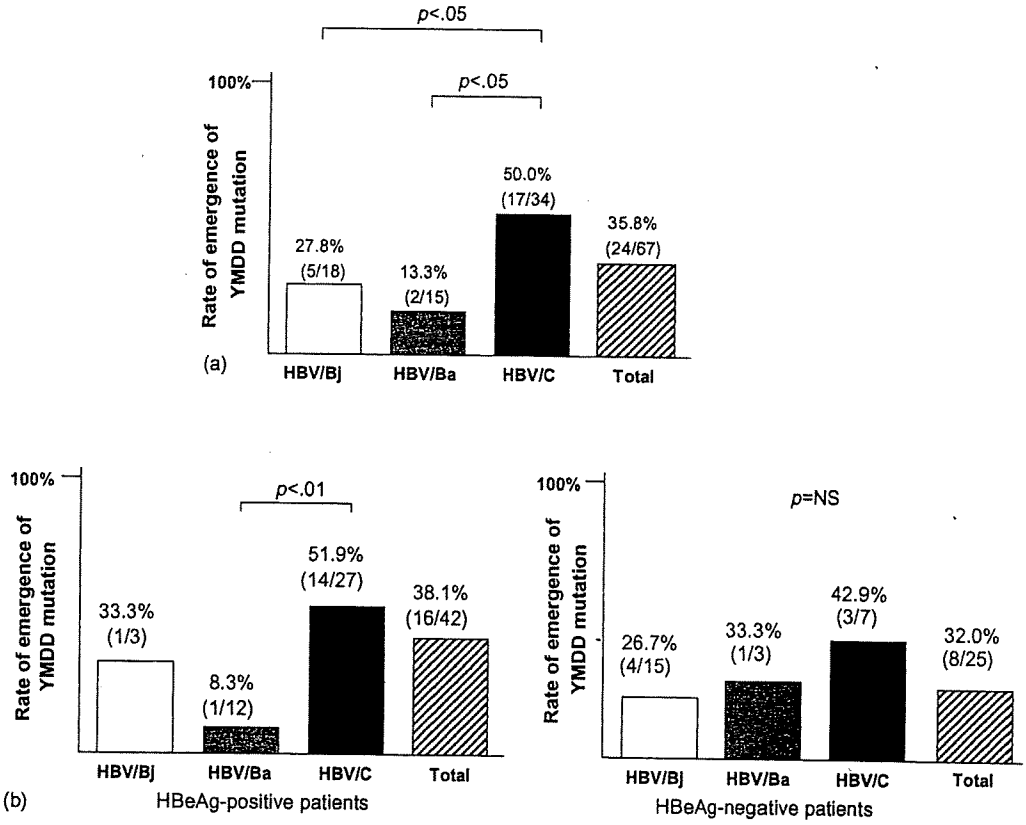

Fig. 2. The emergence rate of the YMDD mutation among three different genotype groups (a) and the emergence rate of the YMDD mutation stratified into HBeAg-positive and -negative patients (b).

were almost same between the HBV/Ba and HBV/C groups ($p < 0.01$; Fig. 2b). There was a trend for a higher proportion of the HBV/C group to have the YMDD mutation in the patients with negative HBeAg. However, the statistical differences were obscure because the numbers of the patients were small when they were stratified into HBeAg-positive or -negative groups.

The pretreatment factors predicting the emergence of the YMDD mutation were analyzed among three HBV genotype groups (Table 6). In the patients aged less than 40 years, 57.1% in the HBV/C group emerged the YMDD mutation compared to 12.5% in the HBV/Ba group ($p < 0.05$). In the patients with wild pre-C, the YMDD mutation emerged more frequently in the HBV/C group than in the HBV/Ba group ($p < 0.05$). In the patients with 7.5 or more log copies/mL of the HBV DNA level, the YMDD mutation emerged more frequently in the HBV/C group than in the HBV/Ba group ($p < 0.05$). Stratified in the positive HBeAg group, 58.9% in the HBV/C group with 7.5 or more log copies/mL of the HBV DNA level emerged the YMDD mutation compared to 40.0% in the HBV/C group with less than 7.5 log copies/mL of the HBV DNA level. However, in the HBV/Ba group with positive HBeAg, there was no difference of the frequency of the YMDD mutation between the higher or the lower HBV DNA groups.

The significant factor predicting emergence of the YMDD mutation by the stepwise logistic regression analysis was HBV/C, compared to HBV/Bj or HBV/Ba (odds ratio: 3.714, $p = 0.02$; Table 7). The other factors such as HBeAg status, HBV DNA levels and ALT levels at baseline were not significant.

3.4. Breakthrough hepatitis

Of all the patients, 11.9% had breakthrough hepatitis at year 2 during therapy (Fig. 3). There was no significant difference in the frequency of breakthrough hepatitis among the three HBV genotype groups.

4. Discussion

It is well-known that the clinical and virologic manifestations of patients with chronic HBV infection depend upon their HBV genotypes [5–8]. However, it is still controversial whether the response or resistance to lamivudine therapy is associated with HBV genotypes. To analyze the relationship between the response or resistance to lamivudine therapy and pretreatment factors, for the first time we conducted an age and gender matched case–control study among Japanese and

Table 6
Emergence of the YMDD mutation in patients among three HBV genotype groups

Characteristics	HBV genotype			p
	HBV/Bj (n=18)	HBV/Ba (n=15)	HBV/C (n=34)	
Age (year)				
<40	2/5 (40.0%)	1/8 (12.5%)	8/14 (57.1%) *	<.05
≥40	3/13 (23.1%)	1/7 (14.3%)	9/20 (45.0%)	.22
ALT				
<200	3/11 (27.3%)	2/11 (18.2%)	8/12 (66.7%) *	<.05
≥200	2/7 (28.6%)	0/4 (0%)	9/22 (40.9%)	.26
HBV DNA level (log copies/mL)				
<7.5	4/15 (26.7%)	1/2 (50.0%)	7/14 (50.0%)	.41
≥7.5	1/3 (33.3%)	1/13 (7.7%)	* 10/20 (50.0%)	<.01
Pre-C (nt.1896) mutation				
Wild	0/4 (0%)	1/10 (10.0%)	* 14/26 (53.8%)	<.05
Mutant	5/14 (35.7%)	1/5 (20.0%)	3/8 (37.5%)	.77
Core promoter (nt.1762/1764) mutations				
Wild	5/16 (31.3%)	1/13 (7.7%)	1/5 (20.0%)	.30
Mutant	0/2 (0%)	1/2 (50.0%)	15/27 (55.6%)	.31

*$p<0.05$

Table 7
Significant factors associated with emergence of the YMDD mutation by the stepwise logistic regression analysis

Factors	Odds ratio	95% CI	p-Value
HBV genotype			
Bj or Ba	1		
C	3.714	1.272–10.847	0.02

Chinese patients with HBV genotypes Bj, Ba and C. In this study, it was indicated that the significant factors predicting a favorable response to lamivudine therapy do not include the HBV genotype, but a higher pretreatment ALT level or negative HBeAg status and that the significant factor predicting emergence of the YMDD mutation is the HBV genotype (HBV/C).

The natural course of chronic HBV infection is usually affected by age and gender [19]. The natural seroconversion from positive HBeAg to anti-HBe is sometimes observed with the normalization of ALT in younger adults [8,10]. In addition, females often experience seroconversion after delivery [20]. So, age and sex are major factors influencing the natural course of chronic HBV infection. Thus, this report is a first case–control study to investigate the relationship between HBV genotype and response to lamivudine therapy.

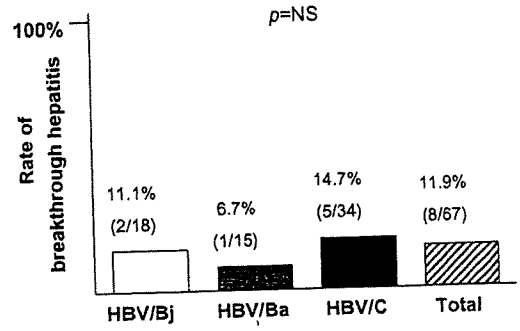

Fig. 3. The rate of breakthrough hepatitis among the three different genotype groups.

In all the patients, there was no significant difference of the rate of favorable response to the therapy among the three HBV genotype groups, even if the patients were stratified into an HBeAg-positive or -negative status. Although this study was an age and gender matched case–control study, the frequency of positive HBeAg was very low in the HBV/Bj group compared with the HBV/Ba or HBV/C groups because a higher proportion of patients with HBV/Bj have negative HBeAg status compared to those with HBV/Ba or HBV/C, as previously reported [12]. So, to investigate the relationship of the HBV genotype with the response to the therapy, we also compared the positive HBeAg patients between the HBV/Ba and HBV/C group. There was no significant difference in the response to the therapy between two groups. In addition, in the patients with negative HBeAg, there was also no significant difference of response to the therapy between the HBV/Bj and HBV/C group. The multiple logistic regression analysis revealed that a higher pretreatment ALT level and pre-C mutation were significant factors predicting complete response in the patients with positive HBeAg, and that higher ALT level and negative HBeAg status were significant factors predicting favorable response in all the patients.

Recently, some papers have reported the relationship between the HBV genotype and the response to lamivudine therapy, while the other paper denied this relationship [21–27]. Therefore, it is still controversial whether the

HBV genotype is associated with the response to lamivudine therapy for more than 1 year. Thus, we conducted a case–control study which revealed no association of HBV genotype with response to therapy. However, there remained to be a significant bias in the baseline features, such as a positive rate of HBeAg. It is ideal that age, gender and HBeAg status are matched. However, there are very few adult patients with HBV/Bj who are positive HBeAg and still have indication for lamivudine therapy, since those patients with HBV/Bj tend to experience seroconversion at a younger age. So, each paper might conduct various results in different backgrounds.

A higher pretreatment ALT level was one of the important predictors for a complete response to lamivudine therapy [28,29]. In this study, an ALT level greater than 200 IU/L and the pre-C mutation were significant factors in the patients with positive HBeAg. It was considered that the pre-C mutation is associated with the induction of a negative HBeAg status [30]. In all the patients, a higher ALT level and a negative HBeAg status were predicting factors for a favorable response, as previously reported [31,32].

It was reported previously that higher HBV DNA level or positive HBeAg status were significant predicting factors for the emergence of the YMDD mutation among the consecutive patients with HBV genotypes A–C in the cross-section studies [22,23]. In general, a higher proportion of Japanese patients with HBV/C tend to have a higher HBV DNA level and a positive HBeAg status which may lead a trend of emergence of the YMDD mutation [22,29,32]. In this study, there was also a trend of higher frequency of emergence of the YMDD mutation in the HBV/C group with positive HBeAg and a higher HBV DNA level. However, the significant predicting factor of emergence of the YMDD mutation was HBV genotype (HBV/C) by the multivariate analysis in the present study. It is still unclear why the viral factors, such as genotype, HBV DNA level or HBeAg status, may affect the chance of the emergence of the YMDD mutation. Further in vitro or vivo studies are warranted.

In this study, the number of patients (only eight) who developed breakthrough hepatitis was too small to investigate the factors affecting breakthrough hepatitis. If we were to observe this cohort of patients for a longer term, we may investigate more information, such as the L180M mutation or other mutations of the HBV genome [33].

The factors associated with a favorable response to lamivudine therapy or the emergence of the YMDD mutation may vary according to the baseline features of the patients studied. In this case–control study, a higher ALT level and negative HBeAg status were significant factors predicting a favorable response to lamivudine therapy, and HBV genotype C was a significant factor predicting the emergence of the YMDD mutation.

However, the patient numbers of this case–control study were not enough to confirm these results. Thus, further studies are warranted with a large number of the patients in each HBV genotype group.

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Molecular Tracing of the Global Hepatitis C Virus Epidemic Predicts Regional Patterns of Hepatocellular Carcinoma Mortality

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Background & Aims: **Molecular evolutionary analysis based on coalescent theory can provide important insights into epidemiologic processes worldwide. This approach was combined with analyses of the hepatitis C virus (HCV) epidemiologic-historical background and HCV-related hepatocellular carcinoma (HCC) in different countries.** ***Methods:*** **The HCV gene sequences of 131 genotype 1b (HCV-1b) strains from Japan, 38 HCV-1a strains from the United States, 33 HCV-1b strains from Spain, 27 HCV-3a strains from the former Soviet Union (FSU), 47 HCV-4a strains from Egypt, 25 HCV-5a strains from South Africa, and 24 HCV-6a strains from Hong Kong isolated in this study and previous studies were analyzed.** ***Results:*** **The coalescent analysis indicated that a transition from constant size to rapid exponential growth (spread time) occurred in Japan in the 1920s (HCV-1b), but not until the 1940s for the same genotype in Spain and other European countries. The spread time of HCV-1a in the United States was estimated to be in the 1960s; HCV-3a in the FSU, HCV-5a in South Africa, and HCV-6a in Hong Kong in the 1960s, mid-1950s, and late 1970s, respectively. Three different linear progression curves were determined by analysis of the relationship between HCV seroprevalence and HCC mortality in different geographic regions; a steep ascent indicated the greatest progression to HCC in Japan, a near horizontal line indicated the least progression in the United States and the FSU, and an intermediate slope was observed in Europe.** ***Conclusions:*** **These findings strongly suggest that the initial spread time of HCV is associated with the progression dynamics of HCC in each area, irrespective of genotype.**

Chronic hepatitis C virus (HCV) infection is an endemic disease affecting millions of individuals worldwide.[1,2] HCV infection usually is clinically mild, but the stages of more than 20% of patients can progress during the clinical course, occasionally culminating in hepatocellular carcinoma (HCC) over the course of 2–3 decades, the latter especially in Japan, Spain, and Italy.[3–6] Because the time lag between HCV infection and cancer development is several decades,[4] it is important to estimate the demographic history of HCV infection to predict the future burden of disease.

HCV is classified into 6 major genotypes.[7–9] Within the genotypes there are many subtypes, with varying geographic distributions and modes of transmission.[7] Subtypes 1a (HCV-1a), 1b, 2a, 2b, and 3a are distributed globally and account for the majority of HCV infections worldwide.[10,11] The rapid spread and global dissemination of these subtypes arises from their efficient parenteral transmission via transfusion of contaminated blood products, medical procedures, and illegal injection drug use. Other endemic and epidemic HCV strains are found in restricted geographic areas, including HCV-4a in Egypt, 5a in South Africa, and 6a in southeast Asia (Hong Kong).[7] Because HCV was not identified until 1989, it is difficult to estimate epidemic dynamics associated with the subtypes prevalent before this time.

Abbreviations used in this paper: FSU, former Soviet Union; IDU, injection drug use; *Sj*, *Schistosoma japonicum*; SRDT, single rate-dated tips.

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Table 1. Characteristics of the Population in Each Country

	United States	Japan *Sj* group	Japan non-*Sj*	Spain	FSU	Egypt	South Africa	Hong Kong
Number	38[a]	64[a]	67[a]	33	27[a]	47[a]	25	24
Mean age	48.2 ± 11.7	69.9 ± 7.7	67.2 ± 8.8	52.9 ± 11.0	24.7 ± 3.6	38.8 ± 9.0	56.8 ± 10.5	49.1 ± 15.4
Sex (M/F)	24/14	34/30	33/34	17/16	22/5	33/14	17/8	12/12
HCV subtypes	1a	1b	1b	1b	3a	4a	5a	6a
Divergence time[b]	1920	1812	1918	1892	1958	1902	1937	1963
Spread time	1965 (1958–1970)	1923 (1890–1937)	1940 (1933–1948)	1942 (1934–1955)	1963 (1958–1974)	1930 (1917–1940)	1955 (1948–1962)	1977 (1968–1982)
Growth rate, y^{-1}	0.15298 (0.106181–0.213943)	0.06667 (0.044460–0.094732)	0.12008 (0.098260–0.149888)	0.09715 (0.074642–0.140650)	0.15625 (0.121928–0.215722)	0.09004 (0.074114–0.115969)	0.19762 (0.107049–0.356198)	0.17120 (0.090839–0.280186)
Risk factors	IDU	PAT	IDU, transfusion, medical	IDU, transfusion, medical	IDU	PAT	Transfusion, medical	IDU

NOTE. 95% confidence intervals shown in parentheses.
PAT, parenteral antischistosomal therapy; transfusion, blood transfusion; medical, medical procedures.
[a]Eight sequences in the United States, 131 in Japan, 20 in the FSU, and 47 in Egypt were obtained from our previous data.[14–17]
[b]Divergence time indicates the most recent common ancestor (MRCA) point of each subtype.

However, using methods based on coalescent theory,[12] the epidemic history of HCV population can be reconstructed from observed genetic diversity of the viral strains.[13] Recently, the molecular clock theory has been applied successfully to estimate the molecular evolutionary rate in long-term serial serum samples obtained from HCV-infected patients in the United States and Japan; a 30-year lag in HCV spread time was shown between these countries. Insofar as a long duration of HCV infection is a critical determinant for the development of HCC, the molecular clock predicted that the incidence of HCC will increase in the United States over the next 2–3 decades[14] and approach the high rates currently observed in Japan.

In a previous study,[13] the spread of HCV-1a, 1b, 4, and 6 infections worldwide was analyzed by the use of HCV sequences obtained from DNA databases; however, corollary clinical and demographic data were limited. In the present study, new sequences from wider geographic regions and with more extensive clinical information are presented. Specifically, HCV-1a strains in the United States, HCV-1b in Japan,[14,15] HCV-1b in Spain, HCV-3a in the former Soviet Union (FSU),[16,17] HCV-4a in Egypt,[18] HCV-5a in South Africa, and HCV-6a in Hong Kong were analyzed by a coalescent-based approach using principles of both population genetics and mathematic epidemiology.[13] Furthermore, the relationship between the estimated spread time and HCC mortality in each country is discussed.

Materials and Methods

HCV Serum Samples From the United States, Spain, FSU, South Africa, and Hong Kong

To elucidate the epidemic history of HCV population in each country, 30 HCV-1a, 33 HCV-1b, 7 HCV-3a, 25 HCV-5a, and 24 HCV-6a samples were obtained from the following blood banks or hospitals, respectively: National Institutes of Health (United States), Hospital Vall d'Hebron (Spain), National Reference Laboratory of Ministry of Health (Uzbekistan), University of the Witwatersrand and National Health Laboratory Services/University of Cape Town (South Africa), and Queen Mary Hospital (Hong Kong). The samples were collected between 2000 and 2003. Along with our previous data,[14–18] the characteristics of the populations studied are shown in Table 1; the mean age of the Japanese population was the oldest and that of the populations from the FSU and Egypt was significantly younger. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethic Committees from each institution. Every patient gave written informed consent to participate in the virologic research of HCV at each blood center or hospital. None of the patients had been treated with interferon therapy for HCV infection.

HCV Gene Sequences

The HCV subtypes studied in each country are shown in Table 1, including 38 HCV-1a sequences from the Untied States (30 newly determined and 8 previously reported sequences[14]); 131 previously reported HCV-1b sequences including 64 from *Schistosoma japonicum* (*Sj*)-positive sera and 67 from *Sj*-negative (non-*Sj*) sera in Japan[15]; 33 HCV-1b sequences from Spain; 27 HCV-3a sequences from the FSU (7 newly determined and 20 previously reported sequences[16,17]); 47 previously reported HCV-4a sequences from Egypt[18]; 25 HCV-5a sequences from South Africa; and 24 HCV-6a sequences from Hong Kong. The GenBank/DNA Data Bank of Japan (DDBJ) accession numbers of the sequences obtained in the present study are AB204592–AB204708. Japanese HCV-1b and Egyptian HCV-4a sequences were obtained from our previous data (AB103424–AB103457 and AF271800–AF271812, respectively).[15,18] Available related sequences in Figure 1 were recruited from the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp).

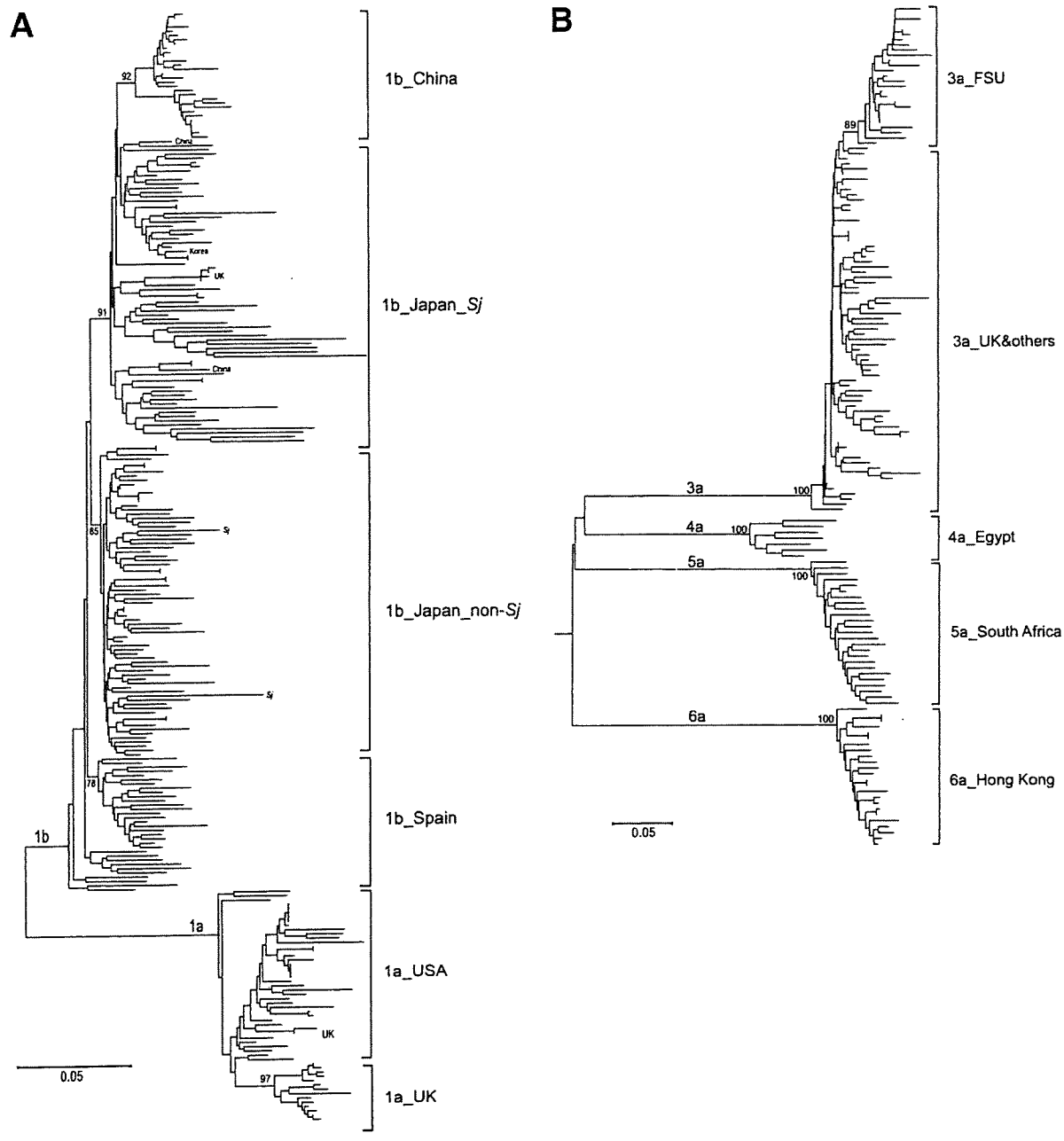

Figure 1. Phylogenetic trees constructed on NS5B sequences of (*A*) HCV-1a and HCV-1b strains and (*B*) HCV-3a, -4a, -5a, and -6a strains. The HCV-1a strains in the United States and HCV-1b strains in Japan (*Sj* and non-*Sj*) and Spain formed each significant cluster, and HCV-3a in the FSU, HCV-4a in Egypt, HCV-5a in South Africa, and HCV-6a in Hong Kong. The numbers in the tree indicate bootstrap reliability by the interior branch test. Exceptional strains were labeled according to their country of origin. Significant clusters were subjected to population history analyses.

Genotyping and Sequencing

Nucleic acids were extracted from the serum samples using a SepaGene RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. Viral RNA was reverse-transcribed to complementary DNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) as described previously.[19] A sequence spanning 339 nucleotides in the NS5B region was amplified by polymerase chain reac-

tion with primers described previously.[14] Polymerase chain reaction products were sequenced directly with Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in polymerase chain reaction, Platinum Pfx DNA Polymerase (Invitrogen Corp.) with a very high fidelity was used. The sequences determined were used to confirm HCV genotypes and to construct phylogenetic trees. To confirm the reliability of the phylogenetic tree, bootstrap reliability was performed by the interior branch test.[20] The overall mean genetic distances in all nucleotide positions and synonymous and nonsynonymous positions were estimated by Molecular Evolutionary Genetics Analysis (MEGA) software, available for free at www.megasoftware.net/index.html, version 3.1.[21]

Analysis of Isolation and Migration of HCV Sequences Among Countries

The phylogeny of the HCV-1a, -1b, and -3a sequences from all of the countries under investigation were estimated by the Neighbor–Joining method using the MEGA software (version 3).[21] The analysis of isolation and migration of the sequences was performed by using a method conducted by Nakano et al.[22] The migration histories of the HCV-1a, -1b, and -3a infections were inferred from the phylogeny by the parsimony method whereby the states are the sampling countries of each sequence and the state changes represent migration events. We calculated the expectations and the statistical significances based on the null distribution generated by 1000 times randomization of the sequences with fixing the topology of the phylogeny, where the null hypothesis is that all of the sequences were sampled from a hypothetic panmictic population, using software we developed ourselves.

Estimating Evolutionary Rates and Dating the Origin of HCV

A reconstructed tree was built on the NS5B sequence of 339 nucleotides by a heuristic maximum-likelihood topology search with stepwise addition and the nearest-neighbor–interchange algorithms. Tree likelihood scores were calculated using the HKY85+G method with the molecular clock enforced by Phylogenetic Analysis Using Parsimony (PAUP) (Sinauer Associates, Inc. Publishers, Sunderland, MA) version 4.0b8. By using the estimated topology, all possible root positions were evaluated under a single rate-dated tips (SRDT) model with the computer software TipDate v1.2 (University of Oxford, Oxford, UK) and the root that yielded the highest likelihood was adopted.[22] The program provided a maximum-likelihood estimate of the rate and also the associated date of the most recent common ancestor of the sequences, using a model that assumed a constant rate of nucleotide substitution. The molecular clock was tested by a likelihood ratio test between the SRDT model and a general unconstrained branch length model (different rate model). To confirm the reliability of the phylogenetic tree, bootstrap resampling tests also were performed 1000 times.

Demographic Model

As estimates of the demographic history, a nonparametric function, known also as the *skyline plot*, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time.[13,23] A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 (University of Oxford, Oxford, UK) to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences.[23] This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood-ratio tests of the parametric maximum-likelihood estimates.[24,25]

Results

Analysis of Isolation and Migration of HCV Sequences Among Countries

Preliminarily, all sequences generated in this study were subjected to phylogenetic analyses together with all previously reported sequences available from GeneBank/DDBJ. The most significant phylogenetic clusters containing a total of 325 representatives of the HCV endemic populations from different regions (Table 1) were determined and subjected to further maximum-likelihood phylogenetic analysis with enforced molecular clock, as previously described.[13,14] Figure 1 shows the phylogenies of the HCV-1a and -1b (Figure 1*A*) and HCV-3a, -4a, -5a, and -6a (Figure 1*B*) sequences obtained in the present study along with closely related sequences. As shown in Figure 1*A*, 4 clusters of HCV-1b sequences were found, and some sequences from China, Korea, and the United Kingdom belonged to the Japanese *Sj* group. Also, 2 *Sj*-positive strains clustered with non-*Sj* strains in Japan. To measure country-wise clustering statistically, the isolation and migration of HCV-1b sequences were analyzed by use of a parsimony method. The estimated number of changes in location between groups (ie, migration events) was 7 for HCV-1b, whereas the expected number of location changes for the 1000 simulated trees created with randomized locations was 40.38 for HCV-1b (Table 2). The observed number of migration events was significantly smaller ($P < .001$) than that expected under the null hypothesis of complete geographic mixing; therefore, this hypothesis can be rejected. This result suggests that there is considerable subdivision by location among the HCV-1b strains sampled. The parsimony analysis also provided clues about the movement of HCV-1b strains among the 6 groups. Table 2 shows the difference between the observed and expected number of changes for each pair of countries. In most cases, the observed number of migration events was

Table 2. Isolation and Migration of HCV Subtype 1b Among 6 Groups

	Japan (*Sj*)	Korea	China	United Kingdom	Spain	Japan (non-*Sj*)
Number of observed changes in tree (total, 7)						
Japan (*Sj*)	—	1	2	1	0	0
Korea	0	—	0	0	0	0
China	0	0	—	0	0	0
United Kingdom	0	0	0	—	0	0
Spain	0	0	0	0	—	0
Japan (non-*Sj*)	2	0	0	0	0	—
Number of expected changes per tree (total, 40.38)						
Japan (*Sj*)	—	0.09	3.30	0.48	3.22	8.16
Korea	0	—	0	0	0	0
China	0.02	0	—	0.01	0.63	1.36
United Kingdom	0.02	0	0.01	—	0.01	0.03
Spain	1.23	0.02	0.63	0.09	—	1.39
Japan (non-*Sj*)	10.27	0.13	4.26	0.65	4.31	—
P value (total, $P < .001$)						
Japan (*Sj*)	—	NS	NS	NS	.027	<.001
Korea	NS	—	NS	NS	NS	NS
China	NS	NS	—	NS	NS	NS
United Kingdom	NS	NS	NS	—	NS	NS
Spain	NS	NS	NS	NS	—	NS
Japan (non-*Sj*)	<.001	NS	.012	NS	.011	—

smaller than expected, indicating no significant movement of HCV-1b strains among these groups. For HCV-1a and -3a, the observed number of migration events was significantly smaller than the expected number, again suggesting significantly less overall migration than would be expected by chance.

The Origin of HCV Subtypes in Each Country

Figure 2 shows a comparison of genetic distances estimated on all synonymous and nonsynonymous nucleotide positions between HCV strains in each country.

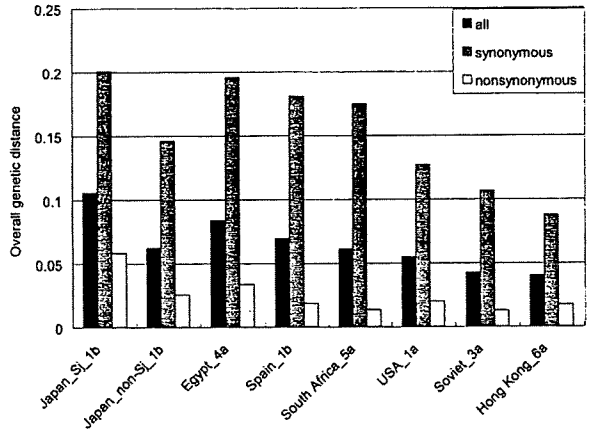

Figure 2. The overall mean genetic distances of all nucleotide positions, synonymous positions, and nonsynonymous positions in each country.

The genetic distances were the greatest among HCV-1b strains in the Japanese *Sj*-positive group, followed by the Egyptian HCV-4a, Spanish HCV-1b, South African HCV-5a, and HCV-1b strains in the Japanese non-*Sj* group. The genetic distances among the US HCV-1a, the FSU HCV-3a, and Hong Kong's HCV-6a were similar and comparatively smaller. These data indicate that the Japanese HCV-1b population is the oldest, and Hong Kong's HCV-6a population is the youngest among the populations studied.

The molecular evolutionary rate was estimated by 2 independent methods. First, our previous linear regression analyses indicated that a molecular evolutionary rate was 0.58 (range, .53–.61) $\times 10^{-3}$ nucleotide substitutions/site/y.[14] Second, TipDate v1.2 was used to compare the different-rate model with the single rate and SRDT models. The SRDT model provides an adequate fit to most datasets ($P > .05$); the rates were similar with the previous ones (range, 0.50–0.72) $\times 10^{-3}$ nucleotide substitutions/site/y. Although the SRDT model was rejected ($P < .05$) in 1 dataset, simulations have shown that even when the molecular clock is rejected, the confidence limits of the substitution rate sometimes may include the true rate.[26] Hence, we used the substitution rate estimated previously: 0.58 $\times 10^{-3}$ substitutions/site/y for the NS5B region.

Based on TipDate, to investigate the origin of HCV subtypes we converted the genetic distance in the phy-

logenetic tree into a timescale by using the molecular clock. According to the timescale, the most recent common ancestor for HCV subtypes in each country was established (Table 1); the divergence time of the most recent common ancestor for HCV-1b in the Japanese *Sj*-group was estimated before 1850, followed by that of Spanish HCV-1b strains (in 1892). The divergence time in the other groups was estimated to be in the 20th century. Notably, HCV-3a and HCV-6a strains have been introduced relatively recently into the FSU (in 1958) and Hong Kong (in 1963), respectively (Table 1).

Historical Analyses of the HCV Population by Using the Coalescent Theory

The level of population subdivision shown in the parsimony analysis described earlier suggests that much of the transmission of the sampled HCV strains occurred within the sampled groups. Therefore, the epidemic history of HCV strains in each group was estimated from separate trees.

Based on the phylogenetic analysis, the effective number of HCV infections through time, N(t), was analyzed using a skyline plot for the HCV strains. The parameters for several models in Genie v3.5 were examined. Time (t) then was transformed to year using the same rate, assuming the collecting time to be the present. Figure 3 shows the skyline plots and HCV population growth in each country according to a piecewise expansion growth model that was evaluated by likelihood-ratio testing (data not shown).[24,25]

$$N(t) = N(0)\exp(-rt) \quad \text{if } t < XN(0)\exp(-rX) \text{ otherwise}$$

This model describes the effective population size at time t in the past [N(t)], the population size at present [N(0)], the exponential growth rate (r), and the transition time (X). An expansion growth model gave the best fit for only the *Sj*-positive HCV-1b population, but because this likelihood ratio was almost the same as that of the piecewise expansion growth model, all populations were applied to the same piecewise expansion growth model. Our estimates of the effective numbers of HCV infections showed a transition from constant size to rapid exponential growth in the 1920s among the Japanese *Sj*-positive HCV-1b population, as we have reported earlier.[15] This indicates the oldest outbreak among all studied populations, whereas the exponential growth among the Japanese *Sj*-negative HCV-1b population was dated in the 1940s,[15] which is close in time to the HCV-1b populations in Spain (Figure 3*A*) and other European countries[27,28] and the HCV-4a population in Egypt.[18] The exponential growth of the HCV-5a population in South Africa occurred in the 1950s (Figure 3*B*), and comparatively recent HCV endemics were dated in the 1960s for both HCV-1a in the United States (Figure 3*C*) and HCV-3a in the FSU (Figure 3*D*), and in the late 1970s for HCV-6a in Hong Kong (Figure 3*E*).

The exponential growth rates also varied among the subtype populations (Table 1). The estimated rates for HCV-5a in South Africa, HCV-6a in Hong Kong, HCV-3a in the FSU, and HCV-1a in the United States were higher than those for HCV-1b in Japan, HCV-1b in Spain, and HCV-4a in Egypt. Hence, our findings indicate that the particular epidemics worldwide, associated with the corresponding HCV subtype, had different patterns in terms of divergence time, exponential spread time, and the dynamic growth rate. The different ages of the studied viral subpopulations, best assessed by synonymous genetic distance values, are shown in Figure 2.

Relative Population Size of HCV Subtypes in Each Country

Current estimates of the HCV subtype distribution in each sampled country were used to transform the epidemic histories shown in Figure 3 and previous data[14,15,18] to reflect the relative historical levels of HCV subtype infection in each country (Figure 4). As shown in Figure 4, 3 different growth patterns were found; one is the oldest historical pattern of HCV-1b in the Japanese *Sj* group, and the second group consists of HCV-1b in the Japanese non-*Sj* group, HCV-1b in Spain, and HCV-4a in Egypt. The last group, with the latest exponential growth, includes 4 different subtypes in independent countries: HCV-1a in the United States, HCV-3a in the FSU, HCV-5a in South Africa, and HCV-6a in Hong Kong.

Discussion

The world map of HCC occurrence still contains many wide gaps owing to difficulties collecting exact clinical and epidemiologic data from many countries. The positive correlation between HCV seroprevalence and HCC mortality was documented in a recent European report.[29] However, most of the HCV seroprevalence data worldwide are derived from studies of blood donors who represent a selective relatively low-risk, younger population. Therefore this approach underestimates the absolute burden of infection[30] and complicates a comparative analysis of the results obtained in different countries, especially for the age-specific HCV seroprevalence.

Because HCC is associated directly with the duration of HCV infection in a given carrier, the time of exposure

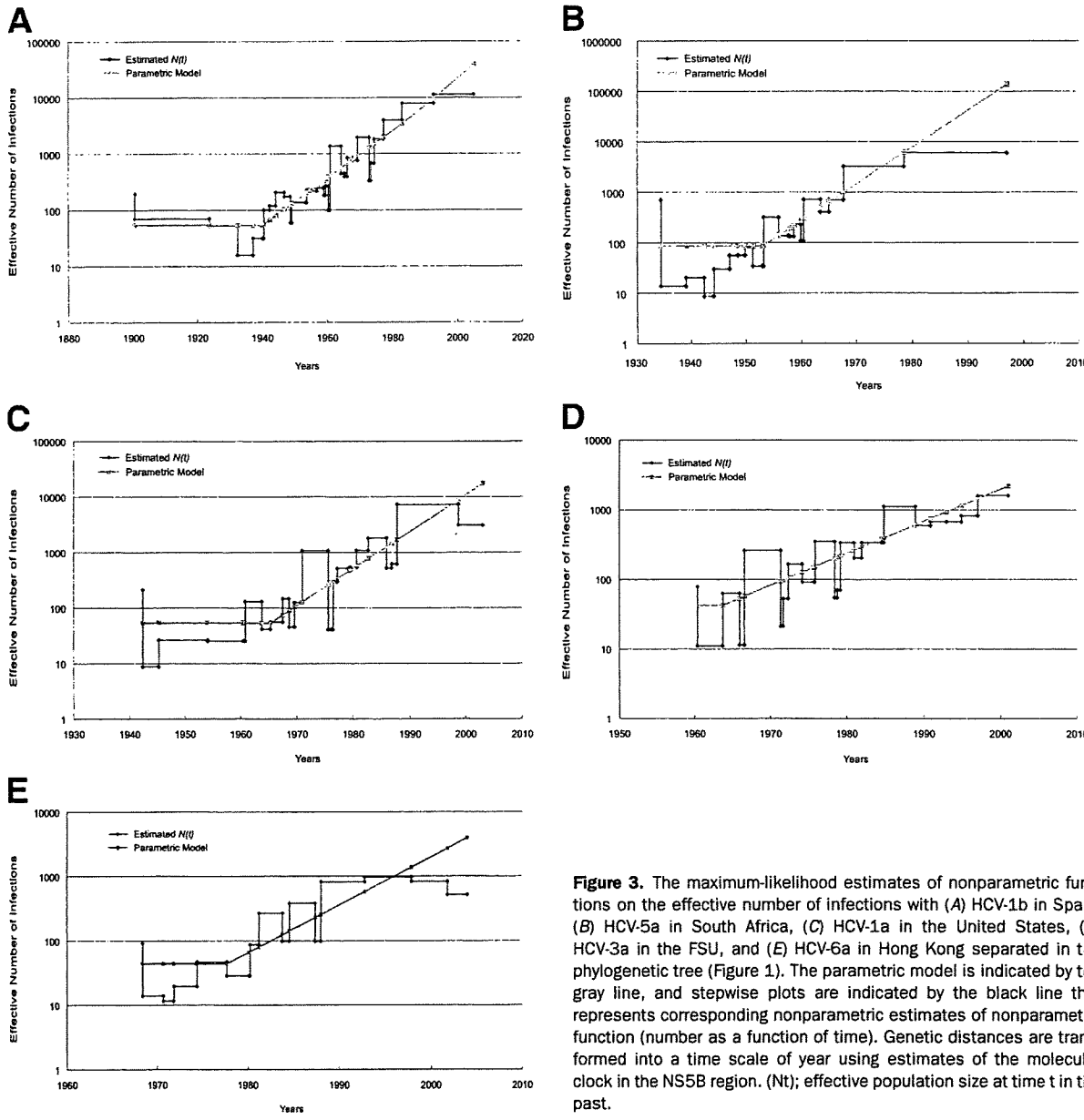

Figure 3. The maximum-likelihood estimates of nonparametric functions on the effective number of infections with (*A*) HCV-1b in Spain, (*B*) HCV-5a in South Africa, (*C*) HCV-1a in the United States, (*D*) HCV-3a in the FSU, and (*E*) HCV-6a in Hong Kong separated in the phylogenetic tree (Figure 1). The parametric model is indicated by the gray line, and stepwise plots are indicated by the black line that represents corresponding nonparametric estimates of nonparametric function (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region. (Nt); effective population size at time t in the past.

to HCV infection is another critical determinant of HCC incidence at the population level. To investigate further the relative role played by the duration of HCV infection, we analyzed current and previously published data from populations throughout the world for the general and age-related HCV seroprevalence, the estimated time of HCV exponential spread, the association with primary risk factors for virus transmission, and HCC mortality.

The past population dynamics of a virus can be inferred from viral gene sequence data using a population genetic model called *the coalescent theory*.[31] The coalescent framework requires a demographic model, denoted N(t), that describes the effective population size through time. A demographic model based on neutral theory, which infers that a constant-size population in the past changes to grow exponentially starting at a specific point in time,[13,24,25] was applied to investigate the HCV population history worldwide. Various HCV genotypes and subtypes circulating in the geographic regions were studied. However, in each

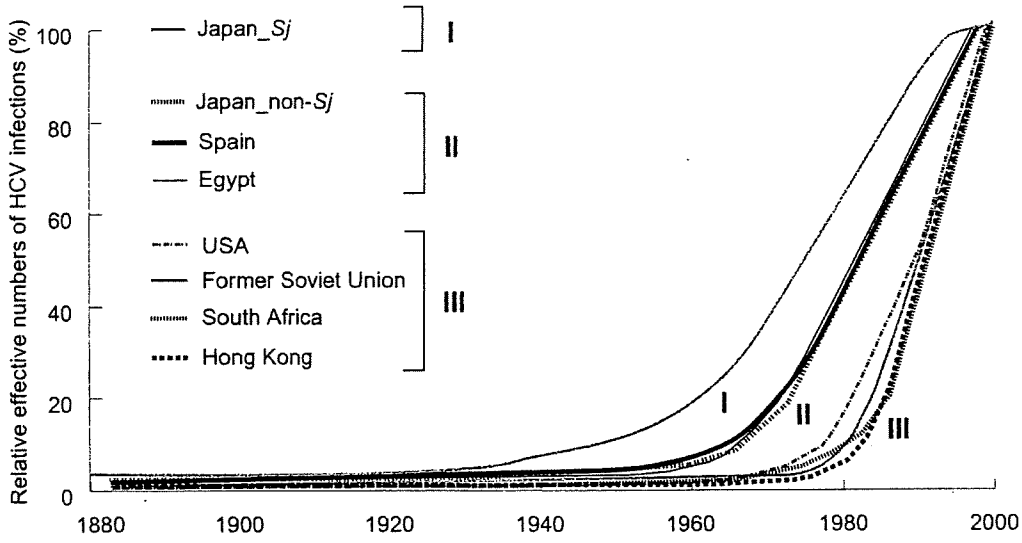

Figure 4. Relative effective numbers of HCV infections in each country. Three different growth patterns were defined as I, II, and III.

country we selected a single subtype representative of the subpopulation conforming to the largest indigenous phylogenetic cluster; this cluster would play an important role in the particular epidemic network (see later), and at the same time fulfill the requirements for calculations by the methods used.[13,14]

In Japan, HCC incidence is exceedingly high and comparatively well studied. Chronic HCV infection is responsible for the majority of HCC cases in Japan even though the overall HCV seroprevalence is relatively low at 1.4%. Notably, however, the highest incidence of HCC occurs in persons older than age 70 (>150 per 100,000 men), in whom HCV prevalence is correspondingly high at approximately 7%.[32] HCV-1b is the predominant genotype in Japan and 2 relatively distinctive waves of its spread were described recently in detail.[15] Briefly, the first wave was associated with treatments for *Sj* beginning in 1921,[33] and the second wave coincided with World War II (1940s) when war-associated injection drug use (IDU), blood transfusions, and medical procedures intensified and contributed to HCV transmission.[15,32,34]

In Europe, HCC mortality rates are highly variable in different countries,[35] and a positive correlation with HCV seroprevalence was documented recently (summarized in Figure 5).[29] Detailed data were obtained in France using a model based on epidemiologic analyses of HCV-infected patients and mortality data from national statistics surveys that allowed tracing of the HCV epidemic back to the 1940s.[27] These data were similar to our results estimated for the epidemic in Spain, where exponential growth of the virus population began after 1940. These data are consistent with the likelihood of HCV transmission during and after the Spanish Civil War in the late 1930s, and with the widespread use of shared needles for penicillin treatments in the early 1940s. In Italy, as in Japan, the prevalence of anti-HCV was the highest among elderly people (age, 75–79 y) suggesting a cohort effect dating to exposure during World War II.[28]

Indeed, in the United States, prevalent subtypes in the general population are HCV-1a (57%) and 1b (17%)[36]; an association of the HCV-1a epidemic with IDU has been reported.[37] Our estimates of the epidemic history of HCV-1a in the United States are consistent with the onset of injection opiate use between 1950 and 1960 and more widespread use in the late 1960s and 1970s.[38] The relative importance of IDU and blood transfusion associated with HCV transmission in the United States has changed over time,[39] and IDU has been the predominant mode since the 1970s.[40]

HCV-1a and 3a are the most prevalent among patients with a history of IDU and appear to be increasing in prevalence worldwide.[10,16,41] In the FSU, HCV-1b was predominant in all population groups until the late 1970s, and was associated primarily with medical procedures and unscreened blood products during and after World War II. After blood screening started in the 1990s, an HCV genotypic shift occurred first in the drug-addicted population and, more recently, has been observed in the general population (Musabaev EI, personal communication). HCV-3a represents a compara-

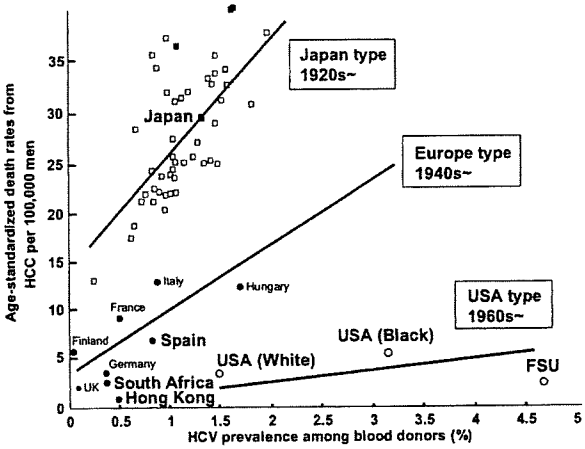

Figure 5. Linear relationship between HCV seroprevalence and HCC annual mortality rates constructed using available data from different countries. Three different patterns were observed: Japan type, European type, and US type. Data from European countries were obtained from a previous report.[29] Japan: HCV seroprevalence was approximately 1.4% in the general population from 1988 to 1992. The age-adjusted mortality rate of HCC among Japanese men was approximately 30 per 100,000 during 1990 (■),[32] which is close to the mean age-adjusted death rates of HCC among the 48 prefectures (27.3 per 100,000 persons in 2001). □ and ■, (*S*/-endemic areas) show the relationship between the age-adjusted annual mortality rates of primary liver cancer and anti-HCV among the general population older than 40 years of age in 2002, and a positive significant correlation was found.[50] Spain: Data were obtained mainly from a previous European study,[29] which was consistent with another recent study performed in Valencia (Spain) (2,172,796 inhabitants in 1998: 1,060,156 males and 1,112,640 female in 2000[51]); the estimated incidence of HCC was 8.2 cases per 100,000 inhabitants. United States: HCV seroprevalence was 3.2% among non-Hispanic blacks and 1.5% among non-Hispanic whites from 1988 to 1994.[36] The age-adjusted mortality rate of HCC among black men was 6.0 per 100,000 in 1991–1995 and 3.4 per 100,000 among white men.[52] FSU: HCV seroprevalence was approximately 5% in blood donors, and the HCV-related HCC mortality rate was estimated at approximately 2.5 per 100,000 men in 1990 (Ruzibakiev R and Musabaev M, personal communication). South Africa: The prevalence of HCV infection among blood donors was .41% during 1992 and 1994.[53] The HCV prevalence was .75% among blacks and .16% among non-Hispanic whites.[54] The age-adjusted incidence of HCC was approximately 30 per 100,000[47] and HCV-related HCC was approximately 10%, indicating 3 per 100,000 during 1986 and 1990 from HCV-related HCC. Hong Kong: the prevalence of HCV was .5% in the general population, and the estimated HCV-related HCC mortality was much rarer than HBV-related HCC mortality.[42,47]

tively recent and growing epidemic associated with IDU in the FSU.[16,41] Our estimates traced that HCV-3a spread from the 1960s, coinciding with the start of mass vaccination campaigns against measles when there were no disposable syringes in the FSU. However, a rapid increase of the estimated viral population size started in the late 1970s, probably associated with the expanded IDU network that was stimulated dramatically by the Afghan War (Ruzibakiev R, personal communication, web information-analytical recourses: http://druglibrary.org/ and http://www.irinnews.org/).

Similar data were obtained from Hong Kong where 2 relatively distinct HCV epidemics were observed: HCV-1b was predominant among older patients with chronic hepatitis whereas HCV-6 was detected predominantly in younger patients with a history of IDU and in young patients with thalassemia major.[42] Age-related prevalence and results of our estimation suggested that HCV-6 infection represents a recent growing epidemic that will have an increasing influence on HCC incidence in the future.

In South Africa (sub-Saharan Africa) and Hong Kong, hepatitis B virus (HBV) infection is much more prevalent than HCV infection and the epidemiology of HCV infection is less well characterized. Our results indicate that both genetic diversity and the growth rate of the HCV-infected population in South Africa are higher than would be expected. This may be explained by the fact that most studied samples were obtained from elder patients in this region, including 8 of 24 patients with HCC (all male blacks; mean age, 65.3 ± 3.3 y). The major risk factors for HCV transmission in South Africa include contaminated blood transfusions and medical procedures performed with inadequately sterilized shared instruments.

In analyzing data on the association between HCC mortality and HCV seroprevalence, 3 general patterns were observed (Figure 5). The first pattern was observed in Japan (Japan type), where the data presented from all geographic parts of the country indicated that HCC mortality was the highest in the world, whereas HCV seroprevalence was comparatively low. The second pattern was observed in European countries (Europe type), where the HCC incidence had a more direct association with HCV prevalence as previously reported,[29] although recent reports in some European countries such as Spain indicated that HCC mortality in men older than 60 years overlapped with the high mortality rate observed in Japan.[43,44] The third pattern was observed in the United States (≈5 million HCV-infected) and the FSU (USA type) with HCV seroprevalence comparable with Japan, but low HCC mortality rates. The greatest progression to HCC in Japan, indicated by the steep ascent in Figure 5, is consistent with the previously reported high annual incidence of HCC (7.9%) among patients with stage F4 fibrosis.[45] Because the HCV epidemic in Japan began early, resulting in a large cohort with a very long duration of infection, more patients in Japan have reached the stage of advanced fibrosis that increases their likelihood of developing HCC. Thus, the slope of the curve is influenced strongly by the age-specific prevalence and the duration of HCV infection. This would predict that as

age and the duration of infection increases in other populations, including the United States, the slope of the HCC mortality curve will increase steeply. The patterns observed in South Africa and Hong Kong, characterized by comparatively low HCV prevalence and low HCV-related HCC mortality, were intermediate between that in Europe and the United States. However, precise data regarding the association of HCV with HCC incidence are not available in these regions, and most cases of HCC in Hong Kong and South Africa probably were associated with HBV infection, which is highly prevalent in these countries.[46,47] A total of 80.3% and 62.7%–70.5% were positive for hepatitis B surface antigen in Hong Kong and South Africa, respectively, whereas a relatively low prevalence of HBV-related HCC (≈20%) was reported in the United States, Europe, and Japan.[48] The relative estimated viral population growth dynamics obtained in different countries are shown in Figure 4. All estimated data were separated into 3 groups according to the time virus exponential spread began; the first rapid spread was associated with schistosomiasis treatments in Japan (*Sj* group) in the 1920s; the second wave occurred in Japan (non-*Sj* group), Spain, and Egypt in the 1940s; and the third wave occurred in the 1960s involving the United States, the FSU, South Africa, and Hong Kong. When we combine these data (Figures 4 and 5), a putative picture of the global HCV epidemic emerges that potentially would allow predictions of HCC dynamics in any region of the world. However, no data on HCC mortality were available from Egypt, where the high general HCV prevalence and the estimated spread time suggest that Egypt might have a very high HCC mortality rate, comparable with that in Japan. A recent report from Egypt showing a high HCC incidence among chronic liver disease patients (4.7%)[49] is consistent with our hypothesis.

It could be argued that our results may not represent the community distribution of HCV strains because of the vast predominance of tertiary institution referral. However, we found no significant difference in the sequences of HCV isolated from blood donors and patients with chronic liver diseases, indicating that little bias would be expected to occur in our molecular evolutionary analyses. Hence, an advantage of the coalescent approach to molecular epidemiology used here is that the entire history of a transmission cluster can be investigated using a relatively small sample of gene sequences. In addition, this approach allows a more complete analysis of global HCC dynamics and a prediction of HCC occurrence rates over time. The implications are that Japan has set the model for HCV-related HCC and that the high HCC incidence in Japan may be replicated by the rest of the world as their HCV-infected population ages and the duration of HCV infection approaches that currently observed in Japan. Clearly, there is a need not only to prevent new HCV infections, but also to eradicate chronic infections with appropriate treatment strategies. Unfortunately, the current costs of antiviral therapy are prohibitive for many of the regions where HCC is likely to escalate in the future. High priority must be given to global prevention of HCV-related cirrhosis, the almost universal predecessor to HCV-induced HCC.

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Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection

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Abstract

Background: Prolonged lamivudine therapy has two major problems: breakthrough hepatitis during treatment and relapse of aminotransferase (ALT) after cessation of the therapy. The aim of this study was to examine factors that could predict ALT flare after stopping lamivudine therapy.
Methods: We analyzed 22 Japanese patients with chronic hepatitis B infection, in whom lamivudine therapy was stopped after HBV DNA level had been gone undetectable (<3.7 LGE/ml) during at least six consecutive months. The post-treatment followed up was carried for 28 months in median (range 9–41). HBV core-related antigen (HBcrAg) assay was assessed using newly developed assay.
Results: After cessation of lamivudine therapy, 11 patients (50%) had relapsed (reactivation of serum ALT >80 IU/l, relapsers) and remaining 11 (50%) did not relapse (non-relapsers). In the univariate comparison of relapsers versus non-relapsers, HBcrAg level at lamivudine cessation point (4.5 ± 1.0 versus 3.4 ± 0.9; $p = 0.0145$) has been shown as a significant predictive factor for non-relapse. All patients with HBcrAg <3.0 log U/ml at the cessation point had no ALT flares. Multivariate analysis on effects of 10 factors (age, sex, cirrhosis, pretreatment ALT level, HBV DNA level, HBcrAg level, mean months till undetectable HBV DNA, duration of undetectable HBV DNA and HBcrAg level at lamivudine cessation point), indicated that HBcrAg level at lamivudine cessation point <3.4 log U/ml was the only independent predictive factor for absence of the post-treatment relapse.
Conclusions: HBcrAg level at lamivudine cessation point might be useful as a prognostic predictor of response to lamivudine therapy cessation. The measurement of HBcrAg is a useful additional test for monitoring chronic HBV infection.

Keywords: Hepatitis B virus; Hepatitis B virus core-related antigen; Lamivudine; Chronic hepatitis B virus infection

1. Introduction

Chronic hepatitis B virus (HBV) infection remains to be one of the major global health problems, affecting an estimate of 400 million people worldwide [1]. In a significant proportion of cases, chorinic infection progress to cirrhosis and liver failure as well as hepatocellular carcinoma (HCC) [2]. Lamivudine monotherapy is considered to be a therapeutic option for patients with chronic hepatitis B, irrespective of hepatitis B e antigen (HBeAg) status [3]. Viral resistance or viral breakthrough frequently associated in with prolongation of lamivudine therapy and caused by drug-resistant HBV mutants [4–7]. The breakthrough hepatitis may develop acute

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hepatitis flares, hepatic decompensation, and fatal hepatic failure [8–10]. Furthermore, the occurrence of lamivudine resistance increases the risk of HCC in antibody to HBeAg (anti-HBe) positive cirrhosis [11]. Hence, the Asia–Pacific consensus on the prevention and management of chronic hepatitis B and C has recommended that full HBeAg seroconversion, defined as undetectable HBeAg and HBV DNA with reappearance of anti-HBe, may be considered a suitable end point for lamivudine therapy. It is also recommended that lamivudine therapy should be maintained for 4–6 months after achieving HBeAg seroconversion to decrease the chance of posttreatment relapse [12].

Our recent study showed that highly sensitive HBV real-time detection direct test (<0.7 log IU/ml) can predict non-relapse after cessation of lamivudine monotherapy for chronic HBV infection [13], but the HBV real-time detection direct test is technically complicated and costly. On the other hand, an enzyme immunoassay (EIA) is a relatively simple method and provides a low cost and quantitative analysis with high reproducibility might have advantages over nucleic acid amplification assay if implied as an alternative. Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed for detection of hepatitis B virus core-related antigen (HBcrAg), which have positive correlation with HBV DNA levels in serum [14,15] and hepatocytes (submitted for publication). Furthermore, HBcrAg was shown as a clinically significant marker in monitoring the antiviral effect of lamivudine therapy [15,16]. Present study evaluated the usefulness of monitoring HBcrAg as a prognostic predictor of lamivudine therapy cessation in HBV-infected patients.

2. Patients, materials, and methods

2.1. Patients

A total of 22 patients with chronic hepatitis B, who were receiving lamivudine therapy during 1996 and 2005 at Nagoya City University Hospital (Nagoya) were enrolled in this study. Cirrhosis was determined mainly by ultrasonography (coarse liver architecture, nodular liver surface, and blunt liver edges) and evidence of hypersplenism (splenomegaly on ultrasonography), a platelet count <100,000 mm^{-3}, or a combination thereof. Written informed consent was obtained from each patient. All were administered 100 mg of lamivudine per day for ≥6 months (median, 12 months; range, 6–43 months). The lamivudine therapy was stopped for each of the patients who had HBV DNA level maintained to be undetectable (<3.7 log genome equivalents [LGE]/ml), as measured by transcription-mediated amplification–hybridization protection assay (TMA–HPA; Chugai Diagnosis science) during >6 consecutive months of the follow up, whereas serum alanine aminotransferase (ALT) level did not exceed 40 IU/l (i.e., the upper limit of normal) during same period. In the HBeAg-positive patients, in addition to undetectable HBV DNA and normal ALT level, HBeAg-seronegative maintained for ≥6 months was used as another criterion for stopping lamivudine therapy.

2.2. Methods

Serum HBsAg, HBeAg, and anti-HBe were measured by commercially available chemiluminescent enzyme immunoassay kit (Fujirebio Inc., Tokyo, Japan). The levels of HBV DNA in serum were determined using the TMA–HPA assay (Fujirebio Inc., Tokyo, Japan), with detection range is 3.7–8.7 LGE/ml and ALT were tested every months. HBcrAg was measured in serum using the CLEIA described previously [14]. Briefly, 150 μl of serum was incubated with 150 μl pretreatment solution containing 15% sodium dodecylsulfate at 60 °C for 30 min. After incubation at 60 ± 4 °C for 30 min, 150 μl pretreated specimen was added to the ferrite micro-particle (coated with monoclonal antibodies (HB44, HB61, and HB114) against denatured HBc and HBe antigens) solution in assay tube. After washing, two other alkaline phosphatase-labelled monoclonal antibodies against denatured HBcAg and HBeAg were added as secondary antibodies. Two hundred microliters substrate (AMPPD: (3-(2′-spiroadamantan)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt) (Applied Biosystems, Bedford, MA, USA) solution was added and the assay tube was incubated for 5 min at 37 °C. HBcrAg assay with The relative chemiluminescence intensity was measured with chemiluminescent enzyme immunoassay (CL-EIA) system for fully automated Lumipulse f CL-EIA analyzer (Fujirebio Inc., Tokyo, Japan), and the HBcrAg concentration was estimated by comparison to a standard curve generated using recombinant HBeAg. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml of HBcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

The end point of follow up was relapse after cessation of lamivudine therapy. Relapse was defined as reappearance of serum HBV DNA (≥3.7 LGE/ml, as measured using the TMA–HPA assay) plus a reactivation in the serum ALT to >80 U/l.

2.3. Statistical evaluation

Data are expressed as mean ± S.D. or median (range). The primary focus of this analysis was to compare patients who experienced relapse with those who did not. The Mann–Whitney *U*-test was utilized to analyze quantitative data, the χ^2 test was used for qualitative data. The effect of age, sex, prevalence of cirrhosis, presence of HBeAg, pretreatment ALT level, pretreatment HBV DNA level, pretreatment HBcrAg level, mean months till undetectable HBV DNA, duration of HBV DNA level of <3.7 LGE/ml, duration of lamivudine therapy, and HBcrAg level at cessation point were assessed by logistic regression analysis. *p*-Value of less than 0.05 were considered to be statistically significant.

Table 1
Characteristics of nominated 22 hepatitis B virus (HBV)-infected patients before lamivudine therapy

Characteristic	Value
Age, mean (years) ± S.D.	45.0 ± 9.3
Male sex, no. (%) of patients	12 (55)
HBeAg positive, no. (%) of patients	6 (27)
Genotype/subgenotype (Bj:Ce)	2:20
Cirrhosis, no. (%) of patients	4 (18)
Pretreatment ALT level, median U/l (range)	118 (47–554)
Pretreatment HBV DNA level, median LGE/ml (range)	6.9 (4.1–8.6)
Pretreatment HBcrAg level, median log U/ml (range)	5.8 (4.2–8.6)
Duration of lamivudine therapy, median months (range)	12 (6–43)
Posttreatment follow-up duration, median months (range)	28 (9–41)

Statistical analyses were performed using STATA Software (Stata Corporation, Texas, USA) version 7.0.

3. Results

Table 1 shows the pretreatment demographic and clinical characteristics of the 22 HBV-infected patients who received lamivudine therapy and stopped it. Before the lamivudine administration, serum HBV DNA was detectable in all patients, and HBeAg was positive in six (27%) of the them. HBV genotype B was determined in two patients and remaining 20 had genotype C-infection. All of the six HBeAg-positive patients stopped lamivudine therapy when their HBeAg became negative and their ALT levels were normalized and their HBV DNA levels were undetected by TMA–HPA (<3.7 LGE/ml) for more than 6 months. For the remaining 16 HBeAg-negative patients lamivudine administration was stopped after ALT levels were normalized and their HBV DNA levels were undetected by TMA–HPA for more than 6 months. After cessation of lamivudine therapy, 11 patients (50%) experienced relapse (relapsers) and 11 patients (50%) did not (non-relapsers). All patients

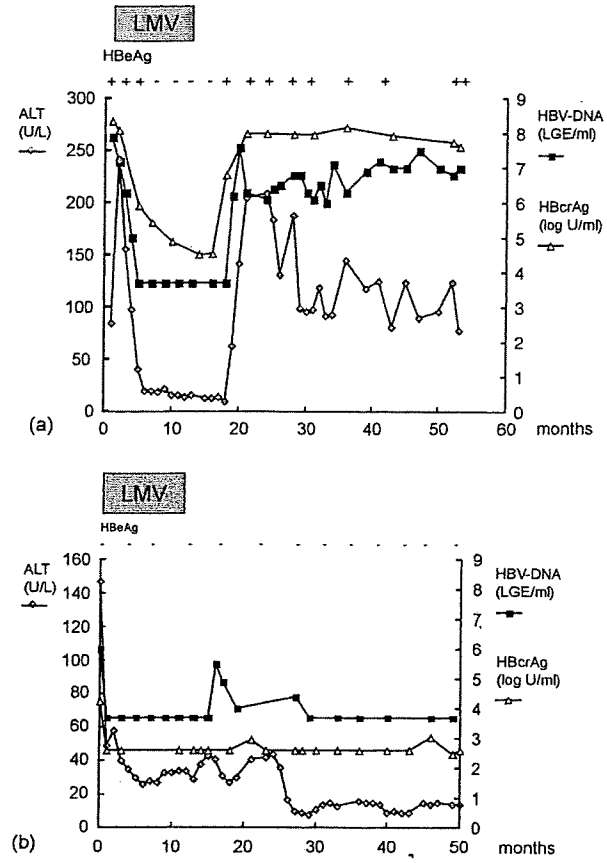

Fig. 1. Featured changes in HBcrAg, HBV DNA and ALT levels observed during clinical courses in (a) patient (no. 1) with ALT relapse and (b) patient (no. 2) without ALT relapse after the cessation of lamivudine therapy.

were then clinically observed after cessation of lamivudine monotherapy (median duration of posttreatment monitoring, 28 months; range, 9–41 months) (Table 2).

Fig. 1 shows typical cases with and without relapse after cessation of lamivudine therapy. Case no. 1 (Fig. 1a): a 46-year-old male with chronic hepatitis B (genotype C). Before receiving lamivudine, his laboratory data were as

Table 2
Characteristics patients with and without relapse after lamivudine therapy

Characteristic	Relapsers ($n = 11$)	Non-relapsers ($n = 11$)	p
Age, mean (years) ± S.D.	44.8 ± 9.3	45.2 ± 9.8	2.82
Male sex, no. (%) of patients	4 (36)	8 (72)	0.08
HBeAg positive, no. (%) of patients	3 (27)	3 (27)	>0.999
Genotype Bj, no. (%) of patients	0 (0)	2 (18)	0.48
Cirrhosis	3 (27)	1 (9)	0.59
Pretreatment ALT level, mean (U/l) ± S.D.	118 ± 78	223 ± 159	0.11
Pretreatment HBV DNA, mean (LGE/ml) ± S.D.	6.6 ± 1.1	6.5 ± 1.3	0.87
Pretreatment HBcrAg level, mean (log IU/ml) ± S.D.	6.0 ± 1.1	5.5 ± 1.3	0.26
Mean months till undetectable HBV DNA (months)	2.3 + 1.6	2.2 + 1.7	0.90
Duration of HBV DNA level of <3.7 mean LGE/ml (months)	13.2 ± 4.2	13.5 ± 11.0	0.15
HBcrAg level at lamivudine cessation point, mean (log U/ml) ± S.D.	4.5 ± 1.0	3.4 ± 0.9	0.0145
Duration of lamivudine therapy, median months (range)	13 (11–28)	11 (6–46)	0.18
Posttreatment follow-up duration, median months (range)	29 (9–39)	23 (9–36)	0.32

follows: HBeAg positive; anti-HBe negative; ALT 242 IU/l; HBV DNA 7.9 LGE/ml; HBcrAg 8.36 log U/ml. The level of ALT decreased and was normalized at 4 months after treatment initiated. The level of HBV DNA decreased rapidly to undetectable at 4 months after treatment initiated, while HBcrAg levels decreased more slowly. At 7 months after treatment initiated, HBeAg became negative. Lamivudine therapy was stopped at 17 months after treatment initiated (undetectable HBV DNA for 13 months). At the point, HBcrAg level was 4.54 log U/ml. One month after treatment stopped, HBeAg was reemerged and HBV DNA was 6.2 LGE/ml. Two months after treatment stopped, the patient experienced ALT relapse (200 IU/l). Case no. 2 (Fig. 1b): a 59-year-old male with chronic hepatitis B (subgenotype Bj). Before receiving lamivudine, his laboratory data were as follows: HBeAg negative; anti-HBe positive; ALT 147 IU/l; HBV DNA 6.0 LGE/ml; HBcrAg 4.25 log U/l. The level of ALT decreased and was normalized at 1 month after treatment initiated. The level of HBV DNA decreased rapidly and became undetectable at 1 month after treatment initiated. HBcrAg level also decreased to less than 3.0 log U/ml next month after treatment start. Lamivudine therapy was stopped at 14th months after treatment initiation. At the point, HBcrAg level was less than 3.0 log U/ml. Next month after treatment stopped, HBV DNA became detectable (5.5 LGE/ml) but has decreased; at 6 months after treatment stopped, the level of the HBcrAg remained less than 3.0 log U/ml. ALT was normal during 36 months after treatment stopped. Interestingly, two patients with subgenotype Bj in this study had no ALT relapse after the cessation of lamivudine therapy, probably due to lower pre-treatment HBV DNA and HBcrAg levels as well as persistently undetectable post-treatment HBcrAg levels (<3.0 log U/ml).

In a univariate comparison of patients who experienced relapse (relapsers) with those who did not (non-relapsers), a predictive factor for the non-relapse after cessation of lamivudine was HBcrAg level at the end of treatment (4.5 ± 1.0 versus 3.4 ± 0.9; $p = 0.0145$). The association with age, sex, presence of HBeAg before treatment, presence of cirrhosis, pretreatment ALT level, pretreatment HBV DNA level, pretreatment HBcrAg level, mean months till undetectable HBV DNA, duration of undetectable HBV DNA level (<3.7 LGE/ml), duration of lamivudine therapy was not significant. Interestingly, all six patient whose HBcrAg level <3 log U/ml at the end of treatment did not experienced relapse. Further multivariate analysis involving the 10 above factors effectors indicated that HBcrAg level of <3.4 log U/ml (OR, 103; 95% CI, 1.3–8242; $p = 0.042$), which was a mean value of non-relapsers, was the only independent predictive factor for absence of posttreatment relapse (Table 3). Six of seven patients whose HBcrAg level <3.4 log U/ml at the end of treatment did not experienced relapse [positive predictive value (PPV), 86%]. Ten of 15 patients whose HBcrAg level ≥3.4 log U/ml at the end of treatment experienced relapse [negative predictive value (NPV), 75%], which was higher than that (58%) when the HBcrAg level ≥3.0 log U/ml was applied as cut-off value.

Table 3
Predictive factors for non-relapse in multivariate analysis

Factor	OR (95% CI)[a]	p
Age (years)	0.16 (0.90–1.10)	0.157
Male sex (%)	0.15 (0.001–17.2)	0.429
Cirrhosis (%)	19.4 (0.08–4932)	0.294
Pretreatment ALT level of >80U/l (%)	1.10 (0.02–63.7)	0.964
Pretreatment HBV DNA (LGE/ml)	0.81 (0.05–14.6)	0.886
Pretreatment HBcrAg level (log U/ml)	1.17 (0.24–5.64)	0.846
Mean months till undetectable HBV DNA (months)	1.32 (0.16–11.1)	0.800
Duration of HBV DNA level of <3.7mean LGE/ml (months)	1.83 (0.15–22.4)	0.636
HBcrAg level of <3.4 log U/ml at lamivudine cessation point	103 (1.3–8242)	0.042
Duration of lamivudine therapy, mean (months) ± S.D.	0.53 (0.42–6.5)	0.616

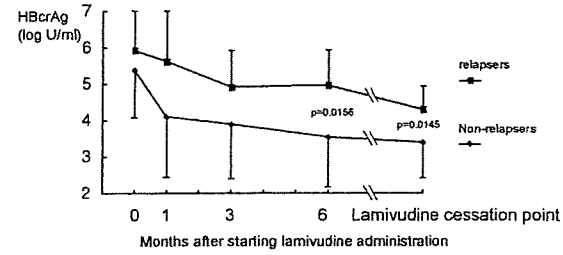

Fig. 2. Linear reduction of the HBcrAg levels is represented in mean value; comparison between relapsers and non-relapsers. At 6 months after treatment initiation and at the cessation point of lamivudine therapy, HBcrAg levels in the patients who experienced relapse were significantly higher than level of HBcrAg in the patients who did not.

Fig. 2 shows comparison of changes in the median levels of HBcrAg during lamivudine administration between relapsers and non-relapsers. The initial median level of HBcrAg in the relapsers was relatively higher than that in the non-relapsers. The levels of HBcrAg in the relapsers were significantly higher than that in the non-relapsers at 6 months after treatment initiation and at cessation points (5.0 ± 1.0 versus 3.5 ± 1.4; $p = 0.0156$ and 4.5 ± 1.0 versus 3.4 ± 0.9; $p = 0.0145$). Interestingly, log-reduction of HBcrAg levels at 6 months after lamivudine administration tended to be greater in non-relapsers than in relapsers (1.7 ± 0.9 versus 1.2 ± 0.8), but no significant. Similarly, log-reduction of HBcrAg at the cessation point also tented to be greater in non-relapsers than in relapsers (2.0 ± 0.9 versus 1.6 ± 1.1), but no significant.

4. Discussion

In this study, we showed that the HBcrAg level <3.4 log U/ml at the point of cessation of lamivudine monotherapy was the only independent predictive factor for absence of post-treatment relapse and better predictive value (PPV 86% NPV 75%) The CLEIA for the HBcrAg is eas-