HBV escapes innate immunity via an excellent mechanism without

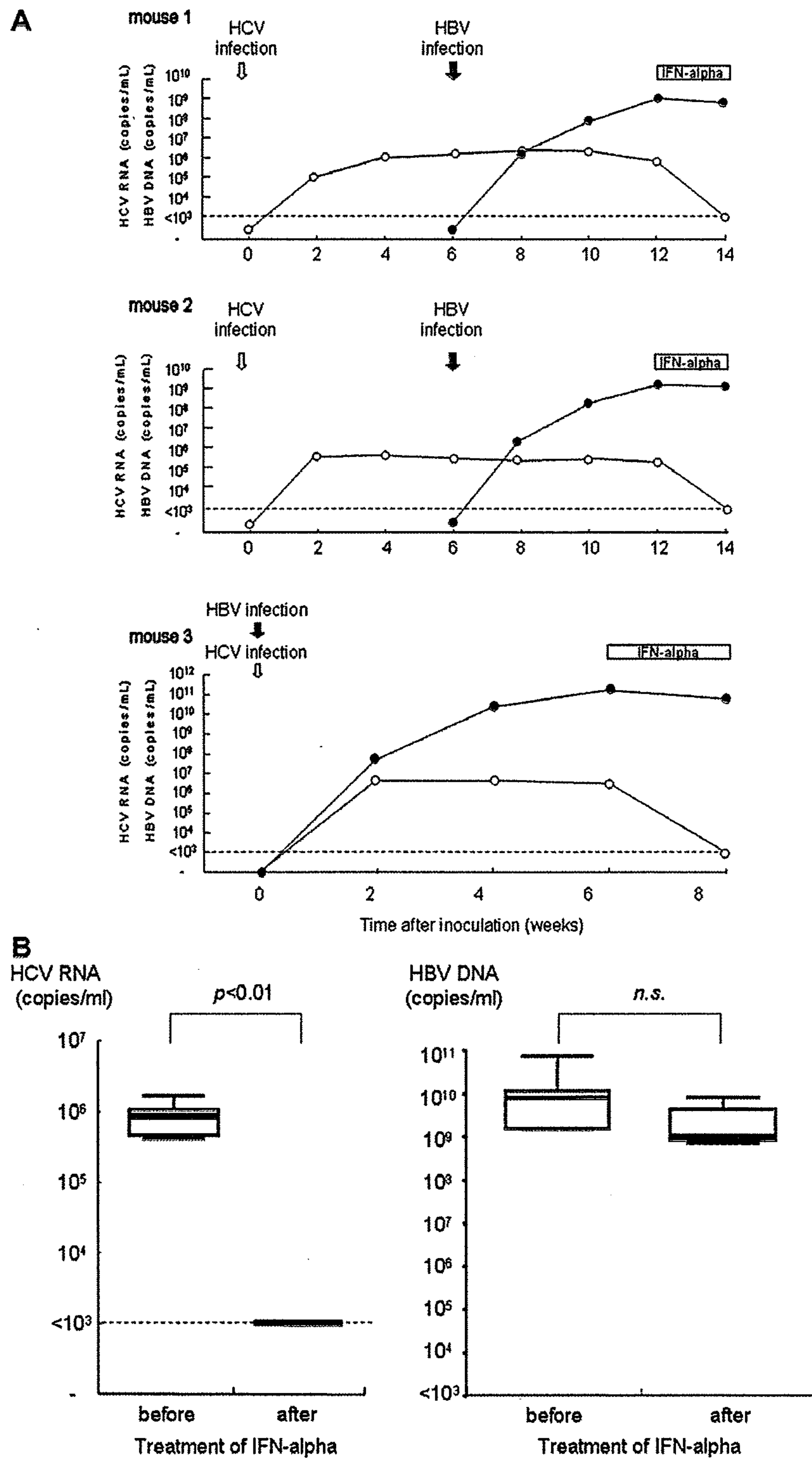

Fig. 3. Changes in serum hepatitis C virus (HCV) RNA and hepatitis B virus (HBV) DNA levels and effects of IFN on HBV–HCV-coinfected mice. Three mice (mouse 1, 2, and 3) were inoculated with both HBV- and HCV-positive human serum samples and treated daily with 7000 IU/g per day of interferon-alpha (IFN-α) intramuscularly for 2 weeks. Mice sera samples were obtained every 2 weeks after injection, and HCV RNA (open circles) and HBV DNA (close circles) were analyzed by quantitative polymerase chain reaction. (A) The horizontal dashed line represents the detectable limit (10^3 copies per milliliter). (B) Serum HCV RNA and HBV DNA titers in mice before and after 2-week IFN-α treatment. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Table 1
Hepatitis B virus (HBV) markers in supernatants of stable HBV-transfected cell lines.

Clone	HBsAg (IU/L)	HBeAg (IU/L)	HBV DNA (log copies per milliliter)
39	0.46	4.57	5.2
42	8.16	1.34	5.3
53	0.08	9.29	5.4

Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

evoking the IFN production system in liver cells. Further study using double-infected mice treated with anti-HBV nucleotide analogs and anti-HCV protease inhibitors should be conducted to confirm the present findings.

With regard to the use of IFN as a treatment, we initially assumed that HBV infection would prevent the effect of IFN on HCV and possibly vice versa in double-infection mice. Unexpectedly, the reduction of HCV by IFN therapy was quite similar in mice infected with HCV only and in those coinfected with HBV and HCV (Figs. 1 and 3). This finding indicated that HBV does not disturb the effect of IFN through signal transduction from the IFN receptor through the Jak-STAT pathway. It was, however, considered possible that HBV and HCV infect different liver cells in mice and replicated without being affected by each other. It has been reported that the same liver cell could be infected with both HBV and HCV [20,26], but it was difficult in the present study to confirm that these two viruses replicate in the same liver cell of mice because it is difficult to visualize HCV antigen and RNA in pathologic sections of the mouse liver. To address this issue, we transfected HCV to stable HBV-producing cell lines (Fig. 4). We thought that both HCV and HBV were produced from successfully HCV RNA transfected cells because transfected cells were stable HBV-producing cells. Presence of the both hepatitis viruses in the same hepatocytes has also been shown by a recent report by Bellecave et al. [20]. We showed in our cell line experiments that only HBV-transfected cell lines produced HBV and that cells cotransfected with HBV and HCV did not show a clear effect of HCV replication on HBV production (Fig. 4A). Similarly, stable production of HBV did not alter the replication of HCV (Fig. 4B). These data are consistent with a recent report [20] that showed that HCV could infect cells producing HBV and suggest a lack of interference between the two viruses in liver cells.

Using HCV-transfected HBV-producing cell lines, we demonstrated that presence of HBV did not disturb the actions of IFN on HCV (Fig. 5C). HCV utilizes certain machinery to disrupt the innate immune system; however, once exposed a large concentration of IFN, the virus shows high sensitivity, as shown in the replicon system [16,27]. Thus, HCV seems to have a relatively weak ability to disturb the antiviral actions of IFN compared with HBV. In contrast, HBV showed strong resistance against IFN in cells with diminished HCV replication [28]. The fact that HBV does not disturb IFN signaling but resists the actions of IFN suggests that HBV counteracts the actions of IFN at IFN-induced antiviral product levels.

Although the culture environment is different from the replicon system, the JFH1 strain seems relatively resistant to IFN [29]. This suggests that the core and envelope proteins, which are absent in the replicon system, might play a role in IFN resistance; however, we could not show any effect for HCV infection on the actions of IFN on HBV replication. This finding sug-

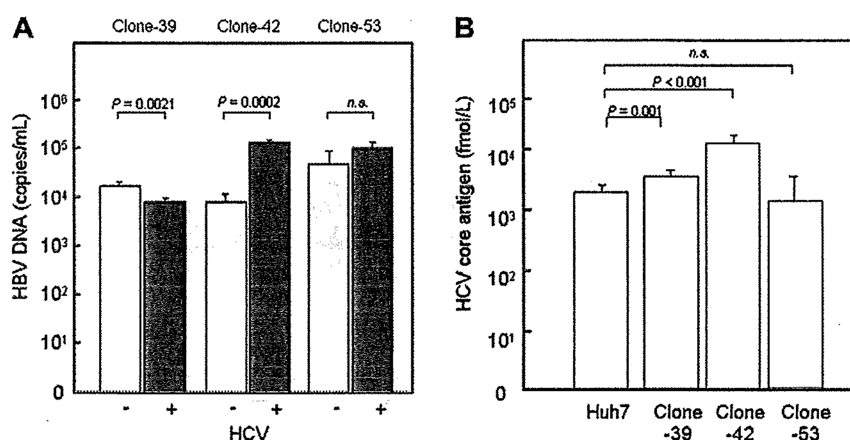

Fig. 4. Virus titers in supernatants of hepatitis B virus (HBV)–transfected or hepatitis C virus (HCV)–transfected cell lines. Huh7 cells were initially stably transfected with 1.4 genome-length HBV DNA. Three cell lines (Clone-39, -42, and -53) producing HBV DNA into the supernatant were selected. (A) HBV DNA levels in supernatants of HBV-producing cell lines 72 hours after transfection with JFH1 RNA (HCV positive) or control plasmid (HCV negative). (B) HCV core antigen levels in the supernatant of parental Huh7 cells and HBV-producing cell lines 72 h after transfection with JFH1 RNA. Data are mean plus or minus standard deviation (n = 3).

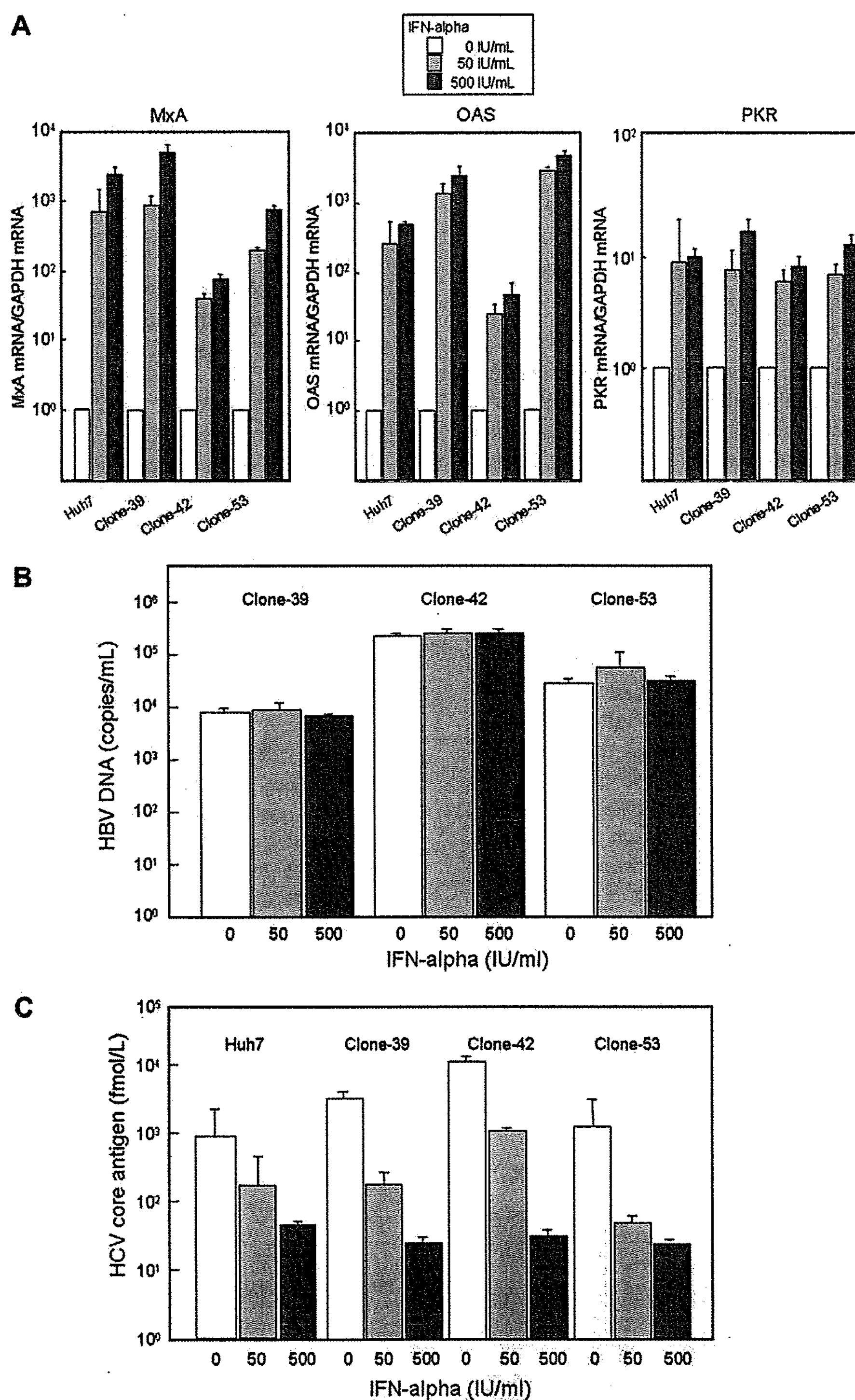

Fig. 5. Effects of interferon (IFN) treatment on hepatitis B virus (HBV) and hepatitis C virus (HCV) *in vitro*. Parental Huh7 cells and three HBV-transfected Huh7 cell lines (Clone-39, -42, and -53) were transfected with JFH1 RNA. Immediately after JFH1 transfection, the cell lines were treated with IFN-α (0, 50, and 500 IU/mL) for 72 h. (A) Intracellular gene expression levels of mixovirus resistance protein A (MxA), 2′,5′-oligoadenylate synthetase (OAS), and RNA-dependent protein kinase (PKR) were measured. RNA levels were expressed relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) messenger RNA. (B) HBV DNA and (C) HCV core antigen in supernatants were measured. Data are mean plus or minus standard deviation ($n = 3$).

gests that the core and envelope proteins have only a weak effect on IFN resistance.

In clinical practice, HBV shows high resistance against IFN therapy. This is also the case in the cell culture system, as we showed in this study and has been reported in previous studies [20,28]. The mechanism by which hepatitis viruses resist IFN needs to be clarified in order to develop new and effective therapies for eradication of these viruses.

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