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ORIGINAL ARTICLE

Efficacy and resistance of entecavir following 3 years of treatment of Japanese patients with lamivudine-refractory chronic hepatitis B

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Abstract

Purpose Lamivudine treatment of chronic hepatitis B (CHB) is associated with frequent resistance and loss of clinical benefit. We present outcomes of lamivudine-refractory Japanese patients treated with entecavir for 3 years.

Methods Eighty-two patients refractory to lamivudine therapy received entecavir 0.5 or 1 mg daily for 52 weeks in phase II study ETV-052, directly entered rollover study ETV-060, and received entecavir 1 mg daily. Responses were evaluated among patients with available samples.

Results After 96 weeks in ETV-060 (148 weeks total entecavir treatment time), 55% (36/65) of patients had hepatitis B virus (HBV) DNA of >400 copies/mL, 85% (52/61) had alanine aminotransferase (ALT) of $\geq 1 \times$ upper limit of normal (ULN), and 14.6% (7/48) achieved HBe seroconversion. A subset of 42 patients received entecavir 1 mg from phase II baseline through 148 weeks: 54% (19/35) had HBV DNA of >400 copies/mL, 84% (27/32) had ALT of $\geq 1 \times$ ULN, and 15% (4/27) achieved HBe seroconversion. Sixteen patients in the 1-mg subset had baseline and week 148 evaluable biopsy pairs: 81% (13/16) showed histologic improvement and 38% (6/16) showed

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improvement in fibrosis. Genotypic resistance to entecavir emerged in 31 patients for a 3-year cumulative resistance probability of 35.9%. Entecavir was generally well tolerated during ETV-060, with no on-treatment ALT flares.

Conclusions Long-term entecavir treatment of lamivudine-refractory CHB resulted in virologic suppression, ALT normalization, and improvements in liver histology. Resistance was consistent with that observed in worldwide studies.

Keywords Japanese · Chronic hepatitis B · Entecavir · Lamivudine refractory · Lamivudine resistant

Introduction

Chronic hepatitis B (CHB) infection is a global public health problem that is estimated to cause between 500,000 and 1.2 million deaths annually [1–3]. Three-quarters of all chronically infected individuals live in the Asia–Pacific region, where hepatitis B virus (HBV) is the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [4]. In Japan, the prevalence of HBV infection was estimated to be 0.8% in 2000, and the vast majority of individuals are infected with HBV genotype C [4–6]. Genotype C virus has been associated with high rates of progression to the complications of CHB, including cirrhosis and HCC [7–11]. In addition to genotype, the level of HBV DNA in the serum is strongly associated with liver disease progression [12, 13]. Persistently detectable and elevated viral loads predict the highest risk of progression to cirrhosis and HCC [12–14]. Suppression of HBV replication with antiviral therapy may reduce the risk of complications and improve the long-term outcomes of CHB patients [15].

Lamivudine has been widely used for the treatment of CHB since its development and initial approval 10 years ago [16, 17]. Lamivudine has demonstrated efficacy and long-term safety and was shown to result in histologic improvement when administered for up to 3 years [16, 18, 19]. However, resistance to lamivudine emerges at a rate of approximately 20% per year and is found in approximately 70% of patients after 4 years of therapy [20, 21]. The emergence of lamivudine resistance may be associated with increases in HBV DNA and alanine aminotransferase (ALT) levels, and loss of histologic response [16, 18, 22]. In patients with cirrhosis, lamivudine resistance may lead to hepatic decompensation and HCC [15, 23, 24]. Recently published CHB treatment guidelines no longer recommend lamivudine as first-line therapy for treatment-naive patients because of the problems that resistance introduces in the management of individual patients and the negative impact that lamivudine resistance has on the subsequent use of other antivirals [25].

Entecavir is a guanosine nucleoside analog that has demonstrated efficacy against nucleoside-naive and lamivudine-refractory CHB [26–29]. In global clinical studies, patients with lamivudine-refractory CHB treated with entecavir 1 mg daily for 48 weeks experienced reduction in HBV DNA levels of more than 5 log copies/mL and improvements in hepatic necroinflammation and fibrosis [28, 29]. Treatment for up to 96 weeks resulted in continued improvement of virologic, biochemical, and serologic end points [30]. In contrast to the nucleoside-naive population, emergence of resistance to entecavir occurred more frequently in the lamivudine-refractory population [30, 31]. To date, there are limited data on the efficacy of entecavir treatment beyond 96 weeks in the lamivudine-refractory patient population. A phase II study in Japan (ETV-052) demonstrated the efficacy and safety of entecavir in Japanese patients who were refractory to lamivudine therapy [32]. Immediately following completion of treatment in study ETV-052, patients were eligible to enroll in rollover study ETV-060 and receive entecavir 1 mg daily for up to 96 weeks. We present efficacy, safety, and resistance results for all patients treated in ETV-052 who rolled over into study ETV-060 for a total entecavir treatment time of up to 3 years (148 weeks). A subset of this cohort received the recommended dose of entecavir (1 mg daily) continuously from ETV-052 baseline, and results for this subset are also reported.

Materials and methods

Study design

Study ETV-060 was a long-term rollover study designed to provide open-label entecavir to lamivudine-refractory patients who completed treatment in the phase II study ETV-052 in Japan. In study ETV-052, 84 patients were randomized 1:1 to entecavir 0.5 mg ($n = 41$) or 1 mg ($n = 43$) daily for 52 weeks [32]. At baseline in this study, all patients had detectable lamivudine-resistance substitutions. Patients who completed 52 weeks of dosing in ETV-052 could enroll in ETV-060 and receive entecavir 1.0 mg daily in an open-label fashion. After completing 96 weeks of treatment in study ETV-060, patients could discontinue therapy or were eligible to receive commercially available entecavir that was approved by Japanese health authorities while ETV-060 was ongoing. The current analysis reports results for patients who completed ETV-052 and were subsequently treated in ETV-060 ($n = 82$) for a total entecavir treatment time (ETV-052 plus ETV-060) of up to 148 weeks. This cohort is termed the *lamivudine-refractory, long-term treatment cohort* (Fig. 1).

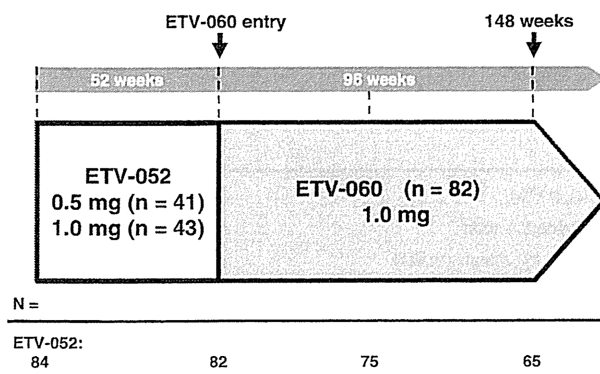

Fig. 1 Lamivudine-refractory, long-term treatment cohort. Eighty-two patients completed 52 weeks of treatment in study ETV-052 and entered rollover study ETV-060, with no interruption or gap in treatment. Sixty-five patients remained on treatment (entecavir 1.0 mg daily) through 96 weeks in study ETV-060, for a total entecavir treatment time of 148 weeks

During study ETV-060, clinical and laboratory measurements (serum chemistries, hematology, prothrombin time/international normalized ratio, and urinalysis) were assessed at baseline, weeks 2 and 4, and every 4 weeks thereafter throughout the dosing period. HBV DNA by PCR and HBV serologies were assayed at baseline, weeks 12 and 24, and subsequently every 24 weeks until week 96 or end of dosing. Liver biopsy specimens were obtained and scored for all patients at baseline and end (48 weeks) of study ETV-052, and repeat biopsy specimens were obtained at week 96 of study ETV-060 (148 weeks total entecavir treatment time) for patients who consented. Biopsy specimens were evaluated using the Knodell necroinflammatory and fibrosis scores and the corresponding New Inuyama classifications [33, 34].

Written informed consent was obtained from all patients, and the study was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice Guidelines, and Articles/Notifications of the Ministry of Health and Labor in Japan.

Patients

The inclusion criteria for study ETV-052 have been fully described elsewhere [32]. Eligible patients were adults with CHB infection and either evidence of active viral replication (HBV DNA $\geq 10^5$ copies/mL) despite at least 24 weeks of lamivudine therapy that was ongoing at the time of randomization or documented evidence of infection with HBV expressing lamivudine-resistance mutations. Patients could be hepatitis B e antigen (HBeAg)-positive or -negative and were required to have elevated levels of ALT [(1.3–10) × upper limit of normal (ULN)] and compensated liver disease. Exclusion criteria included coinfection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus; other forms of liver disease; therapy with any anti-HBV medication other than lamivudine within 24 weeks prior to randomization; and more than 12 weeks of therapy with a nucleoside or nucleotide analog (other than lamivudine) with activity against HBV. Pregnant and breast-feeding women were also excluded. All patients who completed 52 weeks of dosing in study ETV-052 were eligible to enroll in study ETV-060.

Efficacy and safety end points

Efficacy end points included the proportion of patients who achieved undetectable HBV DNA by PCR assay (<400 copies/mL), the proportion achieving ALT normalization (ALT $\leq$ 1.0 × ULN) among those with abnormal ALT at baseline, and the proportion with HBeAg loss and HBe seroconversion among those who were HBeAg-positive at baseline. Histologic results are presented for the cohort of patients who received entecavir 1 mg daily from phase II baseline and had evaluable liver biopsy pairs. Histologic improvement was defined as a $\geq$2-point decrease in the Knodell necroinflammatory score and no worsening of fibrosis (worsening: $\geq$1-point increase in the Knodell fibrosis score). Improvement in fibrosis was defined as a $\geq$1-point decrease in the Knodell fibrosis score. Histologic results were also assessed by the New Inuyama classification [34].

Safety analyses included the incidence of adverse events, serious adverse events, laboratory abnormalities, and discontinuations due to adverse events of treatment during study ETV-060, including results for patients treated beyond 96 weeks. ALT flare was defined as an on-treatment ALT measurement of more than 2 × baseline and more than 10 × ULN.

Resistance assessment

Genotypic analysis was performed on serum samples from all patients at baseline of study ETV-052 for evidence of the lamivudine-resistance substitution M204V/I in the HBV polymerase/reverse transcriptase. During study ETV-052, genotypic analysis to detect substitutions associated with entecavir resistance (at residues L180, T184, S202, M204, or M250 in the HBV polymerase/reverse transcriptase) was performed for patients with virologic breakthrough, defined as an increase in HBV DNA of $\geq$1 $\log_{10}$ copies/mL from nadir in two consecutive measurements or the last on-treatment measurement. During study ETV-060, serum samples were subjected to genotypic analysis to detect substitutions associated with entecavir resistance for patients who had HBV DNA of more than 400 copies/mL at week 100 or 148 (from study

ETV-052 baseline), or at the end of treatment (for patients who discontinued prior to week 148), and for patients who experienced virologic breakthrough.

Assay methods

All clinical laboratory tests, including HBV DNA levels, HBV serologies, and genotypic analyses, were performed at a central laboratory designated by the sponsor (SRL, Inc., Tokyo, Japan). Serum HBV DNA levels were determined by the Roche Amplicor™ PCR assay (limit of quantification = 400 copies/mL; Roche Diagnostics K.K., Tokyo, Japan). Lamivudine-resistance substitutions were identified using a PCR enzyme-linked minisequence assay (Medical & Biological Laboratories Co., Ltd., Aichi, Japan). On-treatment resistance testing was carried out by extraction of HBV DNA followed by PCR amplification and sequencing of codons 1–344 of the reverse transcriptase encoding region.

Statistical analysis

Descriptive summaries were performed. Analyses of efficacy and safety end points were based on patients who received at least one dose of study medication in study ETV-060. For binary end points, patients with missing on-treatment measurements were treated as missing (non-completer = missing analysis). Parameters represented by continuous variables were summarized by means and standard errors. Analyses of HBV DNA as a continuous parameter were applied after $\log_{10}$ transformation.

Table 1 Pretreatment baseline demographics and disease characteristics of the lamivudine-refractory long-term treatment cohort ($n = 82$)

Characteristic	ETV-060 Etecavir 1.0 mg, $n = 82$
Male, *n* (%)	71 (86.6)
Age, years, mean	43
Weight, kg, mean (±SD)	66.81 (10.58)
HBV DNA, mean $\log_{10}$ copies/mL (±SD)	7.69 (0.91)
HBeAg-positive, *n* (%)	62 (75.6)
ALT, IU/L, mean (±SD)	134.7 (111.3)
ALT > 1.0 × ULN, *n* (%)	78 (95.1)
M204V/I mutation present, *n* (%)	82 (100)
HBV genotype, *n* (%)	
A	1 (1.22)
B	2 (2.44)
C	77 (94)
Others	2 (2.44)

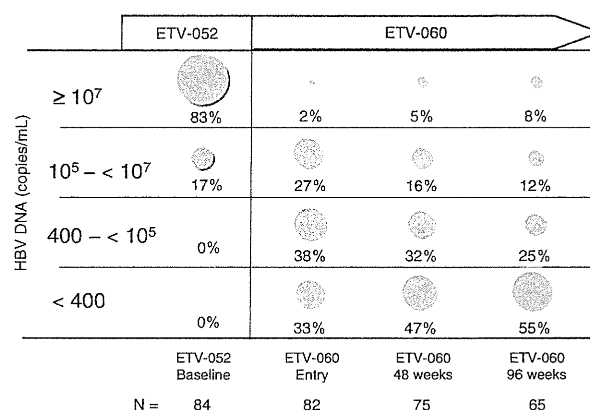

Fig. 2 Distribution of HBV DNA over time in the lamivudine-refractory, long-term treatment cohort. The proportion of patients with HBV DNA of >400 copies/mL increased through ETV-060 week 96 (148 weeks of total entecavir treatment time)

Results

Study population

Eighty-four patients were treated with entecavir in phase II study ETV-052, and 82 patients entered ETV-060, constituting the lamivudine-refractory, long-term treatment cohort (Fig. 1). Seventeen patients discontinued treatment during ETV-060 for the following reasons: adverse event ($n = 8$), protocol violation ($n = 1$), loss to follow-up ($n = 1$), and insufficient effect in the judgment of the investigator ($n = 7$). Sixty-five patients completed 96 weeks of treatment in ETV-060 for a total of 148 weeks of entecavir from ETV-052 baseline through ETV-060 (Fig. 1). Baseline (pretreatment) demographics and disease characteristics of this cohort ($n = 82$) are shown in Table 1. Eighty-seven percent (71/82) of patients were men, and mean age was 44 years. Mean HBV DNA level was 7.69 $\log_{10}$ copies/mL, mean ALT level was 135 IU/L, and 76% (62/82) of patients were HBeAg positive. All patients had documented lamivudine-resistance substitutions at M204. Ninety-four percent (77/82) of patients were infected with HBV genotype C.

Virologic response

HBV DNA was suppressed and decreased rapidly during phase II study ETV-052 [32]. For the 82 patients who entered ETV-060 after completing ETV-052, mean HBV DNA level decreased from 7.69 $\log_{10}$ copies/mL at pretreatment baseline to 3.99 $\log_{10}$ copies/mL at ETV-060 entry (after 52 weeks of entecavir treatment). HBV DNA was further suppressed during 96 weeks of treatment in ETV-060. At baseline of study ETV-060, 33% of patients (27/82) had HBV DNA of >400 copies/mL (Fig. 2), and

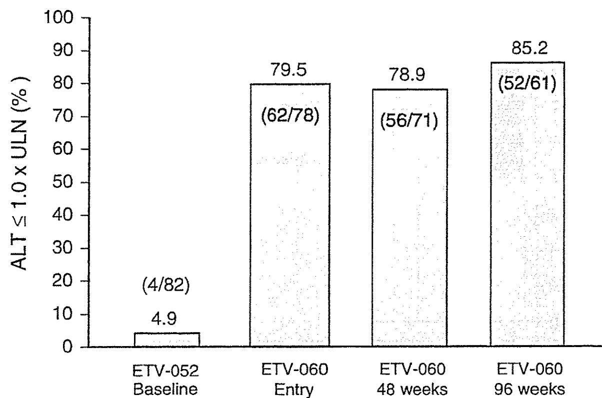

Fig. 3 Proportions of patients with normal ALT (ALT $\leq$ 1.0 $\times$ ULN) over time in the lamivudine-refractory, long-term treatment cohort. Seventy-eight patients had abnormal ALT (ALT > 1.0 $\times$ ULN) at pretreatment baseline. At week 96 of study ETV-060, patients had received a total of 148 weeks of entecavir therapy

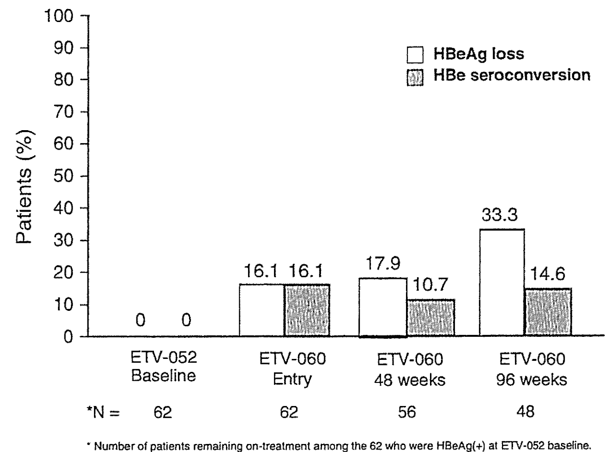

Fig. 4 Proportions of patients with HBeAg loss and HBe seroconversion over time in the lamivudine-refractory, long-term treatment cohort. Sixty-two patients were HBeAg positive at pretreatment baseline. At week 96 of study ETV-060, patients had received a total of 148 weeks of entecavir therapy

this proportion increased to 55% (36/65) by week 96 of ETV-060 (148 weeks total entecavir treatment time). Of the 17 patients who discontinued treatment during ETV-060, one patient had HBV DNA of >400 copies/mL at the last on-treatment measurement.

Biochemical response

At pretreatment baseline, 95.1% (78/82) of patients had abnormal ALT (ALT > 1.0 $\times$ ULN; Table 1; Fig. 3). After 52 weeks of treatment in ETV-052, 79.5% (62/78) of patients had normalized ALT. After 96 weeks of further treatment in ETV-060 (148 weeks total entecavir treatment time), ALT had normalized in 85.2% (52/61) of patients.

Serologic response

Sixty-two patients (76%) were HBeAg-positive at pretreatment baseline (Table 1; Fig. 4). At ETV-060 entry, 16.1% (10/62) of these patients had achieved HBe seroconversion and the same number had lost HBeAg (Fig. 4). After 96 weeks in ETV-060 (148 weeks total entecavir treatment time), 33.3% of patients (16/48) had lost HBeAg and 14.6% (7/48) had undergone HBe seroconversion.

Resistance analysis

No substitutions associated with entecavir resistance emerged during study ETV-052 [32]. Eighty-one of 82 patients were monitored for resistance from ETV-052 baseline through to the end of treatment in ETV-060 (1 patient refused consent for resistance testing). Thirty-one patients developed genotypic resistance to entecavir during the second or third year of treatment, of whom 21 experienced virologic breakthrough. The 3-year cumulative probability of resistance was 35.9% [35].

Safety

Mean exposure to entecavir during study ETV-060 was 101.3 weeks (range 7.1–148). All patients experienced at least one adverse event, and 11% (9/82) experienced serious adverse events (Table 2). One patient was diagnosed with HCC at week 57 of ETV-060. Eight patients (9.8%) discontinued treatment during ETV-060 because of adverse events, such as increased ALT, virologic breakthrough, and genotypic resistance emergence. Five of these eight patients had received entecavir 0.5 mg daily during phase II study ETV-052, and three received entecavir 1 mg from phase II baseline. There were no ALT flares during ETV-060, and no deaths were reported during the study.

Entecavir 1-mg cohort

A subset of 42 patients (42/82) received the recommended 1-mg dose of entecavir for lamivudine-refractory CHB from phase II baseline through to the end of treatment in study ETV-060. In this subset, among patients with available samples, 54% (19/35) had HBV DNA of >400 copies/mL, 84% (27/32) had ALT of $\geq$1 $\times$ ULN, and 15% (4/27) achieved HBe seroconversion after 3 years of continuous treatment with entecavir 1 mg daily. Genotypic resistance emerged in 13 patients in this cohort, and 9 of 13 patients experienced virologic breakthrough. The cumulative 3-year probability of resistance was 30.4%.

Table 2 Summary of safety during ETV-060 in the lamivudine-refractory long-term treatment cohort

	n (%) ETV-060 Entecavir 1.0 mg *n* = 82 (%)
Any adverse event	82 (100)
Clinical adverse events	78 (95.1)
Clinical serious adverse events	6 (7.3)
Grade 3–4 clinical adverse events	2 (2.4)
Most frequent clinical adverse events	
Nasopharyngitis	57 (69.5)
Headache	21 (25.6)
Diarrhea	12 (14.6)
Back pain	8 (9.8)
Laboratory adverse events	77 (93.9)
Laboratory serious adverse events	3 (3.7)
Grade 3–4 laboratory adverse events	15 (18.3)
ALT increased	24 (29.3)
ALT flare[a]	0
Discontinuations due to adverse events	8 (9.8)
Deaths	0

[a] ALT > 2 × baseline and >10 × ULN

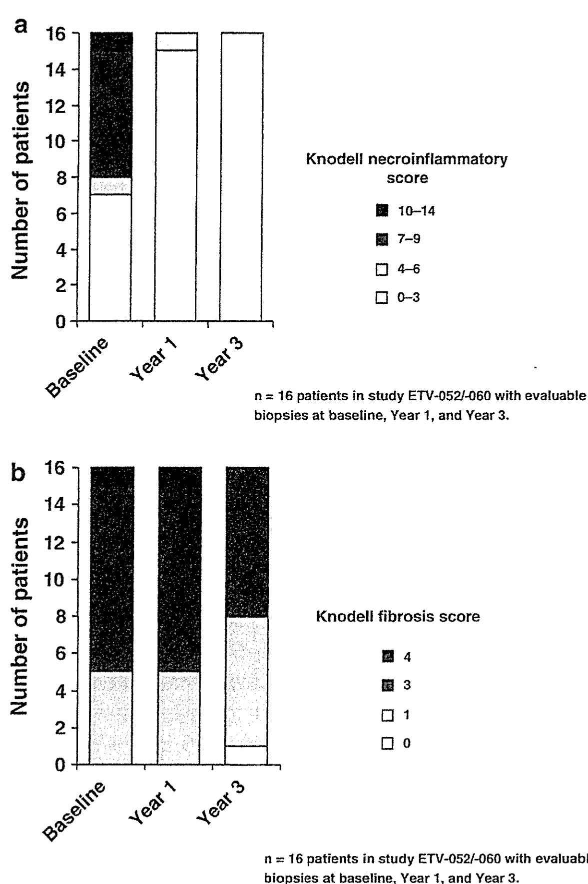

Fig. 5 Distribution of Knodell necroinflammatory scores (**a**) and Knodell fibrosis scores (**b**) at baseline, year 1 (48 weeks), and year 3 (148 weeks) for the 16 patients who had evaluable liver biopsies at all 3 time points

Sixteen (16/42) patients in the 1-mg cohort had paired evaluable liver biopsies from three time points: pretreatment (phase II) baseline, week 48, and week 148 total entecavir treatment time (ETV-060, week 96). Of these, 81% (13/16) demonstrated histologic improvement from baseline through week 148. The mean Knodell necroinflammatory score improved from 6.06 at baseline to 1.44 at week 148, and all patients (16/16) exhibited minimal necroinflammation (a Knodell necroinflammatory score of ≤3 points) at week 148 (Fig. 5a). Knodell fibrosis scores improved in 38% (6/16) of patients from baseline through week 148, and the mean Knodell fibrosis score decreased from 2.44 at baseline to 1.94 at week 148 (Fig. 5b). Liver biopsy assessments using the New Inuyama classification system confirmed the results obtained using the Knodell classification system (data not shown).

Discussion

This report describes the results of 3 years of continuous entecavir therapy in a lamivudine-refractory patient population. All patients in the lamivudine-refractory, long-term treatment cohort had highly elevated levels of HBV DNA with documented lamivudine-resistance mutations at baseline, and 94% were infected with HBV genotype C. This represents a population with potentially poor long-term outcomes. Patients with lamivudine-resistant HBV may have cross-resistance to other antivirals, and genotype C infection is associated with low rates of HBe seroconversion and high rates of liver disease progression [7, 25, 36]. These results show that entecavir therapy for up to 3 years for this population resulted in durable HBV DNA suppression and ALT normalization. More than 50% of patients in the cohort achieved undetectable HBV DNA and almost 90% normalized ALT by year 3. Similar levels of HBV DNA suppression and ALT normalization were observed for the subset of patients who received entecavir 1 mg daily throughout the treatment period. Among patients with liver biopsies from three time points (all of whom received the recommended 1-mg dose of entecavir from phase II baseline), substantial improvements in liver histology were observed: more than 80% of patients demonstrated histologic improvement at year +++3 and slow improvements in fibrosis were observed in 38% of patients.

In previously published results of a multinational clinical trial, entecavir demonstrated potent inhibition of viral

replication in HBeAg-positive, lamivudine-refractory patients infected with a variety of HBV genotypes (A–D) [28, 30]. In that trial, after 48 weeks of treatment with entecavir 1 mg daily, the mean change from baseline in HBV DNA was $-5.11 \log_{10}$ copies/mL, and 19% of patients achieved HBV DNA of >300 copies/mL. Among patients who continued to a second year of entecavir therapy, the mean change from baseline in HBV DNA increased to $-5.9 \log_{10}$ copies/mL, and 40% of patients achieved HBV DNA of >300 copies/mL. In the current study in Japanese patients, 54% achieved HBV DNA of >400 copies/mL. The higher proportion of Japanese patients suppressing HBV DNA to below the PCR limit of quantification in the current study likely reflects the effect of an additional year of entecavir therapy, as well as the lower baseline HBV DNA (7.69 $\log_{10}$ vs. 9.59 $\log_{10}$ copies/mL in the multinational study). The relatively low rate of HBe seroconversion observed in this study (15%) may be related to infection with genotype C virus. In studies in Japan and elsewhere in Asia, HBV genotype C has been associated with lower seroconversion rates than with other HBV genotypes [7, 36–38].

Achieving and maintaining HBV DNA suppression is a principal goal of CHB therapy [25, 39]. Data from prospective long-term studies have shown that elevated HBV DNA levels are associated with the development of long-term complications including cirrhosis and HCC [12–14]. Other research has correlated durable HBV DNA suppression with improved liver histology among antiviral-treated patients [19, 40]. Liaw et al. [15] showed that lamivudine therapy benefits CHB patients with advanced liver disease by reducing the risk of liver disease progression, including the development of HCC. In the present study, the reduction in hepatic necroinflammation and fibrosis observed in a subset of patients through 3 years, along with the durable virologic suppression observed in the larger cohort, suggests that entecavir helps halt or reverse liver disease progression that can lead to poor long-term outcomes.

The emergence of lamivudine resistance can lead to serious clinical consequences, including elevated levels of HBV DNA, exacerbations of hepatitis, and hepatic decompensation [18, 22, 23, 41]. While early studies of patients with lamivudine-resistant HBV suggested that switching to adefovir was efficacious, subsequent work demonstrated the rapid emergence of adefovir resistance in this patient population [42–44]. The emergence of adefovir resistance in this setting can be associated with viral rebound and hepatic decompensation [45]. Adding adefovir to ongoing lamivudine for patients who have developed lamivudine resistance has been recommended as a strategy to reduce the subsequent emergence of adefovir resistance [25, 46]. This strategy is most efficacious in patients with low HBV DNA levels and requires continued resistance surveillance [47, 48]. Studies evaluating the combination of entecavir with adefovir in lamivudine-resistant patients are currently in progress.

The rate of genotypic resistance to entecavir reported here is consistent with the rate that has been observed in multinational populations of lamivudine-refractory patients [49]. In nucleoside-naive patients, emergence of entecavir resistance is rare because of entecavir's potent viral load reduction and high genetic barrier to resistance [49, 50]. Substitutions at M204 ± L180 were detected at baseline for all patients described in this report and have been shown in previous studies to reduce in vitro susceptibility to entecavir by approximately eightfold [51]. Resistance to entecavir requires the presence of the rtM204V/I lamivudine-resistance substitution plus at least one additional amino acid substitution at rtT184, rtS202, or rtM250. In the current study, for the subset of patients who received entecavir 1 mg daily throughout the treatment period, the cumulative rate of entecavir resistance was 30% through 3 years. This is consistent with the rate observed in the entire lamivudine-refractory, long-term treatment cohort and in multinational studies of lamivudine-refractory patients through 3 years (36%) [49]. Combining entecavir with an antiviral with a different resistance profile, such as tenofovir or adefovir, may result in less frequent resistance emergence.

Entecavir was well tolerated during treatment in study ETV-052, with no discontinuations due to adverse events and three early on-treatment flares that were transient and associated with declining levels of HBV DNA [32]. Throughout the extended treatment period during ETV-060, entecavir continued to be well tolerated with relatively few discontinuations and no ALT flares observed. There were no deaths during the study, and one patient was diagnosed with HCC at week 57 of ETV-060. The extent to which long-term treatment with entecavir may reduce development of HCC in CHB patients remains under investigation.

In summary, these results show that treatment with entecavir for up to 3 years in lamivudine-refractory CHB results in continued benefit beyond the first year, including durable HBV DNA suppression and progressive improvements in liver histology, with a resistance profile consistent with that observed in other studies. Entecavir at the recommended dose of 1 mg daily is an option for patients with lamivudine-refractory CHB. Additional research evaluating the combination of entecavir plus adefovir or tenofovir in this patient population is ongoing.

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References

1. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 2004;11:97–107
2. Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733–1745
3. World Health Organization. Hepatitis B Fact Sheet WHO/204. Geneva: World Health Organization; 2000 [cited 2008 June 30]. http://www.who.int/mediacentre/factsheets/fs204/en/.
4. Merican I, Guan R, Amarapuka D, Alexander MJ, Chutaputti A, Chien RN, et al. Chronic hepatitis B virus infection in Asian countries. J Gastroenterol Hepatol 2000;15:1356–1361
5. Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the pre S2-region product. J Virol Methods 1999;80:97–112
6. Hou J, Liu X, Gu F. Epidemiology and prevention of hepatitis B virus infection. Int J Med Sci 2005;2:50–57
7. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, et al. A case-control study of clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Hepatology 2001;33:218–223
8. Jang JW, Lee YC, Kim MS, Lee SY, Bae SH, Choi JY, et al. A 13-year longitudinal study of the impact of double mutations in the core promoter region of hepatitis B virus on HBeAg seroconversion and disease progression in patients with genotype C chronic active hepatitis. J Viral Hepat 2007;14:169–175
9. Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, et al. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. J Natl Cancer Inst 2005;97:265–272
10. Orito E, Mizokami M. Differences of HBV genotypes and hepatocellular carcinoma in Asian countries. Hepatol Res 2007;37(Suppl 1):S33–S35
11. Chan HLY, Hui AY, Wong ML, Am Tse, Hung LC, Wong VW, et al. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 2004;53:1494–1498
12. Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ, et al. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. Gastroenterology 2006;130:678–686
13. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 2006;295:65–73
14. Yuen MF, Yuan HJ, Wong DK, Yuen JC, Wong WM, Chan AO, et al. Prognostic determinants for chronic hepatitis B in Asians: therapeutic implications. Gut 2005;54:1610–1614
15. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004;351:1521–1531
16. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256–1263
17. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, et al. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61–68
18. Lok AS, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 2003;125:1714–1722
19. Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003;124:105–117
20. Chang TT, Lai CL, Chien RN, Guan R, Lim SG, Lee CM, et al. Four years of lamivudine treatment in Chinese patients with chronic hepatitis B. J Gastroenterol Hepatol 2004;19:1276–1282
21. Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, et al. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis 2003;36:687–696
22. Papatheodoridis GV, Dimou E, Laras A, Papadimitropoulos V, Hadziyannis SJ. Course of virologic breakthrough under long-term lamivudine in HBeAg-negative precore mutant HBV liver disease. Hepatology 2002;36:219–226
23. Di Marco V, Marzano A, Lampertico P, Andreone P, Santantonio T, Almasio PL, et al. Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. Hepatology 2004;40:883–891
24. Andreone P, Gramenzi A, Cursaro C, Biselli M, Cammà C, Trevisani F, et al. High risk of hepatocellular carcinoma in anti-HBe positive liver cirrhosis patients developing lamivudine resistance. J Viral Hepat 2004;11:439–442
25. Lok ASF, McMahon BJ. Chronic hepatitis B. Hepatology 2007;45:507–539
26. Chang TT, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med 2006;354:1001–1010
27. Lai CL, Shouval D, Lok AS, Lai CL, Shouval D, Lok AS, et al. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. N Engl J Med 2006;354:1011–1020
28. Sherman M, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, et al. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. Gastroenterology 2006;130:2039–2049.
29. Chang TT, Gish RG, Hadziyannis SJ, Cianciara J, Rizetto M, Schiff ER, et al. A dose-ranging study of the efficacy and

tolerability of entecavir in lamivudine-refractory chronic hepatitis B patients. Gastroenterology 2005;129:1198–1209
30. Sherman M, Yurdaydin C, Simsek H, Silva M, Liaw YF, Rustgi VK, et al. Entecavir therapy for lamivudine-refractory chronic hepatitis B: improved virologic, biochemical and serology outcomes through 96 weeks. Hepatology 2008;48:99–108.
31. Gish RG, Lok AS, Chang TT, de Man RA, Gadano A, Sollano J, et al. Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. Gastroenterology 2007;133:1437–1444.
32. Suzuki F, Toyoda J, Katano Y, Sata M, Moriyama M, Imazeki F, et al. Efficacy and safety of entecavir in lamivudine-refractory patients with chronic hepatitis B: randomized controlled trial in Japanese patients. J Gastroenterol Hepatol 2008;23(9):1320–1326.
33. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981;1:431–435.
34. Ichida F, Tsuji T, Omata M, Ichida T, Inoue K, Kamimura T, et al. New Inuyama classification: new criteria for histological assessment of chronic hepatitis. Int Hepatol Commun 1996;6:112–119
35. Yokosuka O, Kumada H, Toyota J, Takaguchi K, Kobashi H, Shindo M, et al. Three-year assessment of entecavir (ETV) resistance in nucleoside-naïve and lamivudine (LVD) refractory Japanese patients with chronic hepatitis B (CHB). Hepatol Int 2008;2:A161. Abstract No.: FP067
36. Nakayoshi T, Maeshiro T, Nakayoshi T, Nakasone H, Sakugawa H, Kinjo F, et al. Difference in prognosis between patients infected with hepatitis B virus with genotype B and those with genotype C in the Okinawa Islands: a prospective study. J Med Virol 2003;70:350–354
37. Duong TN, Horiike N, Michitaka K, Yan C, Mizokami M, Tanaka Y, et al. Comparison of genotypes C and D of the hepatitis B virus in Japan: a clinical and molecular biological study. J Med Virol 2004;72:551–557
38. Kao JH, Wu NH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes and the response to interferon therapy. J Hepatol 2000;33:998–1002
39. Liaw YF, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, et al. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. Hepatol Int 2008;2:263–283
40. Mommeja-Marin H, Mondou E, Blum MR, Rousseau F. Serum HBV DNA as a marker of efficacy during therapy for CHB infection: analysis and review of the literature. Hepatology 2003;37:1309–1319.
41. Liaw YF, Chien RN, Yeh CT. No benefit to continue lamivudine therapy after emergence of YMDD mutations. Antivir Ther 2004;9:257–62
42. Peters MG, Hann Hw H, Martin P, Heathcote EJ, Buggisch P, Rubin R, et al. Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. Gastroenterology 2004;126:91–101
43. Fung SK, Chae HB, Fontana RJ, Conjeevaram H, Marrero J, Oberhelman K, et al. Virologic response and resistance to adefovir in patients with chronic hepatitis B. J Hepatol 2006;44:283–290
44. Lee YS, Suh DJ, Lim YS, Jung SW, Kim KM, Lee HC, et al. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. Hepatology 2006;43:1385–1391
45. Fung SK, Andreone P, Han SH, Rajender Reddy K, Regev A, Keeffe EB, et al. Adefovir-resistant hepatitis B can be associated with viral rebound and hepatic decompensation. J Hepatol 2005;43:937–943
46. Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. Hepatology 2007;45:307–313
47. Lampertico P, Vigano M, Manenti E, Iavarone M, Lunghi G, Colombo M. Adefovir rapidly suppresses hepatitis B in HBeAg-negative patients developing genotypic resistance to lamivudine. Hepatology 2005;42:1414–1419
48. Lampertico P, Marzano A, Levrero M, Santantonio T, Di Marco V, Brunetto M, et al. Adefovir and lamivudine combination therapy is superior to adefovir monotherapy for lamivudine-resistant patients with HBeAg-negative chronic hepatitis B. J Hepatol 2007;46(Suppl 1):S191
49. Tenney DJ, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, et al. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. Hepatology 2009;49:1503–1514
50. Colonno RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, Yu CF, et al. Entecavir resistance is rare in nucleoside-naïve patients with hepatitis B. Hepatology 2006;44:1656–1665
51. Baldick CJ, Tenney DJ, Mazzucco CE, Eggers BJ, Rose RE, Pokornowski KA, et al. Comprehensive evaluation of hepatitis B reverse transcriptase substitutions associated with entecavir resistance. Hepatology 2008;47:1473–1482

BRIEF REPORT

Effects of Hepatitis B Virus Infection on the Interferon Response in Immunodeficient Human Hepatocyte Chimeric Mice

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Complementary DNA microarray analysis of human livers cannot exclude the influence of the immunological response. In this study, complementary DNA microarray analysis was performed under immunodeficient conditions with human hepatocyte chimeric mice, and gene expression profiles were analyzed by hepatitis B virus (HBV) infection and/or interferon treatment. The expression levels of 183 of 525 genes upregulated by interferon treatment were significantly suppressed in response to HBV infection. Suppressed genes were statistically significantly associated with the interferon signaling pathway and pattern recognition receptors in the bacteria/virus recognition pathway ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). HBV infection attenuated virus recognition and interferon response in hepatocytes, which facilitated HBV escape from innate immunity.

Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Interferon α (IFN-α) has been used for the treatment of chronic hepatitis B, and many large clinical trials and meta-analyses have demonstrated the effectiveness of interferon [1–3]. However, the effect of IFN-α therapy is unsatisfactory, and the molecular basis for tolerance to IFN-α is not clearly defined.

DNA microarray technology has enabled genome-wide analysis of gene transcript levels with the use of clinical tissues and animal models, which has yielded insights into the molecular features of several liver diseases [4–6]. However, it has been difficult to determine whether the changes in gene expression were caused by viral interference or by the human immune response, because all of these studies that used clinical and experimental samples were analyzed under the influence of adaptive immune responses. Recently, Mercer and colleagues developed a human hepatocyte chimeric mouse model [7]. These mice were derived from severe combined immunodeficiency (SCID) mice, which are severely immunocompromised, and the mouse liver cells were extensively replaced with human hepatocytes [7, 8]. With the use of this chimeric mouse model, in which HBV can continuously infect human hepatocytes, the effect of drugs and the response of viral infection can be analyzed in human hepatocytes under immunodeficient conditions [9]. In this study, we performed microarray analysis with human hepatocyte chimeric mouse livers to assess the direct impacts of HBV infection and IFN treatments on gene expression profiles. We successfully demonstrated that HBV infection attenuated the expression of IFN-stimulating genes under immunodeficient conditions, which suggests that HBV proteins might afford escape mechanisms from cellular innate immunity.

METHODS

A serum sample was obtained from a HBV carrier after obtaining written informed consent for the donation and evaluation of the blood sample. The inoculum was positive for Hepatitis B surface and Hepatitis B e antigens, with slightly elevated levels of serum alanine aminotransferase and high-level viremia (HBV DNA load, 7.1 log copies/mL). The studied patient was infected with HBV genotype C. The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (approval ID: D08-9).

The $uPA^{+/+}/SCID^{+/+}$ mice were prepared and the human hepatocytes were transplanted as described elsewhere [8]. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Sixteen chimeric mice, in which >90% of the liver tissue was replaced with human hepatocytes, were divided into

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4 experimental groups. Group A contained 4 mice that were neither infected with HBV nor treated with IFN. Group B consisted of 3 mice that were treated with IFN-α for 6 h (7,000 IU per gram of body weight) just before being humanely killed but were not infected with HBV. Mice in groups C and D were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. After inoculation, we collected mouse serum samples every 2 weeks and analyzed HBV DNA titers by real-time polymerase chain reaction (PCR) and human albumin levels by means of a human albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories), as described elsewhere [9]. Virus and human albumin titer levels are shown in Supplementary data 1. All 9 mice developed measurable viremia 4 weeks after inoculation. Eight weeks after inoculation, 4 of the 9 infected mice (group C) were humanely killed without IFN treatment and the remaining 5 mice (group D) were humanely killed after 6 h of IFN-α treatment (7,000 IU per gram of body weight). The mice were infected, had serum samples extracted, and were killed humanely under ether anesthesia, as described elsewhere [8].

All 16 chimeric mice were killed humanely, and human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA later solution (Applied Biosystems). Total RNA was extracted with TRIzol reagent (Invitrogen) and labeled with cyanine 3 by use of a low RNA input linear amplification kit (Agilent Technologies) after amplification. Cyanine-3–labeled complimentary RNA was hybridized to a 44-K whole human genome oligo microarray (Agilent). Detailed protocols are described in Supplementary data 2.

Gene expression profiles were analyzed using GeneSpring GX software (version 10.0.2; Tomy Digital Biology). The detailed protocol is described in Supplementary data table 3. Complete linkage hierarchical clustering analysis was applied using Euclidean distance, and differentially expressed genes were annotated using information from the Gene Ontology (GO) Consortium. Global molecular networks and comparisons of canonical pathways were generated using Ingenuity Pathway Analysis (IPA) software (version 8.6; Ingenuity Systems).

Total RNA was extracted from the implanted human hepatocytes in the mouse livers by use of an RNeasy mini kit (Qiagen) and was reverse transcribed. The selected messenger RNA (mRNA) was quantified by real-time PCR using the 7300 real-time PCR system (Applied Biosystems), and the expression of glyceraldehyde-3-phosphate dehydrogenase served as a control. The amplification protocol and primer sequences are described in Supplementary data 4 and 5.

RESULTS

To analyze the direct effects of IFN in human hepatocytes, we compared the gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment). Of the 1403 genes that remained after screening with the Welch *T* test, 685 genes showed a >3.0-fold change between groups. Of these 685 genes, 525 genes were up-regulated and the other 160 genes down-regulated by IFN. The top 20 IFN-regulated genes are listed in Supplementary data table 6. GO analysis revealed that 8 (40%) of the top 20 genes that were upregulated with IFN treatment were related to immune response.

To analyze the effect of HBV infection in human hepatocytes, we compared the gene expression profiles between groups A (mice without HBV infection) and C (mice with HBV infection). Among the 1,714 genes hat remained after screening, 373 genes showed a >3.0-fold change between groups. Of these 373 genes, 159 genes were up-regulated and the other 214 genes down-regulated by HBV. The top 20 HBV-regulated genes are listed in Supplementary data table 7. Several oncogenic genes such as growth differentiation factor 15 and glial cell derived neurotrophic factor were included in the top group. Most of the top 20 genes that were downregulated with HBV infection were associated with transcriptional regulation.

To examine whether HBV infection may alter the effect of IFN response in human hepatocytes, we compared gene expression profiles among all groups. As mentioned above, 525 genes were upregulated by >3.0-fold by IFN in the absence of HBV infection. A comparison of groups C (mice with HBV infection but no IFN treatment) and D (mice with both HBV infection and IFN treatment) revealed that 183 (34.9%) of the 525 genes showed statistically significantly reduced IFN response with HBV infection ($P < .01$) (Supplementary data 8A). The top 20 genes in which IFN response was significantly changed by HBV infection are shown in Table 1. The mRNA expression levels of 11 selected genes among the 183 genes with reduced IFN response were also analyzed by real-time PCR, and the reductions in IFN response by HBV infection were verified (Supplementary data 8B). Additionally, we used IPA software to analyze the influence of HBV infection on the IFN response of these 183 genes by means of a pathway-oriented approach. Pathway analysis revealed that several pathways were affected by HBV infection (Table 2). The IFN response was statistically significantly attenuated by HBV infection in the pathways related to IFN signaling and pattern recognition of bacteria and viruses ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively).

DISCUSSION

Elsewhere we have demonstrated a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [9–11]. This mouse model facilitates analysis of the effect of viral infection and the response to medication under immunodeficient conditions. In this study, we performed complementary DNA microarray analysis using the chimeric mouse model and obtained gene expression profiles to analyze

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Table 1. Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus (HBV) Infection

Gene symbol	GenBank accession no.	Function	Fold change in expression level		P
			Without HBV infection	With HBV infection	
ENST00000322831	None	Unknown	4.52	−1.45	4.15×10^{-7}
AA593970	AA593970	EST	9.70	1.61	5.58×10^{-7}
THC2533996	None	Unknown	3.74	−2.50	6.97×10^{-7}
LOC388532	None	Unknown	3.11	−2.48	1.61×10^{-6}
ZNF267	NM_003414	Transcription regulator	7.66	1.79	2.30×10^{-6}
ZNF217	NM_006526	Transcription regulator	3.69	1.03	3.62×10^{-6}
CRSP3	NM_015979	Transcription regulator	7.50	−1.02	4.06×10^{-6}
MGC39372	BC025340	Hypothetical protein	30.92	7.03	5.74×10^{-6}
BF972140	BF972140	EST	16.91	4.71	5.78×10^{-6}
LOC731599	XR_015536	Hypothetical protein	3.17	−4.18	8.58×10^{-6}
LOC645676	AK126559	Hypothetical protein	3.76	1.35	9.13×10^{-6}
THC2650457	None	Unknown	78.07	6.28	1.29×10^{-5}
ZNF24	NM_006965	Transcription regulator	3.69	1.36	1.64×10^{-5}
CCDC68	NM_025214	Unknown	5.88	−2.83	1.89×10^{-5}
SP110	NM_004510	Transcription regulator	5.00	10.77	2.00×10^{-5}
FLJ21272	AK024925	Hypothetical protein	14.70	2.49	3.18×10^{-5}
PLEKHF1	NM_024310	Unknown	6.65	1.84	4.70×10^{-5}
AK026418	AK026418	Unknown	9.50	2.58	5.02×10^{-5}
hCG_1790262	XM_001133847	Unknown	3.13	−2.94	6.25×10^{-5}
CEBPD	NM_005195	Transcription regulator	8.16	1.56	7.03×10^{-5}
FLJ20273	NM_019027	RNA binding	3.37	1.11	7.11×10^{-5}

NOTE. *P* values were analyzed by the Welch *T* test. *CEBPD,* CCAAT/enhancer binding protein (C/EBP) delta; *CCDC68,* coiled-coil domain containing 68; *CRSP3,* mediator complex subunit 23 (*MED23*); EST, expressed sequence tag; *FLJ20273,* RNA binding motif protein 47 (*RBM47*); *PLEKHF1,* pleckstrin homology domain containing, family F (with FYVE domain) member 1; *SP110,* SP110 nuclear body protein; *ZNF24,* zinc finger protein 24; *ZNF217,* zinc finger protein 217; *ZNF267,* zinc finger protein 267.

the direct influence of HBV infection and IFN-α treatment on human hepatocytes.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice, in which liver tissue is largely (>90%) replaced by human hepatocytes, were used in the present study. However, a small amount of mouse-derived cells, such as interstitial cells, bile duct cells, and vascular cells, still remain in the chimeric mouse livers. Because of high homology between the human and mouse genomes, the signals from microarray analyses may be influenced by cross-hybridization with mouse mRNA. It is difficult to produce $uPA^{+/+}/SCID^{+/+}$ mice >10 weeks old without hepatocyte transplantation, and a previous study demonstrated that it is feasible to use microarray analysis in a functional genomics analysis of chimeric mice [12]. Therefore, to compensate for the contamination, the mice in group A, which were neither infected with HBV nor treated with IFN, were used as negative controls.

To analyze the effect of IFN treatment, we compared gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment); 525 genes with >3.0-fold upregulation following IFN treatment were observed. Among them, chemokine (C-X-C motif) ligand 9, chemokine (C-X-C motif) ligand 10, and chemokine (C-X-C motif) ligand 11, which promote T cell adhesion, were remarkably highly induced with IFN treatment (Supplementary data table 6) [13]. These results suggest that the antiviral effects of IFN might involve not only direct activation of IFN-stimulated proteins such as myxovirus resistance protein A and double strand RNA-dependent protein kinase but also activation of immunity via chemokines.

Second, we compared the profiles between groups A (mice without HBV infection) and C (mice with HBV infection). As shown in Supplementary data table 7, more than half (12) of the top 20 genes upregulated by HBV infection localized to the cell membrane or the extracellular region, but 14 (70%) of the 20 downregulated genes localized to the nucleus. In addition, GO analysis demonstrated that genes related to cell cycle and DNA modification were affected by HBV infection. We speculate that HBV infection promotes cell growth and DNA damage in the hepatocyte nucleus and activates the immune response in the cytoplasm. From the clinical standpoint, some healthy HBV carriers develop hepatocellular carcinoma without chronic hepatitis or cirrhosis. The present results strongly support this observation, showing that most of the affected genes are known to be associated with carcinogenesis.

Clinically, HBV is known to develop tolerance to IFN treatment in patients with chronic hepatitis B, although the mechanism is not clear. We analyzed the IFN response with and

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Table 2. Pathway Analysis of 183 Interferon-Induced Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus Infection

Canonical pathways	P	Genes
Interferon signaling	1.00×10^{-8}	*IFIT3, SOCS1, IFIT1, MX1, IFNGR1, JAK2, STAT1, TAP1, IRF1*
Role of pattern recognition receptors in recognition of bacteria and viruses	1.20×10^{-8}	*IL12A, OAS2, OAS3*(includes EG:4940), *IFIH1, PIK3R3, TLR4, NOD2, TICAM1, DDX58, CASP1, NOD1, TLR3, RIPK2*
Type 1 diabetes mellitus signaling	2.00×10^{-4}	*SOCS1, IL12A, RIPK1, GAD1, SOCS6, SOCS2, IFNGR1, JAK2, STAT1, IRF1*
Prolactin signaling	2.70×10^{-4}	*PIK3R3, SOCS1, SOCS6, SOCS2, NMI, JAK2, STAT1, IRF1*
TREM1 signaling	3.50×10^{-4}	*TLR4, NOD2, ICAM1, CASP1, JAK2, TLR3, CASP5*
Production of nitric oxide and reactive oxygen species in macrophages	3.90×10^{-4}	*PIK3R3, TLR4, RND3, PPP2R2A, PPM1J, RHOU, IFNGR1, MAP3K8, IRF8, JAK2, STAT1, IRF1*
Pathogenesis of multiple sclerosis	1.10×10^{-3}	*CXCL10, CXCL9, CXCL11*
Activation of IRF by cytosolic pattern recognition receptors	2.60×10^{-3}	*IFIH1, RIPK1, DDX58, STAT1, IFIT2, ISG15*
Dendritic cell maturation	2.60×10^{-3}	*B2M, PIK3R3, TLR4, ICAM1, IL12A, IL1RN, IRF8, JAK2, TLR3, STAT1*
Interleukin 12 signaling and production in macrophages	3.60×10^{-3}	*PIK3R3, TLR4, IL12A, IFNGR1, MAP3K8, IRF8, STAT1, IRF1*
Sphingosine-1-phosphate signaling	3.60×10^{-3}	*PIK3R3, S1PR2, RND3, CASP1, RHOU, CASP4, CASP7, CASP5*
JAK-STAT signaling	4.00×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Growth hormone signaling	4.70×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Retinoic acid mediated apoptosis signaling	8.50×10^{-3}	*TNFRSF10B, PARP8, TNFSF10, TIPARP, IRF1*

NOTE. *B2M,* beta-2-microglobulin; *CASP1,* caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase); *CASP4,* caspase 4, apoptosis-related cysteine peptidase; *CASP5,* caspase 5, apoptosis-related cysteine peptidase; *CASP7,* caspase 7, apoptosis-related cysteine peptidase; *CXCL9,* chemokine (C-X-C motif) ligand 9; *CXCL10,* chemokine (C-X-C motif) ligand 10; *CXCL11,* chemokine (C-X-C motif) ligand 11; *DDX58,* DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *GAD1,* glutamate decarboxylase 1 (brain, 67kDa); *ICAM1,* intercellular adhesion molecule 1; *IFIH1,* interferon induced with helicase C domain 1; *IFIT1,* interferon-induced protein with tetratricopeptide repeats 1; *IFIT2,* interferon-induced protein with tetratricopeptide repeats 2; *IFIT3,* interferon-induced protein with tetratricopeptide repeats 3; *IFNGR1,* interferon gamma receptor 1; *IL1RN,* interleukin 1 receptor antagonist; *IL12A,* interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35); IRF, interferon regulatory factor; *IRF1,* interferon regulatory factor 1; *IRF8,* interferon regulatory factor 8; *ISG15,* ISG15 ubiquitin-like modifier; *JAK2,* Janus kinase 2; *MAP3K8,* mitogen-activated protein kinase kinase kinase 8; *MX1,* myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *NMI,* N-myc (and STAT) interactor; *NOD1,* nucleotide-binding oligomerization domain containing 1; *NOD2,* nucleotide-binding oligomerization domain containing 2; *OAS2,* 2′-5′-oligoadenylate synthetase 2, 69/71kDa; *OAS3,* 2′-5′-oligoadenylate synthetase 3, 100kDa; *PARP8,* poly (ADP-ribose) polymerase family, member 8; *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PPM1J,* protein phosphatase, Mg2+/Mn2+ dependent, 1J; *PPP2R2A,* protein phosphatase 2, regulatory subunit B, alpha; *RHOU,* ras homolog gene family, member U; *RIPK1,* receptor (TNFRSF)-interacting serine-threonine kinase 1; *RIPK2,* receptor-interacting serine-threonine kinase 2; *RND3,* Rho family GTPase 3; *S1PR2,* sphingosine-1-phosphate receptor 2; *SOCS1,* suppressor of cytokine signaling 1; *SOCS2,* suppressor of cytokine signaling 2; *SOCS6,* suppressor of cytokine signaling 6; *STAT1,* signal transducer and activator of transcription 1, 91kDa; *TAP1,* transporter 1, ATP-binding cassette, sub-family B (MDR/TAP); *TICAM1,* Toll-like receptor adaptor molecule 1; *TIPARP,* TCDD-inducible poly(ADP-ribose) polymerase; *TLR3,* Toll-like receptor 3; *TLR4,* Toll-like receptor 4; *TNFRSF10B,* tumor necrosis factor receptor superfamily, member 10b; *TNFSF10,* tumor necrosis factor (ligand) superfamily, member 10; *TREM1,* triggering receptor expressed on myeloid cells 1.

without HBV infection, focusing on the 525 upregulated genes with IFN treatment and using all obtained gene expression profiles. Interestingly, 61.3% of the extracted genes maintained an IFN response, but in 34.9% of those genes, IFN responses were attenuated by HBV infection (Supplementary data 8A). Genes corresponding to interferon signaling, including suppressor of cytokine signaling 1 (*SOCS1*) and interferon regulatory factor 1, and those corresponding to pattern recognition of bacteria and viruses, including nucleotide-binding oligomerization domain containing 1 (*NOD1*) and receptor-interacting serine-threonine kinase 2 (*RIPK2*), were statistically significantly associated with HBV-mediated attenuation to IFN response ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). According to these results, HBV infection significantly up-regulated *SOCS1* expression and reduced the IFN responsiveness of *SOCS1*. Thus, *SOCS1* might support chronic infection of HBV in escaping the effects of innate immunity or IFN therapy. On the other hand, genes involved in recognition of viral infection were also inhibited following HBV infection. Both *NOD1* and *RIPK2* are related to innate and adaptive immune responses [14, 15]. We speculated that inhibition of *NOD1* or *RIPK2* expression facilitates HBV survival. Although further study is needed, these results may have important implications for the mechanisms of viral escape from innate immunity.

In conclusion, we performed complementary DNA microarray analysis using human hepatocyte chimeric mice. With this system, we could analyze the direct effects of IFN treatment and HBV infection without the confounding effects of the lymphocyte immunological response and obtained evidence that HBV infection attenuated the virus recognition and IFN response in

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hepatocytes, by which means HBV could evade innate immune detection and response.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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References

1. Hsu HY, Tsai HY, Wu TC, et al. Interferon-alpha treatment in children and young adults with chronic hepatitis B: a long-term follow-up study in Taiwan. Liver Int **2008**; 28:1288–97.
2. Lin SM, Tai DI, Chien RN, Sheen IS, Chu CM, Liaw YF. Comparison of long-term effects of lymphoblastoid interferon alpha and recombinant interferon alpha-2a therapy in patients with chronic hepatitis B. J Viral Hepat **2004**; 11:349–57.
3. Sung JJ, Tsoi KK, Wong VW, Li KC, Chan HL. Meta-analysis: treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. Aliment Pharmacol Ther **2008**; 28:1067–77.
4. Bigger CB, Brasky KM, Lanford RE. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. J Virol **2001**; 75:7059–66.
5. Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Kaneko S. Different signaling pathways in the livers of patients with chronic hepatitis B or chronic hepatitis C. Hepatology **2006**; 44:1122–38.
6. Okabe H, Satoh S, Kato T, et al. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res **2001**; 61:2129–37.
7. Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med **2001**; 7:927–33.
8. Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol **2004**; 165:901–12.
9. Tsuge M, Hiraga N, Takaishi H, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology **2005**; 42:1046–54.
10. Hiraga N, Imamura M, Tsuge M, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. FEBS Lett **2007**; 581:1983–7.
11. Kimura T, Imamura M, Hiraga N, et al. Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. J Gen Virol **2008**; 89:2108–13.
12. Walters KA, Joyce MA, Thompson JC, et al. Application of functional genomics to the chimeric mouse model of HCV infection: optimization of microarray protocols and genomics analysis. Virol J **2006**; 3:37.
13. Luster AD, Jhanwar SC, Chaganti RS, Kersey JH, Ravetch JV. Interferon-inducible gene maps to a chromosomal band associated with a (4;11) translocation in acute leukemia cells. Proc Natl Acad Sci U S A **1987**; 84:2868–71.
14. Tong HH, Long JP, Li D, DeMaria TF. Alteration of gene expression in human middle ear epithelial cells induced by influenza A virus and its implication for the pathogenesis of otitis media. Microb Pathog **2004**; 37:193–204.
15. Viala J, Chaput C, Boneca IG, et al. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. Nat Immunol **2004**; 5:1166–74.

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Impact of Radiation and Hepatitis Virus Infection on Risk of Hepatocellular Carcinoma

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In cohort studies of atomic bomb survivors and Mayak nuclear facility workers, radiation-associated increases in liver cancer risk were observed, but hepatitis B virus (HBV) and hepatitis C virus (HCV) infections were not taken strictly into account. We identified 359 hepatocellular carcinoma (HCC) cases between 1970 and 2002 in the cohort of atomic bomb survivors and estimated cumulative incidence of HCC by radiation dose. To investigate contributions of radiation exposure and hepatitis virus infection to HCC risk, we conducted a nested case-control study using sera stored before HCC diagnosis in the longitudinal cohort of atomic bomb survivors. The study included 224 HCC cases and 644 controls that were matched to the cases on gender, age, city, and time and method of serum storage, and countermatched on radiation dose. The cumulative incidence of HCC by follow-up time and age increased significantly with radiation dose. The relative risk (RR) of HCC for radiation at 1 Gy was 1.67 (95% confidence interval: 1.22-2.35) with adjustment for alcohol consumption, body mass index (BMI), and smoking habit, whereas the RRs for HBV or HCV infection alone were 63 (20-241) and 83 (36-231) with such adjustment, respectively. Those estimates changed little when radiation and hepatitis virus infection were fit simultaneously. The RR of non-B, non-C HCC at 1 Gy was 1.90 (1.02-3.92) without adjustment for alcohol consumption, BMI, or smoking habit and 2.74 (1.26-7.04) with such adjustment. *Conclusion:* These results indicate that radiation exposure and HBV and HCV infection are associated independently with increased HCC risk. In particular, radiation exposure was a significant risk factor for non-B, non-C HCC with no apparent confounding by alcohol consumption, BMI, or smoking habit. (Hepatology 2011;53:1237-1245)

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) are recognized as critically important risk factors for HCC. Our previous study actually showed that about 63% of HCC in atomic bomb survivors is related to HCV infection, 14% to HBV infection, and 2% to both HBV and HCV infections.[1] However, an increase of non-B, non-C HCC without HBV and HCV infection has been noted recently in Japan.[2,3] The etiology of non-B, non-C HCC has been poorly understood, although alcoholic hepatitis, nonalcoholic fatty liver disease (NAFLD) including nonalcoholic steatohepatitis (NASH), and hemochromatosis[4,5] are known as risk factors. In Japan, NAFLD has increased along with Westernization of lifestyle, and most NASH cases have developed due to such lifestyle-related diseases such as obesity, diabetes mellitus, and hyperlipidemia.[6] Obesity and diabetes mellitus, as well as NAFLD, have also recently received increased attention as risk factors for HCC.[1,7-12]

Abbreviations: AHS, Adult Health Study; BMI, body mass index; CI, confidence interval; ERR: excess relative risk; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RERF, Radiation Effects Research Foundation; RR, relative risk.

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An increased risk of liver cancer with radiation dose among atomic bomb survivors has been reported based on tumor registries, mortality studies, and pathology review,[13-16] but hepatitis virus infection status was not taken into account. In three previous HBV studies at the Radiation Effects Research Foundation (RERF), the HBV surface antigen (HBsAg)-positive proportion increased with radiation dose.[17-19] Previous research at RERF demonstrated no increase in the prevalence of anti-HCV antibody (anti-HCV Ab) with radiation dose,[20] but reported supermultiplicative effects between radiation exposure and chronic HCV infection in the etiology of HCC without cirrhosis.[21]

On the other hand, the cohort study in workers at the Mayak nuclear facility demonstrated that the risk of liver cancer mortality was significantly associated with plutonium exposure,[22] and that the incidence of HCC was marginally significantly associated with plutonium exposure.[23] In the latest analysis, a significant plutonium dose-response relationship was observed for liver cancer mortality, with risk reasonably described by a linear function.[24] However, liver cancer in those analyses included hepatoblastoma and intrahepatic cholangiocarcinoma as well as HCC. In addition, hepatitis virus infection status was not taken into account in a strict and in-depth manner, although HCC accounted for most of the liver cancer.

A lifespan study using B6C3F1 mice exposed to continuous low-dose-rate γ rays demonstrated that the incidence of HCC was significantly increased in male mice exposed to total doses equivalent to 8,000, 400, and 20 mGy and in females exposed to 8,000 mGy. However, the incidence of other liver tumors did not significantly increase except for that of hepatoblastoma in males exposed to 400 mGy.[25]

With the aim of determining whether radiation exposure is an independent risk factor for HCC, even after adjusting for hepatitis virus infection, alcohol consumption, body mass index (BMI), and smoking habit, we conducted a nested case-control study among atomic bomb survivors using stored sera. We also evaluated whether radiation, alcohol consumption, increase of BMI, and smoking habit contribute to increased risk for non-B, non-C HCC.

Patients and Methods

Cohorts. The Atomic Bomb Casualty Commission (ABCC) and its successor, the RERF, established the Adult Health Study (AHS) longitudinal cohort in 1958, in which more than 20,000 gender-, age-, and city-matched proximal and distal atomic bomb survivors and persons not present in the cities at the time of bombings are examined biennially in outpatient clinics in Hiroshima and Nagasaki.

Cases and Controls. Incident cancer cases were identified through the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registry, supplemented by additional cases detected by way of pathological review of related diseases.[26] As described in our previous study,[1] 359 primary HCC cases were diagnosed among 18,660 AHS participants between 1970 and 2002 who visited our outpatient clinics before their diagnosis. Of these, 229 cases had serum samples obtained within 6 years before HCC diagnosis. After excluding five cases with inadequate stored serum, 224 cases remained for our study. There were no important differences in characteristics such as gender, age at HCC diagnosis, city, alcohol consumption, BMI, or radiation dose to the liver (among exposed persons) between HCC cases excluded due to nonavailability of stored serum and those included in the present study.

Three control sera per case were selected from the at-risk cohort members matched on gender, age, city, and time and method of serum storage, and countermatched on radiation dose in nested case-control fashion.[27] Countermatching (to increase statistical efficiency for studying joint effects of radiation and other factors) was performed using four strata based on whole-body (skin) dose: zero dose (<0.0005 Gy), <0.05 Gy, <0.75 Gy, and ≥ 0.75 Gy (nonzero categories correspond roughly to tertiles of skin dose among all eligible exposed cases). At the time of each case diagnosis, one control serum was selected for each of the three dose strata not occupied by the case. Although the total number of potential matched control serum samples is 672, due to occasional lack of subjects with stored sera who met the matching and countermatching criteria, the total number of control serum samples actually selected was 644, which comprised 488 sera from unique noncase subjects and 156 sera from subjects sampled on repeated occasions.

Laboratory Tests. Virological assays were performed on 211 case and 640 control sera, because 13 case samples and four control samples had insufficient stored sera for these assays. HBsAg and antibody to hepatitis B core antigen (anti-HBc Ab) were measured by enzyme immunoassay (EIA), and anti-HCV Ab was measured by second-generation EIA as described.[28,29] Qualitative detection of HCV RNA among anti-HCV-positive samples was performed using a thermocycler (Whatman Biometra, Goettingen, Germany) based on the nested polymerase chain reaction (PCR) method, as described.[29] HBV infection (HBV+) status was

defined as positive for HBsAg or having a high titer of anti-HBc Ab. HCV infection (HCV+) status was defined as positive for HCV RNA. Non-B, non-C status was defined as negative for HBsAg and not having a high titer of anti-HBc Ab (HBV−) as well as negative for HCV RNA (HCV−).

Radiation Dose. Radiation dose to the liver was estimated for each subject according to Dosimetry System DS02.[30] A weighted sum of the gamma dose in gray plus 10 times the neutron dose in gray was used. Because of the countermatched selection of cases, direct comparison of doses between cases and controls in the study requires that control doses be weighted by the inverses of their selection probabilities.

Information on Alcohol Consumption, BMI, and Smoking Habit. Information on alcohol consumption was obtained from the 1965 AHS questionnaire when available, with missing data complemented using the 1978 mail survey. Alcohol consumption was quantified as volume of each type of alcoholic beverage; mean ethanol amounts were calculated as grams per day as described.[31] BMI (kg/m^2) was calculated from height and weight measured at the AHS examination. Subjects were classified based on BMI quintiles with cutpoints of 19.5, 21.2, 22.9, and 25.0. Following the recommendations for Asian people by the World Health Organization (WHO), the International Association for the Study of Obesity, and the International obesity Task Force,[32] 21.3 to 22.9 kg/m^2 was considered normal, 23.0 to 25.0 kg/m^2 as overweight, and >25.0 kg/m^2 as obese. We used information on BMI obtained 10 years before the time of HCC diagnosis or control matching because this condition is subject to change due to disease progression in the later stages before development of HCC. Information on smoking habit was obtained from the 1965 questionnaire; subjects were categorized as never, current (at time of survey), or former smoker.

Ethical Considerations. This study (RERF Research Protocol 1-04) was reviewed and approved by the Research Protocol Review Committee and the Human Investigation Committee of RERF.

Statistical Analyses. The nested case-control design was analyzed using a partial likelihood method analogous to that used for cohort follow-up studies,[33] which is in practice the same as the conditional binary data likelihood for matched case-control studies[34] except that the subjects (cases and "controls") in the study are not completely independent due to repeated selection. Cumulative incidence of HCC by follow-up time (year) and age was derived according to the method of Nelson and Aalen, using Cox regression to adjust for age at start of follow-up. Cumulative incidence by radiation dose groups (0-0.0009, 0.001-0.999, and 1.0+ Gray) was compared using the Gehan/Breslow generalized Wilcoxon test. All factors other than radiation were analyzed using relative risks (RRs) estimated by a log-linear model. Although radiation exposure could have been adjusted by matching on radiation dose as an additional matching factor in the control selection,[35] in addition to assessing effects of lifestyle factors and viral hepatitis, another purpose of the present study was to examine the effects of radiation exposure after adjustment for possible confounding and interaction by these factors, so matching on radiation—which precludes analysis of radiation risk—was not desirable; rather, we countermatched on radiation.[27,33,36] Radiation risk was analyzed using an excess relative risk (ERR) model (ERR = RR-1) as done previously.[37] The cumulative hazard estimator and comparisons by radiation dose groups were computed using Stata (StataCorp, College Station, TX; v. 11.1); all other analyses were conducted using Epicure (HiroSoft International, Seattle, WA; v. 1.81).

Results

Characteristics of Cases and Controls. Characteristics of the 224 HCC cases and 644 matched controls are shown in Table 1. HCC cases and controls were comparable with respect to gender, age, city, and time and method of serum storage by design. Prevalence of HBV and/or HCV infection status in HCC cases is higher than those in controls. Higher proportions of HCC cases had a history of alcohol consumption of more than 40 g of ethanol per day, were obese (BMI >25.0 kg/m^2), and were current smokers, compared with the controls. HCC cases also received on average higher radiation doses to the liver, compared with the controls.

Cumulative Incidence of HCC by Radiation Dose. Figure 1A,B shows the cumulative incidence of HCC by radiation dose using either follow-up time (adjusted for age at start of follow-up) or age. Of 359 HCC cases diagnosed among 18,660 AHS subjects between 1970 and 2002, the analysis was performed using 322 HCC cases, based on 16,766 subjects with known radiation dose. A significant increase with radiation dose was seen with cumulative incidence both by follow-up time ($P = 0.028$) (Fig. 1A) and by age ($P = 0.0003$) (Fig. 1B). The effect of radiation was especially evident at age 60 years or later.

Risk of HCC for Radiation and Hepatitis Virus Infection. Table 2 shows risk of HCC with and without adjustment for categorical alcohol consumption,

Table 1. Characteristics of HCC Cases and Controls

Study Variables	HCC Cases		Controls	
	Number with Complete Data	n (%)	Number with Complete Data	n (%)
Matched variables				
Gender	224		644	
Male		136 (60.7)		387 (60.1)
Female		88 (39.3)		257 (39.9)
Age at HCC diagnosis (yr)	224	67.6 (10.1)*	–	–
City	224		644	
Hiroshima		155 (69,2)		444 (68.9)
Nagasaki		69 (30.8)		200 (31.1)
Age at serum storage (yr)	224	66.4 (10.2)*	644	63.7 (9.8)*
Unmatched variables				
Viral etiology	211		640	
HBV−/HCV −		45 (21.3)		579 (90.5)
HBV+/HCV −		29 (13.7)		18 (2.8)
HBV−/HCV +		132 (62.6)		41 (6.4)
HBV +/HCV +		5 (2.4)		2 (0.3)
Alcohol consumption (g ethanol/day)	199		577	
None		97 (48.7)		315 (54.6)
0< <20		37 (18.6)		130 (22.5)
20≤ <40		20 (10.1)		64 (11.1)
≥40		45 (22.6)		68 (11.8)
BMI (kg/m^2)	210		633	
10 yrs before diagnosis				
≤19.5		38 (18.1)		122 (19.3)
19.6 - 21.2		33 (15.7)		136 (21.5)
21.3 - 22.9		36 (17.2)		142 (22.4)
23.0 - 25.0		49 (23.3)		124 (19.6)
>25.0		54 (25.7)		109 (17.2)
Smoking habit	199		578	
Never		80 (40.2)		283 (49.0)
Current smoker		107 (53.8)		262 (45.3)
Former smoker		12 (6.0)		33 (5.7)
Radiation dose to the liver (Gy)	204	0.46 (0.69)*	606	0.34 (0.56)*,†

*Mean (SD).
†Weighted mean radiation dose (among controls), calculated by weighting according to their countermatching selection probabilities.

BMI, and smoking habit based on all cases of HCC. The analysis was performed using 186 HCC cases and 600 controls, both separately (radiation only or hepatitis virus infection only) and jointly (radiation and hepatitis virus infection were fit simultaneously), based on subjects with known radiation dose and known HBV and HCV infection status. In analyses where effects of radiation and hepatitis virus infection were fitted separately, unadjusted RR at 1 Gy of HCC for radiation was 1.40 (95% confidence interval [CI], 1.07-1.89, $P = 0.013$), whereas unadjusted RRs of HCC for HBV+/HCV− status and HBV−/HCV+ status were 34 (95% CI, 13-106, $P < 0.001$) and 57 (95% CI, 27-140, $P < 0.001$), respectively. After adjustment for categorical alcohol consumption, BMI, and smoking habit, significant association was found between HCC and radiation dose or hepatitis virus infection, resulting in an RR at 1 Gy of 1.67 (95% CI, 1.22-2.35, $P < 0.001$) for radiation and RRs of 63 (95% CI, 20-241, $P < 0.001$) for HBV+/HCV− status and 83 (95% CI, 36-231, $P < 0.001$) for HBV−/HCV+ status. The above estimates changed little when radiation and hepatitis virus infection were fit simultaneously.

Risk of HCC for Radiation After Excluding Persons with Either or Both Hepatitis Virus Infections. After excluding subjects with either or both hepatitis virus infections, the RRs at 1 Gy of HCC for radiation were estimated as shown in Table 3. There were 161 cases including 119 HCV-infected individuals and 452 matched controls including 29 HCV-infected individuals without HBV infection only. There were 66 cases including 24 HBV-infected individuals and 176 matched controls including 5 HBV-infected individuals without HCV infection only. The adjusted analyses indicated that radiation exposure was significantly associated with increased risks for HCC,

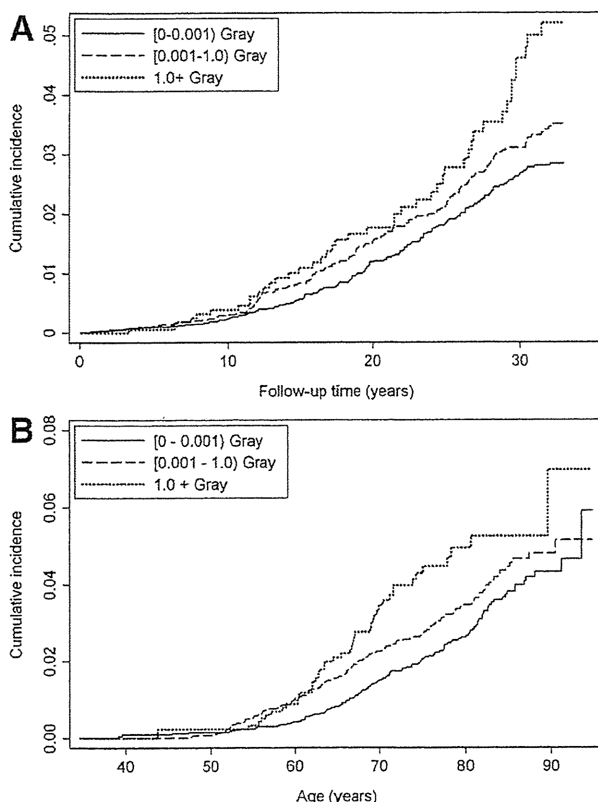

Fig. 1. Cumulative incidence of HCC (1970-2002) by radiation dose. Dotted line: radiation dose $\geq$1.0 Gy; dashed line: radiation dose 0.001 $\leq$ <1.0 Gy; solid line, radiation dose 0 $\leq$ <0.001 Gy. Cumulative HCC incidence by follow-up time (A) and age (B) increased significantly ($P = 0.028$, $P = 0.0003$, respectively) with radiation dose.

even after excluding HBV- or HCV-infected individuals. Furthermore, significant association was found between non-B, non-C HCC and radiation dose, resulting in an RR at 1 Gy of 1.90 (95% CI, 1.02-3.92, $P = 0.041$) for radiation without adjustment for categorical alcohol consumption, BMI, and smoking habit and 2.74 (95% CI, 1.26-7.04, $P = 0.007$) with such adjustment.

Risk of Non-B, Non-C HCC. Effects of alcohol consumption, BMI, and smoking habit on non-B, non-C HCC risk with or without adjustment for radiation dose were estimated using continuous and categorical covariates as shown in Table 4. RRs for continuous covariates are for a one-unit difference in the factor. Risk of non-B, non-C HCC for alcohol consumption per 20 g of ethanol per day was significant with a log-linear model (adjusted RR 1.64, 95% CI, 1.05-2.81, $P = 0.029$), but was limited to the category $\geq$40 g of ethanol per day (adjusted RR 5.49, 95% CI, 0.98-39.2, $P = 0.052$). Significant log-linear association was not found with continuous BMI, and even the category BMI >25.0 kg/m^2 (obese) 10 years before diagnosis did not evidence significant risk despite a rather large estimate of RR (adjusted RR 3.17, 95% CI, 0.92-12.3, $P = 0.068$). Current smoking evidenced significant risk (adjusted RR 5.95, 95% CI, 1.34-33.2, $P = 0.018$), but there were no continuous data on amount smoked. These results indicate that alcohol consumption per 20 g of ethanol per day, current smoking, and perhaps BMI of >25.0 kg/m^2 10 years before diagnosis are associated independently with increased risk for non-B, non-C HCC.

Discussion

The present study confirmed that radiation is associated with increased incidence of HCC among atomic bomb survivors. Additionally, the nested case-control study indicates that radiation and HBV and HCV infection are associated with increased risk for HCC, and that radiation remains an independent risk factor for HCC after taking into account hepatitis virus infection, alcohol consumption, BMI 10 years before HCC diagnosis, and smoking habit. Furthermore, significant association was observed between non-B, non-C HCC and radiation dose, alcohol consumption, and smoking, whereas obesity 10 years before diagnosis was marginally significantly associated with increased risk for non-B, non-C HCC.

In the analysis (Table 2) in which radiation dose and hepatitis virus infection were fitted separately, radiation was significantly associated with increased risk

Table 2. Risk of HCC for Radiation and HBV or HCV Infection Status

Variables	Number of Cases/Controls	Unadjusted RR (95% CI)		Adjusted* RR (95%CI)	
		Alone†	Joint‡	Alone†	Joint‡
Radiation (at 1Gy)	186/600	1.40 (1.07-1.89)	1.39 (0.93-2.26)	1.67 (1.22-2.35)	1.82 (1.09-3.34)
HBV+/HCV −	24/14	34 (13-106)	30 (11-91)	63 (20-241)	50 (16-184)
HBV−/HCV +	119/35	57 (27-140)	58 (28-147)	83 (36-231)	87 (37-251)

Abbreviations: CI, confidence interval; RR, relative risk.

*Adjusted for categorical alcohol consumption, BMI 10 yrs before diagnosis, and smoking habit.

†Radiation dose to the liver and hepatitis virus infection status were fit separately.

‡Radiation dose to the liver and hepatitis virus infection status were fit simultaneously.

Table 3. Risk of HCC for Radiation After Excluding Persons Infected with HBV and/or HCV

Subjects	Number of Cases/Controls	Unadjusted RR at 1 Gy (95% CI)	Adjusted* RR at 1 Gy (95% CI)
Exclude HBV+ (no HCV adjustment)	161/452	1.48 (1.10-2.05)	1.91 (1.34-2.81)
(adjust for HCV)		1.60 (0.997-2.78)	2.32 (1.25-4.76)
Exclude HCV+ (no HBV adjustment)	66/176	1.61 (1.003-2.76)	1.91 (1.13-3.48)
(adjust for HBV)		1.68 (0.96-3.23)	2.16 (1.12-4.76)
Exclude both HBV+ and HCV+†	42/108	1.90 (1.02-3.92)	2.74 (1.26-7.04)

Abbreviations: CI, confidence interval; RR, relative risk.
*Adjusted for categorical alcohol consumption, BMI 10 yrs before diagnosis, and smoking habit.
†Non-B, non-C status.

for HCC with or without adjustment for alcohol consumption, BMI, and smoking habit. Although this finding is in agreement with our previous understanding that liver cancer risk is significantly associated with radiation without adjustment for hepatitis virus infection among atomic bomb survivors, it is difficult to compare the HCC risk estimates between the previous and current study results.[13-16] The difficulty is caused by the inclusion of hepatoblastoma and intrahepatic cholangiocarcinoma in addition to HCC as liver cancer cases in analyses of tumor registry-based liver cancer risk (ERR at 1 Sv = 0.49),[13] mortality study- and tumor registry-based[15,16] liver cancer mortality risk (male: ERR per Sv = 0.39, female: ERR per Sv = 0.35), and liver cancer risk (male: ERR per Gy = 0.32, female: ERR per Gy = 0.28), despite the fact that the majority of liver cancer cases were HCC. Because a relatively large fraction of liver cancer cases was included that were diagnosed only on the basis of death certificates,[13,16] complete exclusion of metastatic liver tumor cases from such cases may not have been possible. Metastatic liver tumor cases were excluded in an analysis of pathological review-based liver cancer risk (ERR per Gy = 0.81), but hepatoblastoma and intrahepatic cholangiocarcinoma were included with HCC.[14]

In the current analyses adjusted for alcohol consumption, BMI, and smoking habit, the RR estimates for radiation increased slightly and showed statistical significance with adjustment for HBV and HCV infection status. HBV infection may be considered an intermediate risk factor for HCC, because three of four previous HBV screenings demonstrated that HBsAg prevalence increases with radiation dose[17-19,38]; therefore, adjustment for HBV infection status might be expected to result in a decreased radiation risk estimate. However, such interpretation is difficult because

Table 4. Risk of Non-B, Non-C HCC for Alcohol Consumption, BMI, and Smoking Habit

Variables	Number of Cases/Controls	Unadjusted RR (95% CI)†	Adjusted* RR (95% CI)†
Continuous			
Alcohol consumption (per 20 g ethanol per day)	37/96	1.51 (0.98-2.60)	1.64 (1.05-2.81)
BMI 10 yrs before diagnosis (per +1 kg/m^2 difference)	41/107	1.06 (0.95-1.18)	1.06 (0.95-1.19)
Categorical			
Alcohol consumption (g ethanol per day)			
None	22/58	1	1
$0 < \ < 20$	5/21	0.98 (0.24-3.60)	0.85 (0.18-3.48)
$20 \leq \ < 40$	2/10	0.78 (0.09-4.49)	0.68 (0.08-4.07)
≥ 40	8/7	5.25 (1.04-33.5)	5.49 (0.98-39.2)
BMI (kg/m^2) 10 yrs before diagnosis			
≤ 19.5	8/18	1.64 (0.45-6.20)	1.66 (0.42-6.83)
19.6 - 21.2	3/22	0.74 (0.12-3.66)	0.80 (0.13-4.15)
21.3 - 22.9	6/25	1	1
23.0 - 25.0	10/24	1.76 (0.42-7.93)	2.37 (0.52-11.5)
> 25.0	14 /18	2.85 (0.86-10.5)	3.17 (0.92-12.3)
Smoking habit			
Never	17/58	1	1
Current smoker	19/38	3.78 (0.99-17.1)	5.95 (1.34-33.2)
Former smoker	1/3	2.83 (0.10-52.3)	4.67 (0.16-93.7)

Abbreviations: CI, confidence interval; RR, relative risk.
*Adjusted for radiation dose to the liver.
†Alcohol consumption, BMI, and smoking habit were fit simultaneously, either as continuous (alcohol and BMI only) or categorical factors.