21. Maeng CY, Ryu CJ, Gripon P, Guguen-Guillouzo C, Hong HJ. Fine mapping of virus-neutralizing epitopes on hepatitis B virus PreS1. Virology 2000;270:9–16.
22. Penna A, Bertoletti A, Cavalli A, Valli A, Missale G, Pilli M, et al. Fine specificity of the human T cell response to hepatitis B virus core antigen. Arch Virol Suppl 1992;4:23–8.
23. Park JH, Cho EW, Lee YJ, Shin SY, Kim KL. Determination of the protective effects of neutralizing anti-hepatitis B virus (HBV) immunoglobulins by epitope mapping with recombinant HBV surface-antigen proteins. Microbiol Immunol 2000;44:703–10.
24. Jung MC, Diepolder HM, Spengler U, Wierenga EA, Zachoval R, Hoffmann RM, et al. Activation of a heterogeneous hepatitis B (HB) core and e antigen-specific CD4+ T-cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. J Virol 1995;69:3358–68.
25. Tsai SL, Chen MH, Yeh CT, Chu CM, Lin AN, Chiou FH, et al. Purification and characterization of a naturally processed hepatitis B virus peptide recognized by CD8+ cytotoxic T lymphocytes. J Clin Invest 1996;97:577–84.
26. Wakita T, Kakumu S, Shibata M, Yoshioka K, Ito Y, Shinagawa T, et al. Detection of pre-C and core region mutants of hepatitis B virus in chronic hepatitis B virus carriers. J Clin Invest 1991; 88:1793–801.
27. Akarca US, Lok AS. Naturally occurring core-gene-defective hepatitis B viruses. J Gen Virol 1995;76 (Pt 7):1821–6.
28. Yuan TT, Lin MH, Qiu SM, Shih C. Functional characterization of naturally occurring variants of human hepatitis B virus containing the core internal deletion mutation. J Virol 1998;72:2168–76.
29. Tai PC, Suk FM, Gerlich WH, Neurath AR, Shih C. Hypermodification and immune escape of an internally deleted middle-envelope (M) protein of frequent and predominant hepatitis B virus variants. Virology 2002;292:44–58.
30. Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S, et al. Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. J Virol 1994;68: 2671–6.
31. Hsieh YH, Su IJ, Wang HC, Chang WW, Lei HY, Lai MD, et al. Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage. Carcinogenesis 2004;25:2023–32.

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2007, p. 3191–3197 Vol. 45, No. 10
0095-1137/07/$08.00+0 doi:10.1128/JCM.00411-07

Influence of Hepatitis B Virus X and Core Promoter Mutations on Hepatocellular Carcinoma among Patients Infected with Subgenotype C2▽

Noboru Shinkai,[1,2] Yasuhito Tanaka,[1] Kiyoaki Ito,[2] Motokazu Mukaide,[1,3] Izumi Hasegawa,[4] Yasuhiro Asahina,[5] Namiki Izumi,[5] Hiroshi Yatsuhashi,[6] Etsuro Orito,[2] Takashi Joh,[2] and Masashi Mizokami[1]*

Departments of Clinical Molecular Informative Medicine[1] and Internal Medicine and Bioregulation,[2] Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, SRL Inc., 51 Komiya, Hachiouji, Tokyo 192-8535,[3] Department of Gastroenterology, Chukyo Hospital, Nagoya 457-8510,[4] Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Tokyo 180-8610,[5] and Clinical Research Center, National Hospital Organization Nagasaki Medical Center, Oomura, Nagasaki 856-8562,[6] Japan

Received 22 February 2007/Returned for modification 30 April 2007/Accepted 23 June 2007

Hepatitis B virus (HBV) genotypes/subgenotypes and their related mutations in the HBV genome have been reported to be associated with hepatocellular carcinoma (HCC). To determine the HCC-associated mutations of the HBV genome in the entire X, core promoter, and precore/core regions, a cross-sectional control study was conducted comparing 80 Japanese patients infected with HBV C2 and suffering from HCC with 80 age-, sex-, and hepatitis B e antigen (HBeAg) status-matched patients without HCC (non-HCC group). Each HBeAg-positive group (31 with HCC; 29 without HCC) and HBeAg-negative group (49 with HCC; 51 without HCC) was also matched with respect to age and sex. The C1479, T1485, H1499, A1613, T1653, V1753, T1762/A1764, and A1896 mutations were frequent in this population. The prevalences of the T1653 mutation in the box α region and the V1753 and T1762/A1764 mutations in the basal core promoter region were significantly higher in the HCC group than in the non-HCC group (56% versus 30%, 50% versus 24%, and 91% versus 73% [$P = 0.0013$, $P = 0.0010$, and $P = 0.0035$, respectively]). The platelet count was significantly lower for the HCC group than for the non-HCC group ($10.7 \times 10^4 \pm 5.1 \times 10^4$ versus $17.3 \times 10^4 \pm 5.1 \times 10^4$ platelets/mm^3 [$P < 0.0001$]). Regardless of HBeAg status, the prevalence of the T1653 mutation was higher in the HCC group (52% versus 24% [$P = 0.036$] for HBeAg-positive patients and 59% versus 33% [$P = 0.029$] for HBeAg-negative patients). In the multivariate analysis, the presence of T1653, the presence of V1753, and a platelet count of $\leq 10 \times 10^4$/mm^3 were independent predictive factors for HCC (odds ratios [95% confidence intervals], 4.37 [1.53 to 12.48], 7.98 [2.54 to 25.10], and 24.39 [8.11 to 73.33], respectively). Regardless of HBeAg status, the T1653 mutation increases the risk of HCC in Japanese patients with HBV/C2.

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated prevalence of >500,000 cases worldwide per year (36). It is accepted that hepatitis B virus (HBV) has carcinogenic potential in humans. HBV has been classified into eight major genotypes (A to H) by using the complete nucleotide sequence of the viral genome (34). HBV genotypes have distinct geographical distributions and correlate with the severity of liver disease (17, 18). Genotypes B and C are prevalent in Asia, and genotype C causes more-serious liver disease than genotype B (5, 35). There are two subtypes (subgenotypes) of genotype B with distinct geographical distributions, provisionally designated Ba ("a" stands for Asia) and Bj ("j" stands for Japan) (43), and clinical differences between patients infected with HBV/Ba and HBV/Bj are becoming clear (1, 42). Recently, a phylogenetic analysis revealed two major groups within genotype C: one for strains from Southeast Asia, including Vietnam, Myanmar, Thailand, and Hong Kong (named HBV/C1), and another for strains from (Far) East Asia, including Japan and China (named HBV/C2) (6, 12, 47). Chan et al. (6) designated the two subgenotypes HBV/Cs, for Southeast Asia, and HBV/Ce, for (Far) East Asia; they not only have different geographic distributions but also different nucleotide sequences in the precore region (6, 47).

Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there has been increasing evidence of an association between molecular alteration and the development of HCC in patients with HBV infection. Mutations in the basal core promoter (BCP) region at nucleotides (nt) 1762 and 1764 (T1762/A1764) and in the precore region at nt 1896 (A1896) are associated with HBV e antigen (HBeAg) seroconversion and persistent viral replication. It is noteworthy that both BCP and precore mutations are often found in patients with advanced liver disease (e.g., HCC) (2, 3, 16, 19, 23, 25, 38). The T1762/A1764 mutations alter HBeAg production at the transcriptional level, and the A1896 mutation in the precore region terminates the translation of precursor protein, abrogates HBeAg production, and results in seroconversion. The T1653 mu-

* Corresponding author. Mailing address: Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan. Phone: 81-52-853-8292. Fax: 81-52-842-0021. E-mail: mizokami@med.nagoya-cu.ac.jp.

▽ Published ahead of print on 25 July 2007.

TABLE 1. Positions and sequences of primers used for PCR amplification and sequencing

Name	Nucleotide sequence (5′ to 3′)	Positions (nt)	Polarity
HBx1360F	TACACCTCCTTYCCATGGCTGCT	1360–1383	Sense
HB7R-2	CCTGAGTGCTGTATGGTGAGG	2062–2072	Antisense
HB6R	AACAGACCAATTTATGCCTA	1803–1784	Antisense
HB7F-2	CATGGAGACCACCGTGAACGC	1607–1627	Sense

tation in the box α region has been reported to increase the risk of HCC in HBeAg-negative patients infected with HBV/C (14, 44). As well, specific mutations in the enhancer II/core promoter of HBV were differently associated with HCC in the context of HBeAg status among HBV/C1/Cs and HBV/C2/Ce carriers (46). There have been many studies involving viral mutations associated with clinical features, but most previous studies have either ignored age, sex, HBeAg status, and HBV genotype/subgenotype or have examined relatively short regions.

Here we performed a cross-sectional control study of 160 age-, sex-, and HBeAg status-matched Japanese patients infected with HBV/C2 to determine the HCC-associated mutations of the HBV genome in the entire X, core promoter, and precore plus core regions.

MATERIALS AND METHODS

Serum samples. A total of 160 serum samples were obtained from chronic HBV C2/Ce carriers who visited the Nagoya City University Hospital, Musashino Red Cross Hospital, or National Hospital Organization Nagasaki Medical Center in Japan. Of these, 140 samples were newly obtained and 20 samples were previously used (14). The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the ethics committees of the institutions listed above, and informed consent was obtained from each carrier. None of the patients had a history of hepatitis C virus coinfection. In Japan, because a main transmission route of HBV is vertical, the duration of HBsAg carriage of all or most patients would correspond with their age. Hassan et al. reported that heavy alcohol consumption (>80 g ethanol per day) contributes to the majority of HCC cases (10). Heavy drinking is less prevalent in Japan; 3 of 80 patients with HCC are heavy alcohol drinkers, and 2 of 80 patients without HCC are heavy alcohol drinkers, indicating that no significant difference in heavy alcohol consumption was found in this population.

Serological assays for HBV markers. HBeAg and anti-HBe were detected by a chemiluminescent enzyme immunoassay (Lumipulse f; FUJIREBIO Inc., Tokyo, Japan).

Amplification and sequencing of the entire X, core promoter, and precore plus core regions. Nucleic acids were extracted from 100 μl of serum using a QIAamp DNA blood minikit (QIAGEN Inc., Hilden, Germany). HBV genes (665 bp) of the entire X, core promoter, precore, and core regions were amplified by PCR with heminested primers. The first-round PCR was performed with sense primer HBX1360F (TACACCTCCTTYCCATGGCTGCT) and antisense primer HB7R-2. The second-round PCR was performed with two sets of sense primers and antisense primers. One set comprised HBX1360 (sense) and Ia1 (antisense), and the second set comprised HB7F-2 (sense) and HB7R-2 (antisense) (Table 1). Thereafter, the PCR products were sequenced directly with a Prism BigDye Terminator cycle sequencing kit (Applied Biosystems) using an ABI 3100 DNA automated sequencer (Applied Biosystems). The sequences covered the entire X region, the enhancer II/core promoter (Fig. 1), and the precore genes, which are associated with HBeAg production, viral replication, and disease progression.

Case-control study. According to the *Gui* guidelines for diagnosis of HCC (39), 80 patients received a diagnosis of HCC on the basis of results of abdominal ultrasonography, angiography, computerized tomography, or magnetic resonance imaging, as well as elevated serum α-fetoprotein levels (≥400 ng/ml). We did pathological examinations of 25 of 80 patients with HCC. For a case-control study on age-, sex-, and HBeAg status-matched subjects, another 80 patients without HCC were compared. Among the non-HCC group, 24 patients were asymptomatic carriers and 56 had chronic hepatitis.

Statistical evaluation. Data were expressed as means ± standard deviations (SD). Statistical analyses were performed using a χ^2 test and Fisher's exact test for categorical variables and Mann-Whitney's U test or one-way analysis of variance for continuous variables, as appropriate. In multivariate analysis, we used 40 U of alanine aminotransferase (ALT)/liter as the cutoff. An HBV DNA level of 5 log genome equivalents (LGE)/ml and a platelet count of $10 \times 10^4/mm^3$ were assessed as cutoff values, because these were used as predictors for disease progression (13, 21, 26–28, 30). Multivariate analysis with logistic regression was used to determine the independent factors associated with HCC. Differences were considered significant for P values less than 0.05. The statistical analysis software used was Stata software, version 8.0 (Stata Corp.).

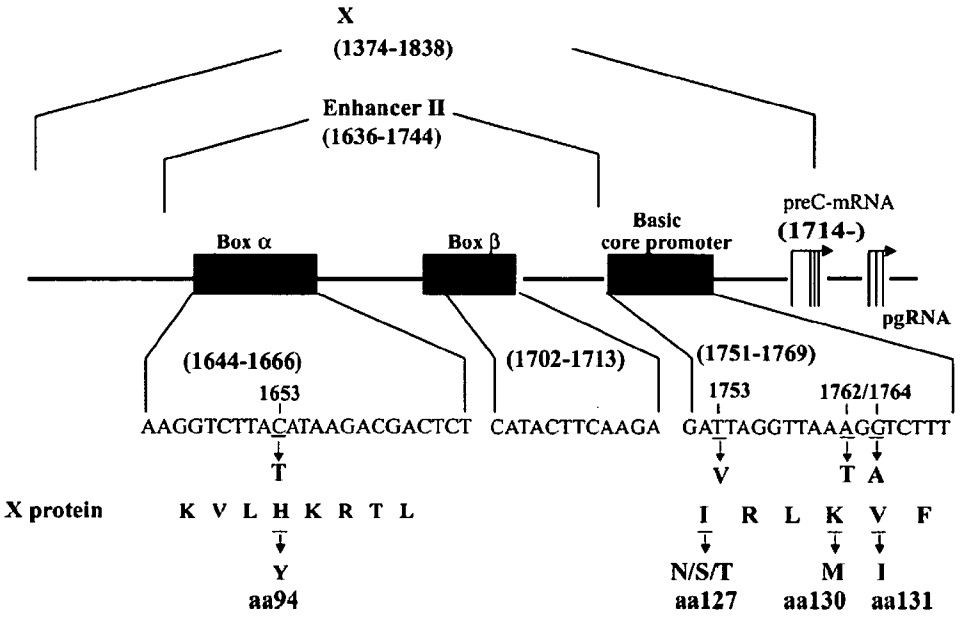

FIG. 1. C1653T, T1753V (where V stands for "not T"), and A1762T/G1764A mutations in the X region, including the enhancer II/core promoter region.

TABLE 2. Clinical and virologic characteristics of patients infected with HBV subgenotype C2/Ce who were matched for age, sex, and HBeAg status

Variable	Value for group		P
	Non-HCC (n = 80)	HCC (n = 80)	
Mean age (yr) ± SD	54 ± 8	55 ± 8	Matched
No. (%) of male patients	67 (84)	68 (85)	Matched
No. (%) of HBeAg-positive patients	29 (36)	31 (39)	Matched
ALT level (mean U/liter ± SD)	66 ± 99	85 ± 133	0.31
HBV DNA level (mean LGE/ml ± SD)	5.7 ± 1.7	5.7 ± 1.4	0.84
Mean platelet count (10^4/mm^3) ± SD	17.3 ± 5.1	10.7 ± 5.1	<0.0001
No. (%) of patients with a mutation in the X region			
T1479C (T to P at aa 36)	39 (49)	45 (56)	0.43
C1485T (P to S at aa 38)	18 (22)	22 (28)	0.58
G1499H	57 (71)	46 (58)	0.10
G1613A	25 (31)	32 (40)	0.32
C1653T (in box α)	24 (30)	45 (56)	0.0013
T1753V (in the core promoter)	19 (24)	40 (50)	0.0010
A1762T/G1764A (in the core promoter)	58 (73)	73 (91)	0.0035
No. (%) of patients with the G1896A mutation in the precore region	41 (51)	43 (54)	0.8743

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank/DDBJ/EMBL databases (accession numbers AB307808 to AB307967).

RESULTS

When we examined HBV DNA sequences in the entire X region as well as the enhancer II/core promoter and precore regions, C1479, T1485, H1499, A1613, T1653, V1753, T1762/A1764, and A1896 mutations were frequent in our population. Table 2 compares ALT levels, HBV DNA levels, and platelet counts, as well as mutations in the X region, box α (enhancer II), the core promoter (Fig. 1), and the precore region, among 80 patients with HCC (HCC group) and 80 patients without HCC (non-HCC group), matched for age, sex, and HBeAg status, in a case-control study. The platelet count was significantly lower for the HCC group than for the non-HCC group ($10.7 \times 10^4 \pm 5.1 \times 10^4$ versus $17.3 \times 10^4 \pm 5.1 \times 10^4$ platelets/mm^3; $P < 0.0001$). The frequencies of the T1653 mutation in the box α region and the V1753 and T1762/A1764 mutations in the BCP region were significantly higher for the HCC group than for the non-HCC group (56% versus 30%, 50% versus 24%, and 91% versus 73%; $P = 0.0013$, $P = 0.0010$, and $P = 0.0035$, respectively). No other significant mutations were observed in this study. Table 3 compares ALT levels and HBV DNA levels, as well as mutations in the X region, box α (enhancer II), the core promoter, and the precore region, among 60 HBeAg-positive patients (29 non-HCC and 31 HCC) matched for age and sex in a case-control study. Among HBeAg-positive patients, the platelet count was significantly lower for the HCC group than for the non-HCC group (10.6 ×

TABLE 3. Clinical and virologic characteristics of patients infected with HBV subgenotype C2/Ce who were matched for age and were positive for HBeAg

Variable	Value for HBeAg-positive group		P
	Non-HCC (n = 29)	HCC (n = 31)	
Mean age (yr) ± SD	53 ± 8	53 ± 7	Matched
No. (%) of male patients	21 (72)	23 (74)	Matched
No. (%) of HBeAg-positive patients	29 (100)	31 (100)	Matched
ALT level (mean U/liter ± SD)	99 ± 128	98 ± 190	0.98
HBV DNA level (mean LGE/ml ± SD)	6.8 ± 1.4	6.4 ± 1.0	0.20
Mean platelet count (10^4/mm^3) ± SD	13.5 ± 5.3	10.6 ± 6.0	0.0062
No. (%) of patients with a mutation in the X region			
T1479C (T to P at aa 36)	13 (45)	14 (45)	>0.9999
C1485T (P to S at aa 38)	6 (21)	5 (16)	0.74
G1499H	25 (86)	20 (65)	0.075
G1613A	8 (28)	11 (35)	0.59
C1653T (in box α)	7 (24)	16 (52)	0.036
T1753V (in the core promoter)	5 (17)	10 (32)	0.24
A1762T/G1764A (in the core promoter)	23 (79)	29 (94)	0.14
No. (%) of patients with the G1896A mutation in the precore region	6 (21)	9 (51)	0.56

TABLE 4. Clinical and virologic characteristics of patients infected with HBV subgenotype C2/Ce who were matched for age and were HBeAg negative

Variable	Value for HBeAg-negative group		P
	Non-HCC (n = 51)	HCC (n = 49)	
Mean age (yr) ± SD	54 ± 9	55 ± 4	Matched
No. (%) of male patients	44 (86)	47 (88)	Matched
No. (%) of HBeAg-positive patients	0 (0)	0 (0)	Matched
ALT level (mean U/liter ± SD)	47 ± 73	76 ± 79	0.048
HBV DNA level (mean LGE/ml ± SD)	5.1 ± 1.5	5.2 ± 1.4	0.67
Mean platelet count (10^4/mm^3) ± SD	19 ± 3.5	11 ± 4.5	<0.0001
No. (%) of patients with a mutation in the X region			
T1479C (T to P at aa 36)	26 (51)	31 (63)	0.23
C1485T (P to S at aa 38)	12 (24)	17 (35)	0.27
G1499H	32 (63)	26 (53)	0.42
G1613A	17 (33)	21 (43)	0.41
C1653T (in box α)	17 (33)	29 (59)	0.016
T1753V (in the core promoter)	14 (27)	30 (61)	0.0012
A1762T/G1764A (in the core promoter)	35 (69)	44 (90)	<0.0001
No. (%) of patients with the G1896A mutation in the precore region	35 (69)	34 (69)	>0.9999

10^4 ± 6.0 × 10^4 versus 13.5 × 10^4 ± 5.3 × 10^4 platelets/mm^3; P = 0.0062). The frequency of the T1653 mutation in the box α region was significantly higher for the HCC group than for the non-HCC group (52% versus 24%; P = 0.036) (Table 3). Among 100 HBeAg-negative patients (51 non-HCC and 49 HCC) matched for age and sex in a case-control study, the platelet count was significantly lower for the HCC group than for the non-HCC group (11 × 10^4 ± 4.5 × 10^4 versus 19 × 10^4 ± 3.5 × 10^4 platelets/mm^3; P < 0.0001), and the frequencies of the T1653 mutation in the box α region and the V1753 and T1762/A1764 mutations in the BCP region were significantly higher for the HCC group than for the non-HCC group (59% versus 33%, 61% versus 27%, and 90% versus 69%; P = 0.016, P = 0.0012, and P < 0.0001, respectively) (Table 4).

In the multivariate analysis among all 160 patients, the presence of T1653, the presence of V1753, and a platelet count of ≤10 × 10^4/mm^3 were independent predictive factors for HCC (odds ratios [95% confidence intervals], 4.37 [1.53 to 12.48], 7.98 [2.54 to 25.10], and 4.39 [8.11 to 73.33], respectively) (Table 5). The presence of H1499 was identified as a significant negative factor for HCC (odds ratio, 0.243 [95% confidence interval, 0.078 to 0.76]).

DISCUSSION

Many studies have reported that the clinical course of chronic HBV infection may be modified by several specific HBV mutations (7, 11, 25, 49), although the significance of such specific mutations in patients with chronic hepatitis B remains controversial. Since the viral mutations might be influenced by age, sex, HBeAg status, and HBV genotype/subgenotype, in this study we investigated sequences bearing the entire X region as well as enhancer II, BCP, and precore/core regions in order to find the specific mutations associated with HCC by comparing matched control groups (HCC versus non-HCC).

The most significant result in this study was that the presence of T1653 and the presence of V1753 were independent predictive factors for HCC in the multivariate analyses. The T1653 mutation was first found in fulminant hepatitis patients (15, 33, 48) and in cases of chronic hepatitis with acute exacerbation (32). Some studies reported that the T1653 or V1753 mutation was associated with HCC (14, 44–46). Poussin et al. also reported that the T1653 mutation was found in HCC (tumor) lesions from two patients, but interestingly, wild-type 1653 was found in nontumor lesions from the same patients (37). T1653 is located in box α, which is a strong activation element of both enhancer II and the core promoter (50), and the α box elements (nt 1646 to 1668) individually stimulate promoter activity more than 100-fold (50). The T1653 mutation converts the box α binding site for C/EBP and related factors (29, 50) into the perfect palindromic sequence 1648-T CTTATATAAGA, which might enhance binding affinity and enhancer II/core promoter activity. Hence, the T1653 mutation could influence HBeAg production and viral replication through BCP activity. Although a number of studies have reported the role of the BCP mutations in viral features, the exact mechanism of HCC development still remains unclear, particularly with respect to the effect of the mutation in the X protein. In this study, the T1762/A1764 mutations were frequent in both the non-HCC and HCC groups. The T1762/A1764 mutations had been found to be highly frequent in older HBV/C carriers (≥50 years) regardless of clinical status (14); however, these results do not contradict the possibility that T1762/A1764 is associated with hepatocarcinogenesis, because the poor prognosis of HBV/C compared to HBV/B (Ba and Bj) correlates with the high prevalence of T1762/A1764 (16). Study of a prospective cohort of 1,638 high-risk individuals in Qidong (China) showed that T1762/A1764 mutation was detected in 8 of the 15 HCC cases (53.3%) before HCC development (20), suggesting that the T1762/A1764 double mutation indicates a high potential risk for hepatocarcinogenesis. The T1653 or V1753 mutation, in addition to the T1762/A1764 mutations, may be one of the promoters of HCC development.

Several functional analyses had been already reported;

TABLE 5. Multivariate analysis of variables with independent predictive value for HCC among a group of 160 patients with HBV infection

Factor	OR (95% CI)[a]	P[b]
Age		
<55	1	NS
≥55	1.37 (0.53–3.53)	
Sex		
Female	1	NS
Male	0.55 (0.15–2.02)	
HBeAg		
Negative	1	NS
Positive	1.22 (0.32–4.73)	
ALT (U/liter)		
<40	1	NS
≥40	1.98 (0.74–5.33)	
HBV DNA level (LGE/ml)		
<5	1	NS
≥5	0.35 (0.10–1.24)	
Platelet count/mm^3		
$>10 \times 10^4$	1	<0.0001
$\leq 10 \times 10^4$	24.39 (8.11–73.33)	
Mutation in the X region		
T1479C mutation		
Absence	1	NS
Presence	2.24 (0.83–6.07)	
C1485T mutation		
Absence	1	NS
Presence	0.89 (0.29–2.72)	
G1499H mutation		
Absence	1	0.015
Presence	0.243 (0.078–0.76)	
G1613A mutation		
Absence	1	NS
Presence	0.48 (0.17–1.40)	
C1653T mutation		
Absence	1	0.0059
Presence	4.37 (1.53–12.48)	
T1753V mutation		
Absence	1	0.0004
Presence	7.98 (2.54–25.10)	
A1762T/G1764A mutation		
Absence	1	NS
Presence	2.93 (0.73–11.87)	
G1896A mutation in the precore region		
Absence	1	NS
Presence	0.68 (0.22–2.07)	

[a] OR, odds ratio; CI, confidence interval.
[b] NS, not significant.

Günther et al. (9) analyzed T1653, C1753, T1762, and A1764 mutations (genotype D; accession number AF043594) in the context of an in vitro study involving wild-type HBV, and they reported that precore mRNA and HBeAg secretion were reduced, but the amount of progeny virus DNA in the cells and in the culture medium increased only marginally (if at all), as determined by Southern blot analysis. Lin et al. (24) analyzed HBV replicative efficiency in vitro. HBV isolated from HCC lesions included T1653, V1753, T1762, A1764, and A1896 mutations (genotype C2; accession number AF182804). An HBV isolate from the same patient's serum 4 years before HCC diagnosis included T1762, A1764, and A1896 mutations (genotype C2; AF182802). The clone from HCC showed higher replicative efficiency than the clone before HCC development (AF182802) by Southern blot analysis. However, considering that the mutant type includes not only T1653, C1753, T1762, and A1764 mutations but also other mutations, it is possible that some other mutation influenced the results in the earlier study. Further functional analyses of HBV/C strains with the T1653 mutation are needed in vitro and in vivo.

HBx, the nonstructural regulatory protein of HBV, has been strongly associated with the development of liver cancer in some HBx-transgenic mouse strains or with increased progression to liver cancer in other toxin-exposed HBx-transgenic mouse strains (reviewed in reference 4). Many functions have been ascribed to HBx, but the precise molecular mechanism(s) responsible for its activities, and how these activities affect viral replication and possibly liver cell transformation, remains poorly defined. The T1653 mutation resulted in a histidine-to-tyrosine amino acid substitution at codon 94 of the X protein, which is the center of the immunodominant antigenic domain of amino acids (aa) 85 to 110 mapped by Stemler et al. (41). Sirma et al. (40) reported that wild-type HBx inhibited the clonal outgrowth of cells and induced apoptosis but that the mutants (same sample as in reference 37), which include the T1653 mutation derived from HCC, did not. Indeed, codon 94 (nt 1653 to 1655) is within the function domain of the X protein, which has been reported to play a central role in transactivation (22). Cong et al. (8) also reported that one of the activation domain of X (aa 90 to 122) is required for binding to XAP3 (protein kinase C-binding protein). Protein kinase C is a large family of phospholipid-dependent kinases involved in cell growth, differentiation, and carcinogenesis. It is of interest whether the T1653 mutation affects the protein-protein interactions between the X protein and XAP3. The V1753 mutation and the T1762/A1764 double mutation resulted in an isoleucine-to-asparagine/serine/threonine substitution at codon 127, a lysine-to-methionine substitution at codon 130, and a valine-to-isoleucine substitution at codon 131, respectively. These amino acid changes in the X region may also affect the function of the X protein. The occurrence of multiple mutations may represent a strategy of HBV to escape immune surveillance and thus contribute to the process of multiple steps in hepatocarcinogenesis.

With regard to other X amino acid substitutions that might be associated with HCC, Yeh et al. reported that a serine-to-alanine amino acid substitution at codon 31 (a T-to-G mutation at nt 1464) was more frequent in an HCC group than in a chronic hepatitis group (49), but the substitution at codon 31 was found for only two HCC patients (and for no patients in the non-HCC group) in the present study. The discrepancy might depend on different HBV genotypes/subgenotypes between Japan and Taiwan. Very recently, Muroyama et al. reported that a serine-to-alanine amino acid substitution at codon 38 (a C-to-T mutation at nt 1485) was more frequent in an HCC group than in a chronic hepatitis group (31). Indeed, the T1485 mutation was frequent in this study, but no significant difference between the HCC and non-HCC groups was observed. On the other hand, the H1499 mutation was a sig-

nificant negative factor in the multivariate analysis, even though it was not significant in the univariate analysis. The H1499 mutation does not result in an amino acid substitution in the X protein, and a previous report by Takahashi et al. showed that the frequency of the H1499 mutation was 73% among 40 HCC patients (44). The frequency was similar to that of the non-HCC group (71%) in this study; thus, the clinical significance of the H1499 mutation remains unclear.

Finally, among HBeAg-positive patients, the T1653 and T1762/A1764 mutations or the V1753 and T1762/A1764 mutations were frequent in the HCC group (sensitivity, 69%; specificity, 72%). Also among HBe-negative patients, the T1653 and T1762/A1764 mutations or the V1753 and T1762/A1764 mutations were frequent in the HCC group (sensitivity, 67%; specificity, 81%). Hence, we would predict the groups at high risk for HCC by the combination of enhancer II/core promoter mutations with X amino acid substitutions as well as by HBV genotypes/subgenotypes. Further prospective studies in countries where HBV genotype C is endemic are required to confirm whether the accumulation of these mutations causes liver disease progression.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for Scientific Research C from the Ministry of Education, Culture, Sports, Science, and Technology (18590741), a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan (H16-kanen-3), the Toyoaki Foundation, and the Foundation for Promotion of Cancer Research in Japan.

We declare no conflict of interest.

REFERENCES

1. **Akuta, N., F. Suzuki, M. Kobayashi, A. Tsubota, Y. Suzuki, T. Hosaka, T. Someya, M. Kobayashi, S. Saitoh, Y. Arase, K. Ikeda, and H. Kumada.** 2003. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J. Hepatol. **38**:315–321.
2. **Baptista, M., A. Kramvis, and M. C. Kew.** 1999. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology **29**:946–953.
3. **Blackberg, J., and K. Kidd-Ljunggren.** 2003. Mutations within the hepatitis B virus genome among chronic hepatitis B patients with hepatocellular carcinoma. J. Med. Virol. **71**:18–23.
4. **Bouchard, M. J., and R. J. Schneider.** 2004. The enigmatic X gene of hepatitis B virus. J. Virol. **78**:12725–12734.
5. **Chan, H. L., A. Y. Hui, M. L. Wong, A. M. Tse, L. C. Hung, V. W. Wong, and J. J. Sung.** 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut **53**:1494–1498.
6. **Chan, H. L., S. K. Tsui, C. H. Tse, E. Y. Ng, T. C. Au, L. Yuen, A. Bartholomeusz, K. S. Leung, K. H. Lee, S. Locarnini, and J. J. Sung.** 2005. Epidemiological and virological characteristics of 2 subgroups of hepatitis B virus genotype C. J. Infect. Dis. **191**:2022–2032.
7. **Chu, C. M., C. T. Yeh, C. S. Lee, I. S. Sheen, and Y. F. Liaw.** 2002. Precore stop mutant in HBeAg-positive patients with chronic hepatitis B: clinical characteristics and correlation with the course of HBeAg-to-anti-HBe seroconversion. J. Clin. Microbiol. **40**:16–21.
8. **Cong, Y. S., Y. L. Yao, W. M. Yang, N. Kuzhandaivelu, and E. Seto.** 1997. The hepatitis B virus X-associated protein, XAP3, is a protein kinase C-binding protein. J. Biol. Chem. **272**:16482–16489.
9. **Günther, S., N. Piwon, and H. Will.** 1998. Wild-type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653)→T, A(1762)→T and G(1764)→A mutations in the core promoter. J. Gen. Virol. **79**:375–380.
10. **Hassan, M. M., L. Y. Hwang, C. J. Hatten, M. Swaim, D. Li, J. L. Abbruzzese, P. Beasley, and Y. Z. Patt.** 2002. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. Hepatology **36**:1206–1213.
11. **Huy, T. T., H. Ushijima, V. X. Quang, T. T. Ngoc, S. Hayashi, T. Sata, and K. Abe.** 2004. Characteristics of core promoter and precore stop codon mutants of hepatitis B virus in Vietnam. J. Med. Virol. **74**:228–236.
12. **Huy, T. T., H. Ushijima, V. X. Quang, K. M. Win, P. Luengrojanakul, K. Kikuchi, T. Sata, and K. Abe.** 2004. Genotype C of hepatitis B virus can be classified into at least two subgroups. J. Gen. Virol. **85**:283–292.
13. **Ikeda, K., Y. Arase, S. Saitoh, M. Kobayashi, T. Someya, T. Hosaka, N. Akuta, Y. Suzuki, F. Suzuki, H. Sezaki, H. Kumada, A. Tanaka, and H. Harada.** 2006. Prediction model of hepatocarcinogenesis for patients with hepatitis C virus-related cirrhosis. Validation with internal and external cohorts. J. Hepatol. **44**:1089–1097.
14. **Ito, K., Y. Tanaka, E. Orito, M. Sugiyama, K. Fujiwara, F. Sugauchi, T. Kato, H. Tokita, N. Izumi, M. Kato, M. F. Yuen, C. L. Lai, R. G. Gish, R. Ueda, and M. Mizokami.** 2006. T1653 mutation in the box alpha increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. Clin. Infect. Dis. **42**:1–7.
15. **Kaneko, M., T. Uchida, M. Moriyama, Y. Arakawa, T. Shikata, K. Gotoh, and S. Mima.** 1995. Probable implication of mutations of the X open reading frame in the onset of fulminant hepatitis B. J. Med. Virol. **47**:204–208.
16. **Kao, J. H., P. J. Chen, M. Y. Lai, and D. S. Chen.** 2003. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology **124**:327–334.
17. **Kidd-Ljunggren, K., Y. Miyakawa, and A. H. Kidd.** 2002. Genetic variability in hepatitis B viruses. J. Gen. Virol. **83**:1267–1280.
18. **Kidd-Ljunggren, K., E. Myhre, and J. Blackberg.** 2004. Clinical and serological variation between patients infected with different hepatitis B virus genotypes. J. Clin. Microbiol. **42**:5837–5841.
19. **Kobayashi, M., Y. Arase, K. Ikeda, A. Tsubota, Y. Suzuki, S. Saitoh, M. Kobayashi, F. Suzuki, N. Akuta, T. Hosaka, T. Someya, M. Matsuda, J. Sato, K. Takagi, Y. Miyakawa, and H. Kumada.** 2003. Precore wild-type hepatitis B virus with G1896 in the resolution of persistent hepatitis B virus infection. Intervirology **46**:157–163.
20. **Kuang, S. Y., P. E. Jackson, J. B. Wang, P. X. Lu, A. Munoz, G. S. Qian, T. W. Kensler, and J. D. Groopman.** 2004. Specific mutations of hepatitis B virus in plasma predict liver cancer development. Proc. Natl. Acad. Sci. USA **101**:3575–3580.
21. **Kubo, S., H. Tanaka, T. Shuto, S. Takemura, T. Yamamoto, T. Uenishi, S. Tanaka, M. Ogawa, K. Sakabe, K. Yamazaki, and K. Hirohashi.** 2004. Correlation between low platelet count and multicentricity of hepatocellular carcinoma in patients with chronic hepatitis C. Hepatol. Res. **30**:221–225.
22. **Kumar, V., N. Jayasuryan, and R. Kumar.** 1996. A truncated mutant (residues 58–140) of the hepatitis B virus X protein retains transactivation function. Proc. Natl. Acad. Sci. USA **93**:5647–5652.
23. **Laskus, T., J. Rakela, M. J. Nowicki, and D. H. Persing.** 1995. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology **109**:1618–1623.
24. **Lin, X., G. S. Qian, P. X. Lu, L. Wu, and Y. M. Wen.** 2001. Full-length genomic analysis of hepatitis B virus isolates in a patient progressing from hepatitis to hepatocellular carcinoma. J. Med. Virol. **64**:299–304.
25. **Lindh, M., C. Gustavson, K. Mardberg, G. Norkrans, A. P. Dhillon, and P. Horal.** 1998. Mutation of nucleotide 1,762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. J. Med. Virol. **55**:185–190.
26. **Lok, A. S., E. J. Heathcote, and J. H. Hoofnagle.** 2001. Management of hepatitis B: 2000–summary of a workshop. Gastroenterology **120**:1828–1853.
27. **Lok, A. S., and B. J. McMahon.** 2001. Chronic hepatitis B. Hepatology **34**:1225–1241.
28. **Lok, A. S., and B. J. McMahon.** 2004. Chronic hepatitis B: update of recommendations. Hepatology **39**:857–861.
29. **López-Cabrera, M., J. Letovsky, K. Q. Hu, and A. Siddiqui.** 1990. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: *trans*-activation and repression by CCAAT/enhancer binding protein. Proc. Natl. Acad. Sci. USA **87**:5069–5073.
30. **Murata, K., K. Sugimoto, K. Shiraki, and T. Nakano.** 2005. Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection. World J. Gastroenterol. **11**:6848–6852.
31. **Muroyama, R., N. Kato, H. Yoshida, M. Otsuka, M. Moriyama, Y. Wang, R. X. Shao, N. Dharel, Y. Tanaka, M. Ohta, R. Tateishi, S. Shiina, M. Tatsukawa, K. Fukai, F. Imazeki, O. Yokosuka, Y. Shiratori, and M. Omata.** 2006. Nucleotide change of codon 38 in the X gene of hepatitis B virus genotype C is associated with an increased risk of hepatocellular carcinoma. J. Hepatol. **45**:805–812.
32. **Nishizono, A., K. Kohno, Y. Takita-Sonoda, M. Hiraga, H. Terao, T. Fujioka, M. Nasu, and K. Mifune.** 1997. Sequential analyses of the mutations in the core upstream and precore regions of hepatitis B virus genome in anti-HBe positive-carriers developing acute exacerbation. J. Med. Virol. **53**:266–272.
33. **Ogata, N., R. H. Miller, K. G. Ishak, and R. H. Purcell.** 1993. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology **194**:263–276.
34. **Okamoto, H., F. Tsuda, H. Sakugawa, R. I. Sastrosoewignjo, M. Imai, Y. Miyakawa, and M. Mayumi.** 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J. Gen. Virol. **69**:2575–2583.
35. **Orito, E., M. Mizokami, H. Sakugawa, K. Michitaka, K. Ishikawa, T. Ichida, T. Okanoue, H. Yotsuyanagi, S. Iino, et al.** 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Hepatology **33**:218–223.

36. **Parkin, D. M.** 2004. International variation. Oncogene **23**:6329–6340.
37. **Poussin, K., H. Dienes, H. Sirma, S. Urban, M. Beaugrand, D. Franco, P. Schirmacher, C. Brechot, and P. Paterlini Brechot.** 1999. Expression of mutated hepatitis B virus X genes in human hepatocellular carcinomas. Int. J. Cancer **80**:497–505.
38. **Raimondo, G., R. Schneider, M. Stemler, V. Smedile, G. Rodino, and H. Will.** 1990. A new hepatitis B virus variant in a chronic carrier with multiple episodes of viral reactivation and acute hepatitis. Virology **179**:64–68.
39. **Ryder, S. D.** 2003. Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults. Gut **52**(Suppl. 3):iii, 1–8.
40. **Sirma, H., C. Giannini, K. Poussin, P. Paterlini, D. Kremsdorf, and C. Brechot.** 1999. Hepatitis B virus X mutants, present in hepatocellular carcinoma tissue abrogate both the antiproliferative and transactivation effects of HBx. Oncogene **18**:4848–4859.
41. **Stemler, M., T. Weimer, Z. X. Tu, D. F. Wan, M. Levrero, C. Jung, G. R. Pape, and H. Will.** 1990. Mapping of B-cell epitopes of the human hepatitis B virus X protein. J. Virol. **64**:2802–2809.
42. **Sugauchi, F., E. Orito, T. Ichida, H. Kato, H. Sakugawa, S. Kakumu, T. Ishida, A. Chutaputti, C. L. Lai, R. G. Gish, R. Ueda, Y. Miyakawa, and M. Mizokami.** 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology **124**:925–932.
43. **Sugauchi, F., E. Orito, T. Ichida, H. Kato, H. Sakugawa, S. Kakumu, T. Ishida, A. Chutaputti, C. L. Lai, R. Ueda, Y. Miyakawa, and M. Mizokami.** 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J. Virol. **76**:5985–5992.
44. **Takahashi, K., Y. Akahane, K. Hino, Y. Ohta, and S. Mishiro.** 1998. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. Arch. Virol. **143**:2313–2326.
45. **Takahashi, K., Y. Ohta, K. Kanai, Y. Akahane, Y. Iwasa, K. Hino, N. Ohno, H. Yoshizawa, and S. Mishiro.** 1999. Clinical implications of mutations C-to-T1653 and T-to-C/A/G1753 of hepatitis B virus genotype C genome in chronic liver disease. Arch. Virol. **144**:1299–1308.
46. **Tanaka, Y., M. Mukaide, E. Orito, M. F. Yuen, K. Ito, F. Kurbanov, F. Sugauchi, Y. Asahina, N. Izumi, M. Kato, C. L. Lai, R. Ueda, and M. Mizokami.** 2006. Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma. J. Hepatol. **45**:646–653.
47. **Tanaka, Y., E. Orito, M. F. Yuen, M. Mukaide, F. Sugauchi, K. Ito, A. Ozasa, T. Sakamoto, F. Kurbanov, C. L. Lai, and M. Mizokami.** 2005. Two subtypes (subgenotypes) of hepatitis B virus genotype C: a novel subtyping assay based on restriction fragment length polymorphism. Hepatol. Res. **33**:216–224.
48. **Uchida, T., T. Saitoh, and H. Shinzawa.** 1997. Mutations of the X region of hepatitis B virus and their clinical implications. Pathol. Int. **47**:183–193.
49. **Yeh, C. T., C. H. Shen, D. I. Tai, C. M. Chu, and Y. F. Liaw.** 2000. Identification and characterization of a prevalent hepatitis B virus X protein mutant in Taiwanese patients with hepatocellular carcinoma. Oncogene **19**: 5213–5220.
50. **Yuh, C. H., Y. L. Chang, and L. P. Ting.** 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J. Virol. **66**:4073–4084.

Journal of General Virology (2007), **88**, 2513–2519 DOI 10.1099/vir.0.82974-0

Increasing genetic diversity of hepatitis C virus in haemophiliacs with human immunodeficiency virus coinfection

Yasuhito Tanaka,[1] Kousuke Hanada,[2] Hideji Hanabusa,[3] Fuat Kurbanov,[1] Takashi Gojobori[2] and Masashi Mizokami[1]

Correspondence
Yasuhito Tanaka
ytanaka@med.nagoya-cu.ac.jp

[1]Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan

[2]National Institute of Genetics, Yata 1111, Mishima, Shizuoka, Japan

[3]Ogikubo hospital, Tokyo, Japan

Patients with inherited bleeding disorders who received clotting factor concentrates before 1987 have high rates of hepatitis C virus (HCV) or HCV/human immunodeficiency virus (HIV) infection. To determine whether the persistent nature of HIV affects the genetic diversity of HCV by less selective pressure through the immunosuppression of HIV/HCV-coinfected patients, both the change of genetic diversity and selective pressure were examined in the HCV envelope genes (E1 and E2) of 325 genotype 1a subclones from eight HIV-positive and five HIV-negative patients with two time points (more than 6 years apart). To infer the genetic diversity of HCV in each patient, we used two approaches. One method was to estimate the difference of total evolutionary distances in the phylogenetic tree between the two time points, and another was to estimate the changes of genetic diversity along the time based on the coalescence theory. The two results indicate that the HIV-positive group has significantly more diverse population structure than the HIV-negative group. A comparative analysis of the synonymous and non-synonymous substitutions found that the HIV-positive group was subject to less selective pressure than the HIV-negative group. In conclusion, HIV-positive patients would have a more diversified HCV population than HIV-negative patients due to less selective pressure from the immune system.

Received 6 March 2007
Accepted 3 May 2007

INTRODUCTION

Increased rates of progression to end-stage liver disease, mortality and reduced treatment response rates have been well documented in haemophiliac and other groups of chronic hepatitis C virus (HCV) carriers with human immunodeficiency virus (HIV) coinfection (Bica *et al.*, 2001; Goedert *et al.*, 2002; Braitstein *et al.*, 2004). Although the mechanism of liver disease progression in HIV-infected patients remains unclear, one of the important roles is assigned to immunosuppression (Goedert *et al.*, 2002).

The estimated HCV virion half-life time was, on average, 2.7 h with pre-treatment production and clearance of 10^{12} virions per day (Neumann *et al.*, 1998). Such a high rate of HCV replication, combined with lack of an error correction mechanism, results in the development of genetically diverse clones in a patient. The genetic diversity of HCV has provided critical insights into short-term outcomes, including early spontaneous viral clearance (Farci *et al.*, 2000), interferon-associated viral clearance (Farci *et al.*, 2002; Pawlotsky *et al.*, 1999) and HCV emergence following liver transplantation (Lyra *et al.*, 2002). To infer the genetic diversity of HCV in a patient, we applied two approaches. One method simply assumed that the genetic diversity of HCV is of different divergence of synonymous distance between two time points. The other method applied to coalescent analysis of genetic diversity along the time, assuming that the genetic diversity of HCV represents a heterogeneous viral population in a given carrier. To evaluate the influence on the HCV evolution exerted by the immunosuppression during persistent HIV coinfection, we examined a cohort of HCV carriers, comparing the HIV-positive and -negative groups.

Determination of the antigen-recognition regions associated with HCV-specific immune positive selection is important for understanding selective pressures underlying the evolution of HCV as well as putative therapeutic targets. In this study, we evaluated the genetic diversity of HCV and determined genomic regions associated with positive selection by comparative analysis of selective forces between HIV-positive and -negative groups in a cohort of haemophilia patients followed for more than 6 years.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this study are AB245555–AB245873.

METHODS

Selection of patients. The patients enrolled in the present study were a subset of a well-characterized cohort of 166 patients with haemophilia who had received non-heated plasma-derived coagulation products before 1987 and had been observed regularly since 1995 at Ogikubo hospital (Tokyo, Japan). Plasma samples from patients with known HCV and HIV serological status were stored at –80 °C. Of these patients, 57 were positive and 109 were negative for anti-HIV. After exclusion of HCV-RNA-negative and interferon-treated patients, and those with a mixture or shift in HCV subtypes during the follow-up, 13 HCV-1a-RNA-positive patients (eight HIV-positive and five HIV-negative patients) were selected at random for this study. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of each institution. Every patient gave written informed consent to participate in the virological research.

Laboratory tests. Laboratory evaluation included complete blood cell count and serum transaminases [alanine aminotransferase (ALT)]. $CD4^+$ cell counts were examined by fluorescence-activated cell sorting at SRL Inc. (Tokyo, Japan). Serum HCV-RNA levels and HIV-RNA levels were measured by a commercial PCR assay (Amplicor HIV-1 Monitor and Amplicore HCV monitor; Roche Diagnostics). The detection limits of PCR for HCV-RNA and HIV-RNA were 500 IU ml^{-1} [0.5 kilo international unit (KIU) ml^{-1}] and 50 copies ml^{-1}, respectively.

HCV-RNA isolation and amplification from the core, E1 and E2 regions. Nucleic acids were extracted from serum samples using a SepaGene RV-R Nucleic Acid Extracting kit (Sanko Junyaku) in accordance with the manufacturer's protocol. Viral RNA was reverse-transcribed to cDNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and random hexamer primer (Takara Shuzo) as described previously (Ohno *et al.*, 1997).

Partial core, E1 and E2 fragments were amplified by using PCR with primers as described previously (Tanaka *et al.*, 2002). To reduce the number of artificial substitutions arising during PCR, Platinum *Pfx* DNA Polymerase (Invitrogen) with a very high fidelity was used.

Cloning and sequencing of cDNA. The amplified products were ligated into pCR-Blunt II-TOPO Vector and used to transform DH5-α high-efficiency competent cells according to the manufacturer's protocol (Invitrogen). The plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen) and the presence of the inserts confirmed by digestion with *Eco*RI. Sequencing was performed on more than 10 clones per patient at the baseline (1995–1997) and the end point (2002–2003). All clones were sequenced with Prism Big Dye (Applied Biosystems) in an ABI 3100 DNA automated sequencer.

Construction of phylogenetic trees. Nucleotide sequences of HCV were aligned by using the program CLUSTAL_X and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetic Analysis software (MEGA version 3.0; Kumar *et al.*, 2001). The MEGA algorithms were used to calculate the mean Tamura–Nei pairwise distance for all clones as well as a matrix of Tamura–Nei pairwise distances for each patient. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times.

Genetic diversity of HCV over a time course. Two approaches were used to infer the genetic diversity of HCV in each patient. In the first approach, total evolutionary distances among a heterogeneous viral population were compared between the baseline and end point for each patient in the phylogenetic tree. The phylogenetic tree of genetic diversity was constructed by using the maximum-likelihood (ML) method and the ancestral sequence was inferred at every node using the ML method (Yang *et al.*, 1995). As the evolutionary distance in each branch, the number of synonymous substitutions per synonymous site (synonymous distance) was estimated by the modified Nei–Gojobori method. Total synonymous distances were assumed to represent the genetic diversity of a heterogeneous viral population in each patient.

The other approach is the coalescence theory based on estimation of the genetic diversity. A consensus sequence based on the sequences of all HCV clones isolated from each patient was used as an outgroup to locate the position of the root in each phylogenetic tree. The topology of the phylogenetic tree was estimated by the neighbour-joining method (PHYLIP). Based on the topology, we constructed the phylogenetic tree and inferred the evolutionary rate by the ML method under the premise of the molecular clock (TipDate) (Rambaut, 2000). Based on the trees and the evolutionary rates estimated by TipDate, the coalescent analysis of genetic diversity was conducted for each patient using the Genie v3.5 software (Pybus *et al.*, 2001; Pybus & Rambaut, 2002). In brief, time t was transformed to year using the HCV molecular evolutionary rate, assuming the sample-collection time to be the present. Function $N(t)$ (effective numbers of HCV infections through time) was estimated by the ML method to infer the genetic diversity of HCV (Pybus & Rambaut, 2002). Although there are several models to infer $N(t)$, the best-fit model was different among patients. Therefore, we chose a simplified model in which the genetic diversity was assumed to be exponentially increased over time (expansion model).

Identification of positively selective regions. Positively selected regions were identified using the modified method of Suzuki & Gojobori (2001). In brief, a phylogenetic tree of sequences from HCV clones was reconstructed in each patient by the ML method. The ancestral sequence was inferred at every node in the phylogenetic tree using the ML method (Yang *et al.*, 1995). Then, synonymous and non-synonymous substitutions throughout the phylogenetic tree were estimated in each branch for each codon site. Here, to see the differences in selective pressure for HCV between the HIV-positive and -negative groups, we independently summed the total numbers of synonymous (N_s) and non-synonymous (N_n) substitutions occurring at each codon site of the HCV clones from either eight patients infected with HIV or five patients without HIV infection. The mean numbers of synonymous (C_s) and non-synonymous (C_n) sites were calculated for each codon site by the modified Nei–Gojobori method. The genetic distance of synonymous (d_S) and non-synonymous (d_N) was calculated as N_s/C_s and N_n/C_n, respectively. Although the ratio d_N/d_S is usually used for estimating selective pressure, we used $(d_N+0.5)/(d_S+0.5)$ ratio instead in the present study, because no synonymous substitution was found in several codon sites. The ratio was calculated along with the sequence by the sliding-window analysis. Each window size consisted of three codons.

RESULTS

Comparison of clinical characteristics between HCV patients with and without HIV infection

When we compared clinical data between HCV patients with HIV (HIV-positive group) and without HIV (HIV-negative group), there were no significant differences of mean age, sex, putative duration of HCV infection or mean peak ALT levels (116 vs 146) (Table 1). Changes of ALT levels also were not different between these two groups. Mean peaks of HCV-RNA levels in the HIV-positive group (2300 ± 668 KIU ml^{-1}), however, were significantly higher than those in the HIV-negative group (936 ± 423, $P=$

Table 1. Clinical characteristics among HCV patients in this study

All patients are male. HCV genotype of all patients is 1a. LC, Liver cirrhosis; ALT, alanine aminotransferase; NT, not tested; +, positive; −, negative.

ID	Age	Putative date of HCV infection	LC	HIV	AIDS	HIV-RNA (copies ml^{-1})	CD4 at baseline (μl^{-1})	HCV-RNA (KIU ml^{-1})			ALT (U l^{-1})	
								Range	Peak	Increase ×2	Range	Increase ×2
NT211	29	1982	−	+	+	130 000	20	130–2100	2100	yes	20–156	yes
GM248	39	1986	−	+	−	23 000	110	290–1200	1200	yes	34–96	yes
OT33	34	1982	−	+	−	2300	286	170–2000	2000	yes	40–43	no
HH127	33	1980–1982	−	+	−	22 000	270	330–3200	3200	yes	27–168	yes
TA92	32	1984	−	+	−	98 000	271	1300–2900	2900	yes	29–34	no
KY48	31	1980–1983	−	+	−	55 000	242	730–2700	2700	yes	66–213	yes
NK112	28	1982	+	+	+	100 000	27	2300–2600	2600	yes	16–28	no
YY321	27	1987	−	+	+	95 000	35	1200–1700	1700	no	181–186	yes
KK202	19	1987	−	−	−	NT	NT	310–1600	1600	yes	21–41	no
KN201	45	1982	+	−	−	NT	NT	300–710	710	no	38–98	no
TS246	20	1984	−	−	−	NT	NT	230–1000	1000	yes	27–37	no
SH265	20	1985	−	−	−	NT	NT	340–470	470	no	130–470	yes
ST251	26	1984	−	−	−	NT	NT	590–900	900	no	38–83	yes

0.0019), which is consistent with previous reports (Eyster *et al.*, 1994). Additionally, seven of eight patients in the HIV group had HCV-RNA elevation more than twice during follow-up, whereas only two patients in the HIV-negative group had HCV-RNA elevation.

For the eight HCV patients in the HIV-positive group, HIV-RNA and CD4 are shown in Table 1. Three of the patients had already developed AIDS and had very low CD4 counts (20, 27, 35 μl^{-1}), and the remaining five patients with HIV also had relatively low CD4 levels (110–286) at the baseline (1995–1997) before initiating highly active anti-retroviral therapy (HAART). Thereby, all HIV-infected patients studied were considered to be in an immunity-suppressed condition. Four patients with a CD4 count less than 200, including the three AIDS patients, received anti-HIV treatments.

Long-term intra-host diversity of HCV evaluated on distinct genomic regions

It has been shown previously that the genetic diversity of HCV changes in an oscillatory manner during the natural course of the infection (Devereux *et al.*, 1997). Taking into account that the genetic diversity of HCV analysed at a single time point might not accurately reflect the dynamic profile of the population over time, we have examined 26 serum samples collected from 13 patients at two distinct time points with intervals of at least 6 years (6–8 years). At least 11 HCV clones were isolated from a single patient at the baseline (1995–1997) and at the end point (2002–2003) of the follow-up. Overall, 325 HCV clones were thus isolated and analysed. All of them belonged to genotype 1a. Phylogenetic relation of the HCV clones isolated from all patients is shown in Fig. 1. Assuming that HCV is composed of a heterogeneous viral population, which is evolving throughout time in a given host (carrier), we aimed to estimate the size and heterogeneity of the population. Two different methods were used to attain this aim.

First, we directly compared the genetic diversity of a heterogeneous viral population between the two time points. To do that, we estimated totals (for all patients in each of the two groups) of intra-host synonymous distances at each of the two time points. This estimation was done independently in both E1 and E2 genomic regions (Fig. 2). The increased difference from the baseline to the end point between the HIV-positive and HIV-negative groups was tested by the regression analysis, and the genetic diversity of the HIV-positive group is significantly higher than that of the HIV-negative group (P=0.043).

Second, the coalescent analysis of genetic diversity of HCV was conducted for each patient. Further, mean curves of the effective numbers of HCV infections were compared between HIV-positive and -negative groups (Fig. 3). Although the estimated mean number was initially relatively lower in the HIV-positive group, the rapid change to exponential growth, was observed several years after HIV infection in this group, whereas in the HIV-negative group, the effective number was gradually increasing throughout the period of time. The difference of exponential growth is significant (P=0.04). Hence, the result obtained by either method indicated the HIV-positive group to have higher genetic diversity of the heterogeneous viral population than the HIV-negative group, suggesting that this was due to the exposure of HIV infection.

Putative positively selective regions in the E1 and E2 regions

Since the higher genetic diversity of HCV was observed in HIV-positive patients, we further examined genetic evi-

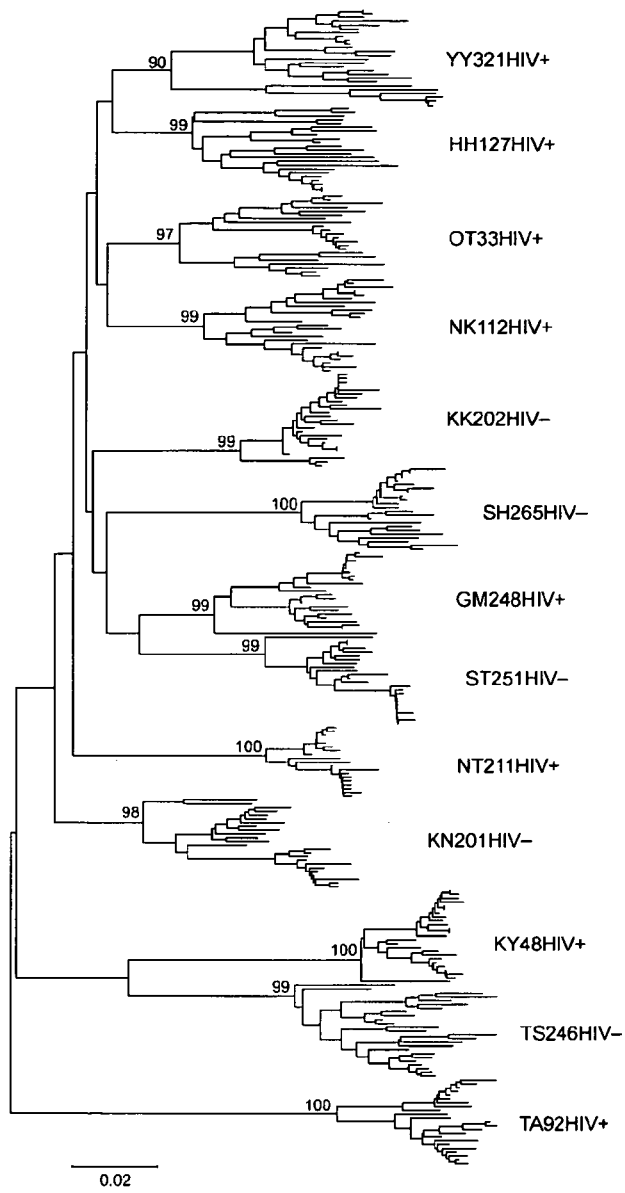

Fig. 1. A phylogenetic tree of E1 and E2 regions of the 325 HCV clones isolated from 13 patients. The significant phylogenetic cluster was observed in each of the eight HIV-positive (HIV+) and five HIV-negative (HIV−) patients. Numbers at nodes indicate bootstrap values of 1000 replications.

dence of the selective immune pressure in both groups. Selective immune pressure was estimated in each, E1 and E2, gene. Some differences were observed between the HIV-positive and -negative groups (Table 2, Fig. 4). Immune epitopes (11 aa segments in the E1 and 5 aa segments in the E2 region) that were observed only in the HIV-negative group might have relatively weak antigenicity. Some of the segments were previously recognized as HCV-specific potential immunogenic targets such as

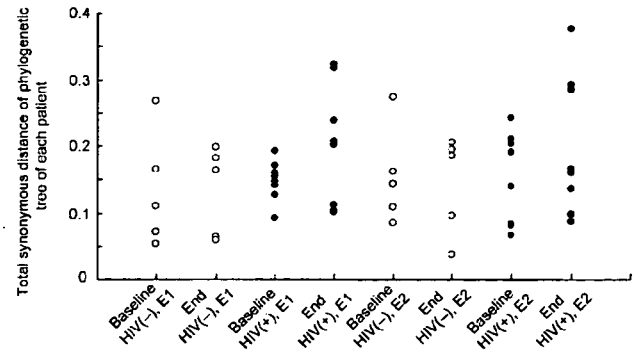

Fig. 2. Total synonymous distances of the phylogenetic tree of each time point. *y*-axis indicates total synonymous distance of the phylogenetic tree constructed by the sequences isolated at each time point. *x*-axis indicates two time points [baseline and end point (End)] and two groups [HIV-positive (HIV+) and -negative (HIV−)]. Each dot represents a patient.

cytotoxic T lymphocyte (CTL) epitopes (URL: http://hcv.lanl.gov/content/immuno/tables/ctl_summary.html), indicating that the positively selected segments estimated in the present study are associated with the immune response. On the other hand, positively selected segments around the hypervariable region (HVR1) regardless of HIV infection should have strong antigenic epitopes, suggesting little influence of the HIV coinfection on the natural immune selection targeting this region.

DISCUSSION

A previous meta-analysis showed a significantly elevated relative risk of severe liver disease in patients coinfected

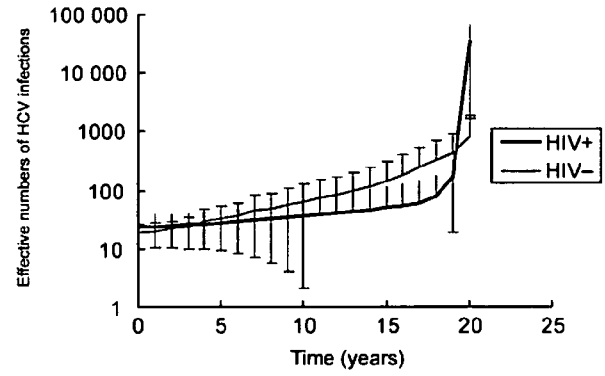

Fig. 3. The mean effective numbers of HCV infections in HIV-positive (HIV+) and -negative (HIV−) groups over the years from the baseline. Two lines indicate the dynamics of the mean effective numbers of HCV infections (*y*-axis) estimated in the E2 region and the bars indicate standard deviations. *x*-axis indicates number of years from the end point.

Table 2. Putative positively selective regions

Unknown epitopes identified only in the HIV-negative group are shown in bold.

Region	PS value*	HCV with HIV	HCV without HIV		
E1	PS>1.0	366–368	**192–195**,	**203–205**,	212–215,
			223–229,	234–237,	**245–247**,
			255–257,	295–299,	**346–349**
			353–358,	**359–361**	
E2	PS>1.0	383–412, 414–418, 434–438	382–410,	418–420,	432–436,
			443–446,	463–465,	**480–482**,
			486–488	496–500	

*PS (positive selection)=$(d_N+0.5)/(d_S+0.5)$.

with HIV and HCV (Braitstein *et al.*, 2004; Graham *et al.*, 2001). Another cohort study of HIV/HCV-coinfected patients also indicated association of low CD4 cell count, alcohol consumption rate and age at HIV/HCV coinfection with acceleration of the liver fibrosis (Benhamou *et al.*, 1999). The increased HCV replication in the HIV/HCV-coinfected patients would induce an intermediate immune response that is large enough to induce hepatic cell destruction and fibrosis but not enough to eradicate the virus from its reservoirs (Lai *et al.*, 2003; Poynard *et al.*, 2003).

In the present study, the genetic diversity of HCV was higher in the HIV-positive group compared with the HIV-negative group, which could be associated with either or both higher rate of HCV replication and HIV-associated immunosuppression, leading to less selective pressure on HCV in the HIV-positive group. The increased HCV replication by immune dysregulation in the HIV/HCV-coinfected patients might damage liver cells through apoptosis and other means such as cytokine disruption as reported previously (Puoti *et al.*, 2001). Although several

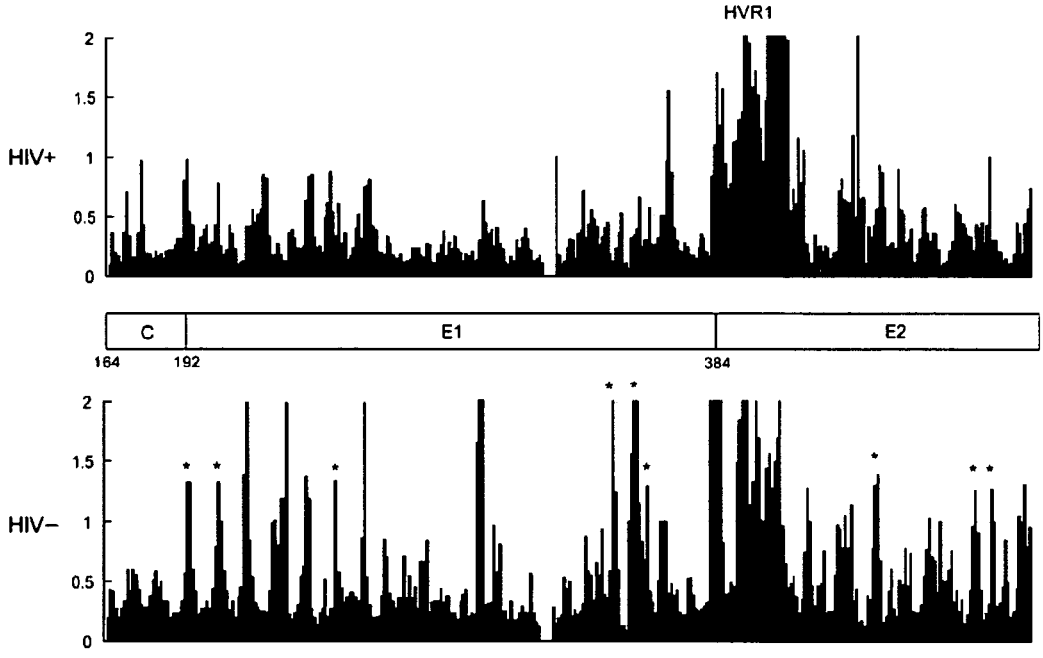

Fig. 4. Comparative histograms of the E1 and E2 regions of the HCV clones from HIV-positive (HIV+) and -negative (HIV−) patients. *y*-axis indicates $(d_N+0.5)/(d_S+0.5)$ ratio, columns exceeding value 1.0 represent the putative positively selective regions. Regions outside of any known CTL epitopes are indicated by asterisks and probably indicate unknown epitopes. *x*-axis indicates the schematic position in the HCV genes: C, core; HVR1, hypervariable region.

studies showed that HCV diversity decreases with the degree of HIV-related immunosuppression (Babik & Holodniy, 2003; Mao *et al.*, 2001; Martell *et al.*, 1992; Qin *et al.*, 2005; Roque-Afonso *et al.*, 2002; Toyoda *et al.*, 1997), this might be associated with the different parameters assessed for the genetic diversity; the conclusions in most previous studies were made by analysing total numbers of HCV clones and overall genetic distances at the amino acid level.

To assess the discrepancy with our results by coalescence-based estimation, we further examined the role of HCV-targeted immune pressure. The HCV nucleotide substitution pattern was compared between subjects with intact immune system versus those with HIV-associated immunosupression using a recently designed approach. Previous reports had used the mean of pairwise synonymous and non-synonymous distances within isolates (Ray *et al.*, 2000; Blackard *et al.*, 2004). Since most isolates do not have an independent evolutionary process, the mean may not represent overall genetic diversity of the heterogeneous viral population. To examine the non-redundant evolutionary process, recent methods have inferred the evolutionary process throughout the phylogenetic tree (Sheridan *et al.*, 2004; Suzuki & Gojobori, 2001; Hanada *et al.*, 2006). In the present study, we have applied a new approach to examine selection of HCV affected by HIV. The approach supported the theory that the diversified population of HIV-positive patients is due to less selective pressure and allows identification of specific regions indicating the presence of positive selection in HIV-negative patients compared with HIV-positive patients with immunosuppression. Although these positively selective segments, which were observed only in the HIV-negative group, might have relatively weak antigenicity, most of them were located inside potential immunogenic targets and others might be somewhat new antigen-recognition regions associated with HCV-specific immune responses. Interestingly, no influence by HIV coinfection was observed in the HVR1, which contains sequence-specific immunological B-cell epitopes that induce the production of antibodies restricted to the specific viral isolate (Kato *et al.*, 1993), indicating that the positively selected segments regardless of HIV infection should have strong antigenic epitopes. Taken together, our findings indicate that defenceless HCV clones that are extinct in usual conditions can survive in HIV-positive patients because of less immune pressure leading to HIV infection. Consequently, the genetic diversity of HCV will be greater in HIV-positive patients. In fact, the diversity of both synonymous and non-synonymous substitutions was larger in HIV-positive patients than HIV-negative patients (data not shown).

One limitation of the present study is the general lack of functional immunological data. In this study, HCV-specific CD4 ELISPOT responses were not detected in all subjects and CD8 cell counts were not measured. Therefore, we chose to use HIV load and CD4 cell counts as surrogate markers of immune suppression. Further investigations of HCV diversity in conjunction with HCV-specific cellular responses will be required when more-sensitive immunological assays are available. Another potential limitation of the study is that only two time points were sampled for each individual. However, as we inferred the evolutionary process based on the phylogenetic tree constructed using the number of clones that were isolated through the long period of follow-up (more than 6 years), we believe that the genetic diversity of the heterogeneous viral population may represent an actual evolutionary process.

In conclusion, HIV-positive patients have more diversified HCV populations than HIV-negative patients, possibly because of reduction of selective pressure from the immune system. The positively selective regions determined in this study might be antigen-recognition regions associated with HCV-specific immune responses.

ACKNOWLEDGEMENTS

Financial support: the work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Science, Sports of Japan and Japan Science and Technology Agency. Potential conflicts of interest: we do not have any commercial or other association that may pose a conflict of interest.

REFERENCES

Babik, J. M. & Holodniy, M. (2003). Impact of highly active antiretroviral therapy and immunologic status on hepatitis C virus quasispecies diversity in human immunodeficiency virus/hepatitis C virus-coinfected patients. *J Virol* 77, 1940–1950.

Benhamou, Y., Bochet, M., Di Martino, V., Charlotte, F., Azria, F., Coutellier, A., Vidaud, M., Bricaire, F., Opolon, P. & other authors (1999). Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group. *Hepatology* 30, 1054–1058.

Bica, I., McGovern, B., Dhar, R., Stone, D., McGowan, K., Scheib, R. & Snydman, D. R. (2001). Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection. *Clin Infect Dis* 32, 492–497.

Blackard, J. T., Yang, Y., Bordoni, P., Sherman, K. E., Chung, R. T. & AIDS Clinical Trials Group 383 Study Team (2004). Hepatitis C virus (HCV) diversity in HIV-HCV-coinfected subjects initiating highly active antiretroviral therapy. *J Infect Dis* 189, 1472–1481.

Braitstein, P., Palepu, A., Dieterich, D., Benhamou, Y. & Montaner, J. S. (2004). Special considerations in the initiation and management of antiretroviral therapy in individuals coinfected with HIV and hepatitis C. *AIDS* 18, 2221–2234.

Devereux, H. L., Brown, D., Dusheiko, G. M., Emery, V. C. & Lee, C. A. (1997). Long-term evolution of the 5′UTR and a region of NS4 containing a CTL epitope of hepatitis C virus in two haemophilic patients. *J Gen Virol* 78, 583–590.

Eyster, M. E., Fried, M. W., Di Bisceglie, A. M. & Goedert, J. J. (1994). Increasing hepatitis C virus RNA levels in hemophiliacs: relationship to human immunodeficiency virus infection and liver disease. Multicenter Hemophilia Cohort Study. *Blood* 84, 1020–1023.

Farci, P., Shimoda, A., Coiana, A., Diaz, G., Peddis, G., Melpolder, J. C., Strazzera, A., Chien, D. Y., Munoz, S. J. & other authors (2000).

The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* **288**, 339–344.

Farci, P., Strazzera, R., Alter, H. J., Farci, S., Degioannis, D., Coiana, A., Peddis, G., Usai, F., Serra, G. & other authors (2002). Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. *Proc Natl Acad Sci U S A* **99**, 3081–3086.

Goedert, J. J., Eyster, M. E., Lederman, M. M., Mandalaki, T., De Moerloose, P., White, G. C., II, Angiolillo, A. L., Luban, N. L., Sherman, K. E. & other authors (2002). End-stage liver disease in persons with hemophilia and transfusion-associated infections. *Blood* **100**, 1584–1589.

Graham, C. S., Baden, L. R., Yu, E., Mrus, J. M., Carnie, J., Heeren, T. & Koziel, M. J. (2001). Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis. *Clin Infect Dis* **33**, 562–569.

Hanada, K., Gojobori, T. & Li, W. H. (2006). Radical amino acid change versus positive selection in the evolution of viral envelope proteins. *Gene* **385**, 83–88.

Hanada, K., Tanaka, Y., Mizokami, M., Gojobori, T. & Alter, H. J. (2006). A reduction in selective immune pressure during the course of chronic hepatitis C correlates with diminished biochemical evidence of hepatic inflammation. *Virology* **361**, 27–33.

Kato, N., Sekiya, H., Ootsuyama, Y., Nakazawa, T., Hijikata, M., Ohkoshi, S. & Shimotohno, K. (1993). Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. *J Virol* **67**, 3923–3930.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* **17**, 1244–1245.

Lai, C. L., Ratziu, V., Yuen, M. F. & Poynard, T. (2003). Viral hepatitis B. *Lancet* **362**, 2089–2094.

Lyra, A. C., Fan, X., Lang, D. M., Yusim, K., Ramrakhiani, S., Brunt, E. M., Korber, B., Perelson, A. S. & Di Bisceglie, A. M. (2002). Evolution of hepatitis C viral quasispecies after liver transplantation. *Gastroenterology* **123**, 1485–1493.

Mao, Q., Ray, S. C., Laeyendecker, O., Ticehurst, J. R., Strathdee, S. A., Vlahov, D. & Thomas, D. L. (2001). Human immunodeficiency virus seroconversion and evolution of the hepatitis C virus quasispecies. *J Virol* **75**, 3259–3267.

Martell, M., Esteban, J. I., Quer, J., Genesca, J., Weiner, A., Esteban, R., Guardia, J. & Gomez, J. (1992). Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* **66**, 3225–3229.

Neumann, A. U., Lam, N. P., Dahari, H., Gretch, D. R., Wiley, T. E., Layden, T. J. & Perelson, A. S. (1998). Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* **282**, 103–107.

Ohno, T., Mizokami, M., Wu, R. R., Saleh, M. G., Ohba, K., Orito, E., Mukaide, M., Williams, R. & Lau, J. Y. (1997). New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* **35**, 201–207.

Pawlotsky, J. M., Germanidis, G., Frainais, P. O., Bouvier, M., Soulier, A., Pellerin, M. & Dhumeaux, D. (1999). Evolution of the hepatitis C virus second envelope protein hypervariable region in chronically infected patients receiving alpha interferon therapy. *J Virol* **73**, 6490–6499.

Poynard, T., Yuen, M. F., Ratziu, V. & Lai, C. L. (2003). Viral hepatitis C. *Lancet* **362**, 2095–2100.

Puoti, M., Bonacini, M., Spinetti, A., Putzolu, V., Govindarajan, S., Zaltron, S., Favret, M., Callea, F., Gargiulo, F. & other authors (2001). Liver fibrosis progression is related to CD4 cell depletion in patients coinfected with hepatitis C virus and human immunodeficiency virus. *J Infect Dis* **183**, 134–137.

Pybus, O. G. & Rambaut, A. (2002). GENIE: estimating demographic history from molecular phylogenies. *Bioinformatics* **18**, 1404–1405.

Pybus, O. G., Charleston, M. A., Gupta, S., Rambaut, A., Holmes, E. C. & Harvey, P. H. (2001). The epidemic behavior of the hepatitis C virus. *Science* **292**, 2323–2325.

Qin, H., Shire, N. J., Keenan, E. D., Rouster, S. D., Eyster, M. E., Goedert, J. J., Koziel, M. J. & Sherman, K. E. (2005). HCV quasispecies evolution: association with progression to end-stage liver disease in hemophiliacs infected with HCV or HCV/HIV. *Blood* **105**, 533–541.

Rambaut, A. (2000). Estimating the rate of molecular evolution: incorporating non-contemporaneous sequences into maximum likelihood phylogenies. *Bioinformatics* **16**, 395–399.

Ray, S. C., Mao, Q., Lanford, R. E., Bassett, S., Laeyendecker, O., Wang, Y. M. & Thomas, D. L. (2000). Hypervariable region 1 sequence stability during hepatitis C virus replication in chimpanzees. *J Virol* **74**, 3058–3066.

Roque-Afonso, A. M., Robain, M., Simoneau, D., Rodriguez-Mathieu, P., Gigou, M., Meyer, L. & Dussaix, E. (2002). Influence of CD4 cell counts on the genetic heterogeneity of hepatitis C virus in patients coinfected with human immunodeficiency virus. *J Infect Dis* **185**, 728–733.

Sheridan, I., Pybus, O. G., Holmes, E. C. & Klenerman, P. (2004). High-resolution phylogenetic analysis of hepatitis C virus adaptation and its relationship to disease progression. *J Virol* **78**, 3447–3454.

Suzuki, Y. & Gojobori, T. (2001). Positively selected amino acid sites in the entire coding region of hepatitis C virus subtype 1b. *Gene* **276**, 83–87.

Tanaka, Y., Hanada, K., Mizokami, M., Yeo, A. E., Shih, J. W., Gojobori, T. & Alter, H. J. (2002). A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci U S A* **99**, 15584–15589.

Toyoda, H., Fukuda, Y., Koyama, Y., Takamatsu, J., Saito, H. & Hayakawa, T. (1997). Effect of immunosuppression on composition of quasispecies population of hepatitis C virus in patients with chronic hepatitis C coinfected with human immunodeficiency virus. *J Hepatol* **26**, 975–982.

Yang, Z., Kumar, S. & Nei, M. (1995). A new method of inference of ancestral nucleotide and amino acid sequences. *Genetics* **141**, 1641–1650.

Risk for hepatocellular carcinoma with respect to hepatitis B virus genotypes B/C, specific mutations of enhancer II/core promoter/precore regions and HBV DNA levels

M-F Yuen,[1] Y Tanaka,[2] N Shinkai,[2] R T Poon,[3] D Yiu-Kuen But,[1] D Y-T Fong,[4] J Fung,[1] D Ka-Ho Wong,[1] J Chi-Hang Yuen,[1] M Mizokami,[2] C-L Lai[1]

[1] Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong; [2] Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; [3] Department of Surgery, The University of Hong Kong, Queen Mary Hospital, Hong Kong; [4] Department of Nursing Studies, The University of Hong Kong, Queen Mary Hospital, Hong Kong

Correspondence to: Dr M F Yuen, Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong; mfyuen@hkucc.hku.hk

Revised 20 April 2007
Accepted 24 April 2007
Published Online First 3 May 2007

ABSTRACT

Background/aim: To examine the risks for hepatocellular carcinoma (HCC) with respect to hepatitis B virus (HBV) genotypes, specific viral mutations (MT), serum HBV DNA levels, and cirrhosis.

Methods: HBV genotypes, 1653/1753/core promoter (CP)/precore MT and HBV DNA levels were determined in 248 HBV patients with HCC and 248 HBV controls.

Results: Genotype C, CP-MT, T1653, HBV DNA levels ≥ 4 $\log_{10}$ copies/ml and cirrhosis had a higher risk for HCC compared to patients with genotype B ($p = 0.001$, OR 1.9), CP wild-type (WT) ($p<0.001$, OR 4.1), C1653 ($p = 0.028$, OR 2.4), HBV DNA <4 $\log_{10}$ copies/ml ($p = 0.003$, OR 2.1) and without cirrhosis ($p<0.001$, OR 4.0) respectively. Multivariate analysis showed that CP-MT, T1653, HBV DNA ≥ 4 $\log_{10}$ copies/ml and cirrhosis were independent factors for HCC (all $p<0.05$). A receiver operating characteristics curve showed no cut-off HBV DNA level associated with minimal chance of HCC. Patients with CP-MT and cirrhosis had a 22.2-fold increased risk of HCC compared to patients with CP-WT and without cirrhosis. Patients with CP-MT and HBV DNA levels ≥ 4 $\log_{10}$ copies/ml had a 7.2-fold increased risk of HCC compared to patients with CP-WT and HBV DNA levels <4 $\log_{10}$ copies/ml. Patients with CP-MT and T1653 had a 9.9-fold increased risk of HCC compared to patients with wild-type for both regions.

Conclusions: CP-MT, T1653, HBV DNA levels ≥ 4 $\log_{10}$ copies/ml and cirrhosis are independent factors for development of HCC. The risks increased substantially in patients having these factors in combination.

Hepatocellular carcinoma (HCC) is a disease of global concern, occurring in over 20% of the 400 million people with chronic hepatitis B infection (CHB). While the exact mechanisms of hepatocarcinogenesis with CHB infection remain elusive, several virological factors have been identified to be possibly associated with a higher risk of development of HCC. These include hepatitis B virus load (HBV DNA) levels, HBV genotypes, core promoter and precore mutations. These factors are also associated with the development of cirrhosis and its complications.[1 2]

The majority of the published studies examining HBV genotypes compare genotypes B and C in relation to the disease profile of CHB because these are the two main genotypes prevailing in Asia, a region contributing around 75% of the world's population of CHB. However, while some studies suggest genotype C has a higher risk of development of HCC,[3–5] this observation is not substantiated by others.[6–8] One large study conducted in Taiwan shows that genotype B is more commonly found in patients with HCC developed at a young age.[9] In the Caucasian and Indian populations, genotype D is associated with a greater risk for HCC than genotype A.

Concerning the common naturally occurring mutations at the precore (G1896A) and core promoter (A1762T and G1764A) regions, some studies show that patients with precore mutants have more aggressive disease including reactivation of CHB and fulminating course of the disease,[10 11] These observations have not been substantiated in other studies partly because the predominant genotypes are different between Asia and Europe/USA.[12 13] For core promoter mutations, some studies report a higher risk of development of HCC in patients with core promoter mutations compared to those with wild-type.[3 6 7 14–16] Again, this has not been confirmed by other studies.[5 17] In addition to these two common mutations, two other mutations, C to T at 1653 in the enhancer II region and T to C/A/G (V) at 1753 in the core promoter region, have recently been found to be associated with the development of HCC.[18–20]

The uncertainty as to whether these virological factors are genuine risk factors for the development of HCC may be due to several reasons. Most of the studies only have a limited number of patients. These studies often examine only specific virological factors; for example, genotypes without considering the possible confounding effect of other parameters, such as viral mutations and HBV DNA levels. Indeed the associations between genotype B with precore mutations and genotype C with core promoter mutations have been shown to be confounding factors.[21] Whether there are any additive or synergistic effects on the risks of HCC development with different combinations of genotypes/precore/core promoter and mutations in the enhancer II region and HBV DNA levels have not been studied. Finally, the risks for development of HCC of these factors in the setting of cirrhosis have not been examined.

Therefore we sought to examine the risks of HBV DNA levels, HBV genotypes, core promoter/precore/T1653/V1753 mutations and cirrhosis individually and in combination for the development of HCC in a large population study.

PATIENTS AND METHODS

Patients

A total of consecutive 248 Chinese CHB patients with HCC were recruited from Department of Medicine and Department of Surgery, The University of Hong Kong, Queen Mary Hospital, Hong Kong from 2000 to 2004. All patients had the diagnosis of HCC for the first time, during regular follow-up in our centre ($n = 198$) or in other hospitals ($n = 50$). Patients with recurrent HCC were excluded from the present study. One hundred and twenty patients had histologically proven HCC. The remaining 128 patients had elevated α-fetoprotein (AFP) with typical imaging features in computerised tomography and/or magnetic resonance imaging and/or hepatic angiogram.

During the same period of recruitment of patients with HCC, 4825 CHB Chinese patients without HCC were being followed up in the University Liver Clinic of Queen Mary Hospital, Hong Kong. A consecutive 248 CHB patients without HCC were recruited as controls. These control patients were matched individually with each patient with HCC for gender, age (less than 2 years difference) and hepatitis B e antigen (HBeAg)/antibody to HBeAg (anti-HBe) status in a 1:1 ratio. The absence of HCC was assured by the absence of any space occupying lesion by ultrasonography performed on two separate occasions 1 year apart.

All patients were positive for hepatitis B surface antigen (HBsAg) checked by radioimmunoassay (AUSRIA II, Abbott Laboratories, North Chicago, IL) for at least 6 months. HBeAg/anti-HBe was also determined by the same assay. Patients with other concomitant diseases including hepatitis C or D virus infection, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and fatty liver (diagnosed by ultrasonography) were excluded.

Liver cirrhosis is defined by the score of >2 according to the aspartate aminotransferase (AST) to platelet ratio index (APRI) calculated from the following formula: ([AST/upper limit of normal]/platelet count [$\times 10^9$/litre]) × 100.[22]

Methods

Stored serum at −70°C were thawed for the determination of the HBV DNA levels, HBV genotypes, core promoter and precore mutations and finally the mutations at the enhancer II region. The HBV DNA levels were measured by Cobas Amplicor HBV Monitor test (Roche Diagnostics, Branchburg, NJ) with a lower limit of detection of 300 copies/ml.

HBV genotypes were determined by the enzyme linked immunosorbent assay (ELISA). The detailed methodology of the assay was described in our previous study.[23] The sequence of core promoter and precore regions including A1762T/G1764A (core promoter mutations) and G1896A (precore mutation) were determined by direct sequencing. The methodology was described in our previous study.[20] The two recently identified HCC-related mutations at the enhancer II and core promoter regions namely, C to T at 1653 and T to C/A/G (V) at 1753, were also sequenced according to the methods described in our previous study[20] in 140 patients with HCC and 100 control patients with adequate sera available for sequencing. There were no differences in the median age (range), male to female and HBeAg: anti-HBe ratios between these two subgroups of 140 and 100 patients [56.6 years (29–83.7) vs. 59.8 years (24.8–81.6), p = 0.13 for age; 114:26 vs. 79: 21, p = 0.65 for male to female ratio; and 40:100 vs. 23:77, p = 0.28 for HBeAg: anti-HBe ratio).

Statistical analysis

All statistical analyses were performed using the SPSS 14.0 for Windows, SPSS Inc., Chicago, IL). The Mann–Whitney U test was used to compare continuous variables between patients with HCC and control patients. The χ^2 test with Yates correction factor or Fisher's exact test was used to compare categorical variables between two groups. A receiver operating characteristic (ROC) curve was used to determine whether there is a cut-off HBV DNA which was associated with no risk of HCC. Logistic regression was adopted to determine independent risk factors for HCC. The adjusted odds ratios (OR) for development of HCC of different combinations of variables were also calculated by the logistic regression analysis with a selected combination defined as the reference. All estimates were accompanied by a 95% confidence interval (CI), where appropriate and a p-value <0.05 was considered as statistical significance.

RESULTS

Demographics

The demographic data for 248 patients with HCC and 248 control patients are listed in table 1. Patients with HCC had a significantly poorer liver biochemical parameters and higher median AFP level compared to control patients. Patients with HCC also had a higher prevalence of liver cirrhosis compared to control patients. The OR for patients with cirrhosis was 4.0 [95% CI, 2.8 to 5.9].

HBV genotypes

A total of 478 out of 496 (96.2%) samples had positive genotype results from EIA test, but this test gave indeterminate results for the remaining 18 samples (10 from patients with HCC, eight from control patients). Of the 238 patients with HCC with genotype results, 67 (28.2%) had genotypes B, 170 (71.4%) had genotypes C and one (0.4%) had genotype D. Of the 240 control patients with genotype results, 100 (41.7%) had genotypes B, 135 (56.3%) had genotype C, three (1.3%) had genotypes D and two (0.8%) had mixed genotypes.

Comparing patients with either genotypes B or C, patients with HCC had a higher prevalence of genotype C compared to control patients [170/237 (71.2%) vs. 135/235 (57.4%) respectively; p = 0.001; OR 1.9; 95% CI, 1.3 to 2.8].

Core promoter and precore mutations

Of all the 496 samples, direct sequencing failed to generate results for 70 samples for core promoter region and 61 samples for precore region.

Table 1 Demographic data for the study population

	Patients with HCC (n = 248)	Control patients (n = 248)
Sex (M:F)	199:49	199:49
Age (years)	57.5 (24.8–83.7)	57.7 (24.8–81.8)
HBeAg:anti-HBe (%)	61:187 (24.6%:75.4%)	61:187 (24.6%:75.4%)
Albumin (g/l)	37 (16–59)*	43 (17–53)*
Bilirubin (μmol/l)	17 (5–531)†	12 (2–96)†
ALT (U/l)	57 (4–1154)‡	46 (9–920)‡
AFP (ng/ml)	136.5 (1–1 060 000)§	5 (1–200)§
Presence of cirrhosis (%)	170 (68.5%)¶	87 (35.1%)¶

*,†,‡,§,¶p<0.001.
Continuous variables are expressed in median (range).
ALT, alanine aminotransferase; AFF, α-fetoprotein.

Results of core promoter mutations were successfully obtained in 194 (78.2%) samples of patients with HCC and in 232 (93.5%) samples of control patients. Patients with HCC had a higher prevalence of core promoter mutations compared to patients without HCC [173/194 (89.2%) vs. 155/232 (66.8%), respectively; p<0.001; OR, 4.1 (95% CI, 2.4 to 6.9)].

Results of precore mutations were successfully obtained by the direct sequencing in 198 (79.8%) samples of patients with HCC and in 237 (95.6%) samples of patients without HCC. There was no significant difference in the prevalence of precore mutations between patients with and without HCC [72/198 (36.4%) vs. 106/237 (44.7%), respectively; p = 0.10].

Relationship between HBV genotypes and core promoter/precore mutations

Patients with genotype B had a higher chance of harbouring precore mutations compared to patients with genotype C [105/144 (72.9%) vs. 67/267 (25.1%), respectively; p<0.001; OR, 8.0; 95% CI, 5.1 to 12.7)]. Patients with genotype C had a higher chance of harbouring core promoter mutations compared to patients with genotype B [237/264 (89.8%) vs. 76/141 (53.9%), respectively; p<0.001; OR, 7.5; 95% CI, 4.5 to 12.6].

HBV DNA levels

To determine whether there is an exact HBV DNA level below which HCC is unlikely to occur, the HBV DNA levels of all the patients with or without HCC were entered in the ROC curve analysis (fig. 1). The ROC nearly overlapped with reference line with the area under the curve (AUC) of 0.51 (p = 0.75; 95% CI, 0.46 to 0.56) indicating that there existed no cut-off HBV DNA level that was associated with minimal risk of HCC. Further separate analysis of patients who had HBeAg seroconversion (anti-HBe positive) with less fluctuation of HBV DNA levels during the course of the disease was performed. The AUC was only 0.56 (p = 0.054; 95% CI, 0.50 to 0.62). This suboptimal value confirmed that there was no HBV DNA level that was associated with minimal risk of HCC even for anti-HBe-positive patients.

Though a "safe" lower limit of HBV DNA level could not be identified, a higher proportion of patients with HCC had high viral load defined by HBV DNA level ≥4 $\log_{10}$ copies/ml compared to that of control patients [218/248 (87.9%) vs. 193/248 (77.8%), respectively; p = 0.003; OR, 2.1; 95% CI, 1.3 to 3.4].

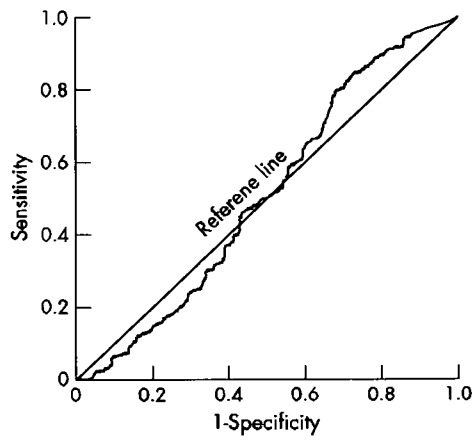

Figure 1 Receiver operating characteristics curve (ROC) of HBV DNA levels and development of HCC (area under curve = 0.51) (p = 0.75; 95% CI, 0.46 to 0.56).

T1653 and V1753 mutations

Results of T1653 and V1753 were successfully obtained by the direct sequencing in 133 (95%) out of 140 samples of patients with HCC and in 99 out of 100 (99%) samples of control patients. The reason for samples with no obtainable results for these two mutations was due to the failure of generation of sequence with good quality by direct sequencing. Patients with HCC had a significantly higher prevalence of T1653 mutations compared to control patients [19.5% (26/133) vs. 9.1% (9/99), respectively; p = 0.028; OR, 2.4; 95% CI, 1.1 to 5.4]. There was no difference in the prevalence of T1753 mutations between patients with HCC and control patients [42.1% (56/133) vs. 44.4% (44/99), respectively; p = 0.72].

Multivariate analysis on the risk factors for HCC

HBV genotypes, core promoter mutations, T1653 mutations, HBV DNA levels and presence of cirrhosis were entered into the logistic regression analysis. Core promoter mutations, T1653 mutation, HBV DNA levels ≥4 $\log_{10}$ copies/ml and presence of cirrhosis were shown to be independent factors associated with HCC (p = 0.015, 0.044, 0.048 and 0.005, respectively). Genotype C, identified as a significant risk factor in the univariate analysis was *not* an independent risk factor for HCC.

Relationship between core promoter mutations, T1653 mutations, HBV DNA levels and cirrhosis

A higher proportion of patients with core promoter mutations had high viral load (HBV DNA ≥4 $\log_{10}$ copies/ml) compared to that of patients without core promoter mutations [284/328 (86.6%) vs. 71/98 (72.4%), respectively; p = 0.001; OR, 2.6; 95% CI, 1.4 to 4.2]. There was no difference in the prevalence of T1653 mutation between patients with core promoter mutations and wild-type [28/174 (16.1%) vs. 6/43 (14.0%), respectively; p = 0.91]. Patients with core promoter mutations had a significantly higher prevalence of cirrhosis compared to patients with core promoter wild-type [177/328 (54.0%) vs. 39/98 (39.8%), respectively; p = 0.014; OR, 1.8; 95% CI, 1.1 to 2.8].

Adjusted risks for patients with core promoter mutations stratified according to HBV DNA levels, 1653 mutations and cirrhosis

Stratifying core promoter mutations, 1653 mutations, HBV DNA levels and presence of cirrhosis to assess the combined risk for the development of HCC resulted in 16 different groups of patients with certain groups having fewer than five patients, thus precluding reliable statistical analysis. Therefore separate analyses were performed by stratifying (1) core promoter mutations according to HBV DNA levels, (2) core promoter mutations with or without concomitant 1653 mutations and (3) core promoter mutations according to presence or absence of cirrhosis. The adjusted odds ratios for the development of HCC are shown in tables 2, 3 and 4, respectively.

DISCUSSION

To our knowledge, the present study is the largest study examining the individual role as well as the possible interacting effects of HBV genotypes, the two commonly occurring mutations (core promoter and precore mutations), mutations at the enhancer II (T1653) and at the more upstream core promoter region (V1753), HBV DNA levels, and liver cirrhosis

Table 2 Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according to the HBV DNA levels

Core promoter	HBV DNA (log_{10} copies/ml)	Number of patients	Odds ratio (95% CI)	p Value
Wild-type	<4	27	Reference	–
Wild-type	≥4	77	1.8 (0.6 to 6.0)	0.33
Mutant	<4	44	3.1 (0.9 to 10.6)	0.07
Mutant	≥4	426	7.2 (2.4 to 21.4)	<0.001

CI, confidence interval.

Table 4 Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according presence or absence of cirrhosis

Core promoter	Cirrhosis	Number of patients	Odds ratio (95% CI)	p Value
Wild-type	No	59	Reference	–
Wild-type	Yes	39	7.5 (2.5 to 23.0)	<0.001
Mutant	No	151	6.0 (2.3 to 15.9)	<0.001
Mutant	Yes	177	22.2 (8.4 to 58.4)	<0.001

CI, confidence interval.

on the development of HCC. This relatively large number of patients would allow any possible links or associations between these factors contributing to the development of HCC to be defined more unequivocally. One of the limitations of the present study is that the role of deletions in the pre-S region of HBV genome which have been recently shown to be associated with the development of HCC has not been studied.[24]

An epiphenomenon observed in the present study was the higher risk of HCC in patients with genotype C compared to patients with genotype B (all were subgenotype B2 in our locality according to our previous study).[25] This is apparently consistent with other studies.[3–5] However, genotype C was not found to be an independent factor for HCC when tested in the multivariate analysis. Core promoter mutations, T1653 mutations, high HBV DNA levels and presence of cirrhosis were independent risk factors for HCC. This is not an unusual finding because of the strong association of genotype C with core promoter mutations (89.8%), and genotype B with precore mutations (72.9%). Though it is well proven that patients with genotype B have an earlier HBeAg seroconversion,[21 26] it appears neither genotype B nor C has any major influential effects on the life-time risk of HCC, a finding in concordance with other studies.[6 27] We have recently shown that the earlier HBeAg seroconversion with genotype B is related to the more intense immunogenic stimulation during the immunoclearance phase.[28] The effects exerted by HBV genotypes B and C on the disease progression of CHB subsequent to HBeAg seroconversion appear to be similar.

However, there are at least two documented effects accompanying core promoter mutations on the development of HCC. Mutations in the core promoter region result in a shift change of the viral pregenomic secondary structure which may enhance the viral replication.[29] Viral replication can also be further enhanced by a second mechanism in which the transcription of the pregenomic RNA will be increased through the removal of the nuclear receptor binding site and creation of a hepatocytes nuclear binding factor.[30] These changes increase the core RNA transcription with enhanced core protein, DNA polymerase, pre-genomic RNA synthesis, but suppress the precore RNA transcription whose normal function is to decrease pregenomic RNA packaging.[31 32] This is in complete concordance with the finding of the present study and of Chauhan and colleagues.[33] HBV DNA levels were higher in patients with core promoter mutations compared to those without core promoter mutations.

In the present study, by setting the patients without core promoter mutations and HBV DNA <4 log_{10} copies/ml as a reference, the adjusted odds ratio for HCC for patients with core promoter mutations at the same viraemic level was 3.1 (95% CI, 0.9 to 10.6), with a borderline p value of 0.07 (table 2). It is possible that the higher risk of HCC in patients with core promoter mutations may also be mediated through another additional pathway independent of the increase in viral replication. The possible carcinogenic mechanisms require further *in vitro* studies and functional analyses to delineate.

The present study demonstrated that the risk of HCC was substantially increased in patients harbouring core promoter mutations and having liver cirrhosis, a 22.2-fold increase when compared to patients with core promoter wild-type and without cirrhosis (table 4). Similarly, patients with core promoter mutations with high HBV DNA levels of ≥4 log_{10} copies/ml had a 7.2-fold increase risk of HCC when compared to patients with core promoter wild-type with HBV DNA levels <4 log_{10} copies/ml (table 2).

In the present study, we found that T1653 was an independent risk factor for the development of HCC. According to our previous studies,[20 34] T1653 mutation is associated with HCC in patients with genotype C. In the present study, we further confirmed with larger number of patients that T1653 was an independent risk factor for HCC irrespective of HBV genotypes. 1653 is located in the box alpha of the enhancer II region of HBV genome. The C to T mutation at 1653 converts histidine to tyrosine at amino acid 94 of the X protein which may explain its association with the hepatocarcinogenesis. According to Takahashi and colleagues, the frequency of T1653 mutation increases with the progression of liver disease from chronic hepatitis to cirrhosis.[19] It occurs later than, and independent of, core promoter mutations in chronic hepatitis B disease. However, when both viral mutations, that is, core promoter and T1653 mutations, co-existed, the risk of HCC was substantially increased to 9.9-fold when compared to patients with wild-type at both genomic regions (table 3).

Finally, the present study showed that there was no reliable cut-off HBV DNA level associated with low risk of HCC. This means that maximal viral suppression to the lowest possible HBV DNA levels should be the target for future management of CHB disease.

In conclusion, core promoter mutations, T1653 mutations, HBV DNA levels ≥4 log_{10} copies/ml and presence of cirrhosis were independent factors for the development of HCC. The risk increased substantially in patients who carried these factors in combination. Future studies should consider these factors in conjunction with age and gender of patients to formulate the risk of HCC in CHB patients.

Table 3 Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according mutations at 1653

Core promoter	1653	Number of patients	Odds ratio (95% CI)	p Value
Wild-type	Wild-type	37	Reference	–
Wild-type	Mutant	6	2.7 (0.5 to 15.6)	0.27
Mutant	Wild-type	146	3.6 (1.6 to 7.9)	0.02
Mutant	Mutant	28	9.9 (3.1 to 31.5)	<0.001

CI, confidence interval.

Competing interests: None.

REFERENCES

1. **Chen CH,** Lee CM, Lu SN, *et al*. Clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations affecting HBV e antigen expression in Taiwan. *J Clin Microbiol* 2005;**43**:6000–6.
2. **Lin CL,** Liao LY, Wang CS, *et al*. Basal core-promoter mutant of hepatitis B virus and progression of liver disease in hepatitis B e antigen-negative chronic hepatitis B. *Liver Int* 2005;**25**:564–70.
3. **Fang ZL,** Yang J, Ge X, *et al*. Core promoter mutations (A(1762)T and G(1764)A) and viral genotype in chronic hepatitis B and hepatocellular carcinoma in Guangxi, China. *J Med Virol* 2002;**68**:33–40.
4. **Tsubota A,** Arase Y, Ren F, *et al*. Genotype may correlate with liver carcinogenesis and tumor characteristics in cirrhotic patients infected with hepatitis B virus subtype adw. *J Med Virol* 2001;**65**:257–65.
5. **Chan HL,** Hui AY, Wong ML, *et al*. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut* 2004;**53**:1494–8.
6. **Yuen MF,** Tanaka Y, Mizokami M, *et al*. Role of hepatitis B virus genotypes Ba and C, core promoter and precore mutations on hepatocellular carcinoma: a case control study. *Carcinogenesis* 2004;**25**:1593–8.
7. **Liu CJ,** Chen BF, Chen PJ, *et al*. Role of hepatitis B virus precore/core promoter mutations and serum viral load on noncirrhotic hepatocellular carcinoma: a case–control study. *J Infect Dis* 2006;**194**:594–9.
8. **Kao JH.** Hepatitis B virus genotypes and hepatocellular carcinoma in Taiwan. *Intervirology* 2003;**46**:400–7.
9. **Kao JH,** Chen PJ, Lai MY, *et al*. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000;**118**:554–9.
10. **Ozasa A,** Tanaka Y, Orito E, *et al*. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006;**44**:326–34.
11. **Omata M,** Ehata T, Yokosuka O, *et al*. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med* 1991;**324**:1699–704.
12. **Laskus T,** Persing DH, Nowicki MJ, *et al*. Nucleotide sequence analysis of the precore region in patients with fulminant hepatitis B in the United States. *Gastroenterology* 1993;**105**:1173–8.
13. **Karayiannis P,** Alexopoulou A, Hadziyannis S, *et al*. Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: importance of host factors. *Hepatology* 1995;**22**:1628–34.
14. **Baptista M,** Kramvis A, Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. *Hepatology* 1999;**29**:946–53.
15. **Kao JH,** Chen PJ, Lai MY, *et al*. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology* 2003;**124**:327–34.
16. **Sakamoto T,** Tanaka Y, Orito E, *et al*. Novel subtypes (subgenotypes) of hepatitis B virus genotypes B and C among chronic liver disease patients in the Philippines. *J Gen Virol* 2006;**87**:1873–82.
17. **Blackberg J,** Kidd-Ljunggren K. Mutations within the hepatitis B virus genome among chronic hepatitis B patients with hepatocellular carcinoma. *J Med Virol* 2003;**71**:18–23.
18. **Takahashi K,** Akahane Y, Hino K, *et al*. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. *Arch Virol* 1998;**143**:2313–26.
19. **Takahashi K,** Ohta Y, Kanai K, *et al*. Clinical implications of mutations C-to-T1653 and T-to-C/A/G1753 of hepatitis B virus genotype C genome in chronic liver disease. *Arch Virol* 1999;**144**:1299–308.
20. **Tanaka Y,** Mukaide M, Orito E, *et al*. Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma. *J Hepatol* 2006;**45**:646–53.
21. **Yuen MF,** Sablon E, Yuan HJ, *et al*. Significance of hepatitis B genotype in acute exacerbation, HBeAg seroconversion, cirrhosis-related complications, and hepatocellular carcinoma. *Hepatology* 2003;**37**:562–7.
22. **Wai CT,** Greenson JK, Fontana RJ, *et al*. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003;**38**:518–26.
23. **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.
24. **Chen BF,** Liu CJ, Jow GM, *et al*. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. *Gastroenterology* 2006;**130**:1153–68.
25. **Yuen MF,** Sablon E, Tanaka Y, *et al*. Epidemiological study of hepatitis B virus genotypes, core promoter and precore mutations of chronic hepatitis B infection in Hong Kong. *J Hepatol* 2004;**41**:119–25.
26. **Chu CJ,** Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. *Gastroenterology* 2002;**122**:1756–62.
27. **Sumi H,** Yokosuka O, Seki N, *et al*. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003;**37**:19–26.
28. **Yuen MF,** Wong DKH, Zheng BJ, *et al*. Difference in T helper responses during hepatitis flares in HBeAg-positive patients with genotypes B and C: Implication for early HBeAg seroconversion. *J Viral Hep* 2006 (in press).
29. **Kidd AH,** Kidd-Ljunggren,K. A revised secondary structure model for the 3'-end of hepatitis B virus pregenomic RNA. *Nucleic Acid Res* 1996;**24**:3295–3301.
30. **Li J,** Buckwold VE, Hon MW, Ou JH. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. *J Virol* 1999;**73**:1239–1244.
31. **Buckwold VE,** Xu Z, Chen M, *et al*. Effects of a naturally occurred mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol* 1996;**70**:5845–51.
32. **Fang ZL,** Ling R, Wang SS, *et al*. HBV core promoter mutations prevail in patients with hepatocellular carcinoma from Guangxi, China. *J Med Virol* 1998;**56**:18–24.
33. **Chauhan R,** Kazim SN, Bhattacharjee J, *et al*. Basal core promoter, precore region mutations of HBV and their association with e antigen, genotype, and severity of liver disease in patients with chronic hepatitis B in India. *J Med Virol* 2006;**78**:1047–54.
34. **Ito K,** Tanaka Y, Orito E, *et al*. T1653 mutation in the box alpha increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. *Clin Infect Dis* 2006;**42**:1–7.