**Table 1. Clinical Characteristics of Patients Acutely Infected With HBV of Distinct Genotypes/Subgenotypes**

| Features | Genotypes/Subgenotypes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Aa (n = 10) | Ae (n = 33) | Ba (n = 22) | Bj (n = 22) | Cs (n = 11) | Ce (n = 192) | D[a] (n = 5) | G[a,b] (n = 6) |
| Age (years) | 42.2 ± 13.1 | 31.2 ± 10.3[d] | 41.5 ± 10.7[e] | 43.5 ± 19.1 | 38.5 ± 11.1 | 36.3 ± 15.0 | 38.6 ± 20.8 | 42.7 ± 17.5 |
| Men | 8 (80%) | 30 (91%)[f] | 19 (86%)[g] | 9 (41%) | 7 (64%) | 122 (64%) | 2 (40%) | 6 (100%) |
| HBeAg positive | 7 (70%) | 26 (79%)[h] | 11 (50%) | 8 (36%) | 8 (73%) | 101 (53%) | 1 (20%) | 4 (67%) |
| ALT (IU/L) | 1875 ± 759 | 2070 ± 1113[i] | 2523 ± 1185 | 3472 ± 2720 | 2269 ± 995 | 2610 ± 1719 | 2559 ± 1672 | 2142 ± 722 |
| Duration of elevated ALT (weeks)[c] | 7.9 ± 5.8 | 9.5 ± 6.2 | 8.8 ± 3.7[j] | 6.0 ± 2.5 | 10.1 ± 7.5 | 7.7 ± 5.1 | 5.7 ± 2.1 | 9.8 ± 1.5 |
| Total bilirubin (mg/dL) | 14.1 ± 10.3 | 9.0 ± 7.2 | 9.3 ± 5.9 | 10.9 ± 9.0 | 11.0 ± 13.8 | 9.8 ± 10.7 | 8.2 ± 2.2 | 13.0 ± 7.8 |
| HBV DNA (log copies/mL) | | | | | | | | |
| Median | 4.76 | 6.08[k] | 5.15 | 4.93 | 5.61 | 4.94 | 5.91 | 5.97 |
| (range) | (2.90-8.08) | (2.00-8.46) | (2.00-8.19) | (2.00-8.44) | (2.00-8.50) | (2.00-9.06) | (2.00-8.37) | (3.35-7.11) |
| <2.00 (undetectable) | 0 (0%) | 1 (3%) | 2 (9%) | 3 (14%) | 2 (18%) | 28 (15%) | 1 (20%) | 0 (0%) |
| Medication with | | | | | | | | |
| Lamivudine | 1 (10%) | 9 (27%) | 2 (9%) | 5 (23%) | 2 (18%) | 28 (15%) | 4 (80%) | 2 (33%) |
| Steroid | 0 | 3 (9%) | 0 | 5 (23%) | 1 (9%) | 16 (8%) | 0 | 0 |

[a]Patients with HBV genotype D or G were not included in the analysis.
[b]All patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.
[c]Exclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.
[d]$P = .0001$, Ae vs. Ba. $P < .01$. Ae vs. Aa. $P < .05$, Ae vs. Bj or Cs.
[e]$P < .05$, Ba vs. Ce.
[f]$P = .0001$, Ae vs. Bj. $P < .005$, Ae vs. Ce.
[g]$P < .005$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[h]$P < .005$, Ae vs. Bj. $P < .01$, Ae vs. Ce. $P < .05$, Ae vs. Ba.
[i]$P < .05$, Ae vs. Bj.
[j]$P < .01$, Ba vs. Bj. $P < .05$. Ba vs. Ce.
[k]$P < .005$, Ae vs. Ce. $P < .05$, Ae vs. Bj.

the peak ALT level tended to be high in patients with HBV/Bj.

Presumed infection routes of 301 patients were sexual transmission in 172 (57%), blood transfusion in 4 (1%), medical accidents in 17 (6%), and unknown in the remaining 108 (36%).

***Clinical Outcome of Patients With Acute Hepatitis B.*** Fulminant hepatitis developed in 40 (13%) patients. To cope with severe acute liver disease, lamivudine and steroid were administered to 53 (18%) and 25 (8%) patients, respectively. Fulminant hepatitis led to death in 16 (5%) patients, and three (1%) received liver transplantation. Exclusive of the 40 patients with fulminant hepatitis who received various treatments and five without clinical data, 256 (85%) were followed for the chronic outcome (Fig. 1). Serum ALT levels stayed elevated for longer than 24 weeks for the diagnosis of chronic hepatitis in eight (3%) of them. Among them, five had cleared HBsAg from serum until then, and therefore, their liver function abnormality was not attributed to persistent HBV infection. Table 2 summarizes persistence of HBV infection in the 256 patients with acute hepatitis; 253 (99%) lost serum HBsAg by 6 months. Hence, HBV infection evolved into chronicity in only 3 of the 256 (1%) patients, representing 2 of the 32 (6%) infected with HBV/Ae and 1 of the 21 (5%) with Ba. All of the three with chronic outcome had low-titered IgG anti-HBc at the presentation, and two of them had been negative for HBsAg before the presentation. None of them had received lamivudine or steroid treatment during their acute phase of illness. Of the patients without antiviral therapy, chronic outcome was significantly more frequent in those infected with HBV/Ae than non-Ae genotypes (9% 2/23 vs. 0.5% 1/187, $P = .032$).

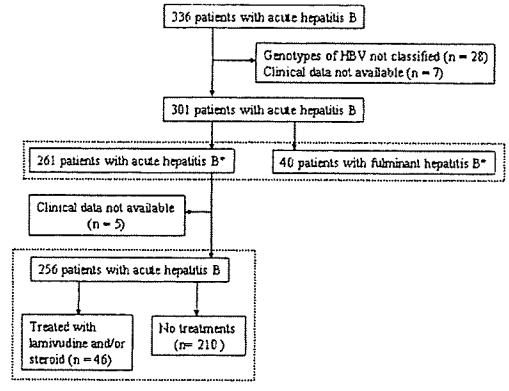


Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including 27 with acute and 10 with fulminant hepatitis.

**Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid**

| Treatment | Total | Genotypes/Subgenotypes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Aa (n = 8)[a] | Ae (n = 32)[a] | Ba (n = 21)[a] | Bj (n = 10)[a] | Cs (n = 10)[a] | Ce (n = 167)[a] | D (n = 3)[a] | G (n = 5)[a] |
| Total (n = 256) | 3/256 (1.2%) | 0 | 2/32 (6%)c | 1/21 (5%) | 0 | 0 | 0 | 0 | 0 |
| Lamivudine (n = 36)[b] | 0/36 (0%) | 0/1 (0%) | 0/9 (0%) | 0/2 (0%) | 0 | 0/1 (0%) | 0/19 (0%) | 0/2 (0%) | 0/2 (0%) |
| Steroid (n = 16)[b] | 0/16 (0%) | 0 | 0/3 (0%) | 0 | 0 | 0/1 (0%) | 0/12 (0%) | 0 | 0 |
| Neither | 3/210 (1.4%) | 0/7 (0%) | 2/23 (9%)c | 1/19 (5%) | 0/10 (0%) | 0/8 (0%) | 0/139 (0%) | 0/1 (0%) | 0/3 (0%) |

[a]Exclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.
[b]Six patients received steroid along with lamivudine.
[c]$P < .05$, Ae vs. non-Ae.

***Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis.*** Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P = .0017$), less predominantly male (43% vs. 71%, $P = .0005$) and less often positive for HBeAg (23% vs. 60%, $P < .0001$) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ($P < .0001$), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL, $P = .0178$); the frequency of undetectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%, $P = .0086$). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%, $P = .0121$), whereas Bj was more often (30% vs. 4%, $P < .0001$) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

**Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV**

| Features | Fulminant (n = 40) | Acute (n = 261) | P Value |
|---|---|---|---|
| Age (years) | 44.7 ± 16.3 | 36.0 ± 14.3 | .0017 |
| Men | 17 (43%) | 186 (71%) | .0005 |
| HBeAg positive | 9 (23%) | 157 (60%) | <.0001 |
| ALT (IU/L) | 4315 ± 2889 | 2284 ± 1221 | <.0001 |
| Total bilirubin (mg/dL) | 20.5 ± 16.4 | 8.3 ± 7.3 | <.0001 |
| HBV DNA (log copies/mL) | | | |
| Median | 4.89 | 5.19 | .0178 |
| (range) | (2.00-8.44) | (2.00-9.06) | |
| <2.00 (undetectable) | 10 (25%) | 27 (10%) | .0086 |
| Treatment | | | |
| Lamivudine | 16 (40%) | 37 (14%) | .0003 |
| Steroid | 9 (23%) | 16 (6%) | .0022 |
| Genotypes/subgenotypes | | | |
| Aa | 1 (2.5%) | 9 (3%) | NS |
| Ae | 0 (0%) | 33 (13%) | .0121 |
| Ba | 1 (2.5%) | 21 (8%) | NS |
| Bj | 12 (30%) | 10 (4%) | <.0001 |
| Cs | 1 (2.5%) | 10 (4%) | NS |
| Ce | 22 (55%) | 170 (65%) | NS |
| D | 2 (5%) | 3 (1%) | NS |
| G | 1 (2.5%) | 5 (2%) | NS |
| Mutations[a] | | | |
| nt 1753 and/or nt1754[b] | 11/30 (37%) | 28/234 (12%) | .0003 |
| A1762T/G1764A | 15/30 (50%) | 39/234 (17%) | <.0001 |
| G1896A | 16/30 (53%) | 21/234 (9%) | <.0001 |
| G1899A | 7/30 (23%) | 8/234 (3%) | <.0001 |

[a]Exclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.
[b]T1753C/A/G and/or T1754C/A/G.

**Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis**

| Factors | Odds Ratio | 95% Confidence Interval | P Value |
|---|---|---|---|
| Age (yr) | | | |
| <34[a] | 1 | | |
| ≥34 | 3.472 | 1.094-11.023 | .0347 |
| Sex | | | |
| Male | 1 | | |
| Female | 2.272 | 0.780-6.613 | .1323 |
| HBeAg | | | |
| Positive | 1 | | |
| Negative | 3.344 | 1.065-10.506 | .0387 |
| ALT (IU/L) | | | |
| <2200[a] | 1 | | |
| ≥2200 | 2.094 | 0.683-6.414 | .1957 |
| Total bilirubin (mg/dL) | | | |
| <10.0[a] | 1 | | |
| ≥10.0 | 18.818 | 4.320-81.980 | <.0001 |
| HBVDNA (log copies/mL) | | | |
| <5.00[a] | 1 | | |
| ≥5.00 | 1.042 | 0.367-2.961 | .9383 |
| Treatment | | | |
| Lamivudine (−) | 1 | | |
| Lamivudine (+) | 2.650 | 0.814-8.625 | .1056 |
| Steroid (−) | 1 | | |
| Steroid (+) | 2.515 | 0.668-9.472 | .1728 |
| Genotypes/Subgenotypes | | | |
| Non-Bj | 1 | | |
| Bj | 7.001 | 1.737-28.228 | .0062 |
| Mutations | | | |
| nt 1753 and/or 1754[b] | | | |
| Absent | 1 | | |
| Present | 2.316 | 0.698-7.683 | .1700 |
| A1762T/G1764A | | | |
| Absent | 1 | | |
| Present | 1..013 | 0.295-3.478 | .9841 |
| G1896A | | | |
| Absent | 1 | | |
| Present | 4.157 | 1.265-13.657 | .0189 |
| G1899A | | | |
| Absent | 1 | | |
| Present | 2.525 | 0.534-11.949 | .2427 |

[a]Median values.
[b]T1753C/A/G or T1754C/A/G.

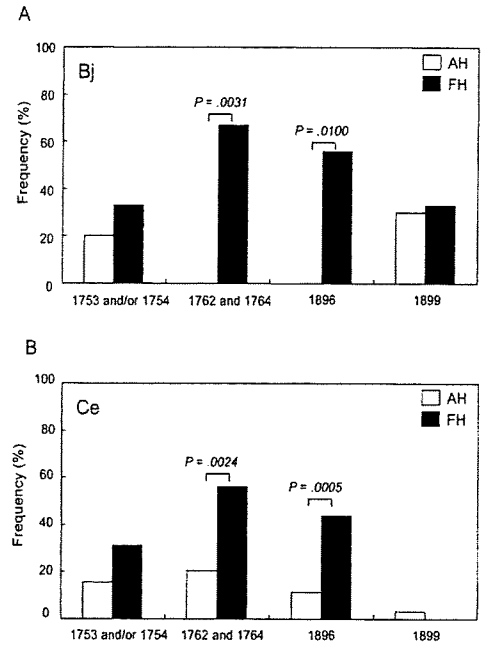


Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively, $P < .0001$ for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ($P = .0003$ and $P < .0001$, respectively).

***Factors Independently Associated With the Development of Fulminant Hepatitis.*** Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02], $P = .035$), HBV/Bj (7.00 [1.74-28.23], $P = .006$), HBeAg-negative (3.34 [1.07-10.51], $P = .039$), total bilirubin ≥10.0 mg/dL (18.82 [4.32-81.98], $P < .0001$) and G1896A (4.16 [1.27-13.66], $P = .019$) were independent risk factors for the development of fulminant hepatitis.

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively, $P \leq .01$ for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

***Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants* In Vitro.** Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj_58 carrying G1896A from a fulminant patient. Figure 3 compares

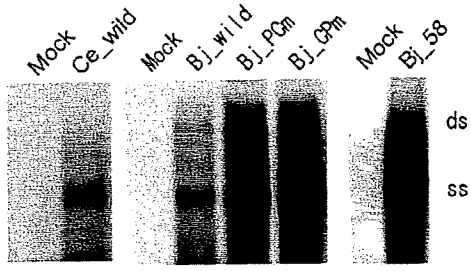


Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ($P < .01$) (Sugiyama M et al., manuscript in submission).

## Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.[26] Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).[27] Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia[28-30] and the Middle East.[31] The failure in detecting these mutations in Western countries[32-35] could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,[36] as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,[37] occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

*In vitro* replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports[38,39]; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.[40] have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/

core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.[41] On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)[42] or patients in an acupuncture-associated outbreak (0/35).[43] In marked contrast, they ranged from 0.2% (14/715) in Greece[44] through 2.7% (1/37) in university students in Taiwan[45] to 10.4% (5/8) in Alaskan Eskimos[46] and 12.1% (7/58) in Germany.[47] HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.[48] In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23% 3/13 vs. 13% 1/8 or 12% 3/25).[49] In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.[49] The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,[49,50] had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae (Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

## References


1. Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733-1745.
2. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059-2073.
3. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 1992;73:1201-1208.
4. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69: 2575-2583.
5. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67-74.
6. Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. HEPATOLOGY 2002;35:1274-1276.
7. Kao JH. Hepatitis B viral genotypes: clinical relevance and molecular characteristics. J Gastroenterol Hepatol 2002;17:643-650.
8. Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329-338.
9. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811-820.
10. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985-5992.
11. Huy TT, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, et al. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 2004;85:283-292.
12. Tanaka Y, Orito E, Yuen MF, Mukaide M, Sugauchi F, Ito K, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. Hepatol Res 2005;33:216-224.
13. Kramvis A, Kew MC. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepatol 2005;12:456-464.
14. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321.
15. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124: 925-932.

16. Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, et al. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. HEPATOLOGY 2004;40:747-755.
17. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus: large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285-1293.
18. Mitamura K. Epidemiology of HDV infection in Japan. Prog Clin Biol Res 1991;364:81-87.
19. Trey C, Lipworth L, Chalmers TC, Davidson CS, Gottlieb LS, Popper H, et al. Fulminant hepatic failure. presumable contribution of halothane. N Engl J Med 1968;279:798-801.
20. Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81-89.
21. Kato H, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, et al. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. HEPATOLOGY 2002;35:922-929.
22. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 1997;78:1719-1729.
23. Hasegawa I, Tanaka Y, Kramvis A, Kato T, Sugauchi F, Acharya SK, et al. Novel hepatitis B virus genotype A subtyping assay that distinguishes subtype Aa from Ae and its application in epidemiological studies. J Virol 2004;78:7575-7581.
24. Sugauchi F, Kumada H, Sakugawa H, Komatsu M, Niitsuma H, Watanabe H, et al. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. Clin Infect Dis 2004;38:1222-1228.
25. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;38:2899-2903.
26. Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, et al. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980-1994. J Gastroenterol Hepatol 2003;18:943-949.
27. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. HEPATOLOGY 2001;33:218-223.
28. Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 1991;100:1087-1094.
29. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991;324:1699-1704.
30. Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241-248.
31. Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 1991;324:1705-1709.
32. Karayiannis P, Alexopoulou A, Hadziyannis S, Thursz M, Watts R, Seito S, et al. Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: importance of host factors. HEPATOLOGY 1995;22:1628-1634.
33. Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J. Nucleotide sequence analysis of the precore region in patients with fulminant hepatitis B in the United States. Gastroenterology 1993;105:1173-1178.
34. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology 1995;109:1618-1623.
35. Liang TJ, Hasegawa K, Munoz SJ, Shapiro CN, Yoffe B, McMahon BJ, et al. Hepatitis B virus precore mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation. J Clin Invest 1994;93:550-555.
36. Parekh S, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, et al. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. J Virol 2003;77:6601-6612.
37. Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, et al. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. Gut 2003;52:1630-1637.
38. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 1996;70:5845-5851.
39. Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology 1993;194:263-276.
40. Bocharov G, Ludewig B, Bertoletti A, Klenerman P, Junt T, Krebs P, et al. Underwhelming the Immune Response: effect of slow virus growth on CD8+-T-lymphocyte responses. J Virol 2004;78:2247-2254.
41. Shimizu M, Ohyama M, Takahashi Y, Udo K, Kojima M, Kametani M, et al. Immunoglobulin M antibody against hepatitis B core antigen for the diagnosis of fulminant type B hepatitis. Gastroenterology 1983;84:604-610.
42. Rinker J, Galambos JT. Prospective study of hepatitis B in thirty-two inadvertently infected people. Gastroenterology 1981;81:686-691.
43. Kent GP, Brondum J, Keenlyside RA, LaFazia LM, Scott HD. A large outbreak of acupuncture-associated hepatitis B. Am J Epidemiol 1988;127:591-598.
44. Tassopoulos NC, Papaevangelou GJ, Sjogren MH, Roumeliotou-Karayannis A, Gerin JL, Purcell RH. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. Gastroenterology 1987;92:1844-1850.
45. Beasley RP, Hwang LY, Lin CC, Ko YC, Twu SJ. Incidence of hepatitis among students at a university in Taiwan. Am J Epidemiol 1983;117:213-222.
46. McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, et al. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis 1985;151:599-603.
47. Schomerus H, Wiedmann KH, Dolle W, Peerenboom H, Strohmeyer G, Balzer K, et al. (+)-Cyanidanol-3 in the treatment of acute viral hepatitis: a randomized controlled trial. HEPATOLOGY 1984;4:331-335.
48. Sherlock S. The natural history of hepatitis B. Postgrad Med J 1987;63:S7-S11.
49. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, et al. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 2002;68:522-528.
50. Suzuki Y, Kobayashi M, Ikeda K, Suzuki F, Arfase Y, Akuta N, et al. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. J Med Virol 2005;76:33-39.

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# Novel subtypes (subgenotypes) of hepatitis B virus genotypes B and C among chronic liver disease patients in the Philippines

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Several hepatitis B virus (HBV) subtypes (subgenotypes), HBV/Aa (A1 : Asia/Africa), Ae (A2 : Europe), Bj (B1 : Japan) and Ba (B2 : Asia), have been reported with respect to clinical differences between patients infected with these subtypes (subgenotypes). HBV genotype distribution among patients with chronic liver diseases was investigated in the Philippines, where such studies have not been carried out previously. One hundred sera were obtained from such patients, consisting of 32 chronic hepatitis (CH), 37 cirrhosis and 31 hepatocellular carcinoma (HCC) patients. Nine complete genomes and 100 core promoter/precore genes of HBV were sequenced directly. Phylogenetic analyses revealed 51 HBV/A (Aa/A1), 22 HBV/B and 27 HBV/C strains. Interestingly, most HBV/C strains in the Philippines formed a specific cluster distinct from previous HBV/C strains (C1–4), indicating a novel subtype (subgenotype), HBV/C5. Moreover, most HBV/B strains fell within the specific cluster of the HBV/B subtype (subgenotype) B5, with viral characteristics of HBV/Ba (B2) carrying a recombination with HBV/C over the precore and core genes. Of the three genotypes, HBV/B and HBV/C were significantly more prevalent than HBV/A in cirrhosis and HCC patients ($P<0{\cdot}02$). The prevalence of the core promoter mutations T1762/A1764 was higher in HCC patients with HBV/B and HBV/C. Multivariate analysis indicated that age [odds ratio (OR) 3·43; 95 % confidence interval (CI) 1·04–11·36; $P=0{\cdot}044$] and the core promoter mutation (OR 14·08; 95 % CI 3·62–4·74; $P<0{\cdot}001$) were significant factors for HCC development. In conclusion, novel HBV subtypes (subgenotypes) C5 and B5 are prevalent in the Philippines, as well as HBV/Aa (A1).

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## INTRODUCTION

*Hepatitis B virus* (HBV) is one of the major causes of chronic liver diseases, including chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Seven major genotypes of HBV have been classified by sequence divergence in the entire genome in excess of 8 %, and they are named A to G (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000). A possible eighth genotype is proposed, with the tentative designation of H (Arauz-Ruiz *et al.*, 2002), but it is closely related phylogenetically to genotype F.

The genotypes of HBV have distinct geographical distributions, which have been associated with anthropological history (Orito *et al.*, 2001). HBV/A is prevalent in Europe, Africa and South-east Asia, including the Philippines (Sugauchi *et al.*, 2004). HBV/B and HBV/C are predominant in Asia, HBV/D is common in the Mediterranean area, the Middle East and India, HBV/E is localized in sub-Saharan Africa and HBV/F (or H) is restricted to Central and South America. All these genotypes occur in the United States, with frequencies dependent on ethnicity (Chu *et al.*, 2003). Moreover, HBV/G has been found in France, Germany and the United States (Chu *et al.*, 2003; Stuyver *et al.*, 2000; Vieth *et al.*, 2002).

The DDBJ/EMBL/GenBank accession numbers of the complete genome sequences of HBV isolates PhCH24, PhLC03, PhLC14, PhHCC15, PhHCC13, PhHCC01, PhCH09, PhHCC03 and PhHCC05 are AB241109–AB241117, respectively.

A neighbour-joining phylogenetic tree showing the relationship of HBV/Aa strains from the Philippines with other HBV/Aa strains and representative strains from other genotypes is available as supplementary material in JGV Online.

HBV strains even of the same genotype may differ both virologically and clinically. There are two subtypes (subgenotypes) of genotype B with distinct geographical distributions, provisionally designated Bj/B1 ('j' for Japan) and Ba/B2 ('a' standing for Asia) (Sugauchi *et al.*, 2002), and clinical differences between patients infected with HBV/Bj (B1) and HBV/Ba (B2) are emerging (Akuta *et al.*, 2003; Sugauchi *et al.*, 2003). Additionally, there has been some evidence for virological and clinical differences between HBV/Aa (A1) in Africa/Asia and HBV/Ae (A2) in Europe/the United States (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004). Infection with HBV/Aa (A1) is associated with low serum levels of HBV DNA as well as a low prevalence of hepatitis B e antigen (HBeAg) in the serum, and is implicated in the high incidence of HBV-related HCC in Africa (Kew *et al.*, 2005; Tanaka *et al.*, 2004). More recently, HBV genotype C (HBV/C) has been classified into two geographically typical subtypes (subgenotypes), HBV/Cs (C1) in South-east Asia and HBV/Ce (C2) in East Asia, and there are virological differences between the two subtypes (subgenotypes) (Chan *et al.*, 2004; Kramvis *et al.*, 2005; Tanaka *et al.*, 2005).

In Asia, HBV genotype B (HBV/B) and C predominate; however, HBV/Aa (A1) is also found in the Philippines. To date, there have been no large population studies on HBV genotypes in the Philippines. The aim of this study is to evaluate HBV genotypes among 100 HBV carriers in the Philippines by molecular evolutionary analysis and to determine the influences of HBV genotypes and viral mutations on clinical characteristics.

## METHODS

**Patients.** A total of 100 serum samples positive for HBsAg were collected from patients with chronic HBV infection in the Philippines. The patients were classified into three clinical groups: (i) patients with chronic liver disease with persistently elevated serum alanine aminotransferase (ALT) levels, such as those with CH ($n=32$); (ii) patients defined as LC with clinical evidence of cirrhosis (e.g. coarse liver architecture, nodular liver surface and blunt liver edge) revealed by evidence of hypersplenism (e.g. splenomegaly revealed by ultrasonography or computed tomography and a platelet count of $<100\,000$ platelets $mm^{-3}$) and clinical complement (e.g. ascites, jaundice, encephalopathy or oesophageal varices) ($n=37$); and (iii) patients who were diagnosed with HCC on the basis of results of abdominal ultrasonography, angiography, computed tomography or magnetic resonance imaging as well as an elevated serum α-fetoprotein (AFP) level ($\geqslant 400$ ng $ml^{-1}$) ($n=31$). Patients who were co-infected with hepatitis C virus or human immunodeficiency virus were excluded. There were no patients who received antiviral treatment during the follow-up period. The study protocol was approved by the Ethics Committees of the participating institutions in accordance with the 1975 Declaration of Helsinki. Informed consent was obtained from each patient prior to any study-related procedures.

**Serological markers of HBV infection.** Hepatitis B surface antigen (HBsAg) was determined by haemagglutination (MyCell; Institute of Immunology Co., Ltd, Tokyo, Japan) or ELISA (Axcysm; Abbott) and HBeAg was detected by chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse *f*; FUJIREBIO Inc.).

**Sequencing of HBV genome.** Nucleic acids were extracted from 100 μl serum using the QIAamp DNA Blood Mini kit (Qiagen). Nine complete genomes and 100 partial HBV genome regions bearing the core promoter and precore/core regions were amplified by PCR with several primer sets, as described previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly using the ABI Prism Big Dye version 3.0 kit (Applied Biosystems) on an ABI 3100 DNA automated sequencer (Applied Biosystems). All sequences were analysed in both the forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX version 11.0 (Software Development Co.).

**Molecular evolutionary analysis of HBV.** Reference sequences were retrieved from the DDBJ/EMBL/GenBank databases with their accession numbers for identification. Nucleotide sequences of HBV were aligned by the program CLUSTAL X and genetic distance was estimated with the 6-parameter method (Gojobori *et al.*, 1982) in the Hepatitis Virus Database (Robertson *et al.*, 1998). Based on these values, phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

**Statistical analyses.** Statistical differences were evaluated using the Mann–Whitney non-parametric test, Fisher's exact probability test and Student's *t*-test where appropriate. Multivariate analyses with logistic regression were used to determine independent factors for the progression to HCC. Differences were considered significant for a $P$ value less than 0·05. The statistical analysis software used was Stata version 8.0 (StataCorp LP).

## RESULTS

### Genotypes of HBV

The distribution of HBV genotypes among 100 carriers in the Philippines indicated 51 HBV/A, 22 HBV/B and 27 HBV/C. All 51 HBV/A were classified into HBV/Aa (A1 : Asia/African type) and all 22 HBV/B strains contained a recombinant sequence of HBV/C in the precore/core region. HBV/D, E, F and G were not found in this study.

### Phylogenetic relatedness among HBV/C and HBV/B in the Philippines

All 27 HBV/C strains found in the Philippines could be amplified and sequenced over the core promoter and precore/core regions spanning 398 bp. Together with 10 HBV/Cs (C1) strains, 10 HBV/Ce (C2) strains, two Vietnamese HBV/C strains (strains 3270 and 8290) that exhibit 4·5–5·7 % genetic difference from other HBV/C strains (Hannoun *et al.*, 2000), two HBV/C strains from Polynesia (HBV/C3), two HBV/C strains from Australian aborigines (HBV/C4), seven HBV/Bj strains (B1), nine original HBV/Ba strains (B2), three HBV/B3 strains and three HBV/B4 strains retrieved from the database, the 27 HBV/C strains, 22 HBV/B strains and two of the 51 HBV/A strains sequenced in the present study were subjected to phylogenetic analyses along with HBV strains representative of HBV/Aa (A1), Ae (A2), D, E, F and G (Fig. 1). Note that 25 (93 %) of the 27 HBV/C strains in this study formed a novel cluster with the two atypical Vietnamese strains (Hannoun *et al.*, 2000), separated from the other HBV/C strains (C1–4). Moreover, 21 (95 %) of the

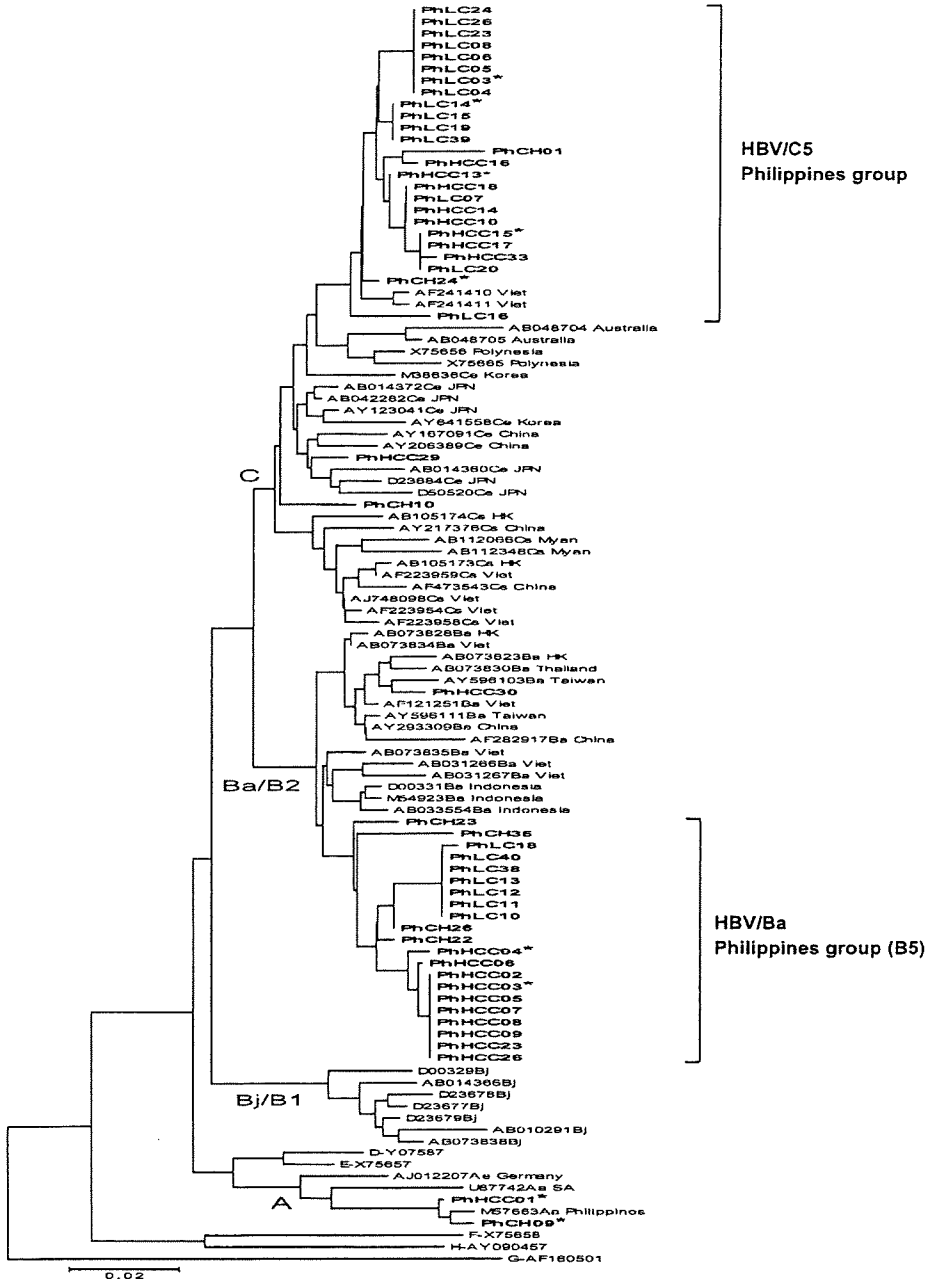


**Fig. 1.** Phylogenetic tree constructed by the neighbour-joining method based on X gene, precore and core gene sequences spanning 398 bp. Fifty-six representative HBV sequences were retrieved from the databases: 10 HBV/Cs (C1) strains, 10 HBV/Ce (C2) strains, six other HBV/C strains, seven HBV/Bj (B1) strains, nine HBV/B2 strains, three HBV/B3 strains, three HBV/B4 strains and eight HBV strains representative of the other six genotypes (Aa/A1, Ae/A2, D–H). Fifty-one strains from this study are shown in bold. Strains from the databases are identified by accession numbers, followed by the subtype (subgenotype) and the country of origin [HK, Hong Kong; JPN, Japan; Myan, Myanmar (Burma); SA, South Africa; Viet, Vietnam]. Bar, 0·02 nucleotide substitutions per site. Strains indicated by asterisks (*) were used for further analyses based on complete genome sequences.

22 HBV/B strains specifically clustered separately from previously reported HBV/B strains. On the other hand, all 51 HBV/A strains, which were just separated from the South African Aa (A1) strains, belonged to the same subtype (subgenotype) as these strains (see Supplementary Fig. S1 in JGV Online).

As shown in Fig. 2, the complete genomes of nine strains (two HBV/A, two HBV/B and five HBV/C) were sequenced successfully (marked by asterisks in Fig. 1). The lengths of the complete genomes corresponding to HBV/A, HBV/B and HBV/C were 3221, 3215 and 3215 nt, respectively. Two of the HBV/C strains had shorter lengths of 3060 nt due to preS2 and core deletions. One of the HBV/B strains had a shorter length of 3179 nt due to preS2 deletions. Phylogenetic analysis of the complete genome sequences revealed five distinct clusters within HBV/C supported by the bootstrap resampling test: HBV/Cs (C1), HBV/Ce (C2), HBV/C3, HBV/C4 and a fifth group consisting of the five HBV/C strains sequenced in the present study and two HBV/C strains from Vietnam (strains 3270 and 8290; Hannoun *et al.*, 2000). Interestingly, 12 of 14 Vietnamese HBV/C strains (86 %) were classified into HBV/Cs (C1), the exceptions being the two strains reported by Hannoun *et al.* (2000), indicating that HBV/C is the predominant type in Vietnam. We named the fifth phylogenetic group HBV/C5.

To find possible recombination in the HBV/C5 strains, phylogenetic analyses were performed in four reading frames, the large S (preS1/preS2/S), X, precore/core and P regions (Fig. 3). A clear separation of HBV/C5 from the other four subtypes (subgenotypes) was found in the tree topology for all four regions. Phylogenetic trees constructed from the four regions revealed significant bootstrap values at the bifurcation of C5. The estimated intergroup nucleotide divergence over the complete genome sequences between C5 and the other subtypes (subgenotypes) was [mean ± SD (range)] 6·4 ± 0·5 % (5·0–7·4 %) versus Cs (C1), 5·7 ± 0·5 % (4·5–6·5 %) versus Ce (C2), 6·5 ± 0·6 % (5·6–7·5 %) versus C3 and 8·1 ± 0·4 % (7·4–8·6 %) versus C4. In the HBV/C5 strains, the nucleotide divergence of the complete genomes between the five HBV/C5 strains determined in the present study and HBV/C5 strains 3270 and 8290 from Vietnam was 3·3 ± 0·4 % (2·8–3·9 %), and the intragroup nucleotide divergence was 1·6 ± 0·5 % (0·8–2·5 %) among the five HBV/C5 strains in the present study.

Phylogenetic analysis of the complete genome sequences also revealed that the HBV/B strains were divided into five subgroups with significant bootstrap values: HBV/Bj (B1), an original HBV/Ba in several Asian countries (B2) and Ba variants HBV/B3 from Indonesia, HBV/B4 from Vietnam and HBV/B5 from the Philippines, represented by two strains in this study. This subtype (subgenotype) numbering is based on the report of Norder *et al.* (2004). Note that 21 of the 22 HBV/B strains from the Philippines were classified into HBV/B5 and one to original HBV/Ba (B2) (Fig. 1). The B5 strains from the Philippines carried the recombination with HBV/C over the precore and core genes that is found in the original HBV/Ba (B2) strains. The estimated intergroup nucleotide divergence over the complete genome sequences between B5 and the other subtypes (subgenotypes) was [mean ± SD (range)] 6·6 ± 0·3 % (6·2–7·1 %) versus B1, 5·3 ± 0·3 % (4·8–5·7 %) versus B2, 3·7 ± 0·1 % (3·6–3·8 %) versus B3 and 5·0 ± 0·08 % (4·9–5·1 %) versus B4.

These complete sequence data mirrored serotyping: all subtype (subgenotype) C5 strains (PhCH24, PhLC03, PhLC14, PhHCC13 and PhHCC15) were *adw2*, the subtype (subgenotype) B5 strains (PhHCC03 and PhHCC05) were *ayw1* and the subtype (subgenotype) Aa (A1) strains (PhCH09 and PhHCC01) were *adw2*.

## Nucleotide and amino acid characteristics of the HBV/C5 strains

A comparison of substitutions between the seven HBV/C5 strains and the consensus sequence of HBV/C, retrieved from the DDBJ/EMBL/GenBank database, over the complete genome showed 22 genotype/subtype (subgenotype) -specific substitutions in the HBV/C5 strains, seven of which were specific for the five HBV/C5 strains from the Philippines (Table 1). As a result of these genotype/subtype (subgenotype) -specific substitutions, 11 amino acid changes were predicted in the HBV/C5 strains. Specific amino acid motifs in the HBV/C5 strains were predicted in the polymerase and preS1/preS2/S region, but not in the X or precore/core regions.

## Comparison of HBV genotypes in different clinical states

Of the three clinical states defined in this study, patients with CH were significantly younger than patients with LC or HCC ($P<0·0001$) (Table 2). The prevalence of HBeAg was significantly higher in patients with CH than in those with LC or HCC ($P<0·005$). The proportion of HBV/A was higher in patients with CH than in those with LC or HCC ($P<0·001$), while HBV/C was significantly more prevalent in LC and HCC than in CH ($P<0·05$).

## Comparison of clinical characteristics among HBV genotypes

Among the three genotypes (A, B and C), there were no significant differences of sex or HBeAg positivity (Table 3); however, patients with HBV/A were significantly younger than those with HBV/B or HBV/C ($P<0·01$). The reason behind this observation is that there were more CH patients with HBV/A than with HBV/B or HBV/C (49, 18 and 11 %, respectively; $P<0·01$). Although no significant differences in HBV genotypes were found between patients with LC and HCC, HBV/B and HBV/C were significantly more prevalent in LC and HCC patients than HBV/A ($P<0·02$).

## Characteristics of mutations in enhancer II, core promoter and precore regions

It has been reported that the T1653 mutation, which is located in the enhancer II upstream core promoter

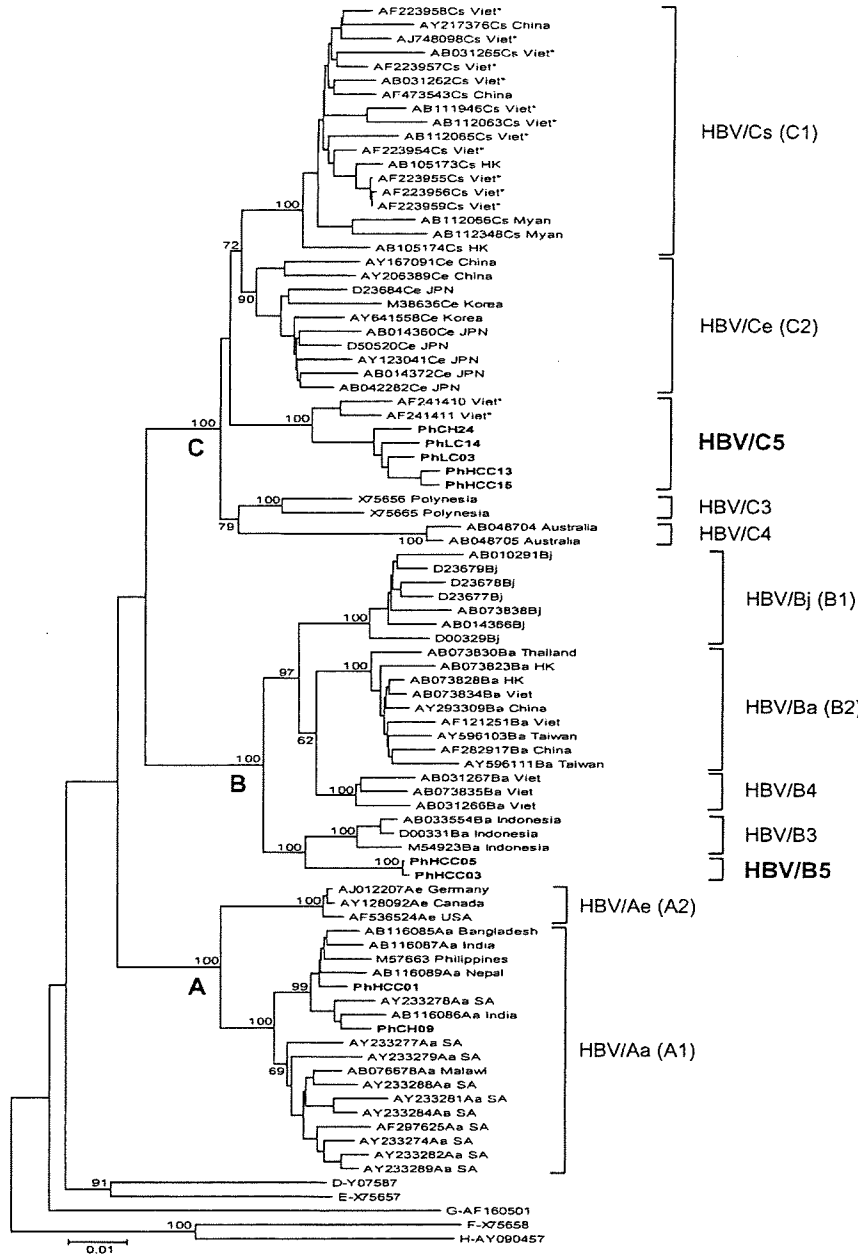


**Fig. 2.** Neighbour-joining phylogenetic tree constructed from complete genome sequences. The five HBV/C5, two HBV/B5 and two HBV/Aa (A1) strains from the Philippines are compared with representative sequences, including 19 HBV/A, 22 HBV/B and 24 HBV/C strains and reference strains of other genotypes. Strains from this study are shown in bold. Strains from the databases are identified as outlined in Fig. 1. Bar, 0·01 nucleotide substitutions per site. Asterisks (*) indicate Vietnamese HBV/C sequences retrieved from GenBank/EMBL/DDBJ.

regulatory region, is associated with disease prognosis in HBV/C (Takahashi *et al.*, 1999; Ito *et al.*, 2006). The frequency of mutations in the core promoter region (T1653 and T1762/A1764) was compared among HBV/A, B and C (Table 3). The T1653 mutation was detected in HBV/A and HBV/C (18 and 22 %), but was not detected in HBV/B. The double mutation in the core promoter region (T1762/A1764) was found to be significantly less frequent in carriers

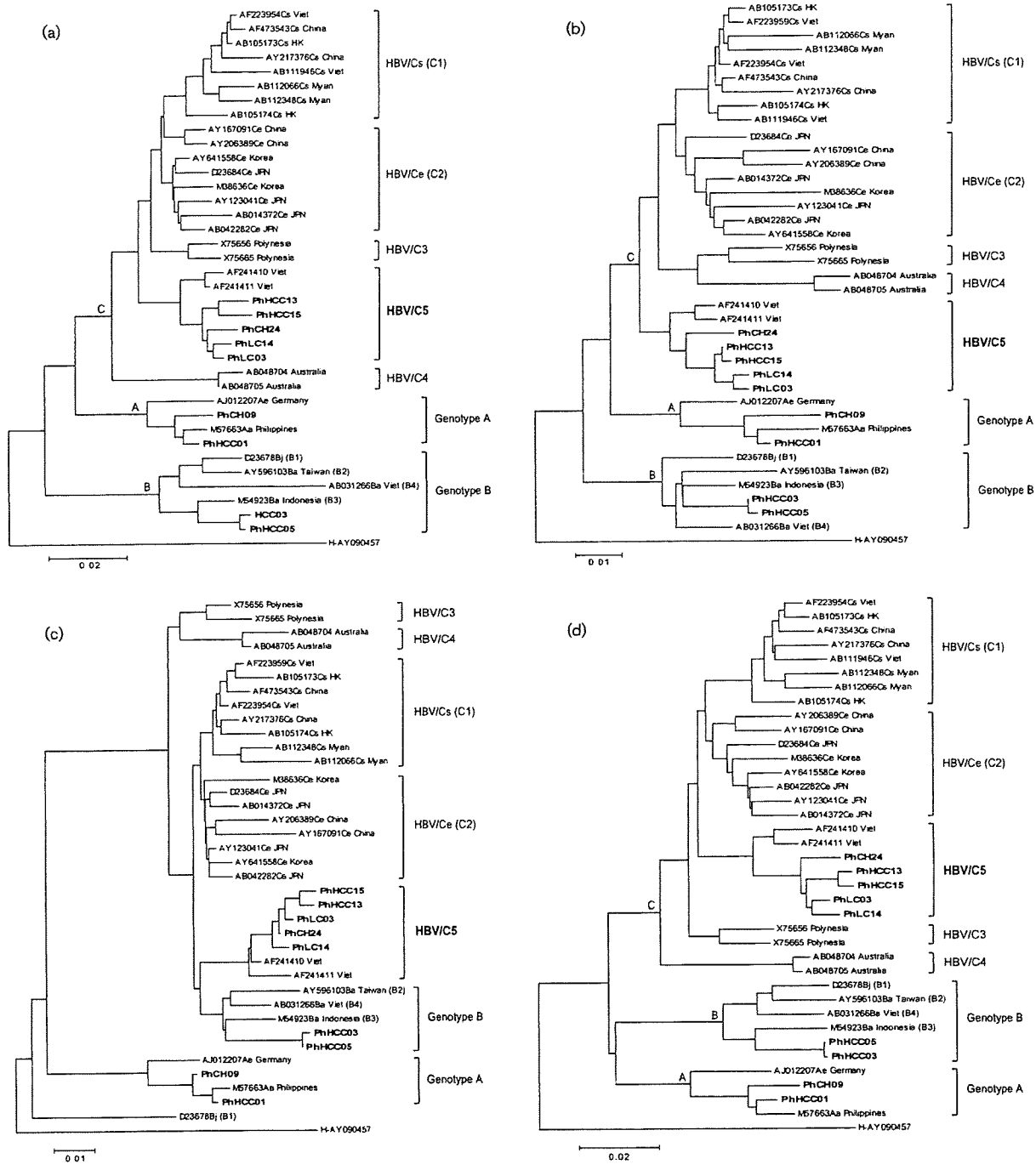


**Fig. 3.** Neighbour-joining phylogenetic trees of the nine completely sequenced strains from this study compared with 29 reference strains based on the ORFs of the large S gene (a), the X gene (b), the precore/core gene (c) and the P gene (d). Strains from this study are shown in bold. Strains from the databases are identified as outlined in Fig. 1. Bars, 0·02 (a, d) or 0·01 (b, c) nucleotide substitutions per site.

**Table 1.** Genotype/subtype- (subgenotype-) specific substitutions in complete genomes of HBV/C5

Non-synonymous changes are shown in bold. Accession numbers of HBV/C genome sequences recovered from the databases that match the HBV/C5 sequence are shown. Amino acids in parentheses are found in a minority of genomes.

| Position | HBV/C consensus | Amino acid(s)/region(s) | HBV/C5 ($n=5$) | Amino acid(s)/region(s) | HBV/C genome(s) that matches HBV/C5 |
|---|---|---|---|---|---|
| 482 | C | **Leu/S, Thr/P** | A | **Ile/S, Asn/P** | AF461043 |
| 1284 | A | Leu/P | T | Leu/P | AY641558 |
| 2269 | A | Gly/C | G | Gly/C | |
| 2284 | T | Thr/C | A | Thr/C | |
| 2293 | T | Ala/C | A | Ala/C | Y18858 |
| 2296 | C | Tyr/C | T | Tyr/C | Y18858 |
| 2299 | A | Arg/C | G | Arg/C | |
| 2606 | A | Thr/P | T | Thr/P | |
| 2609 | T | Val (Ile)/P | A | Val/P | |
| 2648 | G | Arg/P | A | Arg/P | F384371, AY057947 |
| 2653 | A | **Tyr/P** | T | **Phe/P** | AY206376, AY206379 |
| 2750 | A | Leu/P | G | Leu/P | X23684, D23684 |
| 2759 | G | Leu/P | T | Leu/P | D23682, D23683 |
| 2858 | G | **Trp/S**, Leu (Ser)/P | A | **Tyr/S**, Leu/P | AY206381, D23681, AB042284 |
| 2859 | G | **Trp/S, Val/P** | T | **Tyr/S, Phe/P** | AY206381 |
| 2922 | T | **Phe/S, Ser (Pro)/P** | G | **Leu/S, Ala/P** | AB042282, AB042283 |
| 2928 | T | Asp (Gly)/S, **Ser (Ala)/P** | C | Asp/S, **Pro/P** | |
| 2946 | G | Ala/S, **Val, Ile/P** | T | Ala/S, **Phe/P** | Y18858, AY040627 |
| 2997 | T | Asp/S, **Ser/P** | C | Asp/S, **Pro/P** | AY057947 |
| 2999 | A | **His, Gln (Pro)/S, Ser/P** | C | **Pro/S, Pro/P** | AB031265 |
| 3013 | A | **Asn, His (Lys)/S, Lys, Thr/P** | T | **Trp/S, Leu/P** | |
| 3015 | T | **Asn, His (Lys)/S, Ser, Pro/P** | G | **Trp/S, Leu/P** | AY206376, AY206379, X52939 |

of HBV/A than in HBV/B or HBV/C. We also compared the frequency of mutations in the precore region and in Kozak sequences preceding the ATG initiator codon of the precore region among HBV/A, B and C (Table 3). Substitutions in the Kozak sequences (T1809 and T1812) and the mutation in the encapsidation signal of the precore region [T1862 and H1888 (where H means not G)] were identified only in HBV/A. No significant difference in the frequency of the precore stop mutation A1896 was found among HBV/A, HBV/B and HBV/C (0, 9 and 4 %, respectively). All HBV/C5 strains had the C1856 and T1858 mutations, as well as HBV/Ce.

**Table 2.** Comparison of HBV genotypes in different clinical states in the Philippines

| Feature | CH ($n=32$) | LC ($n=37$) | HCC ($n=31$) |
|---|---|---|---|
| Male | 22 (71 %) | 28 (76 %) | 26 (87 %) |
| Age (years) | 30·7 ± 8·7* | 51·3 ± 14·1 | 53·7 ± 15·1 |
| HBeAg | 30 (94 %)* | 21 (57 %) | 17 (55 %) |
| HBV genotype: | | | |
| A | 25 (78 %)* | 15 (41 %) | 11 (35 %) |
| B | 4 (13 %) | 7 (19 %) | 11 (35 %) |
| C | 3 (9 %)* | 15 (41 %) | 9 (30 %) |

*Significantly different ($P<005$).

### Characteristics of HBV genotypes among HCC patients

To clarify the virological and clinical characteristics of HCC patients, we compared several factors, such as age, HBeAg positivity and the frequency of mutations among HCC patients with each of the three genotypes (Table 4). There were no significant differences in age, HBeAg positivity or the frequency of the precore stop mutation A1896 in the three genotypes. The prevalence of the T1653 mutation was significantly higher in HCC patients with HBV/C than in those with HBV/B ($P<0·05$) (Table 4). Logistic regression analysis was performed on age, sex, HBeAg, HBV genotype and the mutations T1653, T1762/A1764 and A1896 to identify factors that might contribute to the progression to HCC. Age [odds ratio (OR) 3·43; 95 % confidence interval (CI) 1·04–11·36; $P=0·044$] and the presence of the T1762/A1764 mutation (OR 14·08; 95 % CI, 3·62–4·74; $P<0·001$) were significant risks for progression to HCC.

## DISCUSSION

The findings of the present study indicate that HBV/A, B and C are prevalent in the Philippines and that HBV/Aa (A1) is the major genotype. A novel HBV/C cluster was found in the Philippines on the basis of complete genome analyses. This cluster is designated HBV/C5, and it includes

**Table 3.** Demographic, clinical and virological characteristics among HBV genotypes

| Feature | HBV/A ($n=51$) | HBV/B ($n=22$) | HBV/C ($n=27$) | $P$ value |
|---|---|---|---|---|
| Male | 42 (86%) | 17 (77%) | 17 (63%) | NS |
| Age (years) | 40·2±14·8* | 52·7±11·8 | 50·9±18·5 | <0·01 |
| HBeAg | 36 (71%) | 17 (77%) | 14 (52%) | NS |
| Clinical status: | | | | |
| CH | 25 (49%)* | 4 (18%) | 3 (11%) | <0·001 |
| LC | 15 (29%)* | 7 (32%) | 15 (56%) | <0·02 |
| HCC | 11 (22%)* | 11 (50%) | 9 (33%) | <0·02 |
| Mutations in the core promoter: | | | | |
| T1653 | 9 (18%) | 0 (0%)* | 6 (22%) | <0·05 |
| T1762/A1764 | 9 (18%)* | 10 (46%) | 11 (41%) | <0·05 |
| Mutations in Kozak sequences preceding the ATG initiator codon: | | | | |
| T1809 | 49 (96%)* | 0 (0%) | 0 (0%) | <0·0001 |
| T1812 | 49 (96%)* | 0 (0%) | 0 (0%) | <0·0001 |
| Mutations in the precore region: | | | | |
| T1858 | 1 (2%)* | 22 (100%) | 27 (100%) | <0·0001 |
| T1862 | 49 (96%)* | 0 (0%) | 0 (0%) | <0·0001 |
| H1888 | 44 (86%)* | 0 (0%) | 0 (0%) | <0·0001 |
| A1896 | 0 (0%) | 2 (9%) | 1 (4%) | NS |

*Significantly different from other genotypes. NS, No significant difference.

two HBV/C strains isolated previously in Vietnam (Hannoun *et al.*, 2000), which are distinct from previous HBV/C strains (C1–4) (Norder *et al.*, 2004). As reported by Hannoun *et al.* (2000), these strains were relatively divergent from previously reported strains, with an overall nucleotide difference of 4–8% justifying the classification of HBV/C5 as a distinct subtype (subgenotype) according to recent proposals on HBV nomenclature. Phylogenetic analyses of the four ORFs (PreS1/PreS2/S, X, P and precore/core) supported the conclusion that the HBV/C5 strains, including the two Vietnamese strains and five strains from this study, formed a specific cluster without recombination. In general, genotypic classification of HBV is likely to correlate with the geographical origin of strains. Interestingly, phylogenetic analyses on the core promoter and precore/core regions showed that most HBV/C strains ($n=25$) from the Philippines belonged to the novel subtype (subgenotype) HBV/C5, whereas only two strains from Vietnam were

**Table 4.** Characteristics of HBV genotypes among HCC patients

| Feature | HBV/A ($n=11$) | HBV/B ($n=11$) | HBV/C ($n=9$) | $P$ value |
|---|---|---|---|---|
| Age (years) | 52·8±17·2 | 55·8±12·7 | 52·7±16·3 | NS |
| HBeAg | 5 (45%) | 7 (64%) | 5 (56%) | NS |
| Mutations in the core promoter: | | | | |
| T1653 | 4 (36%) | 0 (0%)* | 4 (44%) | <0·05 |
| T1762/A1764 | 3 (27%)* | 10 (91%) | 8 (89%) | <0·005 |
| Mutations in Kozak sequences preceding the ATG initiator codon: | | | | |
| T1809 | 10 (91%)* | 0 (0%) | 0 (0%) | <0·0001 |
| T1812 | 11 (100%)* | 0 (0%) | 0 (0%) | <0·0001 |
| Mutations in the precore region: | | | | |
| T1858 | 1 (9%)* | 11 (100%) | 9 (100%) | <0·0001 |
| T1862 | 11 (100%)* | 0 (0%) | 0 (0%) | <0·0001 |
| H1888 | 7 (86%)* | 0 (0%) | 0 (0%) | <0·0001 |
| A1896 | 0 (0%) | 1 (9%) | 0 (0%) | NS |

*Significantly different from other genotypes. NS, No significant difference.

included in this group; most HBV/C strains from Vietnam have been classified into HBV/Cs (C1). Hence, the origin of HBV/C5 might be the Philippines.

Twenty-two genotype/subtype (subgenotype) -specific substitutions were found in the HBV/C5 strains; seven of them were specific for the five HBV/C5 strains from the Philippines. We have already reported a new PCR-RFLP method to distinguish between HBV/C1 and HBV/C2. Using an RFLP with these five specific sites for HBV/C5, it would be possible to discriminate HBV/C5 conveniently among HBV/C. Investigating the mutation pattern of HBV/C5 for distinctive mutations between HBV/C1 and HBV/C2 reported previously (Tanaka *et al.*, 2005), the mutation pattern of HBV/C5 in the encapsidation signal is close to that of HBV/C2; all HBV/C5 strains had C1856 and T1858 mutations, which are also found in HBV/C2. Generally, the precore stop mutation A1896 is accompanied by a C-to-T substitution at nt 1858, forming a base pair with it on the secondary structure of the encapsidation signal, and these mutations can stabilize the ε-loop structure. Although T1858 is frequently found in HBV/C5, only one of 25 HBV/C5 strains possessed the A1896 mutation.

There were also 22 HBV/B strains found in the Philippines, and most of them (21/22, 95 %) formed a specific cluster distinct from previously reported HBV/B strains. It has been reported that genotype B can be classified into two subgroups: HBV/Ba (B2), which is prevalent in Asia, and HBV/Bj (B1), which is restricted to Japan (Sugauchi *et al.*, 2002). Furthermore Norder *et al.* (2004) reported that HBV/B strains could be divided into four subtypes (subgenotypes) confirmed by significant bootstrap support when comparing 72 HBV/B complete genomes from around the world. In our phylogenetic analysis of complete genome sequences, HBV/B strains from the Philippines were newly classified into a fifth group, carrying a recombination with HBV/C over the precore and core genes that is also found in the original HBV/Ba (B2). However, because we could only determine the complete genomes of two HBV/B strains from the Philippines, the clinical and virological differences between HBV/B from the Philippines and other strains were not able to be clarified.

In the Philippines, HBV/Aa (A1) is one of the major genotypes, as it is in South Africa (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2004). HBV/Aa (A1) in South Africa is suggested to be associated with HCC in younger carriers (Kew *et al.*, 2005); however, in the present study, the proportion of HBV/Aa (A1) was higher in patients with CH than in those with LC or HCC. HBV/C or HBV/B in the Philippines was associated with HCC development, suggesting that HBV/Aa (A1) in the Philippines might be different from HBV/Aa (A1) in South Africa. To elucidate the virological differences between HBV/Aa (A1) strains in the Philippines and South Africa, complete genome sequences were compared. Most HBV/Aa (A1) from the Philippines had specific mutations in the encapsidation signal of the precore region (T1862 and H1888) and specific substitutions in the Kozak sequences (T1809 and T1812), as did South African strains (Tanaka *et al.*, 2004; Kimbi *et al.*, 2004). We could not find virological characteristics to distinguish the HBV/Aa (A1) from the Philippines from that in South Africa because only two complete sequences were obtained from HBV/Aa (A1) strains in the Philippines. Further functional studies of HBV/Aa (A1) strains in the Philippines and South Africa will be required in order to establish the difference between these strains.

Finally, we compared other mutations among the three genotypes to find clinical manifestations. Ito *et al.* (2006) reported that the prevalence of T1653 was found to be significantly higher in HCC patients than in CH patients with HBV/C, indicating that T1653 was a predictive factor for HCC in HBV/C. In this study, the prevalence of the T1653 mutation was significantly higher in HCC patients with HBV/C only. These findings indicate that T1653 mutation is one of the critical risk factors for HCC with HBV/C. It was also reported that HBV carriers with the core promoter double mutation T1762/A1764 were at increased risk of HCC and that this mutant may contribute to the pathogenesis of HBV infection. In agreement with previous reports, our data indicate that a high prevalence of the core promoter double mutation was found in HCC patients with HBV/B or HBV/C. HBV/B strains from the Philippines carried the recombination with HBV/C over the core promoter, precore and core genes that is found in original HBV/Ba (B2) strains. Multiple logistic regression analysis showed that age and the presence of the core promoter double mutation were independent risk factors for progression to HCC. However, HBV genotype (HBV/B and C) was not a significant factor for progression to HCC, since both HBV/B and HBV/C were also prevalent in LC patients in this study.

In conclusion, HBV genotypes A, B and C are prevalent in the Philippines. We found novel subtypes (subgenotypes) of HBV/C and HBV/B among chronic liver disease patients in the Philippines. However, few clinical differences among these subtypes (subgenotypes) have been reported. Further clinical studies would be required in a case–control study with large-scale cohorts to evaluate the clinical manifestations of HBV/C5 and HBV/B5.

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## REFERENCES

**Akuta, N., Suzuki, F., Kobayashi, M. & 9 other authors (2003).** The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. *J Hepatol* 38, 315–321.

**Arauz-Ruiz, P., Norder, H., Robertson, B. H. & Magnius, L. O. (2002).** Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* **83**, 2059–2073.

**Bowyer, S. M., van Staden, L., Kew, M. C. & Sim, J. G. (1997).** A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* **78**, 1719–1729.

**Chan, H. L., Hui, A. Y., Wong, M. L., Tse, A. M., Hung, L. C., Wong, V. W. & Sung, J. J. (2004).** Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut* **53**, 1494–1498.

**Chu, C. J., Keeffe, E. B., Han, S. H. & 8 other authors (2003).** Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology* **125**, 444–451.

**Gojobori, T., Ishii, K. & Nei, M. (1982).** Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J Mol Evol* **18**, 414–423.

**Hannoun, C., Norder, H. & Lindh, M. (2000).** An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. *J Gen Virol* **81**, 2267–2272.

**Ito, K., Tanaka, Y., Orito, E. & 12 other authors (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.

**Kew, M. C., Kramvis, A., Yu, M. C., Arakawa, K. & Hodkinson, J. (2005).** Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-Saharan Africans. *J Med Virol* **75**, 513–521.

**Kimbi, G. C., Kramvis, A. & Kew, M. C. (2004).** Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* **85**, 1211–1220.

**Kramvis, A., Weitzmann, L., Owiredu, W. K. & Kew, M. C. (2002).** Analysis of the complete genome of subgroup A′ hepatitis B virus isolates from South Africa. *J Gen Virol* **83**, 835–839.

**Kramvis, A., Kew, M. & Francois, G. (2005).** Hepatitis B virus genotypes. *Vaccine* **23**, 2409–2423.

**Norder, H., Courouce, A. M. & Magnius, L. O. (1994).** Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* **198**, 489–503.

**Norder, H., Courouce, A. M., Coursaget, P., Echevarria, J. M., Lee, S. D., Mushahwar, I. K., Robertson, B. H., Locarnini, S. & Magnius, L. O. (2004).** Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* **47**, 289–309.

**Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, R. I., Imai, M., Miyakawa, Y. & Mayumi, M. (1988).** Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* **69**, 2575–2583.

**Orito, E., Ichida, T., Sakugawa, H. & 11 other authors (2001).** Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* **34**, 590–594.

**Robertson, B., Myers, G., Howard, C. & 14 other authors (1998).** Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. *Arch Virol* **143**, 2493–2503.

**Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

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

**Sugauchi, F., Mizokami, M., Orito, E. & 7 other authors (2001).** A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* **82**, 883–892.

**Sugauchi, F., Orito, E., Ichida, T. & 9 other authors (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.

**Sugauchi, F., Orito, E., Ichida, T. & 10 other authors (2003).** Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* **124**, 925–932.

**Sugauchi, F., Kumada, H., Acharya, S. A. & 10 other authors (2004).** Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* **85**, 811–820.

**Takahashi, K., Ohta, Y., Kanai, K., Akahane, Y., Iwasa, Y., Hino, K., Ohno, N., Yoshizawa, H. & Mishiro, S. (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.

**Tanaka, Y., Hasegawa, I., Kato, T. & 11 other authors (2004).** A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* **40**, 747–755.

**Tanaka, Y., Orito, E., Yuen, M.-F. & 8 other authors (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.

**Vieth, S., Manegold, C., Drosten, C., Nippraschk, T. & Gunther, S. (2002).** Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. *Virus Genes* **24**, 153–156.

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# A case–control study of response to lamivudine therapy for 2 years in Japanese and Chinese patients chronically infected with hepatitis B virus of genotypes Bj, Ba and C

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**Abstract**

*Background/aims:* In eastern Asian countries, hepatitis B virus (HBV) genotype Ba (HBV/Ba), HBV/Bj and HBV/C are prevalent. The aim was to investigate the response or resistance to lamivudine therapy among patients with different HBV genotypes.
*Methods:* Of 67 Japanese and Chinese patients with chronic hepatitis B, 18 patients with HBV/Bj, 15 with HBV/Ba and 34 with HBV/C were selected for a case–control study matched according to gender and age. All the patients were treated with lamivudine for 2 years and evaluated the response or emergence of the YMDD mutation at year 2 during the treatment. HBV genotypes were detected by the restriction fragment length polymorphism. The YMDD mutation was detected by the direct sequencing after amplification by PCR.
*Results:* At year 2 during therapy, 44.8% of the patients showed normalization of ALT and undetectable HBV DNA (favorable response), 35.8% developed the YMDD mutation. There was no significant difference of response to the therapy among the three genotype groups. The emergence of the YMDD mutation was associated with HBV/C. By the multiple logistic regression analysis, however, the significant factor of a favorable response was a higher pretreatment ALT level and negative HBeAg status and the significant factor of the emergence of the YMDD mutation was HBV/C.
*Conclusions:* Higher pretreatment ALT level, HBeAg status or HBV genotype may affect the response or resistance to lamivudine therapy.


*Keywords:* HBV genotype Ba, Bj, C; Lamivudine; Response to therapy; Resistant mutation

## 1. Introduction

Hepatitis B virus (HBV) is one of the major causative agents of acute or chronic liver diseases in Asian countries [1]. All strains of HBV isolated from various countries can be classified into eight HBV genotypes, HBV genotype A

*Abbreviations:* HBV/B, hepatitis B virus genotype B; HBV/Ba, hepatitis B virus subtype Ba; HBV/Bj, hepatitis B virus subtype Bj; HBV/C, hepatitis B virus genotype C

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(HBV/A) to HBV/H, according to their phylogenetic relationship [2–4]. As reported previously, patients with different HBV genotypes show genotype-specific manifestations in their clinical and virologic features [5–8]. In addition, geography-specific distributions of HBV genotypes have been demonstrated among areas and countries in world wide [5,7,9]. In south-east Asian countries, such as Japan, Taiwan or China, HBV/B and HBV/C are most prevalent [6,7,9]. Patients with HBV/B tend to have anti-HBe at a younger age and less chance to develop hepatocellular carcinoma compared to those with HBV/C [6–8,10].

Recently, we have demonstrated that HBV/B strains should be divided into two subtypes, HBV/Ba and HBV/Bj, according to their genetic relationship, and that HBV/Ba is found ubiquitously in Asian countries, while HBV/Bj is found only in Japan [11,12]. We also demonstrated that a higher proportion of patients with HBV/Ba have a HBeAg-positive status and hepatocellular carcinoma at a younger age than those with HBV/Bj [12,13].

Lamivudine is one of the widely available anti-virus drugs for patients with chronic HBV infection, which strongly suppress the reverse transcriptase of HBV. However, some patients develop unsatisfactory anti-viral effects during therapy because of the emergence of lamivudine-resistant strains during therapy [14,15]. It is still controversial whether the efficacy of lamivudine therapy may be associated with the difference of HBV genotypes. Therefore, the aim of this study was to investigate the response to lamivudine therapy for 2 years among patients with different genotypes of HBV/Ba, HBV/Bj or HBV/C.

## 2. Patients and methods

### 2.1. Patient selection

One hundred and twenty-two patients with chronic HBV infection were treated with lamivudine, 100 mg daily, for 2 years in various hospitals in Japan and Hong Kong. All patients were consecutively enrolled in this study, and informed consent was obtained. The patients with normal ALT levels (40 IU/L or less) before commencement of the treatment were excluded. All patients were positive for HBsAg for more than 6 months and negative for both anti-HCV and anti-HIV. All patients were tested by ultrasonography or CT scan to rule out decompensated cirrhosis or hepatocellular carcinoma. For the comparison of the response to lamivudine therapy among different genotypes, 18 patients with HBV/Bj, 15 patients with HBV/Ba and 34 patients with HBV/C were selected and matched according to gender and age for a case–control study. Liver biopsy was performed in the 45 patients who agreed.

For the HBeAg-positive patients, a complete response to lamivudine therapy was defined as the normalization of ALT, undetectable serum HBV DNA by the real-time PCR method, and seroconversion from HBeAg to anti-HBe at year 2 during therapy. For all the patients, including both the HBeAg-positive and -negative groups, a favorable response to lamivudine therapy was defined as the normalization of ALT and undetectable serum HBV DNA at year 2 during therapy.

Lamivudine resistance was defined as the emergence of the YMDD motif mutation (YIDD and/or YVDD) at year 2. Breakthrough hepatitis was designated when emergence of the YMDD mutation and re-elevation of ALT level and HBV DNA level were found.

### 2.2. Virologic assays

HBsAg, HBeAg and anti-HBe were tested by chemiluminescence enzyme immunoassay (CLEIA). The serum HBV DNA level in all the samples was quantified by the real-time PCR method (HBV RTD-Direct test, SRL Inc., Tokyo) in Nagoya City University. The detection limit of this assay was 100 copies/mL. The HBV genotype was determined by restriction fragment length polymorphism as described previously [16]. When the test results were inconclusive, the sequences of the S region were determined directly, then the genotype was decided by phylogenetic analysis [16,17]. The subtypes of HBV/Ba and HBV/Bj were also determined by restriction fragment length polymorphism [11]. The YMDD motif mutations (M204I/V) during lamivudine therapy were detected by the direct sequencing method after amplification by PCR. The Pre-C mutation at nucleotide position 1896 and the basal core promoter double mutations at nucleotide positions 1762 and 1764 were detected using the direct sequencing method [18].

### 2.3. Statistical analysis

The data were statistically analyzed by Student's $t$-test, the non-parametric Mann–Whitney test and the Chi-square test where appropriate. The multivariate analysis was accomplished by multiple logistic regression analysis. A $p$-value less than 0.05 was regarded as statistically significant.

## 3. Results

### 3.1. Baseline differences among the three HBV genotype groups

In the various baseline clinical and virologic factors, there were significant differences among the HBV/Bj, HBV/Ba and HBV/C groups, while age and gender were matched (Table 1). In the HBV/C group, a higher proportion of the patients at the F3 or F4 stage were found compared to the HBV/Bj or HBV/Ba group. The mean ALT level before treatment in the HBV/Ba group was significantly lower than the HBV/Bj or HBV/C group. Only 3% of the HBV/Bj group showed positive HBeAg, compared to 80.0% of the HBV/Ba group and 79.4% of the HBV/C group. The mean serum HBV

Table 1
Baseline clinical and virologic characteristics of the patients studied

| Characteristics | HBV genotype | | | p-Value |
|---|---|---|---|---|
| | HBV/Bj ($n=18$) | HBV/Ba ($n=15$) | HBV/C ($n=34$) | |
| Gender (M:F) | 18:0 | 15:0 | 34:0 | Matched |
| Age (year, mean ± S.D.) | 43.4 ± 11.4 | 40.5 ± 15.2 | 43.2 ± 8.9 | Matched |
| Ethnic (Japanese:Chinese) | 18:0 | 5:10 | 24:10 | <0.01 |
| Stage (F1:F2:F3:F4:ND) | 10:0:2:0:6 | 4:1:1:0:9 | 6:6:7:8:7 | <0.01 |
| ALT (IU/L, mean ± S.D.) | 293.3 ± 353.0 | 152.5 ± 130.8 | 309.9 ± 288.3 | <0.05 |
| Positive for HBeAg | 3 (16.7%) | 12 (80.0%) | 27 (79.4%) | <0.01 |
| HBV DNA level (log copies/mL, mean ± S.D.) | 5.96 ± 1.69 | 8.17 ± 0.81 | 7.40 ± 1.09 | <0.01 |
| Pre-C (nt. 1896) mutation (wild:mutant) | 4:14 | 10:5 | 26:8 | <0.01 |
| Core promoter (nt. 1762/1764) mutations (wild:mutant) | 16:2 | 13:2 | 5:27 (ND:2) | <0.01 |

DNA level of the HBV/Bj group was significantly lower than the HBV/Ba or HBV/C group. A higher proportion of the HBV/Bj group had pre-C mutation compared to the HBV/Ba or HBV/C group. In contrast, a higher proportion of the HBV/C group had the core promoter mutations compared to the HBV/Bj or HBV/Ba group.

### 3.2. Response to lamivudine therapy

Of all the 67 patients, 44.8% showed a favorable response to lamivudine therapy (Fig. 1a). 61.1% of the HBV/Bj group, 40.0% of the HBV/Ba group and 38.2% of the HBV/C group showed a favorable response to the therapy, respectively. However, there was no significant difference in the favorable response rate among the different HBV genotype groups.

When the patients were stratified into the HBeAg-positive or -negative group, 33.3% showed complete response in the patients with positive HBeAg, compared to 60.0% in the negative HBeAg group ($p<0.05$; Fig. 1b). There was no significant difference in the favorable response rate among the three HBV genotype groups, even if the

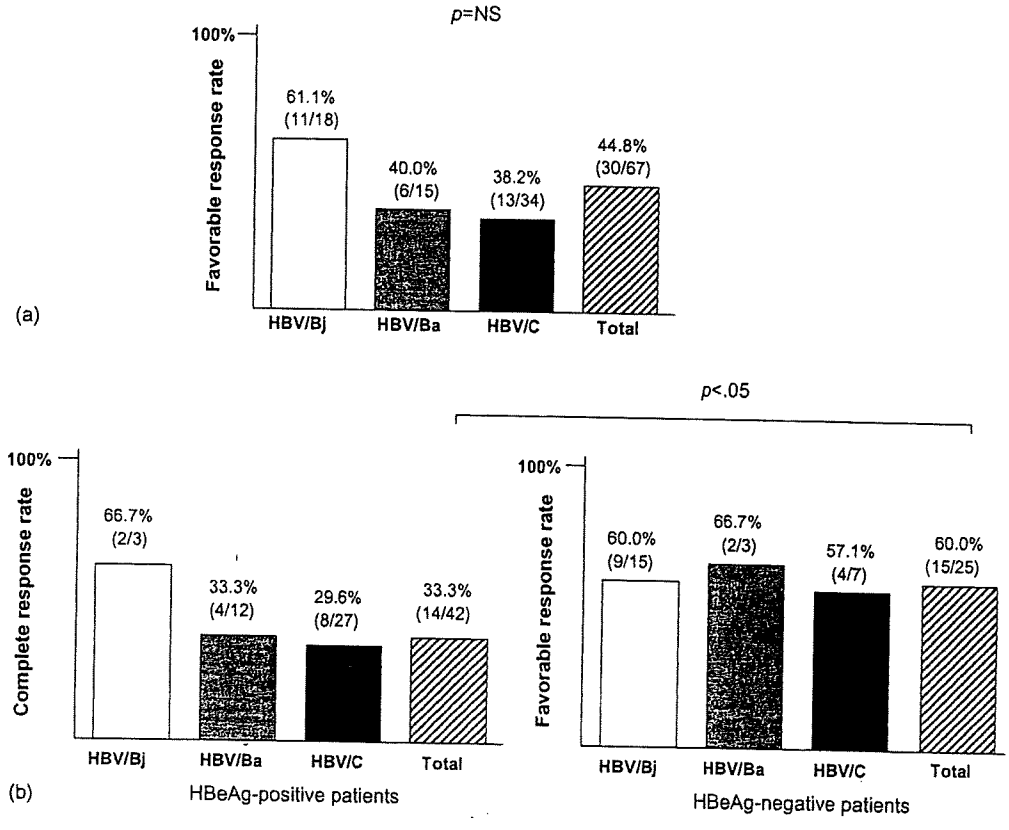


Fig. 1. The rate of favorable responses to lamivudine therapy in both HBeAg-positive and -negative patients among three different genotype groups (a) and the rate of complete response in the HBeAg-positive patients and the rate of favorable response in the HBeAg-negative patients (b).

Table 2
Complete response rate in HBeAg-positive patients with HBV/Ba and HBV/C

| Characteristics | HBV genotype | | p-Value |
|---|---|---|---|
| | HBV/Ba ($n=12$) | HBV/C ($n=27$) | |
| Age (years) | | | |
| <40 | 4/8 (50.0) | 2/13 (15.4) | 0.22 |
| ≥40 | 0/4 (0) | 6/14 (42.9) | 0.31 |
| ALT (IU/L) | | | |
| <200 | 1/9 (11.1)* | 1/9 (11.1) | 0.45 |
| ≥200 | 3/3 (100)* | 7/18 (38.9) | 0.18 |
| HBV DNA level (log copies/mL) | | | |
| <7.5 | 1/1 (100) | 2/10 (20.0) | 0.59 |
| ≥7.5 | 3/11 (27.3) | 6/17 (35.3) | 0.97 |
| Pre-C (nt. 1896) mutation | | | |
| Wild | 2/9 (22.2) | 6/25 (24.9)* | 0.72 |
| Mutant | 2/3 (66.7) | 2/2 (100)* | 0.81 |
| Core promoter (nt. 1762/1764) mutations | | | |
| Wild | 4/12 (33.3) | 1/2 (50.0) | 0.73 |
| Mutant | 0/0 (0) | 7/23 (30.4) | – |

Values in parenthesis are given in percentage.
* $p<0.05$.

patients were stratified into HBeAg-positive or -negative groups.

In the patients with positive HBeAg, pretreatment factors associated with complete response were analyzed in 12 patients in the HBV/Ba group and 27 in the HBV/C group (Table 2). Between the two genotype groups, there were no significant differences of the factors predicting complete response to the therapy in age, ALT level, HBV DNA level, pre-C mutation and core promoter mutations. However, in the HBV/Ba group, the patients who had 200 IU/L or higher ALT levels showed complete response more frequently than those with less than 200 IU/L. In the HBV/C group, a higher proportion of the patients with pre-C mutation tended to have a complete response compared to those with wild pre-C.

Of the patients with negative HBeAg, 15 in the HBV/Bj group and 7 in the HBV/C group, there were no significant differences in the predicting factors for a favorable response to the therapy between the HBV/Bj and HBV/C groups (Table 3).

To investigate the significance of predicting factors associated with complete response to lamivudine therapy in HBeAg-positive patients, stepwise logistic regression analysis was performed (Table 4). A factor of the pretreatment ALT level of 200 IU/L or higher and pre-C mutation were significant factors (odds ratio: 12.056, $p=0.03$ and 25.553, $p=0.03$, respectively).

In all the patients with both positive and negative HBeAg, the significant factors predicting a favorable response to the therapy were a pretreatment ALT level of 200 IU/L or higher (odds ratio: 3.715, $p=0.02$) and a negative HBeAg status (odds ratio: 3.472, $p=0.03$; Table 5). The other factors were not significant.

Table 3
Favorable response rate in HBeAg-negative patients with HBV/Bj and HBV/C

| Characteristics | HBV genotype | | p-Value |
|---|---|---|---|
| | HBV/Bj ($n=15$) | HBV/C ($n=7$) | |
| Age (years) | | | |
| <40 | 1/2 (50.0) | 1/1 (100) | 0.67 |
| ≥40 | 8/13 (61.5) | 3/6 (50.0) | 0.98 |
| ALT (IU/L) | | | |
| <200 | 4/8 (50.0) | 1/3 (33.3) | 0.85 |
| ≥200 | 5/7 (71.4) | 3/4 (75.0) | 0.56 |
| HBV DNA level (log copies/mL) | | | |
| <7.5 | 7/13 (53.8) | 1/4 (25.0) | 0.66 |
| ≥7.5 | 2/2 (100) | 3/3 (100) | – |
| Pre-C (nt. 1896) mutation | | | |
| Wild | 2/9 (22.2) | 1/1 (100) | 0.65 |
| Mutant | 7/13 (53.8) | 3/6 (50.0) | 0.74 |
| Core promoter (nt. 1762/1764) mutations | | | |
| Wild | 7/13 (53.8) | 2/3 (66.7) | 0.81 |
| Mutant | 2/2 (100) | 2/4 (50.0) | 0.76 |

Values in parenthesis are given in percentage.

Table 4
Significant factors associated with complete response to lamivudine therapy in HBeAg-positive patients by the stepwise logistic regression analysis

| Factors | Odds ratio | 95% CI | p-Value |
|---|---|---|---|
| ALT level (IU/L) | | | |
| <200 | 1 | | |
| ≥200 | 12.056 | 1.322–109.907 | 0.03 |
| Pre-C (nt. 1896) mutation | | | |
| Wild | 1 | | |
| Mutant | 25.553 | 1.391–469.526 | 0.03 |

### 3.3. Emergence of YMDD mutation

Of all the patients, 35.8% emerged the YMDD mutation at year 2 during therapy (Fig. 2a). In the HBV/C group, 50.0% had the YMDD mutation compared to 27.8% in the HBV/Bj and 13.3% in the HBV/Ba group ($p<0.05$, respectively). When the patients were stratified into positive or negative HBeAg status, 51.9% of the HBV/C group with positive HBeAg emerged the YMDD mutation compared to 8.3% of the HBV/Ba group with positive HBeAg, even though HBeAg positivity and HBV DNA levels at baseline

Table 5
Significant factors associated with favorable response to lamivudine therapy in both HBeAg-positive and -negative patients by the logistic regression analysis

| Factors | Odds ratio | 95% CI | p-Value |
|---|---|---|---|
| ALT level (IU/L) | | | |
| <200 | 1 | | |
| ≥200 | 3.715 | 1.269–10.875 | 0.02 |
| HBeAg status | | | |
| Positive | 1 | | |
| Negative | 3.472 | 1.156–10.417 | 0.03 |