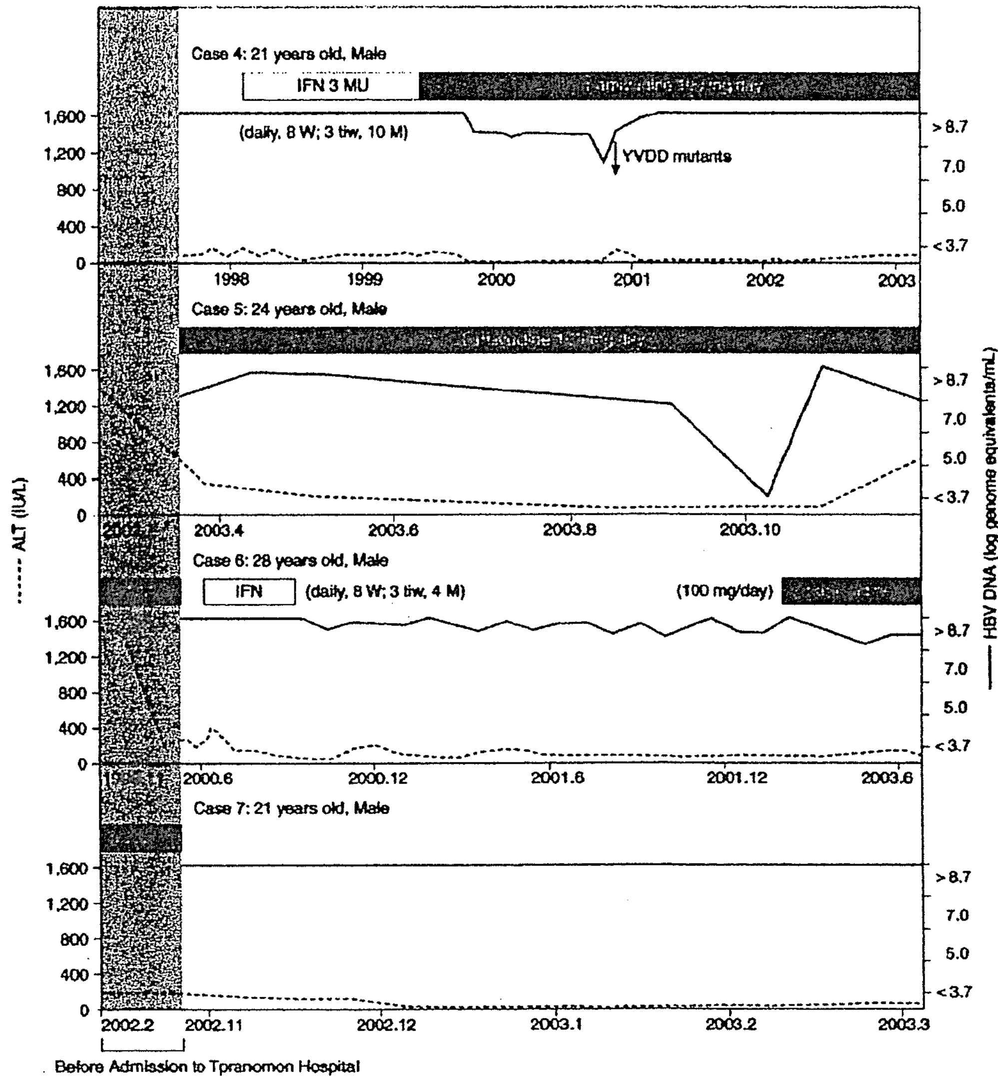

Fig. 2. Clinical courses of the four patients in whom HBeAg persisted after contracting infection with HBV genotype A despite antiviral treatment. The patient in Case 9 did not receive treatment after the admission. Courses and treatment they received before admission to Gastroenterology Department in Toranomon Hospital are shown in shaded areas on the left. IFN, interferon; SNMC, Stronger Neo-Minophagen C.

transfer to the Toranomon Hospital. Cases 4 and 6 did not respond to IFN or lamivudine that was commenced immediately after IFN or at an interval; lamivudine has been continued on them indefinitely. Case 5 was started on lamivudine soon after he was admitted to hospital and had been maintained on it for 1 year; he never responded to lamivudine. Variants with mutations in the YMDD motif of DNA polymerase/reverse-transcriptase developed in Case 4 while he was receiving lamivudine accompanied by a rise in ALT levels.

Although serum ALT returned to normal spontaneously (<50 IU/L) and then elevated only moderately in Case 7, high levels of HBV DNA (>8.7 LGE/ml) persisted through more than 1 year. Antiviral treatment was withheld because of the absence of active hepatitis.

DISCUSSION

There are marked geographical differences in the distribution of HBV genotypes [Magnius and Norder, 1995; Lindh et al., 1997; Miyakawa and Mizokami, 2003]. Of them, genotype A is not indigenous in Japan where genotypes B and C prevail and account for by far the majority of acute as well as chronic HBV infections [Orito et al., 2001; Kobayashi et al., 2002]. Some characteristics of HBV genotype A infection are increasingly coming to the fore in Japan and have aroused concerns in hepatologists at hospitals in urban areas with cosmopolitan populations. Ogawa et al. [2002] found 14 of the 25 (56%) patients with acute hepatitis B in a downtown Tokyo (Shinjuku) were infected with

HBV genotype A. Moreover, the frequency of acute hepatitis induced by HBV genotype A in our hospital is higher after than before 1991 (2/22 [9%] vs. 26/46 [57%], $P < 0.0001$) [Kobayashi et al., 2004]. The present study sums up our experiences on acute infection with HBV genotype A at the Department of Gastroenterology in Toranomon Hospital situated in the Metropolitan Tokyo during the past 28 years, to supplement our previous reports with additional findings and new insights [Kobayashi et al., 2002, 2003, 2004].

First, infection with HBV genotype A spreads principally by extramarital sexual contact in the adulthood in Japan [Kobayashi et al., 2002; Ogawa et al., 2002]. All the 31 patients of acute hepatitis B infected with HBV genotype A in the present series were men, and 16 (52%) of them confided having had extramarital heterosexual or homosexual contacts. Only one mother of 32 patients with acute or chronic infection with HBV genotype A possessed HBV DNA in serum; her genotype was B [Kobayashi et al., 2003], thereby excluding perinatal transmission of genotype A. In a molecular epidemiological survey of HBV in Amsterdam, a cluster of genotype A related in men having sex with men has been recognized [van Steenbergen et al., 2002].

Secondly, acute infection with HBV genotype A tends to persist. Of the 31 patients with acute genotype A infection, seven (23%) failed to clear it within 6 months, in comparison with one of the nine (11%) with acute genotype B or three of the 42 (7%) with acute genotype C infection. In our previous report [Kobayashi et al., 2002], infection persisted in all three patients infected with genotype A, in contrasted to the clearance of HBsAg in all four with genotype B (one) or C (three).

Low maximum ATL levels (<500 IU/L [83%] vs. ≥500 IU/L [4%], $P = 0.0001$) and the high baseline HBV DNA levels (median: >8.7 vs. 6.0 LGE/ml, $P = 0.004$) were predictive of the perpetuation of acute HBV genotype A infection. Hence, compromised immune responses toward lower inflammation activity in the liver and higher viral replication may have a role in evolving HBV genotype A infection. Four of the seven (57%) patients who progressed to chronic were homosexuals. It is tempting to speculate that derangement in cytotocxic T cell response contributed to the failure in clearing acute HBV infection toward persistence [Handzel et al., 1984]. Immunomodulatory treatments to cope with severe acute hepatitis, given to five of the seven (71%) patients before referral to hospital (Figs. 1 and 2), may have promoted the persistence of infection with HBV genotype A. We have reported that acute prolonged HBV infection occurs more often in patients with than without immunomodulatory treatments during acute illness, regardless of genotypes (86% [6/7] vs. 2.4% [1/42], $P = 0.01$).

Thirdly, HBV genotype A infection persisting in patients with acute hepatitis B is not cleared often by antiviral therapy. HBV genotype A infection was terminated in only one of the six (17%) patients who received antiviral treatment. He was one of the three patients who seroconverted with the loss of HBeAg; interferon and lamivudine was given to him early in the course of infection (Cases 1 in Fig. 1). Since most (~95%) patients with acute adulthood hepatitis B resolve infection in Japan, antiviral treatment is rarely used for them. As far as acute infection with HBV genotype A is concerned, however, therapeutic intervention needs to be considered in view of the frequent chronic outcomes. Since many (76% [24/31]) patients even with HBV genotype A can clear infection spontaneously, the timing of starting antiviral therapy would have to be contemplated. The single patient who cleared HBsAg was started on interferon and then lamivudine within 3 months after he was referred to our hospital. It is not certain whether he could have cleared HBV infection, should he never be placed on lamivudine early. HBsAg was not cleared, however, in the remaining two patients with HBeAg seroconversion in whom IFN was started 4 and 2 years, respectively, after they came to our care (Fig. 1).

Early antiviral treatment deserves consideration in patients who are infected with HBV genotype A, especially because of its propensity to become chronic. It is not certain how long patients should receive lamivudine after HBV DNA has disappeared from the circulation. Inasmuch as cccDNA continues to be present in the liver [Brechot et al., 1980; Yotsuyanagi et al., 1998], even after HBsAg is cleared from serum, a therapeutic option would be to continue lamivudine until anti-HBs is detected in serum as in Case 1. In view of the poor immune responses with low ALT levels, which might be inherent to HBV genotype A infection among homosexual, such a special care would have to be taken for its treatment.

There are two genetic subgroups of genotype A designated Ae which is common in Europe (the original genotype A) and Aa which is frequent in Africa as well as Asia [Sugauchi et al., 2003]; Aa is equivalent to subgroup A′ described by Bowyer et al. [1997]. It strikes as a surprise that of the 68 patients who were infected acutely or chronically with HBV genotype A and admitted to the Toranomon Hospital, 54 (79%) possessed HBV of subgroup Ae (European type); HBV of subgroup Aa (African/Asian type) was found in only four (6%) [Kobayashi et al., 2004]; they all were infected persistently. Since subgroup Ae was not found in any patients with acute hepatitis B in our series, it remains unclear whether or not the outcome of primary infection with HBV genotype A would be influenced by subgroup Aa and Ae.

Although acute HBV infection of genotype A tends to persist in comparison with those of the other genotypes, only a minority (7/31 [23%]) develops chronic infection. An efficient therapeutic strategy has to be found, however, since the infection with HBV genotype A was terminated in only one of the six (17%) patients who were treated. Recently, adefovir dipivoxil was found to be effective for the treatment of chronic hepatitis B [Marcellin et al., 2003], and it may offer a reasonable option for resolving persistent HBV genotype A infection.

REFERENCES

Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. 2002. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 83:2059–2073.

Botha JF, Ritchie MJ, Dusheiko GM, Mouton HW, Kew MC. 1984. Hepatitis B virus carrier state in black children in Ovamboland: Role of perinatal and horizontal infection. Lancet 1:1210–1212.

Bowyer SM, van Staden L, Kew MC, Sim JG. 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 78:1719–1729.

Brechot C, Pourcel C, Louise A, Rain B, Tiollais P. 1980. Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. Nature 286:533–535.

Chu CJ, Lok AS. 2002. Clinical significance of hepatitis B virus genotypes. Hepatology 35:1274–1276.

Handzel ZT, Galili-Weisstub E, Burstein R, Berner Y, Pecht M, Netzer L, Trainin N, Barzilai N, Levin S, Bentwich Z. 1984. Immune derangements in asymptomatic male homosexuals in Israel: A pre-AIDS condition? Ann NY Acad Sci 437:549–553.

Heijtink RA, Paulij W, van Roosmalen M, Hellings JA, Niesters HG, Schalm SW, Osterhaus AD. 1999. Characteristics of the early phase of chronicity in acute hepatitis B infection. J Med Virol 57:331–336.

Kao JH. 2002. Hepatitis B viral genotypes: Clinical relevance and molecular characteristics. J Gastroenterol Hepatol 17:643–650.

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Takagi K, Miyakawa Y, Kumada H. 2002. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 68:522–528.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Hosaka T, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Kumada H. 2003. Clinical features of hepatitis B virus genotype A in Japanese patients. J Gastroenterol 38:656–662.

Kobayashi M, Suzuki F, Arase Y, Akuta N, Suzuki Y, Hosaka T, Saitoh S, Kobayashi M, Tsubota A, Someya T, Ikeda K, Matsuda M, Sato J, Kumada H. 2004. Analysis of hepatitis B virus genotype A infection in Japan. J Gastroenterol (~~in press~~Q2). 39, 844-850,

Lee WM. 1997. Hepatitis B virus infection. N Engl J Med 337:1733–1745.

Lindh M, Andersson AS, Gusdal A. 1997. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus-large-scale analysis using a new genotyping method. J Infect Dis 175:1285–1293.

Lindh M, Horal P, Norkrans G. 2000. Acute hepatitis B in Western Sweden—genotypes and transmission routes. Infection 28:161–163.

Magnius LO, Norder H. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology 38:24–34.

Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, Jeffers L, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med 348:808–816.

Miyakawa Y, Mizokami M. 2003. Classifying hepatitis B virus genotypes. Intervirology 46:329–338.

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Q2: Please update. 39: 844-850.

Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. 1992. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 73:1201–1208.

Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, Mito H, Yoshizawa H. 2003. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980–1994. J Gastroenterol Hepatol 18:943–949.

Ogawa M, Hasegawa K, Naritomi T, Torii N, Hayashi N. 2002. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. Hepatol Res 23:167–177.

Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y. 1976. e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N Engl J Med 294:746–749.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 34:590–594.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. 2000. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J Gen Virol 81:67–74.

Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Gish RG, Ueda R, Miyakawa Y, Mizokami M. 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 124:925–932.

Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 85:811–820.

Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 80:97–112.

Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, Mayumi M. 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 87:81–89.

van Steenbergen JE, Niesters HG, Op de Coul EL, van Doornum GJ, Osterhaus AD, Leentvaar-Kuijpers A, Coutinho RA, van den Hoek JA. 2002. Molecular epidemiology of hepatitis B virus in Amsterdam 1992–1997. J Med Virol 66:159–165.

Yao GB. 1996. Importance of perinatal versus horizontal transmission of hepatitis B virus infection in China. Gut 38:S39–S42.

Yotsuyanagi H, Yasuda K, Iino S, Moriya K, Shintani Y, Fujie H, Tsutsumi T, Kimura S, Koike K. 1998. Persistent viremia after recovery from self-limited acute hepatitis B. Hepatology 27:1377–1382.

Journal of Gastroenterology and Hepatology (2005) DOI: 10.1111/j.1400-1746.2005.03798.x

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DOCUMENT TYPE

Virological differences between patients infected with subtypes Ba and Bj of hepatitis B virus genotype B

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Abstract

2

Background: Hepatitis B virus (HBV) genotype B is classified into subtype Ba with the recombination with genotype C in the precore region plus core gene and subtype Bj without it. Virological and clinical differences between infections with subtypes Ba and Bj, however, are yet to be determined. recombination

Methods: During 1976 through 2001, 224 patients visited Toranomon Hospital in Tokyo, Japan who were infected with HBV genotype B. Subtypes of genotype B were determined by sequencing HBV-DNA recovered from sera for detecting recombination with genotype C.

Results: Subtype Ba was detected in 53 patients (24%) and Bj in 167 (75%); subtypes were not able to be determined in the remaining four (1%). The only virological difference was that detection of hepatitis B e antigen at the presentation was more frequent in the patients infected with subtype Ba than those with Bj (63% vs 33%, $P = 0.016$). There were no differences in the distribution of liver disease of various forms between the patients infected with subtypes Ba and Bj at presentation. No differences were noted, either, in the development of liver cirrhosis or hepatocellular carcinoma, or the loss of hepatitis B surface antigen from serum, between the patients infected with subtypes Ba and Bj during follow up of up to 26 years.

Conclusions: Although there were some virological differences between the patients infected with subtypes Ba and Bj of HBV genotype B, they do not seem to influence the long-term clinical outcome.

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Key words: hepatitis B e antigen, hepatitis B surface antigen, hepatitis B virus, genotypes, subtypes.

CORRECTED PROOF

INTRODUCTION

Hepatitis B virus (HBV) is classified into seven genotypes designated by the letters from A to G.[1–3] Recently, an eighth genotype, named H, has been proposed that is closely related to genotype F.[4] Genotypes of HBV have distinct geographic distribution and they influence the clinical course of hepatitis B. Because genotypes A and D frequently occur in Western countries, while genotypes B and C are common in Asia, clinical differences between genotypes A and D, as well as B and C, have been studied extensively.

It has been reported that genotype A induces chronic liver disease more frequently[5] and is associated with better response to interferon than genotype D.[6] Another recent study, however, has found that sustained biochemical remission and clearance of HBV-DNA, as well as the clearance of hepatitis B surface antigen (HBsAg), occurred at a higher rate in genotype A- than in genotype D-infected patients.[7] There have been increasing lines of evidence for more severe liver disease and longer duration of hepatitis B e antigen (HBeAg) in serum, accompanied by delayed seroconversion to antibody to HBeAg (anti-HBe), in infection with HBV genotype C than B.[8,9] Furthermore, hepatocellular carcinoma (HCC) develops more frequently in the patients infected with HBV genotype C than B.[10] Clinical courses may differ, however, even among the patients

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Accepted for publication 28 May 2004.

infected with the same genotypes of HBV. For instance, infection with HBV genotype B in Taiwan induces HCC much more frequently than that in Japan.[10,11]

Recently, two distinct subtypes of genotype B have been reported, designated Ba and Bj. Subtype Ba is ubiquitous in Asia, while Bj is restricted to Japan.[12] Notably, HBV isolates of subtype Ba possess the recombination with genotype C over the precore region plus core gene, while those of subtype Bj do not.[12] For the purpose of evaluating any clinical and virological differences, the 53 patients infected with subtypes Ba were compared with the 167 patients infected with subtype Bj at Toranomon Hospital in Tokyo, Japan.

METHODS

Patients infected with HBV

During 26 years from 1976 to 2001, 1674 patients infected with HBV visited Department of Gastroenterology, Toranomon Hospital in Tokyo Japan, and genotypes of HBV were determined in them. Genotype A was detected in 53 patients (3%), B in 224 (13%), C in 1332 (80%), D in four patients, E in one patient and F in three patients; genotypes were unidentifiable in the remaining 57 patients (3%). Subtypes of genotype B, in terms of Ba and Bj,[12] were determined in sera collected from the 224 patients infected with HBV genotype B at the presentation for evaluating any clinical and virological differences between infections with these two subtypes. Patients were considered to be in the asymptomatic carrier state when alanine aminotransferase (ALT) levels stayed normal (≤50 IU/L) throughout the observation period. Chronic hepatitis was diagnozed by liver biopsies performed under laparoscopy, and liver cirrhosis by liver biopsy as well as ultrasonographic images and laparoscopic findings. Hepatocellular carcinoma was diagnosed by imaging modalities, such as ultrasonography, computed tomography and magnetic resonance imaging, and by liver biopsy if necessary. The study design conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the hospital. An informed consent was obtained from every patient.

Serum markers of HBV infection

The HBsAg was determined with commercial kits by hemagglutination (MyCell, Institute of Immunology, Tokyo, Japan) and radioimmunoassay (AUSRIA II-125, Dinabot, Tokyo, Japan), and antibody to HBV core of IgM class was tested for by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBc-antiM RIA; Dinabot). The HBeAg was determined by ELISA (ELISA, F-HBe; Kokusai Diagnostic, Kobe, Japan). The six major genotypes of HBV (A–F) were determined by ELISA with commercial kits (HBV genotype EIA, Institute of Immunology) after the method of Usuda *et al.*[13,14] It involves the expression of seven preS2 epitopes (*b, f, g, k, m, s and u*) detected by monoclonal antibodies, the combination of which is specific for each of the six HBV genotypes: *bsu* for genotype A; *bm* for B; *bks* for C; *bksu(g)* for D, *bksu* for E and bk for F. Genotype G, which was discovered recently,[3] was determined by the combination of preS2 serotype for genotype D and subtype adw of HBsAg; it is characteristic of this genotype.[15] Serotypes of HBsAg were determined by ELISA with commercial kits (HBs Antigen Subtype EIA, Institute of Immunology).

Determination of subtypes Ba and Bj of genotype B

Nucleic acids were extracted from serum (100 μL), which had been stored at −80°C, with a Smitest EX&R kit (Genome Science, Tokyo, Japan). The core gene of HBV-DNA in extracted nucleic acids were amplified by polymerase chain reaction (PCR) with nested primers. The first-round PCR was performed with BJF3 (sense, 5′-CCG ACC TTG AGG CAT ACT TC-3′ [nt 1690–1709]) and BJF4 (antisense, 5′-GGG TCC CAC AAA TTG CTT AC-3′ [nt 2580–2606]) primers, and the second-round PCR with FJF1 (sense, 5′-GCT GTG CCT TGG GTG GCT TTG-3′ [nt 1876–1897]) and BJR2 (antisense, 5′-GCG ACG CCG TGA TTG AGA CCT-3′ [nt 2398–2411]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, CA, USA). They were then purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced in the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The core-gene sequences from patients were analyzed phylogenetically along with reference Ba and Bj sequences by 6-parameter and neighbor-joining methods.[16,17]

Nucleotide sequences of the precore region and core promoter

For determination of the wild-type or mutants in the precore region, nucleic acids extracted from serum were amplified by PCR with nested primers. The first-round PCR was performed with BCP-F7 (sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ [nt 1580–1599]) and BCP-R8 (antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ [nt 2295–2276]) primers, and the second-round PCR with BCP-F5 (sense, 5′-GCA TGG AAC CAC CGT GAA C-3′ [nt 1606–1625]) and BCP-R6 (antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ [nt 2014–1995]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis, purified and sequenced as described here. Mutations interfering with translation and transcription of HBeAg were sought in the precore region and core promoter, respectively. They included a G-to-A mutation at nucleotide 1896 (A1896) in the precore region and the double mutation in the core promoter converting the codon 1762 from A to T as well as codon 1764 from G to A (T1762/

A1764). Also examined was nt 1858 of T or C in HBV-DNA sequences.

Statistical analysis

Frequencies between groups were compared using the χ^2 test, Fisher's exact test and Mann–Whitney *U*-test. Data analysis was performed using SAS (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered significant. Differences in the progression rate of chronic hepatitis B and the frequency of HBsAg clearance were evaluated by Kaplan–Meier technique and log-rank test.

RESULTS

Clinical manifestations of the patients infected with subtype Ba or Bj of HBV genotype B

The HBV-DNA sequences were determined from nucleic acids extracted from 224 patents infected with HBV genotype B who visited Toranomon Hospital in Tokyo, Japan during 1976 through 2001, and who were subjected to phylogenetic analysis on the core gene. Subtype Ba having the recombination with genotype C was identified in 53 (24%) patients and subtype Bj without such recombination in 167 (75%); distinction between Ba and Bj was not possible in the remaining four patients (1%). Table 1 compares frequencies of acute hepatitis, asymptomatic carrier state, chronic hepatitis and liver cirrhosis with or without HCC, between 53 patients infected with HBV subtype Ba and 167 with Bj. There were no differences in the distribution of liver disease of various forms between the patients infected with subtype Ba and Bj.

Demographic and virological characteristics of patients with chronic hepatitis who were infected with HBV subtype Ba or Bj

Demographic and virological features are compared between patients with chronic hepatitis B, 24 of whom were infected with subtype Ba and 82 with subtype Bj (Table 2). There were no differences in sex, age, duration of follow up and mothers persistently infected with HBV, between the patients infected with subtypes Ba and Bj. At presentation, however, the prevalence of HBeAg in serum was significantly higher in the patients infected with subtype Ba than Bj (63% vs 33%,

Table 1 Distribution of liver disease in patients infected with HBV subtype Ba or Bj

Disease/condition	Subtypes of genotype B		
	Ba ($n = 53$) n (%)	Bj ($n = 167$) n (%)	*P*
Acute hepatitis	0	5 (3)	NS
Asymptomatic carrier state	22 (42)	66 (40)	NS
Chronic hepatitis	24 (45)	82 (49)	NS
Liver cirrhosis or hepatocellular carcinoma	7 (13)	14 (8)	NS

NS, not significant.

Table 2 Comparison between patients with chronic hepatitis who were infected with HBV subtypes Ba or Bj

Features	Subtypes of genotype B		
	Ba ($n = 24$) n (%)	Bj ($n = 82$) n (%)	*P*
Male	19 (79)	73 (89)	NS
Age (years); median (range)	36 (23–62)	37 (21–83)	NS
Follow up (days); median (range)	3363 (50–11 642)	3475 (165–10 679)	NS
Mother with HBsAg	2 (8)	12 (15)	NS
Serotype of HBsAg			
Adw	22 (92)	73 (89)	NS
Adr	1 (4)	1 (1)	NS
Adwr	1 (4)	0	NS
Untypeable	0	9 (11)	NS
HBeAg at presentation	15 (63)	27 (33)	$P = 0.016$
Clearance of HBeAg	10/15 (67)	21/27 (78)	NS
HBV-DNA at presentation (LEG/mL)†	7.5 ± 6.2	4.9 ± 3.6	NS

NS, not significant.
†Log equivalent genome (LEG)/mL by transcription mediated assay.

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$P = 0.016$). In contrast, the prevalence of HBeAg was no different between the asymptomatic carriers with Ba and Bj infections (3/22, 14% vs 6/82, 7%). Falling short of being significant, the mean titer of HBV-DNA was somewhat higher in the patients infected with subtype Ba than Bj.

Figure 1 depicts the development of liver cirrhosis and HCC in patients with chronic hepatitis B during follow up of up to 20 years. There were no differences in the progression of liver disease between the patients infected with subtypes Ba and Bj. No differences were noted, either, in the loss of HBsAg from serum during follow up between them, although HBsAg tended to disappear earlier in patients infected with subtype Ba than Bj up to 15 years (Fig. 2).

Mutations in the core promoter and precore region, which increase with the duration of infection and influence the severity of liver disease, were examined in the patients with chronic hepatitis at the time of presentation. Table 3 compares mutations in the core promoter and precore region between the patients infected with subtypes Ba and Bj. There were no differences in the frequency of the stop-codon mutation in the precore region, or that of the double mutation in the core promoter, between the patients infected with the two different subtypes of genotype B.

Distributions of HBsAg serotypes were no different between the patients infected with subtypes Ba and Bj. The 1858th nucleotide of T or C that influences the precore mutation (A1896) was invariably T in all 18 patients infected with subtype Ba, and in all 70 with Bj who were examined.

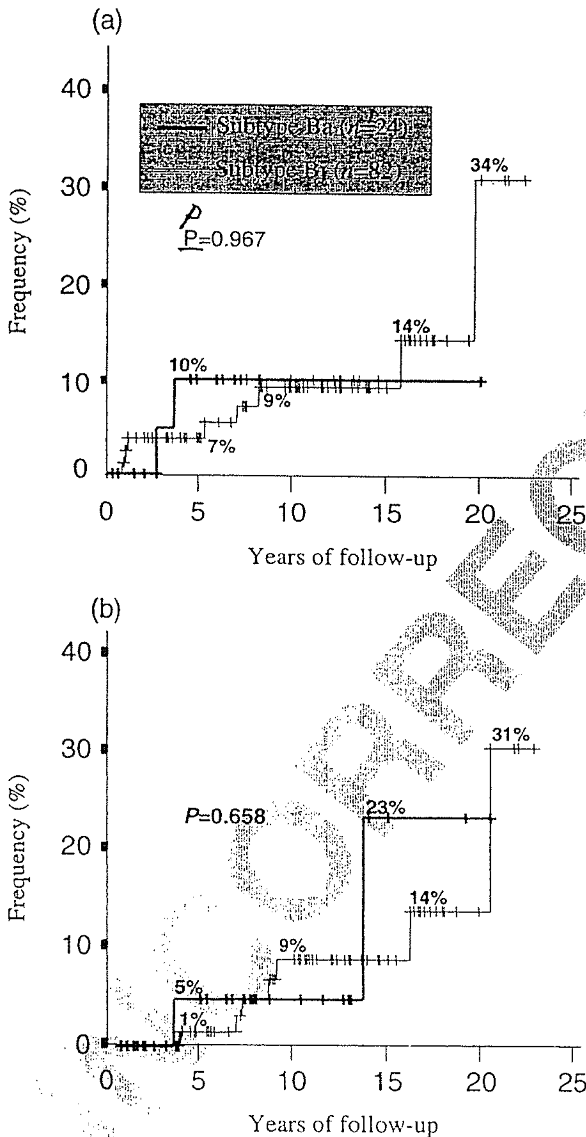

Figure 1 Evolution of chronic hepatitis in patients infected with subtype Ba or Bj of HBV genotype B. Development of liver cirrhosis (a) and hepatocellular carcinoma (b) were compared between the 24 patients infected with HBV genotype Ba and the 82 with Bj during follow up of 20 years or longer. There were no differences in the development of either liver cirrhosis of hepatocellular carcinoma by evalution of the XXX obtained by the product-limit method the log-rank test.

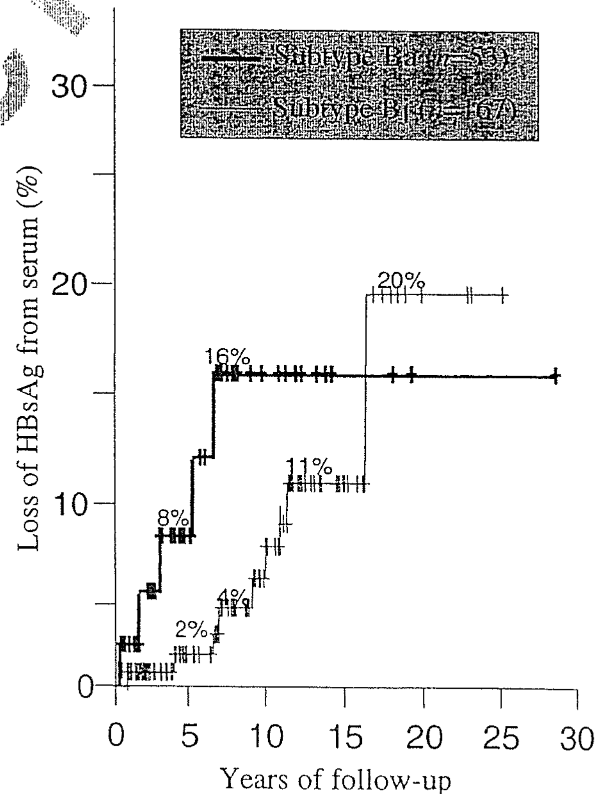

Figure 2 Loss of HBsAg from serum during long-term follow up. The [illegible] patients infected with subtype Ba and the [illegible] with subtype Bj of HBV genotype B, who presented with HBeAg in serum, were compared by the product-limit method the log-rank test.

Clinical and virological characteristics of patients with liver cirrhosis who were infected with subtype Ba or Bj

Table 4 lists demographic, histolgical and virological features of patients with liver cirrhosis, five of whom were infected with subtype Ba and 10 with subtype Bj. As for patients with chronic hepatitis B, the detection of serum HBeAg at presentation was significantly more frequent (60% vs 0%, $P = 0.022$), and the mean titer of HBV-DNA in serum tended to be higher, in the patients infected with subtype Ba than Bj.

DISCUSSION

Recombination between HBV isolates of distinct genotypes has been reported,[18,19] which may endow recombinants with a phenotype for virological characteristics or disease-inducing capacity distinct from parent genotypes. Because genotypes A and D are frequent in Western countries, A/D recombinants are reported there. Likewise, because genotypes B and C are common in Asia, B/C recombinants occur mostly in Asian countries. Not so many such recombinants have been reported, however, probably reflecting uncommon recombination events in the HBV infection.

Two subtypes of genotype B are reported, one of which has recombination with genotype C in the precore region and core gene, while the other does not.[12] It is surprising that essentially all HBV strains from Asian countries other than Japan are of the Ba subtype with the recombination (suffix 'a' representing Asia), in contrast to most of those from Japan that are of subtype Bj, without the recombination (suffix 'j', standing for Japan). Because Japan is unique in that both Ba and Bj subtypes occur in the genotype B infection, we set out to examine any demographic, virological and clinical differences between subtypes Ba and Bj. A study conducted at Toranomon Hospital in Tokyo was carried out to determine whether there would be any differences in subtypes Ba and Bj, in the same epidemiological and clinical setting in the patients of a single ethnic origin.

During 26 years from 1975 to 2001, 224 patients infected with HBV of genotype B presented to Department of Gastroenterolology, Toranomon Hospital located at the center of Tokyo, Japan. Subtypes of genotype B were determined by sequencing HBV-DNA, and Ba was found in 53 (24%) and Bj in 167 (75%); HBV isolates of genotype B from only four patients (1%) were untypeable into Ba or Bj. The 53 patients infected with subtype Ba and the 167 with subtype Bj were compared demographically, clinically and virologically.

The prevalence of subtype Ba (24%) in the patients who visited Toranomon Hospital in Tokyo was higher than that reported by Sugauchi *et al.* from Japan (7/97; 7%).[20] HBV subtype Ba is infrequent in Japan, in contrast to the other Asian countries, where subtype Ba accounts for all genotype B infections.[20] Because Toranomon Hospital is a tertiary referral hospital, selection may have occurred in favor of patients with severe disease or who were refractory to treatment. The frequency of HBsAg in mothers of patients tended to be higher in subtype Bj infection than Ba (15/167, 9% vs 2/53, 4%). Hence, the patients with subtype Ba would have had a higher chance of infection in later life than those with

Table 3 Mutations in the core promoter and precore region in the patients with chronic hepatitis who were infected with HBV subtype Ba or Bj

Mutation	Ba ($n = 18$) n (%)	Bj ($n = 69$) n (%)	P
Core promoter†			
Mutant	4 (22)	15 (22)	NS
Wild-type	14 (78)	54 (78)	
Precore region‡			
Mutant	9 (50)	36 (52)	NS
Wild-type	9 (50)	33 (48)	

NS, not significant.

Examination was possible in 18 of the 24 patients infected with subtype Ba and 69 of the 82 infected with subtype Bj of HBV genotype B.

†Double mutation for T1762/A1764; ‡A1896 mutation for a stop codon at amino acid 28.

Table 4 Comparison between patients with liver cirrhosis who were infected with HBV subtypes Ba or Bj

Features	Subtypes of HBV		
	Ba ($n = 5$) n (%)	Bj ($n = 10$) n (%)	P
Male	5 (100)	7 (70)	NS
Age (years); median (range)	44 (24–50)	37 (21–83)	NS
Follow up (days); median (range)	4505 (2001–8199)	1524 (487–5151)	NS
Mother with HBsAg	0	2 (20)	NS
HBeAg at presentation	3 (60)	0	0.022
Clearance of HBeAg	3/3 (100)	0/0	NS
HBV-DNA at presentation (LEG/mL)†	4.9 ± 4.1	4.1 ± 3.8	NS

NS, not significant.

†Log equivalent genome (LEG)/mL by the transcription mediated assay.

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subtype Bj. The duration of HBV infection therefore may have been shorter in patients with subtype Ba than Bj, which needs to be taken into consideration when evaluating virological differences between them. The prevalence of HBeAg in sera is reported to be higher in patients of the same age who were infected with subtype Ba than Bj,[20] which has been confirmed in the preesnt study. These differences, however, would not readily be attributed to virological differences alone and need to be evaluated with reference to the duration of HBV infection.

Subtypes Ba and Bj did not seem to affect the severity of clinical disease. The distribution of acute hepatitis, asymptomatic carrier state, chronic hepatitis and liver cirrhosis with or without HCC was no different between the patients infected with Ba and Bj in the present series. Subtypes Ba and Bj, however, have been shown to influence resistance to lamivudine as well as virological and biochemical breakthroughs in our previous study.[21]

There was an important virological difference between Ba and Bj infection. The patients with chronic hepatitis or liver cirrhosis infected with subtype Ba possessed HBeAg in serum significantly more frequently than those infected with subtype Bj. Because HBeAg persists longer in patients infected with HBV genotype C than B,[8,9] this trait of genotype C would have borne out in HBV strains of subtype Ba that possess the recombination with genotype C over the precore region and core gene. The persistence of HBeAg over a longer period of time, while the seroconversion to anti-HBe takes place accompanied by hepatitis flares, would results in more severe disease in the patients infected with HBVgenotype C than B.[8]

Mutations in the core promoter and precore region that downregulate and abolish the synthesis of HBeAg, respectively, are under influence of HBV genotypes, and the double mutation in the core promoter (T1762/A1764) is detected more frequently in the patients infected with genotype C than B.[8,22] In so far as the core promoter region of subtype Ba is replaced by that of genotype C,[12] it would be more prone to the mutation for T1762/A1764 than that of subtype Bj. Because the T1762/A1764 mutation is implicated in hepatocarcinogenesis in patients infected with HBV,[23] a high frequency of this mutation in subtype Ba infection would be responsible, at least in part, for HCC in patients in Taiwan who develop this during youth.[10]

Very recently, Sugauchi *et al.* compared 80 patients infected with subtype Ba from Asian countries other than Japan, with 80 patients infected with subtype Bj from Japan while controlling for severity of liver disease.[20] They found a higher frequency of HBeAg in serum and the double mutation in the core promoter (T1762/A1764) in the patients infected with subtype Ba than Bj. Because the Sugauchi *et al.* study was case-controlled on patients with identical distribution of asymptomatic carrier state, chronic hepatitis, liver cirrhosis and HCC, the influence of genotype Ba and Bj on the clinical course of hepatitis B was not within the scope of the study.[20] In the present series of 53 Japanese patients infected with subtype Ba and the 157 infected with Bj, no influence of these subtypes was observed in terms of distribution of liver disease of distinct severity and the development of liver cirrhosis and HCC in patients with chronic hepatitis B during follow up of up to 26 years. These observations come as a surprise, in view of the response to lamivudine being poorer in the patients infected with subtype Ba than Bj.[21]

In conclusion, there is a significant virological difference between HBV infection of subtype Ba and Bj, which seem to be attributable to the recombination with genotype C in HBV isolates of subtype Ba. Persistence of HBeAg would influence the clinical course and response to antiviral therapies in the patients infected with subtype Ba, who would fare worse than those with subtype Bj in the long term. This would need to be confirmed in an extended series of patients who are infected with HBV of Ba or Bj in prospective studies, in view of the small number of studied patients with Ba infection in Japan, where Bj prevails.

ACKNOWLEDGMENT

The present study was surpoted in part by research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1 Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J. Gen. Virol.* 1988; **69**: 2575–83.

2 Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; **198**: 489–503.

3 Stuyver L, De Gendt S, Van Geyt C *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J. Gen. Virol.* 2000; **81**: 67–74.

4 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO, Genotype H. A new Amerindian genotype of hepatitis B virus revealed in Central America. *J. Gen. Virol.* 2002; **83**: 2059–73.

5 Mayerat C, Mantegani A, Frei C. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J. Viral Hepat.* 1999; **6**: 299–304.

6 Zhang X, Zoulim F, Habersetzer F, Xiong S, Trepo C. Analysis of hepatitis B virus genotypes and pre-core region variability during interferon treatment of HBe antigen negative chronic hepatitis B. *J. Med. Virol.* 1996; **48**: 8–16.

7 Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 2002; **123**: 1848–56.

8 Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. *J. Infect. Dis.* 1999; **179**: 775–82.

9 Kao JH, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with

chronic hepatitis B virus infection. *J. Clin. Microbiol.* 2002; **40**: 1207–9.

10 Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000; **118**: 554–9.

11 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590–4.

12 Sugauchi F, Orito E, Ichida T *et al.* Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J. Virol.* 2002; **76**: 5985–92.

13 Usuda S, Okamoto H, Iwanari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J. Virol. Methods* 1999; **80**: 97–112.

14 Usuda S, Okamoto H, Tanaka T *et al.* Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. *J. Virol. Methods* 2000; **87**: 81–9.

15 Kato H, Orito E, Sugauchi F *et al.* Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. *J. Virol. Methods* 2001; **98**: 153–9.

16 Gojobori T, Ishii K, Nei M. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J. Mol. Evol.* 1982; **18**: 414–23.

17 Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 1987; **4**: 406–25.

18 Bollyky PL, Rambaut A, Harvey PH, Holmes EC. Recombination between sequences of hepatitis B virus from different genotypes. *J. Mol. Evol.* 1996; **42**: 97–102.

19 Morozov V, Pisareva M, Groudinin M. Homologous recombination between different genotypes of hepatitis B virus. *Gene* 2000; **260**: 55–65.

20 Sugauchi F, Orito E, Ichida T *et al.* Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* 2003; **124**: 925–32.

21 Akuta N, Suzuki F, Kobayashi M *et al.* The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. *J. Hepatol.* 2003; **38**: 315–21.

22 Orito E, Mizokami M, Sakugawa H *et al.* A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; **33**: 218–23.

23 Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology* 2003; **124**: 327–34.

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Characteristics of Patients with Chronic Hepatitis C who Develop Hepatocellular Carcinoma after a Sustained Response to Interferon Therapy

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Supported in part by a grant 13670544 from the Ministry of Health, Labor, and Welfare, Japan.

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Received October 17, 2003; revision received May 7, 2004; accepted June 21, 2004.

BACKGROUND. The objective of the current study was to determine the characteristic features of sustained responders who develop hepatocellular carcinoma after treatment with interferon for chronic hepatitis C.

METHODS. This study included 3626 patients with chronic hepatitis C who had received interferon monotherapy. Cox proportional hazards analysis was used to compare sustained responders who did and did not develop hepatocellular carcinoma, and nonsustained responders who developed hepatocellular carcinoma in a multicenter, retrospective cohort study.

RESULTS. Among 1197 sustained responders, 27 patients developed hepatocellular carcinoma (2.3%). Compared with sustained responders who did not develop hepatocellular carcinoma, patients who developed disease more often were male ($P = 0.0212$), were older ($P = 0.0068$), and had advanced-stage histologic disease before interferon therapy ($P = 0.0345$). Conversely, compared with patients with hepatocellular carcinoma who were not sustained responders, patients who were sustained responders tended to be older at the time of the initiation of interferon therapy ($P = 0.0552$) and at the time hepatocellular carcinoma was detected ($P = 0.0593$), and they also were predominantly male ($P = 0.0507$). The histologic staging and serum aminotransferase levels at the initiation of interferon therapy, the interval to the detection of tumor, and the tumor size showed no significant differences between the two groups.

CONCLUSIONS. Sustained responders in the group at high risk for developing hepatocellular carcinoma after interferon therapy were older, more often were male, and had more advanced histologic disease stage. Such patients should be followed carefully periodically for > 10 years after they complete interferon therapy. *Cancer* 2004;101:1616–22. *© 2004 American Cancer Society.*

KEYWORDS: chronic hepatitis type C, hepatocellular carcinoma, interferon, sustained responder.

In Japan, chronic hepatitis C (CH-C) with advanced histologic staging often progresses to hepatocellular carcinoma (HCC),[1] although patients who are seropositive for antihepatitis C virus (anti-HCV) antibodies or for HCV RNA do not always progress to cirrhosis or HCC.[2,3] Risk factors for developing HCC in patients with CH-C are advanced histologic stage, irregular regeneration of hepatocytes, heavy drinking, higher serum alanine aminotransferase (ALT) levels or lower serum albumin levels, male gender, and older age.[1,4–7] Since 1992, patients with CH-C commonly have been treated with interferon α (IFN-α) or IFN-β, which are covered by public health insurance in Japan. Because IFN improves hepatic inflammation and inhibits the progression of hepatic fibrosis, it

© 2004 American Cancer Society
DOI 10.1002/cncr.20537
Published online 18 August 2004 in Wiley InterScience (www.interscience.wiley.com).

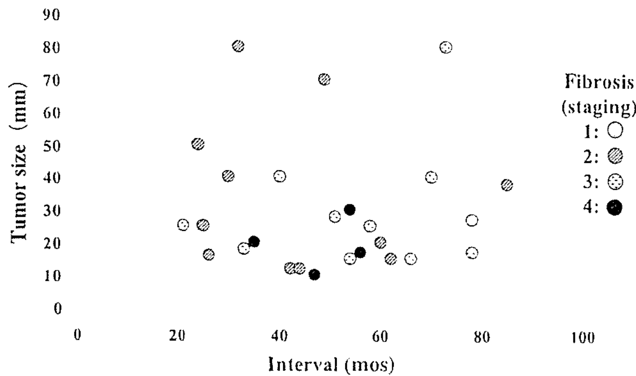

FIGURE 1. The interval from the completion of IFN therapy to the detection of SR HCC statistically did not correlate significantly with the tumor size or hepatic staging.

TABLE 1
Characteristics of Patients with Chronic Hepatitis C who were Treated with Interferon[a]

Characteristic	Sustained responder	Nonsustained responder	P value[b]
No. patients	1197	2429	—
Male:female ratio	776:421	1568:861	0.8826
Age (yrs, mean ± SD)	49.4 ± 11.9	51.2 ± 10.6	< 0.0001
Histologic staging score: No. of patients (%)			
F1	385 (38.6)	522 (25.8)	
F2	322 (32.3)	613 (30.3)	< 0.0001
F3	262 (26.3)	782 (38.6)	
F4	29 (2.9)	109 (5.4)	
Not available	199	403	

SD: standard deviation; IFN: interferon.
[a] All data were determined before interferon therapy.
[b] P values were calculated with the Fisher exact probability test and the Wilcoxon two-sample test.

has been suggested that the incidence of HCC may be reduced by IFN treatment. In fact, IFN therapy reportedly was effective not only for improving liver biochemistry and eliminating HCV RNA but also for reducing the inflammation/fibrosis scores and lowering the risk of HCC, especially in sustained responders (SR patients).[8–14]

Although a significant decrease in the incidence of HCC has been observed in SR patients after IFN therapy,[9–14] HCC is detected in some of them.[15–25] The clinical features of SR patients who develop HCC (SR HCC patients) and the long-term incidence of HCC in SR patients remain unclear, and the optimal duration and frequency of follow-up have not been established. Therefore, we analyzed SR HCC patients to determine their characteristic features compared with SR patients who did not develop HCC (SR non-HCC patients) and non-SRs who developed HCC (non-SR HCC patients).

MATERIALS AND METHODS

Patients

For this study, 3626 patients with CH-C were enrolled (2344 males and 1282 females) who had received IFN therapy between January 1990 and November 2001. Data from these patients were collected from 6 institutions and related hospitals, including 1371 patients from Kyoto Prefectural University of Medicine, 1478 patients from Osaka University, 497 patients from Miyazaki Medical College, 130 patients from Nagoya University, 102 patients from Shinsyu University, and 48 patients from Yamaguchi University. All patients were seropositive for anti-HCV antibodies, positive for serum HCV RNA, and seronegative for hepatitis B virus surface antigen. We excluded patients who had coexisting liver diseases, such as autoimmune hepatitis or primary biliary cirrhosis, and confirmed that they did not abuse alcohol (daily alcohol intake > 60 g of ethanol). No patients were infected with human immunodeficiency virus (HIV). At the time of entry into this study, no patients showed evidence of HCC, as determined by ultrasonography (US) and/or computed tomography (CT) studies. In principle, patients underwent liver biopsy prior to IFN therapy, and the histologic diagnoses were reached according to the classification of Desmet et al.[26] The gender, mean age, and histologic disease stage at the initiation of IFN therapy are shown in Table 1.

Natural IFN-α, recombinant IFN-α-2a, and recombinant IFN-α-2b were used in this study. In general, the IFN treatment protocol was within the range covered by public health insurance in Japan, namely, 3–10 MU of IFN-α for 24 weeks (daily for 2 weeks and 3 times per week for 22 weeks). In a few patients, administration of IFN-α was prolonged to 52 weeks. In some patients who suffered from severe side effects, the therapy period was shortened. In addition, patients for whom the total dose of IFN was < 200 MU were excluded from the study. Patients who had been treated with peginterferon or IFN/Ribavirin also were excluded. There was no difference noted with regard to the treatment protocol among the institutions and their related hospitals. We checked the laboratory findings at the end of IFN therapy and 6 months later. SR patients were defined as those who demonstrated continuous normal serum ALT levels for 6 months after finishing IFN therapy. The remaining patients were regarded as non-SR patients. The patient population included 1197 SR patients and 2429 non-SR patients.

We followed all patients for at least 1 year after the end of IFN therapy. The mean ± standard deviation

(SD) follow-up was 5.9 years ± 1.9 years. In SR patients, in general, we performed biochemical examinations, which sometimes included α-fetoprotein, every 3–12 months after confirming a sustained response. US and/or CT studies were performed at least once annually. However, because the incidence of HCC in non-SR patients—especially those with advanced-stage disease (fibrotic scores of F3 or F4)–was expected to be higher than that in SR patients, US and/or CT studies were performed every 3–6 months in non-SR patients. This strategy was similar in all of the institutions, and the frequency of radiographic examination was calculated to avoid unnecessary cost and not to miss HCC. However, some SR patients and non-SR patients who skipped or stopped visiting the outpatient clinic and some patients who were followed by their home physicians were not followed sufficiently. The diagnosis of HCC was based on appropriate radiologic findings (hepatic angiography, dynamic CT, magnetic resonance imaging).[27] When it was difficult to determine a final diagnosis with the radiologic findings, a histologic diagnosis was reached by tumor biopsy. In 17 of 27 SR HCC patients, a histologic diagnosis of HCC was obtained by the examination of resected hepatic tumors or biopsied tumor specimens. Patients who were diagnosed with HCC within 1 year after the end of IFN therapy were excluded from this study because of the possibility that a small but detectable HCC was missed before IFN therapy. Written informed consent to receive IFN therapy and to participate in this follow-up study was obtained from all patients, and the ethical committees of the participating institutions approved this study.

Statistical Analysis

Statistical analysis was performed using the SAS/PC statistical package (SAS Institute, Cary, NC). The Fischer exact probability test was used to compare the frequencies of gender. The Wilcoxon two-sample test was used to compare age, histologic staging, serum ALT level, interval between the end of IFN therapy and the detection of HCC, and the size of HCC. The independent risk factors for developing HCC in SR patients were examined by Cox proportional-hazards analysis; the variables were gender, age, histologic stage, and serum ALT level. Patients who had missing data were excluded from this analysis. Each variable was transformed into categorical data comprised of two-sample, ordinal numbers for multivariate analysis. P values were two-sided, and P values < 0.05 were considered statistically significant.

RESULTS

Characteristic Features of SR HCC Patients

During the observation of 3626 patients, HCC was detected in 259 patients; however, 19 patients were excluded, because HCC was detected within 1 year after they completed IFN therapy. The distribution of the remaining 240 HCC patients among the 6 institutions was as follows: 109 patients from Kyoto Prefectural University of Medicine (HCC incidence, 8.0%), 102 patients from Osaka University (HCC incidence, 6.9%), 3 patients from Miyazaki Medical College (HCC incidence, 0.6%), 15 patients from Nagoya University (HCC incidence, 11.5%), 8 patients from Shinsyu University (HCC incidence, 7.8%), and 3 patients from Yamaguchi University (HCC incidence, 6.3%). The incidence of HCC did not differ significantly among the institutions, except for Miyazaki Medical College. partly because hepatic fibrosis was less advanced in patients from this institution compared with patients from the other five institutions. Of 240 patients, 27 were SR patients, and 213 were non-SR patients. The ages of the 240 patients at the initiation of IFN therapy ranged from 37–77 years (mean age ± SD, 59.1 years ± 6.6 years) and varied from 39–83 years (63.6 years ± 6.8 years) at the time HCC was detected.

Among the 27 SR HCC patients, 5 patients consumed ≈ 50 g of ethanol daily. By evaluating liver specimens and biochemical examinations, including γ-glutamyl transferase, we excluded the possibility of alcoholic liver diseases in these patients. Serum HCV RNA was evaluated in the SR HCC patients by reverse transcriptase-polymerase chain reaction analysis. Twenty-six SR HCC patients were complete responders (seronegative for HCV RNA both at the end of IFN therapy and 6 months later), and 1 SR HCC patient was a biochemical responder (seropositive for HCV RNA at the end of IFN therapy). In 1 complete responder who developed HCC, serum HCV RNA became positive 12 months after completing IFN therapy.

No correlation could be found between the interval before HCC was detected, tumor size, or hepatic histologic stage among the SR HCC patients (Fig. 1). HCC that was detected long after discontinuing IFN therapy was not always large, and the patients with large HCC did not always show more advanced stage according to liver histology. The greatest dimensions of the 2 largest SR HCC tumors were 80 mm and were detected 32 months and 73 months after the end of IFN therapy. The greatest dimension of SR HCC found after the longest interval (85 months) was 38 mm.

Tumor tissue samples could be examined from 18 of 27 SR HCC patients. Two samples were categorized

TABLE 2
Comparisons between Sustained Responders with and without Hepatocellular Carcinoma[a]

Characteristic	SR HCC	SR non-HCC	P value[b]
No. of patients	27	1170	
Male:female ratio	25:2	751:419	0.0016
Age (yrs, mean ± SD)	60.7 ± 7.5	50.2 ± 12.4	< 0.0001
Serum ALT (IU/L, mean ± SD)	111.7 ± 67.7	122.6 ± 109.9	0.7267
Histologic staging score: No. of patients (%)			
F1	1 (3.7)	384 (39.6)	
F2	11 (40.7)	310 (32.0)	< 0.0001
F3	10 (37.0)	252 (26.0)	
F4	5 (18.5)	24 (2.5)	

SR: sustained responder; HCC: hepatocellular carcinoma; SD: standard deviation; ALT: alanine aminotransferase; IFN: interferon.
[a] All data were determined before interferon therapy.
[b] P values were calculated with the Fisher exact probability test and the Wilcoxon two-sample test.

TABLE 3
Factors Associated with the Development of Hepatocellular Carcinoma in Sustained Responders[a]

Characteristic	Risk ratio	95% CI	P value
Male vs. female	5.498	1.290–23.439	0.0212
Age	7.378	1.737–31.326	0.0068
Stage of liver disease	2.344	1.064–5.164	0.0345
Serum ALT	1.331	0.606–2.923	0.4768

95% CI: 95% confidence interval; ALT: alanine aminotransferase.
[a] All data were determined before interferon therapy. Statistical analysis was performed using the Cox proportional hazards test. The variable for age was set at < 50 years or ≥ 50 years, the variable for stage was set < F3 or ≥ F3, and the variable for the serum alanine aminotransferase level was set at < 88 UI/L or ≥ 88 UI/L. The variables age and serum alanine aminotransferase level were determined as median data. The variable for stage was set to obtain the largest hazard ratio.

as well differentiated HCC, 11 samples were moderately differentiated HCC, 2 samples were poorly differentiated HCC, and 2 samples were undifferentiated HCC. One sample was the necrotic tissue after transcatheter arterial embolization therapy (TAE). Nontumorous liver tissue samples from 18 patients were evaluated for their fibrosis scores in resected HCC or tumor biopsy specimens. Liver fibrosis scores improved in nine patients, did not change significantly in eight patients, and worsened in one patient.

Sixteen of 27 SR HCC patients underwent partial hepatectomy, and 10 patients were treated with TAE and/or percutaneous ethanol injection therapy. Because one patient changed his hospital after the diagnosis of HCC, we could not know his prognosis.

Comparison between SR HCC Patients and SR Non-HCC Patients

We compared 27 SR HCC patients with 1170 SR non-HCC patients. The SR HCC patients included 25 males (92.6%) and 2 females (7.4%), and the SR non-HCC patients included 751 males (63.5%) and 419 females (35.8%). At the time IFN therapy was initiated, the mean age of the SR HCC patients was 60.7 years ± 7.5 years (range, 37–70 years), whereas the mean age of the SR non-HCC patients was 50.2 years ± 12.4 years (range, 17–73 years). Thus, the SR HCC patients more often were male ($P = 0.0016$) and were older ($P < 0.0001$) compared with the SR non-HCC patients (Table 2).

The fibrotic scores in biopsied liver specimens before IFN therapy for the SR HCC patients included 1 F1 specimen (3.7%), 11 F2 specimens (40.7%), 10 F3 specimens (37.0%), and 5 F4 specimens (18.5%); and the fibrotic scores for the SR non-HCC patients included 384 F1 specimens (39.6%), 310 F2 specimens (32.0%), 252 F3 specimens (26.0%), and 24 F4 specimens (2.5%). The 2 female SR HCC patients both had F4 specimens. Among the total SR population, SR HCC patients had more advanced-stage disease ($P < 0.0001$). The mean serum ALT level at the initiation of IFN therapy was 111.7 IU/L ± 67.7 IU/L in the SR HCC patients and 122.6 IU/L ± 109.9 IU/L in the SR non-HCC patients (Table 2).

Cox proportional-hazards analysis of factors associated with the development of HCC in the SR patients was performed with four variables (gender, age, histologic stage, and serum ALT level). In this analysis, the hazard ratios for age, stage, and serum ALT level were calculated between the two groups. The age variable was set at < 50 years or ≥ 50 years, the fibrotic score (stage) variable was set at < F3 or ≥ F3, and the variable for serum ALT level was set at < 88 IU/L or ≥ 88 IU/L. The variables age and serum ALT level were determined as median data. We chose the variable for stage to obtain the greatest hazard ratio. The SR HCC patients more often were male ($P = 0.0212$, 95%CI, 1.290–23.439), were older ($P = 0.0098$, 95%CI, 1.737–31.326), and had advanced-stage disease according to liver histology ($P = 0.0345$; 95%CI, 1.064–5.164) before IFN therapy. Gender, age, and histologic stage before IFN therapy were considered independent risk factors for the development of HCC (Table 3).

Comparison between SR HCC Patients and Non-SR HCC Patients

We compared the clinical characteristics of the 27 SR HCC patients with the 213 non-SR HCC patients. The non-SR HCC patients included 161 males (75.6%) and 52 females (24.4%). The mean age of the non-SR HCC patients at the initiation of IFN therapy was 58.9 years ± 6.5 years (range, 40–77 years), and the mean age at

TABLE 4
Comparisons between Sustained Responders and Nonsustained Responders among Patients with Hepatocellular Carcinoma

Characteristic	SR	Non-SR	P value[a]
No. of patients	27	213	
Male:female ratio	25:2	161:52	0.0507
Age at the initiation of IFN (yrs, mean ± SD)	60.7 ± 7.5	58.9 ± 6.5	0.0552
Age at the detection of HCC (yrs, mean ± SD)	65.1 ± 7.8	63.4 ± 6.7	0.0593
Serum ALT (IU/L)[b]	111.7 ± 67.7	120.5 ± 56.4	0.2027
Histologic staging score: No. of patients (%)[b]			
F1	1 (3.7)	12 (5.6)	
F2	11 (40.7)	36 (16.9)	0.1861
F3	10 (37.0)	135 (63.4)	
F4	5 (18.5)	30 (14.1)	
Interval (mos, mean ± SD)[c]	49.3 ± 18.2	49.7 ± 24.8	0.7484
Tumor size (mm, mean ± SD)	31.2 ± 20.1	21.3 ± 9.9	0.1573

SR: sustained responder; IFN: interferon; SD: standard deviation; HCC: hepatocellular carcinoma; ALT: alanine aminotransferase.
[a] P values were calculated with the Fisher exact probability test and the Wilcoxon two-sample test.
[b] Data were determined before interferon therapy.
[c] The interval was between the completion of interferon therapy and the detection of hepatocellular carcinoma.

time HCC was detected was 63.2 years ± 6.7 years (range, 44–83 years). The mean serum ALT level in the non-SR HCC patients at the start of IFN therapy was 120.5 IU/L ± 56.4 IU/L. The fibrotic scores of biopsied liver specimens obtained from the non-SR HCC patients before IFN therapy included 12 F1 specimens (5.6%), 36 F2 specimens (16.9%), 135 F3 specimens (63.4%), and 30 F4 specimens (14.1%). Thus, concerning gender and age, the SR HCC patients tended to be predominantly male ($P = 0.0507$) and were older (both at the initiation of IFN therapy [$P = 0.0552$] and at the time HCC was detected [$P = 0.0593$]) compared with the non-SR HCC patients; however, the serum ALT levels and the histologic stage before IFN therapy among the SR HCC patients did not differ significantly compared with the non-SR HCC patients (Table 4).

The mean interval between the end of IFN therapy and the detection of HCC for the SR HCC patients was 49.3 months ± 18.2 months (range, 21–85 months), which was not significantly different from that for the non-SR HCC patients (49.7 months ± 24.8 months; range, 12–141 months). The mean greatest dimension of SR HCC was 31.2 mm ± 20.1 mm, which was slightly greater than, but not significantly different from, the mean greatest dimension of non-SR HCC (21.3 mm ± 9.9 mm) (Table 4).

DISCUSSION

In the current study, we compared the clinical characteristics of SR HCC patients with the characteristics of SR non-HCC patients to determine the characteristic features of SR HCC. The incidence of HCC among the 1197 SR patients was 2.3%, and the incidence among the 2429 non-SR patients was 8.8% during the mean follow-up of 5.9 years. In patients with CH-C, aging and advanced hepatic histologic stage reportedly are major risk factors for HCC development.[1,4] This was true for the SR population in our current investigation, because the risk ratio for developing HCC was > 7 times greater in older patients (≥ 50 years) and was more than twice as high in patients who had advanced histologic stage disease (fibrotic score ≥ F3) according to a Cox proportional-hazards analysis. Khan et al. also reported that male gender is an important risk factor for HCC development.[5] In the current study, males were more than five times more likely to develop HCC in the SR population. Thus, older male patients with advanced hepatic fibrosis were considered to be a high-risk group for developing HCC among the SR population (Table 3).

Conversely, compared with the non-SR HCC patients, the SR HCC patients were older at the initiation of IFN therapy ($P = 0.0552$) and at the detection of HCC ($P = 0.0593$), and they were predominantly male ($P = 0.0507$). Although these characteristics may not have differed significantly in the current study, a study of even larger size may show that this indeed is a trend. The histologic staging, the serum ALT level at the initiation of IFN therapy, the interval for the detection of HCC, and the tumor size did not differ significantly between the two groups. The tumor size in SR HCC patients was slightly greater compared with the tumor size in non-SR HCC patients, most likely because of the extended interval of screening for HCC after patients attained a sustained response to IFN therapy (Table 4).

Some previous articles reported that HCV RNA may survive in the hepatic tissues of SR HCC patients[28–30] and may be involved in the carcinogenesis or growth of HCC. Although we could not demonstrate the presence of HCV RNA in tumors and surrounding hepatic tissues from SR HCC patients, eradication of HCV from these tissues, along with the nontumorous hepatic tissues, was confirmed in several previous studies,[15–21] suggesting that the persistence of HCV is not essential for the growth of HCC in SR patients.

To ascertain the time of HCC occurrence, several studies were performed that examined the doubling time (DT) of HCC. Two studies from Japan reported that the DT of HCC measuring < 3 cm in greatest dimension was 93.0 days ± 57.4 days or 195.0 days ± 171.0 days.[31,32] Barbara et al. reported that the DT of HCC measuring < 5 cm in greatest dimension was 204.2 days ± 135 days.[33] Recently, Toyoda et al. re-

ported similar results, assuming that the greatest dimension of occult HCC was 5 mm before IFN therapy.[34] We calculated the growth interval between a single HCC cell and an HCC measuring 1 cm in greatest dimension on the assumption that the DT of HCC was 90 days and concluded that the growth interval may be > 6 years.[8] Because smaller and well differentiated HCCs have a longer DT, the growth interval to reach 1 cm in greatest dimension may be much longer than 6 years. Therefore, it is probable that small HCC may have existed in the liver prior to IFN therapy in the current SR HCC patients.[35]

It cannot be determined with certainty how long SR patients should be followed after they complete IFN therapy. Judging from the results obtained in the current study, we recommend that, when SR patients are male, age > 50 years old, and have F3 or F4 histologic stage, they should be checked by US or CT at least twice per year for > 10 years. Other SR patients with less advanced disease should be checked at least once per year.

REFERENCES

1. Ikeda K, Saitoh S, Suzuki Y, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol.* 1998;28:930–938.
2. Kenny-Walsh E, for the Irish Hepatology Research Group. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. *N Engl J Med.* 1999;340: 1228–1233.
3. Alberti A, Noventa F, Benvegnu L, Boccato S, Gatta A. Prevalence of liver disease in a population of asymptomatic persons with hepatitis C virus infection. *Ann Intern Med.* 2002;17:961–964.
4. Aizawa Y, Shibamoto Y, Takagi I, et al. Analysis of factors affecting the appearance of hepatocellular carcinoma in patients with chronic hepatitis C. A long term follow-up study after histologic diagnosis. *Cancer.* 2000;89:53–59.
5. Khan MH, Farrell GC, Byth K, et al. Which patients with hepatitis C develop liver complications? *Hepatology.* 2000; 31:513–520.
6. Shibata M, Morizane T, Uchida T, et al. Irregular regeneration of hepatocytes and risk of hepatocellular carcinoma in chronic hepatitis and cirrhosis with hepatitis-C-virus infection. *Lancet.* 1998;351:1773–1777.
7. Kasahara A, Hayashi N, Mochizuki K, et al. Clinical characteristics of patients with chronic hepatitis C showing biochemical remission, without hepatitis C eradication, as a result of interferon therapy. The Osaka Liver Disease Study Group. *J Viral Hepatol.* 2000;7:343–351.
8. Okanoue T, Itoh Y, Minami M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. *J Hepatol.* 1999; 30:653–659.
9. Kasahara A, Hayashi N, Mochizuki K, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology.* 1998;27:1394–1402.
10. Okanoue T, Itoh Y, Kirishima T, et al. Transient biochemical response in interferon therapy decreases the development of hepatocellular carcinoma for five years and improves the long-term survival of chronic hepatitis C patients. *Hepatol Res.* 2002;23:62–77.
11. Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. *Ann Intern Med.* 1999;131: 174–181.
12. Imai Y, Kawata S, Tamura S, et al. Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. *Ann Intern Med.* 1998;129:94–99.
13. Ikeda K, Saitoh S, Arase Y, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology.* 1999;29:1124–1130.
14. Tanaka H, Tsukuma H, Kasahara A, et al. Effect of interferon therapy on the incidence of hepatocellular carcinoma and mortality of patients with chronic hepatitis C: a retrospective cohort study of 738 patients. *Int J Cancer.* 2000;87:741–749.
15. Hirashima N, Mizokami M, Orito E, et al. Development of hepatocellular carcinoma in a patient with chronic hepatitis C infection after a complete and sustained response to interferon-alpha. *J Gastroenterol Hepatol.* 1996;11:955–958.
16. Inoue M, Ohhira M, Ohta T, et al. Hepatocellular carcinoma developed in a patient with chronic hepatitis C after the disappearance of hepatitis C virus due to interferon therapy. *Hepatogastroenterology.* 1999;46:2554–2560.
17. Miyano S, Togashi H, Shinzawa H, et al. Case report: occurrence of hepatocellular carcinoma 4.5 years after successful treatment with virus clearance for chronic hepatitis C. *J Gastroenterol Hepatol.* 1999;14:928–930.
18. Tamori A, Kuroki T, Nishiguchi S, et al. Case of hepatocellular carcinoma in the caudate lobe detected after interferon caused disappearance of hepatitis C virus. *Hepatogastroenterology.* 1996;43:1079–1083.
19. Kim SR, Matsuoka T, Maekawa Y, et al. Development of multicentric hepatocellular carcinoma after completion of interferon therapy. *J Gastroenterol.* 2002;37:663–668.
20. Okamura K, Yamazaki K, Ohmura T, et al. A resected case of hepatocellular carcinoma with sustained response to interferon for five years. *Acta Hepatol Jpn.* 2000;41:43–47.
21. Yamada M, Ichikawa M, Matsubara A, Ishiguro Y, Yamada M, Yokoi S. Development of small hepatocellular carcinoma 80 month after clearance of hepatitis C virus with interferon therapy. *Eur J Gastroenterol Hepatol.* 2000;12:1029–1032.
22. Nagano K, Fukuda Y, Nakano I, et al. A case of the development of two hepatocellular carcinoma and a cholangiocarcinoma with cirrhosis after elimination of serum hepatitis C virus RNA with interferon therapy. *Hepatogastroenterology.* 2000;47:1436–1438.
23. Sugo H, Kitayama N, Iwata T, et al. Development of hepatocellular carcinoma in a patients with chronic hepatitis C after a complete response to interferon therapy. *Acta Hepatol Jpn.* 2000;41:195–198.
24. Sugiura N, Sakai Y, Ebara M, et al. Detection of hepatocellular carcinoma after interferon therapy for chronic hepatitis C: clinical study of 26 cases. *J Gastroenterol Hepatol.* 1996;11:535–539.

25. Kubo S, Nishiguchi S, Tamori A, et al. Resected cases of hepatocellular carcinoma detected after interferon therapy for chronic hepatitis C. *Hepatogastroenterology.* 2000;47: 1100–1102.
26. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology.* 1994;19:1513–1520.
27. Okuda K, Kondo Y. Primary carcinoma of the liver. In: Haubrich WS, Schaffner F, Berk JE, editors. Bockus gastroenterology. 5th edition (3), Philadelphia: WB Sanders Company, 1995:2468–2472.
28. Larghi A, Tagger A, Crosignani A, et al. Clinical significance of HCV RNA in patients with chronic hepatitis C demonstrating long-term sustained response to interferon-alpha therapy. *J Med Virol.* 1998;55:7–11.
29. Reichard O, Glaumann H, Fryden A, et al. Two-year biochemical, virological, and histological follow-up in patients with chronic hepatitis C responding in a sustained fashion to interferon alfa-2b treatment. *Hepatology.* 1995;21:918–922.
30. Balart LA, Perrillo R, Roddenberry J, et al. Hepatitis C RNA in liver of chronic hepatitis C patients before and after interferon alfa treatment. *Gastroenterology.* 1993;104:1472–1477.
31. Majima Y. Growth rate of hepatocellular carcinoma by ultrasonography and its clinical significance. *Acta Hepatol Jpn.* 1984;25:754–765.
32. Ebara M, Ohto M, Shinagawa T, et al. Natural history of minute hepatocellular carcinoma smaller than three centimeters complicating cirrhosis. A study in 22 patients. *Gastroenterology.* 1986;90:289–298.
33. Barbara L, Benzi G, Gaiani S, et al. Natural history of small untreated hepatocellular carcinoma in cirrhosis: a multivariate analysis of prognostic factors of growth rate and patient survival. *Hepatology.* 1992;16:132–137.
34. Toyoda H, Kumada T, Honda T, et al. Analysis of hepatocellular carcinoma tumor growth detected in sustained responders to interferon in patients with chronic hepatitis C. *J Gastroenterol Hepatol.* 2001;16:1131–1137.
35. Okanoue T, Itoh Y. Hepatocellular carcinoma in sustained responders of interferon treated chronic hepatitis C. *J Gastroenterol Hepatol.* 2003;18:121–123.

Journal of Viral Hepatitis, 2004, 11, 136–140

Type I interferon receptor and response to interferon therapy in chronic hepatitis C patients: a prospective study

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Received February 2003; accepted for publication July 2003

SUMMARY. The type I interferon (IFN) receptor consists of at least two subunits, IFNAR1 and IFNAR2. We previously found a correlation between IFNAR1 and IFNAR2 expression in liver, and a correlation in IFNAR2 expression, but not in IFNAR1, between liver and peripheral blood mononuclear cells (PBMCs). The aim of this study was to prospectively assess whether IFNAR2 expression levels in PBMCs as well as in liver act as markers for predicting response to IFN therapy in chronic hepatitis C patients. Fifty-two Japanese patients with chronic hepatitis C, were enrolled. IFNAR2 mRNA was quantified using competitive polymerase chain reaction, in liver and PBMC specimens, and of the 52 patients assigned to receive a 6-month course of interferon-α therapy, 36 patients who received more than 300 million units of interferon were analysed. IFNAR2 mRNA expression levels were significantly higher in liver than in PBMCs in all 36 patients ($P = 0.016$). Seventeen sustained virologic responders showed lower pretreatment hepatitis C virus (HCV)-RNA levels ($P = 0.017$) in serum and higher pretreatment levels of IFNAR2 mRNA in liver ($P = 0.007$), but not in PBMCs, compared with nonsustained virologic responders. In multivariate analysis, these factors were independently associated with a sustained virologic response (i.e. HCV-RNA level: odds ratio 0.23, 95% CI 0.038–0.864; and IFNAR2 in liver: odds ratio 1.116, 95% CI 1.015–1.227). Hence, IFNAR2 expression levels in liver, but not in PBMCs, is predictive of response to IFN treatment in chronic hepatitis C patients.

Keywords: IFNAR1, IFNAR2, liver tissue, peripheral blood mononuclear cells, PBMCs.

INTRODUCTION

To date, interferon (IFN) with or without ribavirin is the only therapy known to eradicate the hepatitis C virus (HCV) and induce long-term normalization of aminotransferase levels in patients with chronic hepatitis C. However, this occurs in <50% of the treated patients. Therefore, the identification of prognostic factors predictive of response to IFN therapy is important and several factors have been reported [1–5]. As IFN elicits antiviral activity by binding to receptors on the cell surface [6,7], expression of the type I IFN receptor in liver tissue is likely to be involved in the pathogenesis of viral hepatitis and response to IFN therapy. In fact, recent studies have demonstrated a significant correlation between hepatic expression of the type I IFN receptor and response to IFN therapy in chronic hepatitis C patients [8–12]. A prospective study, however, has not been conducted.

The type I IFN receptor consists of at least two subunits: the IFNAR1 (IFNα receptor) and the IFNAR2 (IFNα/β receptor) [13,14], both of which have been cloned and are directly involved in signal transduction [13,15,16]. We have previously quantified mRNA levels of both subunits, using a competitive polymerase chain reaction (PCR) assay, in liver and peripheral blood mononuclear cells (PBMCs) from chronic hepatitis C patients [17]. We have demonstrated that levels of IFNAR1 expression are strongly correlated with IFNAR2 levels in liver, and further that IFNAR2 expression, but not IFNAR1 expression, in liver is related to that in

Abbreviations: IFN, interferon; IFNAR1, interferon receptor subunit 1; IFNAR2, interferon receptor subunit 2; PBMC, peripheral blood mononuclear cells.

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PBMCs [17]. These results prompted us to determine whether IFNAR2 expression levels in PBMCs as well as in liver act as possible markers for predicting response to IFN therapy in chronic hepatitis C patients. Herein we prospectively examined the expression levels of IFNAR2 in liver and PBMCs in chronic hepatitis C patients and assessed whether expression levels were related to response therapy.

METHODS

Patients

From March 1999 to February 2001, 52 Japanese patients with chronic hepatitis C, who met the study's criteria, were enrolled in this prospective trial by Yamaguchi University Hospital and affiliated institutions. Criteria for enrollment included: persistently elevated serum alanine aminotransferase (ALT) levels for >6 months prior to enrollment; positive results for HCV-RNA in serum; liver histological examination upon informed consent (showing lesions compatible with chronic hepatitis) performed within a month of enrollment; absence of detectable hepatitis B virus surface antigen; exclusion of all other potential causes of chronic liver disease such as autoimmune hepatitis, primary biliary cirrhosis, drug-induced hepatitis, or metabolic liver disease; no history of alcohol abuse, defined as alcohol intake of ≥80 g/day for longer than 3 years; no pregnancy; platelet count ≥70 000/mm^3 and leukocyte count ≥3000/mm^3.

Study design

After providing informed consent, patients were assigned to receive 6–9 million units (MU) of recombinant IFN alpha-2b (Intron A, Schering-Plough, Osaka, Japan) intramuscularly, six times weekly for 2–4 weeks, followed by 6–9 MU three times weekly for 22–24 weeks (total 468–810 MU). Patients were followed up for >6 months after completion or cessation of therapy. Blood chemistry and blood cell counts were measured at the beginning of treatment, and then every 4 weeks during the treatment and follow-up period. HCV serotype and HCV-RNA levels were measured immediately upon collection of the initial sample at commencement of therapy. PBMC samples were available from all patients on the same day as liver biopsy. Expression levels of IFNAR2 mRNA were determined for both liver tissue and PBMCs, using a competitive PCR assay.

Patients were categorized according to IFN response: patients with a sustained virologic response were defined by the normalization of serum ALT during the 6-month period after completion of treatment and the absence of detectable serum HCV-RNA tested 6 months after completion of therapy; those categorized as nonsustained virologic responders did not meet these criteria. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics committee.

Detection of serum HCV-RNA, HCV serotyping and quantification of HCV-RNA

Serum HCV-RNA was detected using the AMPLICOR HCV amplification kit (Roche Diagnostics, Tokyo, Japan). HCV serotypes were determined by the method of Tanaka *et al.* [18]. Serum viral load was quantified by either a branched DNA probe assay, version 1.0 (Quantiplex, Chiron, Emeryville, CA, USA) or AMPLICORE HCV Monitor test, version 1.0 (Roche Diagnostics), and expressed as the logarithm of copy numbers per millilitre. For statistical purposes, HCV-RNA levels below the detection limit of the branched DNA probe assay (350 000 genome equivalents per millilitre) were set at 350 000 genome equivalents per millilitre.

Determination of IFNAR2 expression levels in liver tissue and PBMCs

Liver biopsy specimens were divided into two portions; one for light microscopy, and the other for measurement of IFNAR2 mRNA. This second portion was immediately put into acid guanidinium solution and frozen in liquid nitrogen. PBMC samples were separated from 10 mL of heparinized blood by density gradient centrifugation with Leuko PREP (Becton Dickinson, Lincoln Park, NJ, USA), washed three times with RPMI 1640 culture medium, and stored at −80 °C until use, as were liver specimens. Levels of IFNAR2 mRNA expression were quantified using a competitive PCR assay, as described previously [17]. In brief, competitive PCR fragments were designed to have deletions and to be amplified by the same PCR primers as those for the amplification of IFNAR2 cDNA. Competitive PCR fragments were quantified by the absorbance value at 260 nm of UV, and serial twofold dilutions were prepared. PCR primers were designed on the basis of the sequences published by Novik *et al.* [13] (Genebank X77722). RNA was extracted from the homogenized PBMCs and liver tissue using 900 μL of RNA zol^{TM}B (Biotex Laboratories Inc., Houston, TX, USA). One microgram of total RNA was reverse transcribed, and the resulting cDNA and a known volume (368, 184, 92, 46, or 23 copies) of competitive PCR fragments were co-amplified. The PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and bands of expected size were measured visually under UV. The PCR products of competitive fragments for IFNAR2 cDNA were observed at lengths of 622 bp. The reverse-transcribed PCR products of IFNAR2 mRNA were observed at lengths of 759 bp. The quantity of IFNAR2 mRNA was determined by comparing the density of PCR product from PBMCs or liver with that from competitive fragments, and expressed as copy numbers per 10 ng β-actin mRNA amplified from the same specimen.

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Table 1 Parameters of analysed patients with comparison between sustained virologic responders (SVR) and nonsustained virologic responders (non-SVR)

	Subjects	SVR	Non-SVR	P-value
Number of patients	36	17	19	
Age	49 ± 10	47 ± 9	50 ± 10	0.31
Gender (M/F)	23/13	12/5	11/8	0.43
HCV RNA level (log [copies])	5.5 ± 0.7	5.2 ± 0.7	5.7 ± 0.5	0.017
HCV serotype (1/2/1+2)	20/14/2	8/9/0	12/5/2	0.15
Staging of liver fibrosis (F0/F1/F2/F3/F4)	4/19/7/6/0	2/11/3/1/0	2/8/4/5/0	0.36
Total IFN dose (mega units)	679 ± 180	678 ± 186	679 ± 179	0.99
Expression level of IFNAR2 mRNA*				
Liver	1074 ± 913†	1494 ± 925	698 ± 737	0.007
PBMCs	592 ± 724	715 ± 928	481 ± 478	0.34

HCV, hepatitis C virus; IFN, interferon.
*Expressed as copy numbers per 10 ng of β-actin mRNA.
†$P = 0.016$ *vs* expression level of IFNAR2 mRNA in PBMCs.
Results were expressed as mean ± SD.

using a human β-actin competitive PCR set (Takara Biochemicals, Tokyo, Japan).

Statistical analysis

Results were expressed as mean ± SD. Differences in proportion were tested by the Fisher's exact test. Mean quantitative values were compared by the Student's t-test. Nonparametric data were compared using the Wilcoxon signed rank test. Multiple logistic regression analysis was performed using a multiple regression model to identify factors associated with a sustained virologic response to IFN therapy. Possible associated factors for the sustained virologic response to IFN therapy included eight variables: age, gender, total dose of interferon, HCV subtype, HCV-RNA level, staging for liver fibrosis, expression level of IFNAR2 in liver, and expression level of IFNAR2 in PBMCs. All reported P-values were two-tailed and a P-value of 0.05 was considered to be significant.

RESULTS

Completion of the assigned course of IFN

Three patients refused the assigned course of IFN therapy. Among the remaining 49 patients treated with IFN, seven were obliged to suspend or cease IFN treatment before completion of therapy because of adverse effects. One patient who completed the scheduled course of IFN therapy could not be followed up for more than 6 months. Liver or PBMCs specimens were not available from eight patients. The mean total dose of administered IFN was 609 ± 247 MU for all patients. Consequently, taking the standard total dose of IFN into consideration, 36 patients who received more than 300 MU of IFN were included in the analysis of factors predicting response to IFN.

Clinical and viral characteristics of patient population

Twenty-three men and 13 women (aged 25–64 years, with a mean age of 49 years) were examined in this study. According to the criteria for staging fibrosis (F0, F1, F2, F3, and F4) [19], four showed an F0 staging, 19 showed F1, seven showed F2, and six showed F3. HCV serotype 1 was detected in 20 patients (55.6%), serotype 2 in 14 (38.9%), and both were detected in two (5.5%). The mean logarithm of pretreatment HCV RNA levels was 5.5 ± 0.7 for analysed patients (Table 1).

Expression level of IFNAR2 in liver and PBMCs

Pretreatment IFNAR2 mRNA levels in liver and PBMCs were greater than the detection limit in 33 (92%) and 30 (83%) of the 36 patients examined, respectively. For statistical purposes, IFNAR2 mRNA levels below the detection limit of our assay were set at 23 copies/10 ng β-actin, as the maximum dilution in which competitive PCR fragments were constantly detected included more than 23 copies of cDNA. The mean levels of IFNAR2 mRNA expression were significantly higher in liver (1074 ± 913 copies/10 ng β-actin) than in PBMCs (592 ± 724 copies/10 ng β-actin) ($P = 0.016$) (Table 1) and were weakly correlated between liver and PBMCs ($r = 0.357$, $P = 0.032$) (Fig. 1). Expression levels of IFNAR2 in liver and PBMCs were not correlated with HCV-RNA levels in serum, and did not differ between HCV serotypes or between stagings based on degree of liver fibrosis.

Response to IFN and predictive factors of response

Seventeen patients (47.2%) treated with IFN therapy were sustained virologic responders. Sustained and nonsustained virologic responders did not differ significantly with respect