Determination of Hyaluronic Acid in Serum

Hyaluronic acid was determined by the agglutination of microparticles coated with proteins that specifically bind with it (Elpia-Ace HA, Fujirepio, Tokyo, Japan).

Statistical Analysis

Frequencies were compared between groups by the Mann–Whitney *U*-test and Fisher's exact test, and means by the Wilcoxon signed rank test. Loss of HBeAg or HBsAg was compared in the Kaplan–Meier life table, and differences were evaluated by log-rank test after the production limit method. A *P*-value less than 0.05 was considered significant.

RESULTS

Patients Infected Chronically With HBV Genotype A

There were 45 patients who were infected chronically with HBV genotype A and had been followed for 3 years or longer. Of them, 19 had persistently normal ALT levels (asymptomatic carriers), while the remaining 26 with elevated ALT levels possessed biopsy-proven chronic hepatitis. Table I compares demographic and virological characteristics at the baseline between the 19 asymptomatic carriers and 26 patients with chronic hepatitis. HBeAg was more frequent and the median HBV DNA level higher in patients with chronic hepatitis than asymptomatic carriers. The majority of asymptomatic carriers (79% [15/19]) and patients with chronic hepatitis (73% [19/26]) were infected with subgenotype Ae. There were three (12%) patients infected with subgenotype Aa and two of them had chronic hepatitis. Subgenotypes were not classifiable in the remaining four (21%) asymptomatic carriers and four (15%) patients with chronic hepatitis. Liver disease worsened in a single patient with chronic hepatitis. He was 47 years old at the presentation and infected with subgenotype Ae. Cirrhosis developed followed by hepatocellular carcinoma in him.

HBsAg and HBeAg in Patients With Chronic Hepatitis Infected With HBV Genotype A

Of the 26 patients infected with HBV genotype A, 4 (15%) lost HBsAg during follow-up, in comparison with 16 of the 116 (14%) patients with genotype B and 68 of the 862 (8%) with genotype C. Figure 1 compares seroclearanace of HBsAg among patients with genotype A, B, or C. The loss of HBsAg at 5 years was significantly more frequent in patients with genotype A than B or C (12% vs. 2% or 3%, $P = 0.0395$).

Of the 26 hepatitis patients with genotype A, 19 (75%) possessed HBeAg at the presentation. HBeAg was cleared from serum in 14 (74%) of them during follow-up, in comparison with the seroclearance in 36 of the 41 (88%) patients with genotype B and in 347 of the 562 (62%) with genotype C. Figure 2 compares seroclearance of HBeAg among patients with genotype A, B, or C. At 5 years of follow-up, HBeAg was cleared more frequently in patients with genotype B than in those with genotype A or C (78% vs. 58% or 45%, $P = 0.00001$).

Development of Cirrhosis and Hepatocellular Carcinoma in Patients Infected With HBV of Various Genotypes

Figure 3 compares the development of cirrhosis in patients infected with genotype A, B, or C. Of the patients with genotype A, cirrhosis developed in only one at 5 years, but not any more during follow-up for 20 years. In contrast, cirrhosis increased steadily in patients with genotype B or C; it developed twice more often in patients with genotype C than B (30% vs. 14%).

Hepatocellular carcinoma developed in the single cirrhotic patient with genotype A, but did not in any others with genotype A during follow up for 20 years (Fig. 4). It increased with time, however, in patients with genotype B or C. Hepatocellular carcinoma tended to develop more frequently in patients with genotype C than B at 20 years (15% vs. 11%).

Changes in HBV DNA Levels and Hyaluronic Acid in the Patients Infected With HBV Genotype A

Of the 26 patients with genotype A, 14 (54%) seroconverted for the loss of HBeAg, while 5 (19%) kept it throughout follow-up longer than 3 years; the remaining 7 (27%) patients were without HBeAg at the presentation and thereafter. Table II compares demographic and virological characteristics of the three

TABLE I. Baseline Characteristics of the 45 Patients Infected With HBV Genotype A Who Were Followed for Longer Than 3 Years

Feature	Asymptomatic carriers (n = 19)	Chronic Hepatitis (n = 26)	Differences
Age (years)[a]	29 (11–48)	32 (13–59)	NS[c]
Male	15 (79%)	24 (92%)	NS
Follow-up (years)[a]	6.5 (3.4–17.7)	6.8 (3.5–18.6)	NS
History of transfusion	0 (0%)	1 (4%)	NS
Anti-HCV	0 (0%)	1 (4%)	NS
HBeAg positive	3 (16%)	19 (75%)	$P = 0.0002$
HBV DNA (log copies/ml)	<2.6 (<2.6–5.9)	>7.6 (<2.6–>7.6)	$P = 0.001$
Subgroups (Aa/Ae/ND[b])	0%/79%/21%	12%/73%/15%	NS

[a]Median values are shown with the range in parentheses.
[b]Not determined.
[c]Not significant.

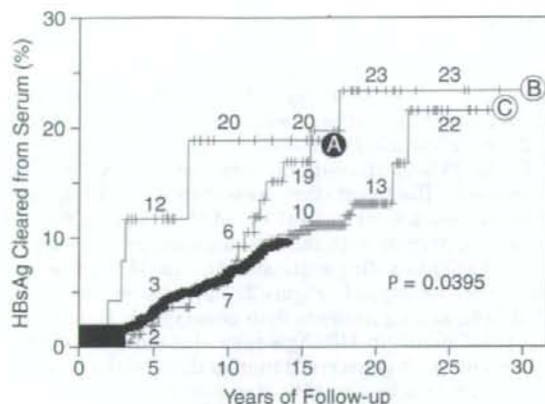

Fig. 1. Seroclearance of HBsAg during follow-up. Clearance rates of HBsAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant between genotype A and genotypes B and C at 5 and 10 years, as well as between genotypes B and C at 20 years by the log-rank test. Seroclearance of HBsAg did not spontaneously occurr in all of them.

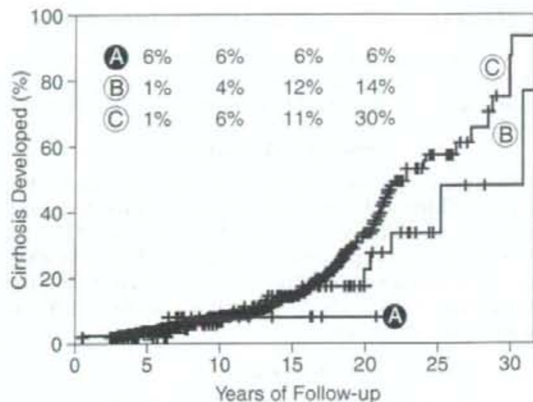

Fig. 3. Development of cirrhosis in patients infected with HBV genotype A, B, or C.

groups of patients at the baseline. Levels of HBV DNA were significantly lower in the patients without HBeAg than in those whom HBeAg persisted or who seroconverted within 3 years ($P = 0.03$).

Figure 5 compares changes in HBV DNA levels among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained negative for HBeAg. HBV DNA levels >7.6 log copies/ml continued for longer than 3 years in four of the five (80%) patients with persistent HBeAg. HBV DNA levels decreased in 13 of the 14 (93%) patients with seroconversion; they slightly changed from 6.7 to 7 log copies/ml in the remaining one patient. HBV DNA decreased to levels below the detection limit in 4 of the 14 (29%) patients with seroconversion and 1 of the 7 (14%) without HBeAg at the baseline. Of the 7 patients without HBeAg, 4 (57%) kept HBV DNA in detectable levels, comparable to 9 of the 14 (64%) patients with seroconversion. Decreases in HBV DNA during follow-up for 3 years or longer were significantly more frequent in the patients with seroconversion and those without HBeAg than in those with persistent HBeAg (93% [13/14] and 86% [6/7] vs. 20% [1/5], $P = 0.0095$ by the Fisher's exact test).

Figure 6 compares serum levels of hyaluronic acid among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained HBeAg-negative. Hyaluronic acid increased in four of the five (80%) patients in whom HBeAg persisted in contrast to only one of the seven (14%) patients without HBeAg. Increases in serum levels of hyaluronic acid ≥10 ng/ml was more frequent in the

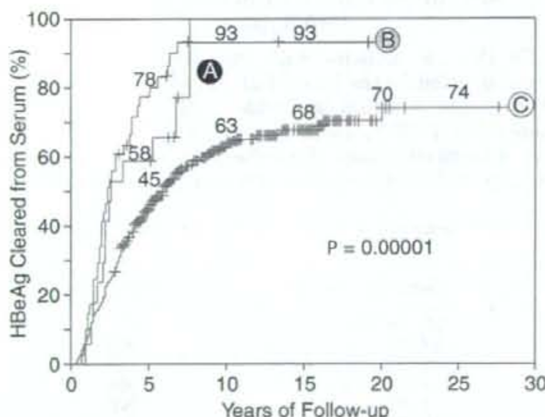

Fig. 2. Seroclearance of HBeAg during follow-up. Clearance rates of HBeAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant among genotypes A–C at 5 years as well as between genotypes B and C since 10 years or later by the log-rank test. Seroclearance of HBeAg did not spontaneously occurr in all of them.

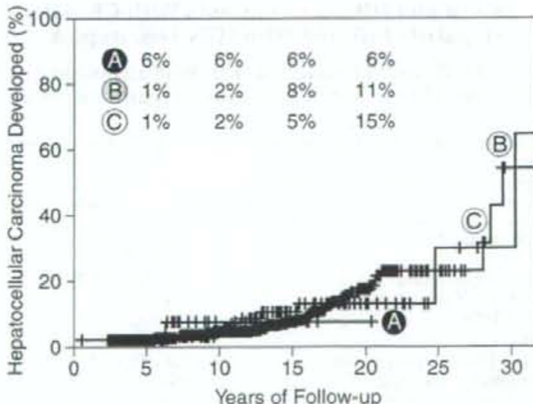

Fig. 4. Development of hepatocellular carcinoma in patients infected with HBV genotype A, B, or C.

TABLE II. Baseline Characteristics of the 26 Patients Infected With HBV Genotype A in Whom HBeAg Persisted, Who Seroconverted and Who Were Without HBeAg at the Presentation

Feature	HBeAg persisted (n = 5)	Seroconverted (n = 14)	Without HBeAg (n = 7)	Differences
Age (years)[a]	49 (24–59)	30 (13–60)	33 (14–41)	NS[c]
Male	5 (100%)	14 (100%)	5 (71%)	NS
Follow-up (years)[a]	6.2 (3.7–7.4)	9.2 (3.0–21)	8.1 (3.9–17)	NS
History of transfusion	0	1 (7%)	0	NS
Anti-HCV	0	0	1 (14%)	NS
HBV DNA (log copies/ml)	>7.6 (all patients)	>7.6 (6.7–>7.6)	4.1 (<2.6–7.1)	$P = 0.03$
Subgroups (Aa/Ae/ND[b])	(0%/80%/20%)	(7%/79%/14%)	(29%/57%/14%)	NS

[a]Median values are shown with the range in parentheses.
[b]Not determined.
[c]Not significant.

patients with persistent HBeAg than in those with seroconversion and those without HBeAg (80% [4/5] vs. 14% [2/14] and 14% [1/7], $P = 0.017$ by the Fisher's exact test).

Of the 19 hepatitis patients presenting with serum HBeAg, 16 received antiviral and/or steroid withdrawal therapies, and 11 (69%) responded by the loss of HBeAg, while the remaining 4 failed to do so (Table III). There were three patients in whom HBeAg disappeared without receiving treatments. In total, therefore, seroconversion was accomplished in 14 of the 19 (74%) patients with genotype A.

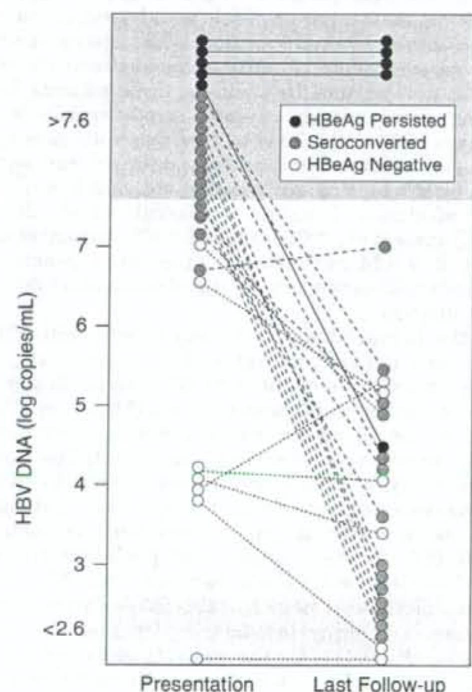

Fig. 5. Changes in serum levels of HBV DNA from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

DISCUSSION

Of the eight genotypes of HBV, E, and F are local, and confined to Central Africa and Central/South America, respectively [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003]. Genotype H is genetically close to F and distributes in Central America [Arauz-Ruiz et al., 2002]. Genotype G occurs very rarely [Stuyver et al., 2000; Chu et al., 2003; Kato et al., 2004], and is always

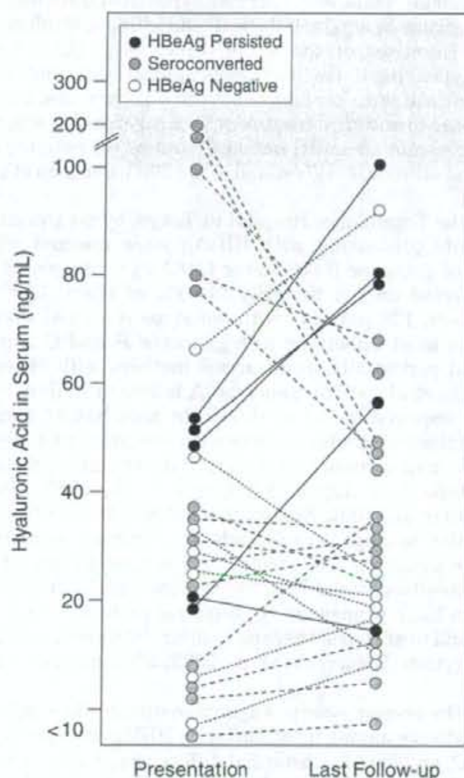

Fig. 6. Changes in serum levels of hyaluronic acid from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

TABLE III. Loss of HBeAg in the 19 Hepatitis Patients Infected With HBV Genotype A Who Had Been Followed for Longer Than 3 Years

Case No.	Sex/age	Pathology	Sub-group	Treatment	HBeAg Lost
1	M23	F1/A1	Ae	Interferon	Yes
2	M33	F2/A1	Ae	Interferon	Yes
3	M44	F3/A1	Ae	Interferon	Yes
4	M57	F2/A1	Ae	Interferon	Yes
5	M13	F1/A1	Ae	Steroid withdrawal	Yes
6	M16	F1/A1	Ae	Steroid withdrawal	Yes
7	M28	F1/A1	ND	Steroid withdrawal	Yes
8	M47	F2/A1	Aa	Steroid withdrawal	Yes
9	M17	F1/A1	Ae	Steroid/Interferon	Yes
10	M29	F1/A1	Ae	Lamivudine	Yes
11	M38	F1/A1	Ae	Lamivudine	Yes
12	M30	F1/A0	Ae	None	Yes
13	M39	F1A1	Ae	None	Yes
14	M47	F3/A2	Ae	None	Yes
15	M24	F2/A2	Ae	Interferon and others[b]	No
16	M43	F2/A1	Ae	Steroid/Interferon	No
17	M48	F1/A2	Ae	Interferon/Lamivudine	No
18	M49	F1/A1	ND[a]	Steroid withdrawal	No
19	M59	F1A1	Ae	Interferon	No

[a]Not determined.
[b]The patient received interferon, lamivudine interferon/lamivudine, and then lamivudine plus entecavir.

co-infected with HBV of the other genotypes [Kato et al., 2002, 2003]. Thus, only four genotypes (A–D) are left for comparison in epidemiological and clinical studies in most countries of the world. Since even these four genotypes have distinct geographical distributions, comparison with respect to severity of liver disease or response to antiviral treatment is hardly feasible among them, except in multi-national studies on patients of diverse ethnicities [Westland et al., 2003; Janssen et al., 2005].

In the Toranomon Hospital in Tokyo, by far the most patients presenting with HBsAg were infected with HBV of genotype B (10.5%) or C (83.3%), and genotype A infected only a minority (3.10%) of them. During 31 years, 128 patients with genotype A visited there. Unlike most infections with genotype B and C transmitted perinatally from carrier mothers with HBeAg [Okada et al., 1976], genotype A infection in Japan is often acquired in the adulthood by men having extramarital sexual contacts either with men or women; there has been no evidence for maternal transmission of HBV genotype A in Japan [Kobayashi et al., 2002, 2003; Ogawa et al., 2002; Suzuki et al., 2005]. HBV infection prevails among homosexuals in Western countries where genotype A is frequent, who poorly respond to vaccines [Goilav and Piot, 1989]. Genotype A infection in Japan has a propensity to become chronic and tends to respond to antiviral therapies better than genotype B or C infection [Kobayashi et al., 2002, 2003 Suzuki et al., 2005].

In the present study, we have compared the virological outcome among infections with HBV genotypes A, B, and C, and found substantial differences. Patients with genotype A fared better than those with genotype B or C in that they cleared HBsAg and HBeAg faster during follow-up (Figs. 1 and 2). It is not certain, however, whether or not the observed differences are influenced by the duration of HBV infection. HBV genotype A is contracted predominantly by men in the adulthood and genotypes B or C had been transmitted perinatally until 1986 when the national immunoprophylaxis started. It needs to be pointed out that this study is retrospective in nature, and most patients with HBeAg had received interferon, lamivudine or steroid withdrawal, or combination thereof. Of the 16 patients with genotype A who received treatment, 11 (69%) responded and cleared HBeAg from serum. In addition, three patients lost HBeAg spontaneously. Hence seroconversion was achieved in 14 of the 19 (74%) patients with genotype A. In view of lamivudine, adefovir dipivoxil, and pegylated interferon that are reported efficacious in treatment of chronic hepatitis B [Perrillo et al., 2000; Hadziyannis et al., 2003; Kumada, 2003; Janssen et al., 2005], it would be unethical to evaluate genotype-dependent differences in the natural course of persistent HBV infection.

Of the 45 individuals chronically infected with HBV genotype A and had been followed for 3 years or longer, HBeAg was more frequent and HBV DNA levels higher in the 26 patients with biopsy-proven chronic hepatitis than in the 19 asymptomatic carriers. Among the 26 patients with genotype A, HBeAg persisted throughout the observation in 5 (19%) and disappeared in 14 (54%); HBeAg remained negative in the other 7 (27%) patients. HBV DNA stayed in high levels more frequently ($P = 0.0095$) in the patients with persistent HBeAg (80% [4/5]) than in those who seroconverted (7% [1/14]) or remained HBeAg-negative (29% [2/7]). Furthermore, increases in serum hyaluronic acid $\geq$10 ng/ml were more frequent ($P = 0.017$) in the patients with persistent HBeAg (80% [4/5]) than in those with seroconversion (14% [2/17]) or HBeAg-negative (14% [1/7]). Although the patients with genotype A fare better than those with genotype B or C, persistent HBeAg refractory to

treatment would predict ongoing liver disease with fibrosis in progress.

Recently, subgenotypes have been recognized and they may influence the biology of HBV and liver disease. For instance, a subgenotype of B having the recombination with genotype C (Ba) induces more severe liver disease with poorer response to lamivudine than that without the recombination (Bj) [Sugauchi et al., 2002, 2003; Akuta et al., 2003]. As for genotype A, there are two subgenotypes with different geographical distributions. Subgenotype Ae is common in Europe and the United States, while Aa is prevalent in Asia and Africa [Bowyer et al., 1997; Sugauchi et al., 2004]. In a case-control study, HBeAg was more frequent and HBV DNA levels higher in carriers of Ae than Aa [Tanaka et al., 2004]. The majority of genotype A strains from our patients (86%) were found to be Ae; they were probably introduced to Japan by immigrants and visitors from foreign countries [Kobayashi et al., 2004]. Cirrhosis and hepatocellular carcinoma developed in only one of the 19 (5%) patients infected with subgenotype Ae, in remarkable contrast to frequent hepatocellular carcinoma in Africa where infection with subgenotype Aa is common during the infancy [Kew et al., 2005].

Although there have been accumulating lines of evidence for virological and clinical influence of HBV genotypes, there are conflicting views on them. Differences between genotypes B and C in Asia [Kao et al., 2000; Orito et al., 2001; Tsubota et al., 2001; Chan et al., 2004; Yu et al., 2005] have not been reproduced, probably due to selection bias for the patients with severe disease [Sumi et al., 2003] or subgenotypes of B different between Japan (Bj) and Hong Kong (Ba) [Yuen et al., 2004]. Liver disease, once advanced beyond a certain severity, will progress spontaneously irrespective of HBV genotypes. Subgenotype Ba having the recombination with genotype C may be endowed with a higher disease-inducing capacity than subgenotype Bj without the recombination [Sugauchi et al., 2002].

Of patients infected with three different genotypes in Japan, the virological outcome of persistent HBV infection was more favorable for those with genotype A than B and C in that order. It is not known where genotype D stands, although it fares worse than genotype A in chronic HBV infection [Thakur et al., 2002; Janssen et al., 2005]. In ranking the four major genotypes (A–D) in disease-inducing capacity and response to antiviral therapies, perinatals, or adulthood transmission, as well as subgenotypes inherent to countries, would have to be taken into considerations [Sugauchi et al., 2002, 2004; Norder et al., 2004].

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Changes in Viral Loads of Lamivudine-Resistant Mutants and Evolution of HBV Sequences During Adefovir Dipivoxil Therapy

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The addition of adefovir dipivoxil (ADV) to ongoing lamivudine therapy is effective against lamivudine-resistant virus in patients with hepatitis B virus (HBV) infection. We studied 39 patients who received ADV added to lamivudine for breakthrough hepatitis. We determined early viral changes (12 weeks) in YMDD mutants (rtM204I [YIDD sequence], rtM204V [YVDD]) and rtL180M in all 39 patients as well as amino acid changes in the polymerase reverse transcriptase (rt) region and precore/core promoter mutations in 15 patients who received long-term treatment (more than 1 year). Changes in rtM204I and rtL180M viral loads were greater than that of the rtM204V, albeit statistically insignificant. Moreover, the greatest change in viral load was seen for rtM204I without hepatitis B e antigen (HBeAg). The precore mutant was replaced with wild-type virus in three of eight patients after 1 year of added ADV therapy. Compared to baseline with lamivudine therapy only, new amino acid mutations were seen in the rt region at baseline with ADV in seven patients. At 1 year after ADV coadministration, the YMDD motif was replaced with wild-type (rt204M) in two patients, in whom mutations were fewer and of a different type. We conclude that the rtM204I may be more sensitive to ADV in vivo. ADV tended to select wild-type virus from precore mutants. Moreover, viruses that were wild-type in the rt region reappeared after 1 year of ADV coadministration in some patients. ***J. Med. Virol. 78:1025–1034, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; breakthrough hepatitis; YMDD mutant; precore; core promoter; reverse transcriptase

INTRODUCTION

The goal of therapy in patients with hepatitis B virus (HBV) is to limit or reverse progression of the disease through the sustained suppression of HBV replication [Conjeevaram and Lok, 2003]. Several studies have reported that various nucleoside analogues such as lamivudine are effective in suppressing HBV replication, improving transaminase levels and liver histology, and enhancing the rate of loss of hepatitis B e antigen (HBeAg) [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. A major problem with the long-term use of lamivudine, however, is its potential to induce viral resistance, with associated increases in HBV DNA and serum transaminases [Honkoop et al., 1997; Chayama et al., 1998; Suzuki et al., 2003].

Adefovir dipivoxil (ADV) is a potent suppressor of both wild-type and lamivudine-resistant HBV in vitro and a suppressor of wild-type HBV in vivo [Hadziyannis et al., 2003; Marcellin et al., 2003]. Clinical trials to date show that the addition of ADV to ongoing lamivudine therapy in lamivudine-resistant patients, or its administration as monotherapy, produces virologic and biochemical improvements [Perrillo et al., 2000, 2004; Hosaka et al., 2004; Peters et al., 2004].

Recently, a rapid, highly sensitive and reproducible method for quantifying mutant HBV virus in lamivudine-treated patients was reported [Punia et al., 2004]. Using a real-time polymerase chain reaction (PCR; LightCycler) with a ResonSense probe, this method detects as little as 0.01% of YMDD mutant DNA among

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10^5-10^9 copies of wild-type DNA. However, there are few reports of changes in YMDD mutant (rtM204I [YIDD sequence], rtM204V [YVDD]) viral loads during ADV treatment added to ongoing lamivudine therapy [Punia et al., 2004].

During the course of chronic HBV infection, natural seroconversion to antibody to HBeAg (anti-HBe) usually correlates with the resolution of viremia and clinical recovery. Mutation in the precore region (nucleotide [nt] 1896) is related to the absence of HBeAg secretion [Carman et al., 1989] and may enhance the stability of the secondary structure of pregenome encapsidation signals, ensuring perpetuation of viral replication and thus contributing to viral persistence [Lok et al., 1994]. Buckword et al. [1996] showed that HBV genome carrying core promoter mutations (nt G1762A and A1764T) influenced viral replication. Cho et al. [2000] and our group [Suzuki et al., 2002] reported that lamivudine therapy resulted in reversion from precore and core promoter mutants to wild-type, but that these mutants reappeared during long-term therapy. However, it is not clear at this stage how ADV influences precore and core promoter mutants of lamivudine-resistant virus.

Analysis of mutations of the reverse transcriptase (rt) domain of HBV polymerase in patients who had received long-term (48 or 60 weeks) ADV monotherapy revealed the presence of several amino acid substitutions [Yang et al., 2002; Westland et al., 2003.]. Other studies showed that selection of the rtN236T polymerase mutant is associated with resistance to ADV [Angus et al., 2003; Villeneuve et al., 2003]. Further elucidation of this process requires the analysis of amino acid substitutions during coadministration of ADV with ongoing lamivudine therapy for lamivudine-resistant virus.

The aims of this prospective study were (1) to determine changes in YMDD mutant (rtM204I, rtM204V) and rtL180M viral loads during coadministration of ADV with ongoing lamivudine therapy in patients with HBV, and (2) to determine viral polymerase (rt region), precore and core promoter mutants during treatment with ADV by analyzing serial serum samples from patients with lamivudine resistance.

PATIENTS AND METHODS

Patients

The subjects were 39 consecutive adult Japanese patients who commenced ADV treatment between November 2002 and June 2004 at the Department of Gastroenterology, Toranomon Hospital. At entry, all 39 patients were being treated with lamivudine for chronic HBV infection when the emergence of YMDD motif mutations indicated the development of breakthrough hepatitis. They had not received other nucleoside analogue drugs before lamivudine and were therefore treated by the addition of ADV to the ongoing lamivudine therapy (Group 1). Moreover, viral load data for another group of nine patients who were previously cotreated with interferon (IFN) in addition to ongoing lamivudine against breakthrough hepatitis (before ADV therapy was instituted in Japan) were compared with the viral load of patients treated with ADV (Group 2). These nine patients received IFN therapy daily for 4 weeks, and then three times a week for 20 weeks (Table I). All patients were negative for hepatitis C serologic markers. Lamivudine and ADV were administered orally at 100 and 10 mg/day, respectively. Chronic hepatitis or cirrhosis was confirmed before lamivudine treatment by peritoneoscopy and/or needle biopsy (n = 23), or clinical features (n = 16) [Suzuki et al., 2003].

Blood Tests and Serum Viral Markers

Routine biochemical tests were performed using standard procedures before and during therapy at least once each month. HBsAg, HBeAg, and anti-HBe were determined by radioimmunoassay kits (Abbot Diagnostics, Chicago, IL) according the instructions provided by

TABLE I. Patient Characteristics at the Start of Therapy for Lamivudine Breakthrough Hepatitis

	ADV (Group 1)	IFN (Group 2)
Total number	39	9
Sex (female/male)	4/35	2/7
Age (years)[a]	48 (26–58)	46 (23–56)
Aspartate aminotransferase (IU/L)[a]	118 (37–478)	158 (42–495)
Alanine aminotransferase (IU/L)[a]	188 (24–858)	234 (72–727)
Bilirubin (mg/dl)[a]	0.8 (0.3–13.7)	0.8 (0.2–2.3)
Albumin (g/dl)[a]	3.7 (2.6–4.5)	3.9 (3.4–4.3)
Liver histology (CH/LC)[b]	23/16	7/2
Serum HBV DNA[c] (Amplicor: log copy/ml)[a]	7.3 (4.4 –> 7.6)	>7.6 (5.9 –> 7.6)
HBeAg (positive/negative)	24/15	7/2
HBV genotype (A/B/C)	2/3/34	0/0/9

[a]Data are median (range).
[b]Liver histology: CH, chronic hepatitis; LC, liver cirrhosis.
[c]HBV DNA levels were measured by Amplicor HBV Monitor assay. HBV DNA values below the lower limit of detection are listed as 2.6 Log copy/ml and those over the upper limit of detection as 7.6 Log copy/ml. For statistical analysis, all HBV DNA values over the upper limit of detection (>7.6 Log copy/ml) were set to 8.0.

the manufacturer. Serum HBV DNA was quantified using the Roche Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN), a PCR-based assay with a lower limit of detection of 400 copies of HBV DNA/ml (2.6 Log copy/ml).

Quantitation of Lamivudine-Resistant Mutants by Real-Time Amplification Refractory Mutation System (ARMS) PCR

DNA was extracted from 100 μl of serum. The assay was performed using a sensitive, real-time PCR-based assay for the detection of lamivudine resistance-associated mutations in the presence of high levels of wild-type virus, as reported recently [Punia et al., 2004]. Briefly, this method is based on ARMS PCR for the detection of single base mutations [Newton et al., 1989] and uses the same ARMS primers, reactions, and cycling conditions on the LightCycler. To prepare the standards (rt204M, rtM204I, and rtM204V), the first PCR product amplified using primers P1 and P2, as reported previously [Günther et al., 1995], was cloned into the plasmid vector pBluescript (Stratagene, La Jolla, CA). The concentration of purified plasmids was based on absorbance at 260 nm (GeneQuant II; Amersham Pharmacia Biotech, Tokyo, Japan). The standards for real-time PCR were prepared by serial dilution of a plasmid of known concentration. DNA values of those mutants below the lower limit of detection were expressed as 2.0 Log copy and those over the upper limit of detection as 9.0 Log copy. Selectivity of this assay was tested as described previously [Punia et al., 2004] using reactions containing 10^9 copies of wild-type DNA (rt204M) template and from 10^9 to 0 copies of mutant virus (rtM204I or rtM204V) template. Under these conditions, the mutant primers (for rtM204I and rtM204V) detected the number of copies of mutant template present within the range of 10^9–10^4 copies. Moreover, one primer (rtM204I or rtM204V) detected the number of copies of mutant template present within the range of 10^9–10^4 copies (mixed with 10^9 copies of the other mutant virus [rtM204V DNA or rtM204I DNA], respectively). The detection limit for mutation of rt180 (rtL180M) was the same. Total HBV DNA levels were measured by real-time PCR as described previously [Punia et al., 2004]. Serum samples were assayed at five time points, namely before (baseline) and at 2, 4, 8, and 12 weeks after the start of coadministration of ADV with ongoing lamivudine therapy. Moreover, in some patients, serum samples were also assayed at two other time points; at 24 and 52 weeks. Data for the time-dependent decline in viral load relative to baseline were log transformed, and thus all results for quantitative HBV level are expressed as Log_{10} copy.

Determination of Nucleotide Sequences of HBV DNA

We determined the nucleotide sequences of HBV DNA of the initial 15 patients who received ADV treatment. Among these 15 patients, 4 received combination therapy of lamivudine, ADV, and IFN because of severe hepatitis. The remaining 11 patients were cotreated with ADV in addition to the ongoing lamivudine therapy and belonged to Group 1. DNA was extracted from 100 μl of serum. PCR reactions for detection of the rt region (nt 130–1161) of HBV DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense: 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense: 5′-GGCAGGATAGCCGCATTGTG-3′), and PreSBamH1 (sense: 5′-CTTGGGATCCAGAGC-TACAGCATGG-3′) and BR112 (antisense: 5′-TTCC GTCGACATATCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification with 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and final extension at 72°C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense: 5′-GGCCAAGTCTG-TACAACATC-3′) and B12R (antisense: 5′-TGCA-GAGGTGAAGCGAAGTG-3′), and B11F and B14R (antisense: 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing. Measurement of sequences in the rt region was performed at three time points; at the start of lamivudine, start of ADV, and 1 year after the start of ADV therapy. Nucleotide sequences of the core promoter and precore regions were determined as described previously [Suzuki et al., 2002], with measurements taken at the same three time points. All HBV genomes analyzed in detail by sequencing were found to be of genotype C. All sequence alignments were performed in comparison with genotype C wild-type sequences (accession no. AB014378, AB014394, AB033550, AB033551, AB0335 56, AB042283).

Mutation of the HBV DNA polymerase gene (rtM204I/V) was determined using PCR and restriction fragment length polymorphism (PCR-RFLP) as described previously [Chayama et al., 1998].

Statistical Analysis

Differences between groups were examined for statistical significance using the χ^2 and Mann–Whitney test (*U*-test) where appropriate. A two-tailed *P*-value less than 0.05 was considered significant.

RESULTS

Changes in Viral Loads of Lamivudine-Resistant Mutants During ADV Therapy

Changes in rtM204I, rtM204V, and rtL180M viral loads were measured in all 39 patients. At the start of ADV coadministration, the number of patients with detectable rtM204I alone, rtM204V alone and mixed-type (rtM204I and rtM204V) among the 39 patients was 17, 4, and 18, respectively. Viral load of rtL180M was detected in 36 patients. Figure 1 shows the median log changes from baseline in rtM204I, rtM204V, and

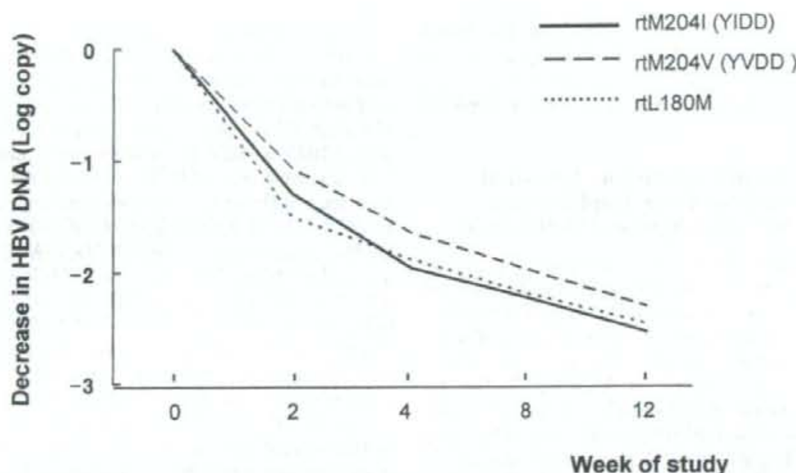

Fig. 1. Median log changes in rtM204I, rtM204V, and rtL180M viral loads from baseline during the initial 12 weeks of coadministration of ADV added to ongoing lamivudine therapy. HBV DNA levels of rtM204I, rtM204V, and rtL180M were measured by real-time PCR. rt, HBV polymerase reverse transcriptase.

rtL180M viral loads during the initial 12 weeks of ADV and lamivudine coadministration. The changes in viral load of rtM204I and rtL180M were greater than that of rtM204V, although the difference was not statistically significant. The rate of decrease of all mutants at 12 weeks was about one-hundredth (1/100) that at baseline. Moreover, the change of viral load of HBV DNA in HBeAg-negative patients was greater than that in HBeAg-positive patients at 12 weeks (median log changes in viral load; HBeAg-positive vs. -negative $= -2.14$: -2.71; $P = 0.077$). The numbers of rtM204I and rtM204V with HBeAg, and rtM204I and rtM204V without HBeAg were 24, 13, 11, and 9, respectively. The change of viral load of rtM204I without HBeAg was the greatest among the groups.

Among the nine patients coadministered IFN with ongoing lamivudine therapy, rtM204I only was detected in three and mixed-type was detected in six patients. Viral load of rtL180M was detected in eight patients. Log changes in rtM204I, rtM204V, and rtL180M viral loads under IFN coadministration are shown in Figure 2. The log viral load change for the rtM204V was greater than that for the rtM204I, although the difference was not statistically significant.

Normalization of alanine aminotransferase (ALT) level at 1 year was noted in 35 of 39 patients of Group

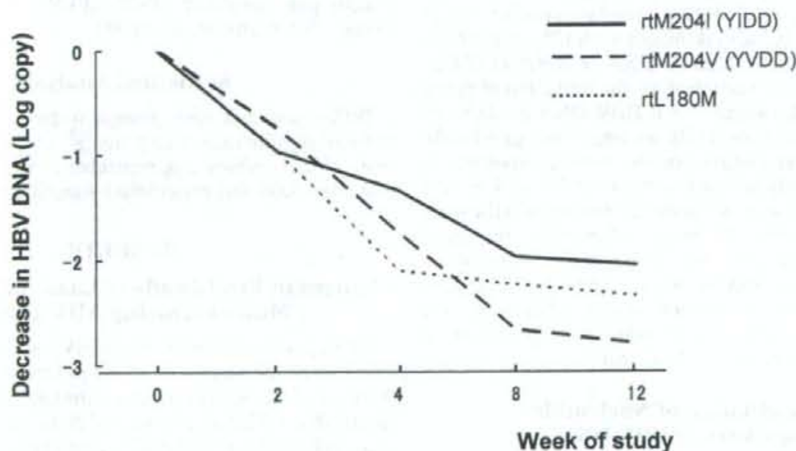

Fig. 2. Median log changes in rtM204I, rtM204V, and rtL180M viral loads from baseline and during the initial 12 weeks of coadministration of IFN added to ongoing lamivudine therapy. HBV DNA levels of rtM204I, rtM204V, and rtL180M were measured by real-time PCR.

1. Moreover, HBV DNA levels in 11 of 39 patients of Group 1 were more than 2.6 Log copy/ml by Amplicor HBV Monitor assay at 52 weeks. Those 11 patients were persistently HBeAg-positive and had mutant viral loads that were over 10^6 copies at the commencement of ADV and lamivudine coadministration. The number of patients with detectable rtM204I alone and mixed-type (rtM204I and rtM204V) was five and six, respectively. The rtM204I and rtM204V viral loads in these 11 patients were also measured at 24 and 52 weeks (Fig. 3). Viral loads of five patients with rtM204I alone gradually decreased but were still detectable at 52 weeks

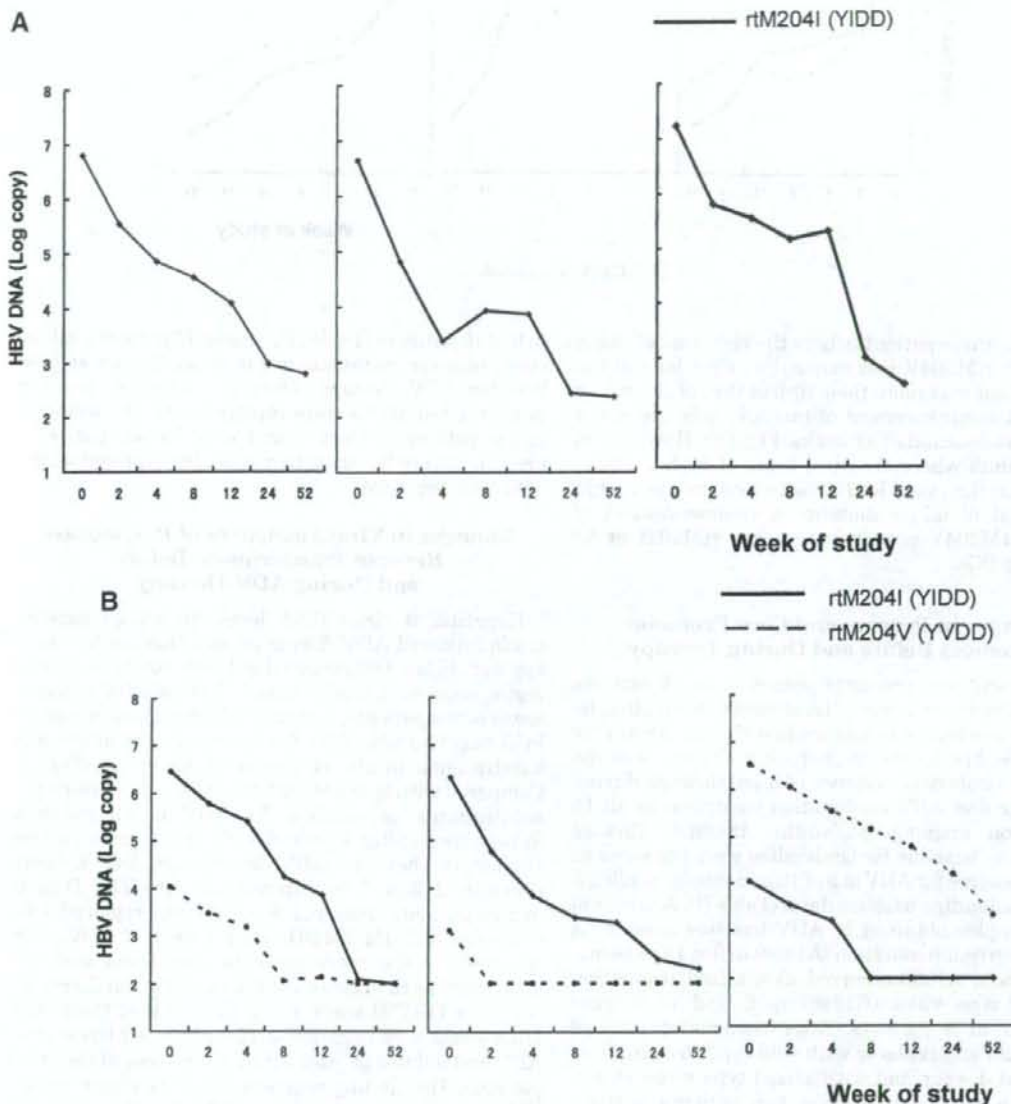

Fig. 3. Changes in rtM204I and/or rtM204V viral loads in nine patients with HBV DNA levels of >2.6 Log copy/ml as determined by Amplicor HBV Monitor assay at 52 weeks during ADV and lamivudine coadministration. HBV DNA levels of rtM204I and rtM204V were measured by real-time PCR. **A**: Viral loads in three of the five patients with rtM204I alone at commencement of ADV plus lamivudine combination therapy. Similar changes in viral loads were noted in the other two patients. Viral loads of these five patients with rtM204I alone gradually decreased but were still detectable at 52 weeks. **B**: Viral loads in three patients in whom either rtM204I or rtM204V was the major mutant (viral load of major mutant was over 10-fold that of minor mutant). Only the major mutant was detected at 52 weeks. **C**: Viral loads in three patients with two similar mutants (viral load of major mutant was within 10-fold that of minor mutant). Mutant rtM204V predominated over rtM204I at 52 weeks.

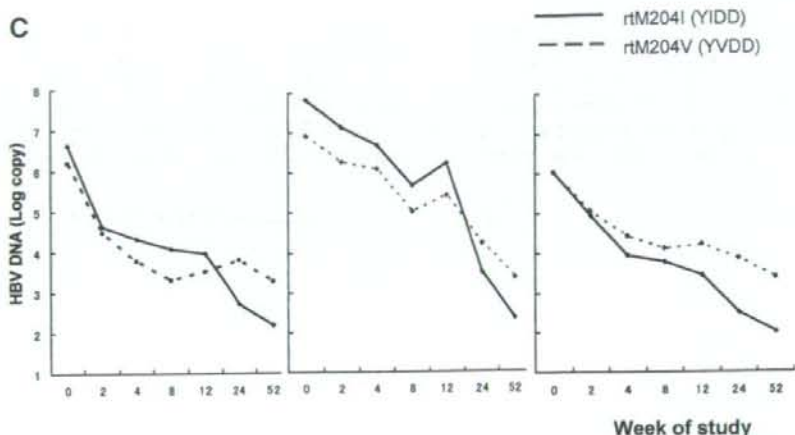

Fig. 3. (*Continued*)

(Fig. 3A). In three patients where the viral load of either rtM204I or rtM204V was major (i.e., viral load of the major mutant was more than 10-fold that of the minor mutant) at commencement of therapy, only the major mutant was detected at 52 weeks (Fig. 3B). However, in three patients where the viral loads of both mutants were similar (i.e., viral load of major mutant was within 10-fold that of minor mutant) at commencement of therapy, rtM204V predominated over rtM204I at 52 weeks (Fig. 3C).

Changes in Precore and Core Promoter Sequences Before and During Therapy

Precore and core promoter sequences in 15 patients were analyzed over 1 year of treatment with coadministered ADV in addition to ongoing lamivudine therapy for lamivudine breakthrough hepatitis. There was no clinical or virological evidence of breakthrough during lamivudine and ADV combination treatment in all 15 patients on ongoing lamivudine therapy. Precore sequences at baseline for lamivudine were the same as those at baseline for ADV in 9 of 10 patients (excluding 5 lacking lamivudine baseline data) (Table II). Analysis of serum samples obtained at ADV baseline revealed a precore stop codon mutation (A1896) in 9 of 15 patients, among whom A1896 occurred as a mixed population with wild-type virus (G1896) in 6 and as a pure population in 3 patients. After coadministration of ADV, A1896 was replaced with wild-type virus in three patients at 1 year and with mixed-type virus in one patient. In particular, among five patients without HBeAg at 1 year, including 2 HBeAg-seronegative patients, A1896 was replaced with wild-type virus in two and by mixed-type virus in one patient. Thus, A1896 was observed in three of eight patients, excluding seven PCR-negative patients, at 1 year.

The core promoter sequences at baseline for lamivudine therapy were the same as those at baseline for ADV in 9 of 10 patients (Table II). Among 15 patients, all had core promoter mutations in samples collected at baseline for ADV therapy. During treatment, the core promoter mutations were replaced with the wild-type in one patient (Patient 6) at 1 year. In this patient, a precore stop codon mutation was also replaced with a wild-type sequence.

Changes in Viral Sequences of Polymerase Reverse Transcriptase Before and During ADV Therapy

Hepatitis B virus DNA levels in all 15 patients coadministered ADV during ongoing lamivudine therapy were below 3,000 copy/ml at 1 year. Analysis of the rt region sequences (amino acid 1–344) of HBV polymerase in seven patients, excluding eight patients who were PCR-negative after ADV for 1 year, showed amino acid substitutions in the rt region in all seven (Fig. 4). Compared with baseline for lamivudine, there were new substitutions at baseline for ADV in all patients. Substitutions after 1 year of ADV, however, were very similar to those at ADV baseline in five patients (Patients 2, 3, 4, 7, 9). Interestingly, the YMDD motif in two patients (Patients 5 and 6) was replaced with wild-type (rt204M/YMDD) after 1 year of ADV. Substitutions in these two patients were fewer and of a different type than those at ADV baseline. Furthermore, Amplicor HBV Monitor assay showed that their HBV DNA levels were negative at 12 weeks after the start of ADV and fell to a greater extent than those of the other patients. This finding suggests that ADV may suppress YMDD mutants more than wild-type virus in some patients.

DISCUSSION

Mutations leading to lamivudine resistance are generally detected by conventional DNA sequencing after PCR amplification of a selected portion of the viral

TABLE II. Serial Precore and Core Promoter Sequences of Patients Treated With Lamivudine and Adefovir Dipivoxil

		Lamivudine					Adefovir dipivoxil									
		Baseline					Baseline					1 Year				
					CP nt					CP nt					CP nt	
Patient	Genotype	eAg	YMDD motif	Precore nt 1896	1762	1764	eAg	YMDD motif	Precore nt 1896	1762	1764	eAg	YMDD Motif	Precore nt 1896	1762	1764
1	C	ND	ND	ND	ND	ND	+	I+V	G/A	T	A	+	I	A	T	A
2	C	+	M	G	T	A	+	I+V	G	T	A	+	I+V	G	T	A
3[a]	C	+	M	G/A	T	A	+	I+V	G/A	T	A	–	V	G	T	A
4	C	+	M	G/A	T	A	+	I+V	G/A	T	A	+	I+V	G	T	A
5	C	+	M	G	T	A	+	I+V	G	T	A	–	M	G/A	T	A
6[a]	C	–	M	G/A	T	A	–	I+V	G/A	T	A	–	M	G	A	G
7	C	–	M	G	T	A	–	I	A	T	A	–	I	G/A	T	A
8	C	+	ND	ND	ND	ND	+	I+V	G	T	A	+	V	N	N	N
9	C	+	M	G	T	A	+	I	G	T	A	+	I	N	N	N
10	A	–	M	G	T	A	–	V	G	T	A	–	N	G	T	A
11	C	ND	ND	ND	ND	ND	+	I+V	G/A	T	A	–	N	N	N	N
12	C	ND	ND	ND	ND	ND	+	I+V	G	T	A	+	N	N	N	N
13[a]	C	+	M	G/A	A/T	G/A	+	I	G/A	T	A	+	N	N	N	N
14[a]	C	+	M	A	T	A	–	I	A	T	A	–	N	N	N	N
15	C	–	ND	ND	ND	ND	–	I+V	A	T	A	–	N	N	N	N

Baseline, time of the beginning of therapy; CP, core promoter; ND, not done; N, PCR-negative; eAg, HBeAg; YMDD motif; M, rt204M; I, rtM204I; V, rtM204V; I+V, mixed type (rtM204I+rtM204V).
[a]Received lamivudine, adefovir dipivoxil, and interferon therapy.

aa.1 — Reverse transcriptase — 344

Patient	Time point	Substitutions (before YMDD)	YMDD motif	Substitutions (after YMDD)
Pat. 2	(1)	I16T S78T D134E		V214A
	(2)	N13Y I16T H55R L80I	M204I	F221Y
	(3)	N13Y I16T H55R L80I	M204I	F221Y
Pat. 3	(1)			H337N
	(2)	V84M K154N L180M V191I	M204V	H337N
	(3)	G52E V84M K154N L180M V191I	M204V	H337N
Pat. 4	(1)	N139Q Y141F V142I L145M		H337N
	(2)	L80V N139Q Y141F V142I L145M	M204I	H337N
	(3)	L80V N139Q Y141F V142I L145M	M204I	H337N
Pat. 5	(1)	F195I		
	(2)	L80I F151L L180M	M204I	
	(3)	S106C		S256C H337N
Pat. 6	(1)	T118A D134N		C303W H337N
	(2)	A96V T118A L180M	M204V	S219A L229F H337N
	(3)			N238H
Pat. 7	(1)	T7A		P325S
	(2)	T7A H55R S106C	M204I	T222A S223A P325S
	(3)	T7A H55R S106C	M204I	
Pat. 9	(1)	S78T		H337N
	(2)	L80I	M204I	H337N
	(3)	L80I	M204I	I265M H337N

Fig. 4. Changes in viral sequences of polymerase reverse transcriptase before and during therapy. Measurements were taken at three time points: (1) start of lamivudine therapy, (2) start of coadministration of ADV with ongoing lamivudine therapy against breakthrough hepatitis, and (3) after coadministration with ADV for 1 year. Patient numbers are the same as in Table II. L180M denotes the substitution of leucine with methionine at amino acid position 180 in the reverse transcriptase region of HBV polymerase.

polymerase gene. The sensitivity of sequencing for minority quasispecies is low, however, with detection in most cases limited to no more than 20% of the total viral population [Gunthard et al., 1999]. Other molecular techniques developed to detect changes associated with lamivudine resistance include PCR-RFLP, a 5′ nuclease assay, and line probe assay technology [Chayama et al., 1998; Stuyver et al., 2001; Whalley et al., 2001].

Punia et al. [2004] first reported that rtM204I, rtM204V, and rt180M viral loads could be measured by real-time ARMS-PCR. However, their report included data from only a few cases. Here, we measured sequential viral loads of mutants during coadministration of ADV in addition to established treatment with lamivudine and showed that the viral loads of rtM204I, especially without HBeAg, decreased at the most rapid rate. This finding indicates that ADV therapy has a more suppressive effect against rtM204I. Moreover, when viral loads of both mutants (rtM204I and rtM204V) were similar at commencement of ADV therapy in patients with mixed-type virus, rtM204V predominated over rtM204I at 52 weeks. Considering these findings, the rtM204I may be more sensitive to ADV in vivo. On the other hand, it was reported that ADV was an equally effective inhibitor of rtM204I and rtM204V replication in vitro, and suppressed the rtL180M to an even greater extent [Chin et al., 2001; Ono et al., 2001]. With respect to the effectiveness of ADV against rtM204I and rtM204V, our data (in vivo) differ from that of previous studies (in vitro). Moreover, suppression of the rt180M was linked to that of the rtM204I or rtM204V and the rt180M viral load was influenced by those of rtM204I or rtM204V in vivo. However, it is not clear why ADV was apparently more effective against the rtM204I in vivo, and further studies are necessary to investigate this issue.

On the other hand, the log viral load change for rtM204V was greater than that for rtM204I during IFN coadministration with ongoing lamivudine, although the difference was not statistically significant. However, the number of patients undergoing IFN therapy was small and further studies of larger population samples are necessary to confirm this finding. On the other hand, our previous study showed that the suppression of lamivudine-resistant virus by long-term IFN + lamivudine therapy was clinically insufficient, with only 38% of patients achieving negative HBV DNA status [by branched DNA assay] after 6 months of IFN (unpublished data). On this basis, the long-term clinical efficacy of ADV added to ongoing lamivudine therapy is apparently superior to that of IFN coadministration.

During lamivudine therapy, precore mutants tend to be replaced with wild-type virus at 1 year, and this

change is unrelated to the emergence of YMDD motif mutations [Cho et al., 2000; Suzuki et al., 2002]. On the other hand, patients who showed mutations in the YMDD motif during long-term lamivudine therapy also exhibited the reappearance of precore mutants [Suzuki et al., 2002]. However, the addition of ADV to ongoing lamivudine therapy appeared to result in the preferential selection of wild-type virus, similar to the case of initial lamivudine therapy, although only a few cases were tested. This finding suggests that antiviral nucleoside analogues, such as lamivudine and ADV, selectively suppress precore mutants over wild-type virus. On the other hand, core promoter mutations at 1 year were replaced with wild-type in only one patient (Patient 6). It has been reported that core promoter mutations during lamivudine therapy also tended to be replaced at 1 year by wild-type virus [Cho et al., 2000; Suzuki et al., 2002], and more recently that three of five seroconverters of HBeAg harbored core promoter mutations at baseline that were progressively replaced with wild-type genome during ADV monotherapy [Werle et al., 2004]. However, our present study showed that, compared to initial lamivudine therapy or ADV monotherapy, coadministration of ADV with ongoing lamivudine therapy might be less effective against core promoter mutants than wild-type virus.

With regard to ADV monotherapy, several mutations in the HBV polymerase rt region have been observed during this treatment [Yang et al., 2002; Westland et al., 2003]. Moreover, selection of the rtN236T polymerase mutant was associated with resistance to ADV [Angus et al., 2003; Villeneuve et al., 2003]. Few data are available on sequencing of the rt region during coadministration of ADV with ongoing lamivudine therapy. Mutations after 1 year of coadministration of ADV and lamivudine were very similar to those at coadministration baseline. However, the YMDD motif mutation in two patients was replaced with wild-type (rt204M) at 1 year after coadministration, and another mutation pattern within the rt region was also changed. Moreover, in Patient 6, precore and core promoter mutations were replaced with wild-type at 1 year after coadministration. These findings suggest that ADV may selectively suppress lamivudine-resistant virus, and that wild-type virus may predominate in patients in whom drug efficacy is high, although the status of the rt region in eight patients whose PCR was negative at 1 year could not be ascertained.

In conclusion, we analyzed changes in rtM204I, rtM204V, and rtL180M viral loads in patients with HBV cotreated with lamivudine and ADV for lamivudine-resistant virus. The changes in rtM204I and rtL180M viral loads were greater than that of rtM204V, although the difference was not statistically significant. This finding was also clarified by analysis of serial changes in rtM204I and rtM204V viral loads. Moreover, the change in rtM204I viral load without HBeAg was greatest. Precore wild-type virus was apparently preferentially selected by the coadministration of ADV with lamivudine, in the same way that it was by initial lamivudine therapy at 1 year. Moreover, analysis of the rt region showed that ADV may suppress lamivudine-resistant virus and that wild-type virus may predominate. A better efficacy of ADV was noted against HBeAg-negative (and/or precore mutant) and lamivudine-resistant virus. Further studies are necessary to correlate virological changes and clinical efficacy during longer coadministration of ADV with ongoing lamivudine therapy for lamivudine-resistant virus.

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Anticarcinogenic Impact of Interferon on Patients with Chronic Hepatitis C: A Large-Scale Long-Term Study in a Single Center

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Key Words

Cirrhosis · Fibrosis · Hepatitis C virus · Hepatocellular carcinoma · Interferon

Abstract

Background: The anticarcinogenic capacity of interferon (IFN) was assessed in a cohort of Japanese patients with chronic hepatitis C en masse. ***Patients and Methods:*** The rate of hepatocarcinogenesis was analyzed in 2,166 patients with chronic hepatitis C, of whom 1,654 had received IFN therapy while 512 had not. ***Results:*** Crude rates of hepatocarcinogenesis in treated and untreated patients were 2.6 and 4.6% at the end of the 5th year, 5.8 and 12.7% at the 10th year and 13.9 and 23.9% at the 15th year (after completion of IFN therapy for those treated) ($p<0.001$). IFN decreased the hazard ratio of carcinogenesis to 0.42 ($p < 0.001$) in multivariate analysis with adjustments for significant covariates including fibrotic stage, γ-glutamyl transpeptidase level, gender, platelet count and age. Among the 1,654 patients treated with IFN, 606 (36.6%) achieved persistent loss of hepatitis C virus (HCV) RNA and an additional 266 (16.1%) gained normal levels of alanine aminotransferase without loss of HCV RNA for 6 months or longer after the completion of IFN therpay. Cumulative rates of hepatocarcinogenesis in sustained virological responders and biochemical responders were 1.4 and 2.0% at the end of the 5th year, 1.9 and 3.6% at the 10th year and 1.9 and 7.5% at the 15th year, respectively. The hazard ratio of sustained virological response was 0.10 ($p < 0.001$), and that of biochemical response was 0.12 ($p<0.001$). Normalization of aminotransferase levels after IFN therapy without loss of serum HCV RNA decreased hepatocarcinogenesis. ***Conclusion:*** IFN significantly decreased the rate of hepatocarcinogenesis in patients with chronic hepatitis C as a whole in Japan, even in those who fail to clear HCV RNA from serum.

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Introduction

In most developed countries, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for the great majority of hepatocellular carcinoma (HCC), with incidence rates dependent on the regional prevalence of these hepatitis viruses. HCV-associated HCC typically develops through a sequence of events that progress from chronic inflammation through fibrosis and cirrhosis accompanying dysplasia and ultimately to HCC. In our previous cohort study on Japanese patients with HCV-related cirrhosis [1], cumulative rates of developing HCC at 5, 10 and 15 years were 21.5, 53.2 and 75.2%, respectively. According to our observations of untreated patients with chronic hepatitis C [2], rates of hepatocarcino-

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genesis at 5, 10 and 15 years were estimated to be 4.8, 13.6 and 26.0%, respectively. The life expectancy of patients with HCV-related cirrhosis is largely influenced by the development of HCC in the clinical course. As the efficacy of radically curative therapies for HCC remains limited at best, and since a severe organ shortage does not provide with sufficient chances for liver transplantation, the prevention of HCC in patients with chronic liver disease is of great importance at the present.

Interferon (IFN) is effective in eliminating HCV and reducing serum levels of alanine aminotransferase (ALT) in some patients with chronic hepatitis C [3–6]. Reduced incidence of HCC in HCV-associated cirrhosis by IFN has been reported by many investigators including ourselves [7–14]; only a few studies have failed to find its benefit [15, 16]. However, many published studies had shortcomings in the study design, in terms of pooling patients who received IFN in diverse regimens, relatively short periods of follow-up despite a long incubation period of HCC, large numbers of dropouts and retrospective studies with historical controls. Moreover, almost all studies evaluated the activity of IFN to prevent HCC by comparing responders and nonresponders to the treatment. Due to difficulties in studying patients with chronic hepatitis C, a number of nonrandomized studies examined the effect of IFN on the incidence of hepatocarcinogenesis [17–20]. With invariable limitations in study design and interpretation of the results, these studies have disclosed useful information as regards the treatment of patients with chronic HCV infection.

In order to evaluate whether IFN can reduce the rate of carcinogenesis in patients with chronic hepatitis C, we compared 1,654 patients with IFN therapy with 512 patients without treatment in a single clinical center, who were adjusted for background features by the multivariate analysis. Therefore, the principal aims of our study were to show the role of IFN in preventing HCC in chronic hepatitis type C en masse and to establish the extent to which IFN decreases the rate of carcinogenesis as a sequel to chronic hepatitis C in a society.

Patients and Methods

Study Population

A total of 2,166 patients with chronic hepatitis were examined, whose initial sera tested negative for hepatitis B surface antigen by radioimmunoassay (Ausria, Dainabot, Tokyo, Japan) and positive for anti-HCV by the second-generation enzyme-linked immunosorbent assay (Dainabot); anti-HCV was tested in sera that had been stored frozen at –80°C. They included 1,421 men and 745 women aged 14–78 with a median of 50 years. They were all diagnosed with chronic hepatitis by liver biopsy with or without peritoneoscopy between 1970 and 2000 at the Department of Gastroenterology in Toranomon Hospital, Tokyo, Japan. Patients who had possibly developed HCC already at the time of diagnosis of hepatitis were strictly excluded from the study. In order to exclusively investigate hepatocarcinogenesis in HCV-related cirrhosis, patients coinfected with HBV were excluded.

Among the 2,166 patients with HCV-related hepatitis, 1,654 (76.4%) received IFN therapy, mostly since 1987 when IFN was available in Japan; new antivirals or anticarcinogenic treatments of viral cirrhosis, except for IFN, were not introduced in 1987 or thereafter in Japan. The remaining 512 patients did not receive IFN or any other antiviral therapies. This is a retrospective cohort study with historical controls composed of patients before 1987 and those who refused or could not receive IFN for various reasons since 1987.

Background and Laboratory Findings

Table 1 shows demographic profiles and laboratory data for the 1,654 patients treated with IFN and the 512 without receiving IFN since they were diagnosed with chronic hepatitis. There were more males, with a median age 3 years lower in treated than in nontreated patients. There were 299 treated patients (18.1%) with a history of alcohol intake ≥500 kg until the diagnosis of chronic hepatitis (corresponding to daily consumption of 3,000 ml of beer or 300 ml of whiskey for 20 years) and 113 (22.1%) untreated patients ($p < 0.001$). Because IFN was introduced to our hospital in 1987, the observation period was significantly shorter in the treated than in untreated patients (median 10.4 vs. 12.3 years; $p < 0.0001$).

Although all patients tested positive for HCV RNA during their clinical courses, tests for the concentration of HCV RNA in the initial serum was possible in 1,863 (86.5%) patients. HCV genotypes were analyzed by the serological typing method with a commercial kit (Kokusai Diagnostic Corporation, Kobe, Japan) in which the serological group 1 represented genotypes 1a and 1b, and group 2 stood for 2a and 2b genotypes. HCV in the serological group 2 was significantly more frequent in patients with IFN treatment than in those without. Concentration of HCV RNA was determined in the initial sera from 1,873 (86.5%) patients by the competitive polymerase chain reaction (PCR) method with the HCV probe assay (Chiron Corp., Emeryville, Calif., USA) or by PCR with Amplicor HCV Monitor kits (Roche Diagnostics Japan Co., Tokyo, Japan). High concentration of HCV (≥10^6 copies/ml by the competitive PCR or ≥10^6 equivalents/ml by the HCV probe assay) was significantly more frequent in untreated than in treated patients ($p < 0.0001$). The stage of hepatic fibrosis was not different between the two groups.

Interferon Treatment and Judgment of the Effect

A total of 1,654 patients underwent IFN therapy in one or more treatment courses: 1,358 patients (82.1%) received IFN once, 240 patients (14.5%) twice, and the remaining 56 patients (3.4%) three times or more. Initial treatment was performed with natural or recombinant IFN-α (n = 1,238), natural IFN-β (n = 386) or both (n = 30). Regimens of IFN were variable: 926 (56.0%) patients received IFN 6–9 million units (MU) daily for 8 weeks, followed by 2 or 3 times per week for 16 weeks; 329 (20.0%) received IFN 6–9 MU daily for 2–4 weeks, followed by 3 times per week for 20–22 weeks; 185 (11.2%) underwent a short-course therapy with IFN

Table 1. Patient profiles and laboratory data at the diagnosis of chronic hepatitis

Factors	Interferon therapy		p value
	yes (n = 1,654)	no (n = 512)	
Male	1,110 (67.1%)	311 (60.7%)	0.024
Age, years	50 (16–72)	53 (21–78)	<0.001
History of transfusion	607 (36.7%)	229 (44.7%)	0.001
Family member with liver disease	426 (25.8%)	140 (27.3%)	0.47
Alcohol intake ≥ 500 kg	299 (18.1%)	113 (22.1%)	0.044
Observation period, year	10.4 (0.1–33.6)	12.3 (0.1–33.6)	<0.001
Laboratory data			
ALT, IU/l	63 (4–1,266)	67 (4–704)	0.098
AST, IU/l	106 (9–1,660)	96 (12–832)	0.0001
γ-GTP, IU/ml	62 (6–1,118)	70 (3–850)	0.39
Platelet counts, $\times 1{,}000/mm^3$	169 (27–433)	165 (35–560)	0.091
ICG R_{15}, %	14 (1–90)	16 (1–95)	0.003
AFP, ng/ml	4 (1–90)	5 (1–1,180)	0.42
HCV serological group			
Group 1, genotypes 1a/1b	1,021 (66.1%)	259 (81.4%)	<0.0001
Group 2, genotypes 2a/2b	488 (31.6%)	48 (15.1%)	
Undetermined	36 (2.3%)	11 (3.5%)	
HCV RNA concentration			
High[a]	937 (58.4%)	191 (71.3%)	<0.0001
Low[b]	668 (41.6%)	77 (28.7%)	
Histological stage of hepatitis			
F1, slight fibrosis	1,029 (62.2%)	298 (58.2%)	0.10
F2/F3, moderate/severe fibrosis	625 (37.8%)	214 (41.6%)	

AST = Aspartate aminotransferase; AFP = α-fetoprotein; ICG R_{15} = retention of indocyanine green at 15 min.

[a] HCV RNA concentration $\geq 10^6$ copies/ml by the competitive PCR or $\geq 10^6$ equivalents/ml by the HCV probe assay.

[b] HCV RNA concentrations less than high concentrations.

daily for 4–8 weeks; 128 (7.7%) were administered with intermittent IFN 3 times per week for 24 weeks; 72 (4.4%) had a prolonged course of IFN for 8–36 months; 8 (0.5%) received IFN-β 6 MU daily for 6–18 months, and the remaining 6 (0.4%) were given IFN-α combined with IFN-β for 4 months. The median dose of 624 MU was administered during the median period of 24 weeks. IFN for 24 weeks or longer was given to 83.2% of the patients. IFN therapy was usually initiated within a few months after the diagnosis of chronic hepatitis, and all patients were started on it within 12 months. The median interval between liver biopsy and initiation of IFN was 9 days.

Almost all the patients given IFN showed varied degrees of fever, chills, myalgia, headache and general malaise after the first injection. Most patients developed leukocytopenia and thrombocytopenia in various degrees. A significant thrombocytopenia $\leq 40{,}000/mm^3$ required a reduction of the IFN dose in 39 patients. IFN therapy was discontinued due to psychosis in 35 patients and ophthalmological symptoms in 12 patients. None of the patients developed decompensated liver disease with ascites, encephalopathy, jaundice or variceal bleeding. Although only 88 (5.3%) patients could not continue injection with IFN, studies for carcinogenesis were analyzed on the intention-to-treat basis.

The efficacy of IFN was judged by the clearance of HCV RNA from serum and ALT levels 12 months after the completion of treatment. Sustained virological response (SVR) was defined as persistent disappearance of HCV RNA after therapy, biochemical response (BR) as normal ALT levels without elimination of HCV RNA for at least 6 months after therapy, and no response (NR) as persistently elevated or transiently normalized ALT levels without loss of HCV RNA lasting for less than 6 months.

Follow-Up of Patients and Diagnosis of HCC

Patients were followed up monthly after diagnosis of chronic hepatitis in our outpatient clinic and monitored for hematological, biochemical and virological parameters. With their admission, during and after the treatment with IFN, weekly or biweekly follow-up was performed in almost all patients who received IFN. Imaging diagnosis was made once or twice per year in the majority of patients with ultrasonography or computed tomography. Angiography was performed only when HCC was highly suspected on imaging by ultrasonography or computed tomography.

When angiography pictured a characteristic hypervascular nodule specific for HCC in patients, histological confirmation was not required in the majority of them. Microscopic examinations of liv-

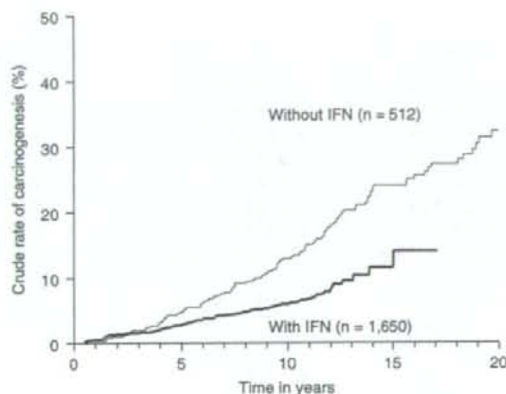

Fig. 1. Crude rates of hepatocarcinogenesis in patients treated with IFN and those untreated. The carcinogenesis rate was significantly lower in treated than in untreated patients (log-rank test, $p < 0.0001$).

er tissues obtained by a fine-needle biopsy were performed in 14 patients whose angiogram could not portray a typical image of HCC. There were 89 patients in whom HCC was confirmed histologically on liver specimens obtained at surgery or autopsy. Detection of serological tumor markers and increase with time were also taken into account in the diagnosis of HCC.

There were 223 (10.3%) patients lost to follow-up, including 164 (9.9%) treated and 59 (11.5%) untreated. Rates of annual dropouts in treated and untreated patients were 0.95 and 0.93%, respectively. In 9 patients, the response to IFN was judged by information on aminotransferase levels determined in other clinics and by persistent HCV RNA, as well as aminotransferase levels at 6 months after the completion of therapy in an additional 3 patients. Therefore, the response to IFN could be judged in all patients including the 12 who were lost to our follow-up early. Since the eventual outcome with respect to the development of HCC was not confirmed in these patients, their data were censored in statistical analyses [21]. Deaths unrelated to liver disease were censored and withdrawn from the analysis. The date of the last follow-up in this study was May 1, 2004, and the median observation period of studied patients was 10.7 years, with a range of 0.1–33.6 years.

Statistical Analysis

Nonparametric Mann-Whitney U test and χ^2 test were used for analysis of background characteristics of patients. The rate of HCC development was calculated by the Kaplan-Meier method [22]; it was based on the duration between diagnosis of chronic hepatitis by liver biopsy and detection of HCC. Differences in slopes of carcinogenesis curves were evaluated by the log-rank test. To gain a robust statistical power for the anticarcinogenic activity of IFN, observation of treated patients was initiated at the commencement of IFN therapy, in lieu of the diagnosis of chronic hepatitis. Independent factors associated with the development of HCC were studied using the stepwise Cox regression analysis [23]. The following 18 variables were analyzed for potential covariates in hepatocarcinogenesis at the time when hepatitis was diagnosed: age, sex, total alcohol intake, family history of liver disease, history of blood transfusion, stage of hepatic fibrosis, aspartic aminotransferase, ALT, albumin, bilirubin, globulin, γ-glutamyl transpeptidase (γ-GTP), platelet count, retention of indocyanine green at 15 min, serological grouping of HCV, HCV RNA level and IFN treatment.

Although continuous variables without conversion of data were evaluated in multivariate analyses, several variables were transformed into categorical data consisting of two or three ordinal numbers in calculating hazard ratios. All factors found to be marginally associated with hepatocarcinogenesis with p values <0.15 were tested by the multivariate Cox proportional hazard model. All analyses of data were performed with the computer program SPSS version 11 [24], and a p value <0.05 was considered significant.

Results

Response to IFN

Response to IFN was judged 12 months after the completion of therapy by both HCV RNA and serial ALT readings. Among the 1,654 patients with IFN treatment, SVR (elimination of HCV RNA) was achieved by 606 (36.6%), BR (ALT normalized for at least 6 months without clearance of HCV RNA from serum) in 266 (16.1%) and NR (elevated or transiently decreased ALT levels without loss of serum HCV RNA) in 782 (47.3%).

Crude Rates of Hepatocarcinogenesis

During the median observation period of 10.7 years, HCC developed in 199 of the 2,166 (9.2%) patients, including 96 of the 1,654 (5.8%) patients treated with IFN and 103 of the 512 (20.1%) patients without IFN (fig. 1). Among the 199 patients with HCC, 140 (70.4%) imaged a typical hypervascular stain on angiography and dynamic computed tomography, while 59 failed to exhibit tumor stains on angiography. HCC in these 59 patients was confirmed histologically on liver specimens obtained at surgery or by fine-needle biopsy.

Crude rates of hepatocarcinogenesis in patients treated with IFN and those untreated were 1.3 and 1.8% at the end of the 3rd year (after the completion of therapy), 2.6 and 4.6% at the end of the 5th year, 5.8 and 12.7% at the 10th year and 13.9 and 23.9% at the 15th year, respectively (fig. 1). The carcinogenesis rate was significantly lower in patients treated with IFN than in untreated patients (log-rank test, $p < 0.0001$).

Impact of IFN on Hepatocarcinogenesis

During the observation period, HCC developed in 96 of the 1,654 (5.8%) patients treated with IFN, including