and results in a marked decrease of HBV DNA and serum transaminase levels, seroconversion of HBe antigen (HBeAg) to anti-HBe, and histopathological improvement [4–9].

The optimal duration of lamivudine administration for HBV-infected patients is still controversial for two main problems; drug resistance and sustainability of the response to treatment. In particular, there is a need to evaluate short-term treatment with respect to post-treatment safety and the sustainability of responses, and long-term treatment with regard to biochemical relapse (breakthrough hepatitis) associated with the emergence of YMDD motif mutant [10–17]. The American Association for the Study of Liver Diseases practice guidelines suggested that lamivudine could be discontinued in patients who had completed one year of treatment and had persistent HBeAg seroconversion on more than one occasion determined 2–3 months apart [18]. However, this is not completely evaluated in Japanese genotype C-dominant hepatitis B patients.

The present study was designed to deal with the following three issues: (1) To compare the virological and biochemical relapse rates according to the continuation or termination of lamivudine monotherapy, and to compare the risk of biochemical relapse after the termination of the treatment and breakthrough hepatitis during long-term treatment; (2) to determine the independent predictive factors at discontinuation of treatment that contributed to early biochemical relapse in discontinuous patients, and (3) to evaluate the efficacy of retreatment with lamivudine monotherapy.

## Patients and Methods

*Patients*

Lamivudine therapy was provided to 394 consecutive patients with chronic hepatitis B who tested positive for HBs antigen at Toranomon Hospital between September 1995 and December 2002. Among these, 269 patients started lamivudine monotherapy at abnormal alanine transferase (ALT) levels (normal for ALT, 6–50 IU/l) and were able to achieve ALT normalization during treatment, and were enrolled in this retrospective study. The latter group consisted of 25 patients who stopped the lamivudine monotherapy during ALT normalization (discontinuous group) and 244 patients who did not stop the lamivudine monotherapy (continuous group), and the discontinuation or not of lamivudine during ALT normalization was selected at their own request. To compare the cumulative virological and biochemical relapse rates between the discontinuous group and continuous group, all 25 patients of the discontinuous group entered this study along with 75 patients of the continuous group. The latter group was selected from among the 244 because they matched patients of the discontinuous group with respect to sex, age, and observation period after the start of lamivudine monotherapy. They had been confirmed to have hepatitis by liver biopsies, were free of decompensated liver cirrhosis and hepatocellular carcinoma. Coinfection and superinfection with hepatitis A, C, and delta viruses, and human immunodeficiency virus were ruled out serologically or genomically using commercially available kits or conventional polymerase chain reaction (PCR)-based assays. None of the patients had a history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, and metabolic disease.

Patients were given a dose of 100 mg of lamivudine once a day. The median period of treatment in the discontinuous group (0.72 years, range; 0.10–5.6 years) was significantly shorter than that of the continuous group (1.8 years, range; 0.71–7.6 years, $p < 0.0001$). The median observation period after the commencement of lamivudine therapy was not significantly different based on the matching of the two groups, and the periods were 2.1 years (range; 0.68–7.9 years) in the discontinuous group and 1.8 years in the continuous group (range; 0.71–7.6 years). In the discontinuous group, the median observation period after discontinuation of lamivudine therapy was 1.4 years (range; 0.15–6.7 years). With regard to the observation period, patients of the discontinuous group who received another course of lamivudine treatment for biochemical relapse and those of the continuous group who received additional interferon treatment for biochemical relapse, were treated as censored data at the time of lamivudine retreatment and additional interferon treatment in the statistical analysis of cumulative relapse rates. The clinical characteristics of enrolled patients are summarized in table 1, and those of discontinuation are shown in table 2.

*Methods*

Our study compared virological and biochemical relapse in continuous and discontinuous lamivudine monotherapy groups, and determined the independent predictive factors at discontinuation that contributed to early biochemical relapse in the discontinuous group. Furthermore, we also evaluated the efficacy of retreatment with lamivudine monotherapy. Patients in whom ALT levels became abnormal (>50 IU/l) after a period of ALT normalization were defined as biochemical relapsers. Patients in whom levels of HBV DNA re-elevated after the minimum levels, ignoring undetectable HBV DNA levels, were defined as virological relapsers. Especially, virological relapse during lamivudine treatment associated with the emergence of YMDD motif mutant were defined as DNA breakthrough, and biochemical relapse associated with DNA breakthrough were defined as breakthrough hepatitis. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by a detailed interview and medical examination at least once every month. Patient compliance with treatment was evaluated by a questionnaire.

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for various laboratory data including ALT levels, HBV DNA levels, and the presence of YMDD motif mutant. The serum samples were stored in aliquots at −80°C until use. HBs antigen and HBeAg/eAb were determined by radioimmunoassay (Abbott Diagnostics, Chicago, Ill., USA). HBV DNA was measured by transcription-mediated amplification and hybridization protect assay (TMA-HPA) (Chugai Diagnostica, Tokyo, Japan). The lower and upper limits of detection of TMA-HPA are $5 \times 10^3$ and $5 \times 10^8$ viral genomic equivalents (GE)/ml, respectively. HBV genotype was determined using a previously reported

**Table 1.** Clinical characteristics of enrolled patients

| | Discontinuous group (n = 25) | Continuous group (n = 75) | p value |
|---|---|---|---|
| Age, years[a] | 33 (19–75) | 34 (20–75) | matched |
| Sex, male/female | 19/6 | 57/18 | matched |
| Period of observation[b] | 2.1 (0.68–7.9) | 1.8 (0.71–7.6) | matched |
| HBV DNA, LGE/ml[a] | 7.6 (<3.7 to >8.7) | 7.5 (<3.7 to >8.7) | NS |
| HBeAg, number of positive | 20 (80.0%) | 45 (60.0%) | NS |
| HBV genotype, number of C | 22 (88.0%) | 61 (81.3%) | NS |
| Liver cirrhosis[c] | 2 (8.0%) | 3 (4.0%) | NS |
| Family history of liver disease[d] | 18 (72.0%) | 59 (78.7%) | NS |
| T-Bil, mg/dl[a] | 0.7 (0.3–20.7) | 0.7 (0.3–10.5) | NS |
| ALT, IU/l[a] | 97 (51–3,168) | 150 (53–2,274) | NS |
| Albumin, g/dl[a] | 3.9 (2.8–4.3) | 3.8 (2.5–4.8) | NS |
| Cholinesterase, ΔpH[a] | 1.0 (0.6–1.5) | 1.1 (0.5–1.7) | NS |
| Duration of lamivudine therapy, years[a] | 0.72 (0.10–5.6) | 1.8 (0.71–7.6) | <0.0001 |

[a] Data expressed as median (range).
[b] Period of follow-up after the start of lamivudine therapy.
[c] Scoring according to the system of Desmet et al. [22].
[d] Family history of positivity for hepatitis B surface antigen including third-degree relatives.
LGE = Logarithm of genome equivalent per millilitre; T-Bil = total bilirubin; ALT = alanine transferase (normal range ≤ 50 IU/l); NS = not significant.

**Table 2.** Characteristics of patients at discontinuation of lamivudine monotherapy

| | |
|---|---|
| Number | 25 |
| Sex, male/female | 19/6 |
| Age, years[a] | 34 (19–75) |
| Number of cirrhosis | 2 (8.0%) |
| HBV genotype, number with genotype C | 22 (88.0%) |
| Family history of liver disease[b] | 18 (72.0%) |
| HBeAg, number of positive | 9 (36.0%) |
| HBV DNA, patients with <3.7 LEG/ml | 18 (72.0%) |
| T-Bil, mg/dl[a] | 0.7 (0.3–1.3) |
| ALT, IU/l[a] | 23 (10–50) |
| BCP nt 1762/1764 (W/M/mi/N) | 5/8/2/10 |
| PC nt 1896 (W/M/mi/N) | 10/3/2/10 |
| Presence of YMDD motif mutant | 3 (12.0%)[c] |
| Duration of lamivudine therapy, years[a] | 0.72 (0.10–5.6) |

[a] Data expressed as median (range), or number of patients.
[b] Family history of positivity for hepatitis B surface antigen including third-degree relatives.
[c] One patient was PCR-negative with serum sample at stop of treatment, but had been already detected before stop.
Abbreviations, as in table 1, BCP = Basic core promoter; PC = precore; nt = nucleotide. W = wild type (BCP; $A^{1762}G^{1764}$. PC; $G^{1896}$); M = mutant type; mi = mixed type of wild and mutant virus; N = PCR-negative.

method [19, 20]. Antibody against HCV was detected with a third-generation enzyme-linked immunoassay (Ortho Diagnostic Japan, Tokyo). YMDD motif mutant was detected using the sensitive PCR-restriction fragment length polymorphism [21].

Liver biopsy specimens were obtained percutaneously or at laparoscopy using a modified Vim Silverman needle of 2 mm internal diameter (Tohoku University style, Kakinuma Factory, Tokyo). Each specimen was scored according to the system of Desmet et al. [22].

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from each patient. The study was approved by the Human Ethics Committee of Toranomon Hospital.

*Nucleotide Sequencing of HBV Basic Core Promoter (nt 1762/1764) and Precore (nt 1896)*

Nucleotide sequences of HBV were compared with the prototype sequences of the HBV genotype C [19]. HBV DNA was extracted with a Smitest EX & R kit (Genome Science, Tokyo). Nucleic acids were amplified by nested PCR using the following primers. Nucleotide sequences of basic core promotor (BCP) nt 1762/1764 and precore (PC) nt 1896: The first-round PCR was performed with BCP-F7 [sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ (nt 1580–1599)] and BCP-R8 [antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ (nt 2295–2276)] primers, and the second-round PCR with BCP-F5 [sense, 5′-GCA TGG AAA CCA CCG TGA AC-3′ (nt 1606–1625)] and BCP-R6 [antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ (nt 2014–1995)] primers. All samples were initially denatured at 95°C for 4 min. Thirty-five cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min

at 55 °C, and extension for 3 min at 72 °C with an additional 7 min for extension. Then 1 μl of the first-round PCR product was transferred to the second-round PCR reaction. Other conditions for the second-round PCR were the same as the first-round PCR, except that the second-round PCR primers were used instead of the first-round PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Perkin-Elmer, Chiba, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [23] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

*Statistical Analysis*

The $\chi^2$ test, Fisher's exact probability test, and Mann-Whitney's U test were used to compare the background characteristics between groups. The cumulative virological and biochemical relapse rates were calculated using the Kaplan-Meier technique, differences between the curves were tested using the log-rank test. Statistical analyses of virological and biochemical relapse periods according to the mode of monotherapy (continuous and discontinuous groups) were calculated using the period from the start of lamivudine monotherapy, and those concerned with the characteristics of the discontinuous group were calculated using the period after discontinuation of the treatment. Stepwise Cox regression analysis was used to determine independent predictive factors at discontinuation of lamivudine monotherapy that contributed to early biochemical relapse after discontinuation of the treatment. We also calculated the odds ratios and 95% confidence intervals. Potential predictive factors associated with early biochemical relapse included the following ten variables at discontinuation of treatment: sex, age, histological stage, HBV genotype, levels of HBV DNA, HBeAg, pattern of BCP and PC, presence of YMDD motif mutant, and duration of lamivudine therapy. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS, Chicago, Ill., USA). All p values <0.05 by the two-tailed test were considered significant.

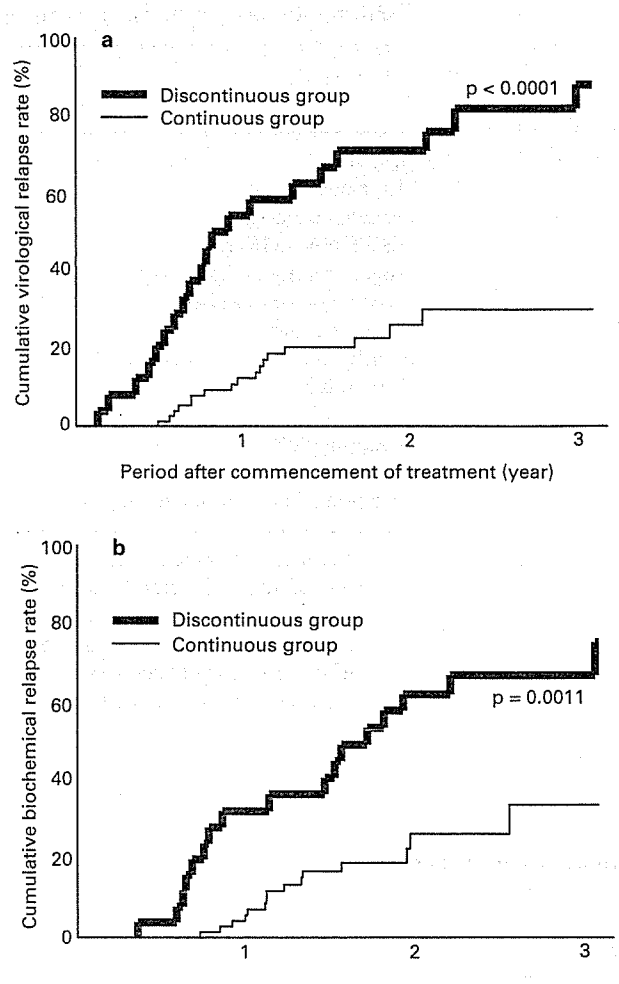


**Fig. 1.** Virological and biochemical relapse rates according to the continuation or discontinuation of lamivudine monotherapy, in patients matched for age, sex, and observation period after the start of treatment. **a** Cumulative virological relapse rates after the commencement of treatment. **b** Cumulative biochemical relapse rates after the commencement of treatment. Virological and biochemical relapse in the discontinuous group emerged significantly earlier than in the continuous group.

## Results

*Virological and Biochemical Relapse*

Virological relapse occurred in 24.0% (18 of 75 patients) of patients of the continuous group and 84.0% (21 of 25) of the discontinuous group. The cumulative virological relapse rates of the continuous and discontinuous group were 12.3 and 54.1% at the end of one year after the commencement of lamivudine monotherapy; 26.0 and 70.8% at 2 years; and 30.1 and 87.8% at 3 years, respectively. Virological relapse in the discontinuous group emerged significantly earlier than the continuous group ($p < 0.0001$; log-rank test) (fig. 1a).

Biochemical relapse occurred in 22.7% (17 of 75 patients) of patients of the continuous group and 68.0% (17 of 25 patients) of the discontinuous group. The cumulative biochemical relapse rates of the continuous and discontinuous group were 4.27 and 32.2% at the end of one year after commencement of lamivudine monotherapy; 26.5 and 61.9% at 2 years; and 33.9 and 66.7% at 3 years, respectively. Biochemical relapse in the discontinuous group emerged significantly earlier than in the continuous group ($p = 0.0011$; log-rank test) (fig. 1b).

**Table 3.** Comparison of ALT and T-Bil levels after biochemical relapse and the emergence of YMDD motif mutant between patients who continued and those who discontinued lamivudine monotherapy

| | Discontinuous group | Continuous group | p value |
|---|---|---|---|
| Biochemical relapse cases (n = 34) | 17 | 17 | |
| Peak T-Bil, mg/dl[a] | 0.9 (0.5–3.8) | 1.1 (0.5–4.9) | NS |
| Peak ALT, IU/l[a] | 384 (191–1,480) | 538 (67–1,736) | NS |
| T-Bil ratio[b] | 1.5 (0.3–3.5) | 2.0 (0.2–12.3) | NS |
| ALT ratio[b] | 3.9 (1.0–18.7) | 3.5 (0.1–10.8) | NS |
| YMDD motif mutant cases (n = 25) | 3 | 22 | |
| Peak T-Bil, mg/dl[a] | 2.1 (0.7–3.8) | 1.1 (0.5–4.9) | NS |
| Peak ALT, IU/l[a] | 441 (50–638) | 236 (26–1,736) | NS |
| T-Bil ratio[b] | 1.0 (0.3–3.5) | 1.8 (0.1–12.3) | NS |
| ALT ratio[b] | 5.9 (0.6–11.2) | 1.7 (0.1–10.8) | NS |

[a] Data expressed as median (range). [b] The ratios of peak levels to pretreatment. For abbreviations, see table 1.

**Table 4.** Predictors of early biochemical relapse after lamivudine monotherapy, determined by multivariate analysis

| Factors | Category | Odds ratio (95% confidence interval) | p |
|---|---|---|---|
| Histology | 1: no cirrhosis | 1 | |
| | 2: cirrhosis | 16.1 (2.30–113) | 0.0052 |
| HBeAg | 1: negative | 1 | |
| | 2: positive | 5.61 (1.77–17.8) | 0.0035 |
| Basic core promoter (A1762G1764) | 1: undetectable mutant | 1 | |
| | 2: detectable mutant | 3.93 (1.31–11.8) | 0.015 |

Variables that achieved statistical significance ($p < 0.05$) on multivariate Cox proportional hazard model are shown.

YMDD mutants were not detected in any of the pretreatment serum samples. Emergence of YMDD motif mutant was noted in 29.3% (22 of 75 patients) of patients of the continuous group and 12.0% (3 of 25 patients) of the discontinuous group. In the continuous group, all of 18 virological relapsers showed DNA breakthrough associated with the emergence of YMDD motif mutant, and all of 17 biochemical relapsers showed breakthrough hepatitis associated with DNA breakthrough.

*ALT and Bilirubin Levels after Biochemical Relapse or Emergence of YMDD Motif Mutant*

The peak levels of serum ALT and bilirubin after biochemical relapse, and the ratios of peak levels to pretreatment were not significantly different between continuation or discontinuation groups (table 3). Likewise, the peak levels of serum ALT and bilirubin after the emergence of YMDD motif mutant, and the ratios of peak levels to pretreatment were also not significantly different between the two groups (table 3).

*Factors Associated with Early Biochemical Relapse after Discontinuation of Lamivudine Monotherapy*

The cumulative biochemical relapse rates of the discontinuous group were 48.0, 64.8, 69.2, and 69.2% at the end of 0.5, 1, 2, and 3 years after discontinuation of lamivudine monotherapy, respectively. Potential predictive factors associated with early biochemical relapse after discontinuation of treatment were explored in 25 patients of the discontinuation group. In univariate analyses, the following six factors tended to or significantly influenced the early biochemical relapse: HBeAg ($p = 0.0048$), levels of HBV DNA ($p = 0.039$), pattern of BCP ($p = 0.026$), pattern of PC ($p = 0.033$), age ($p = 0.083$), and liver cirrhosis ($p = 0.096$). In multivariate analysis using these factors, HBeAg ($p = 0.0035$), liver cirrhosis ($p = 0.0052$), and pat-

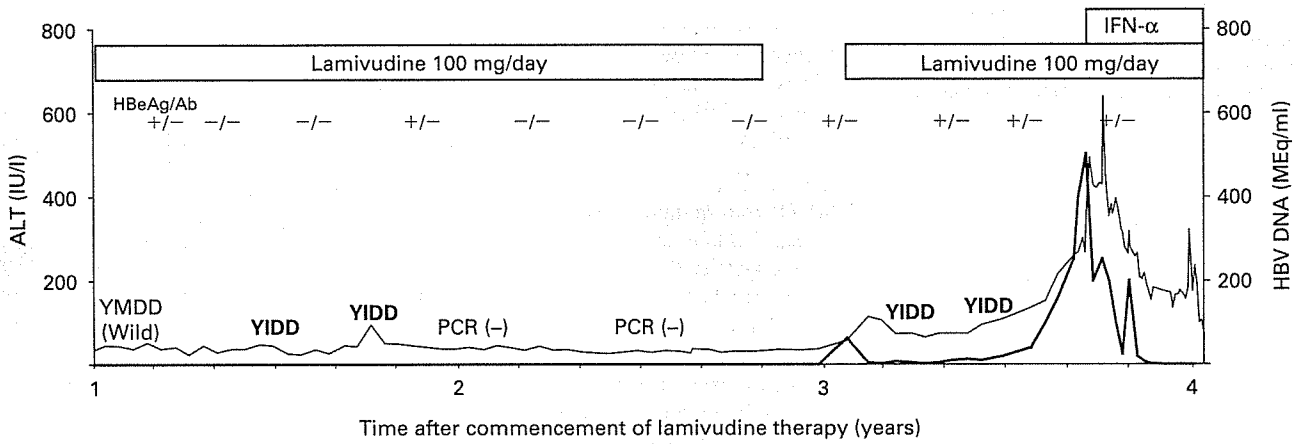


**Fig. 2.** Clinical summary of a 34-year HBeAg-positive male patient infected with HBV-genotype C complicated with liver cirrhosis. The patient was treated with lamivudine monotherapy for 2.8 years, which resulted in HBeAg negativity and a decrease in HBV DNA to undetectable levels as measured by TMA-HPA for one year or more, despite the emergence of YMDD motif mutant (YIDD type). After the discontinuation of lamivudine, however, the patient developed severe biochemical relapse, during retreatment of lamivudine monotherapy. The case was later controlled when combination therapy of lamivudine and IFN-α was used. HBV DNA was indicated by branched DNA signal amplification technology (Chiron Corp., Emeryville, Calif., USA) to show the viral loads of higher ranges, and the results were expressed as $10^6$ genomic equivalents per millilitre (MEq/ml). Thick line = HBV DNA level, thin line = ALT level.

tern of BCP ($p = 0.015$) were independent significant predictors of early biochemical relapse after discontinuation of the treatment (table 4). The odds ratio of liver cirrhosis was 16.1 compared with the absence of cirrhosis. The odds ratio of HBeAg-positive was 5.61 compared with HBeAg-negative. The odds ratio of detectable BCP mutant virus was 3.93 compared with undetectable BCP mutant virus.

*Retreatment for Biochemical Relapse after Discontinuation of Lamivudine Monotherapy*

Eight of 17 patients, who showed relapse after the termination of the treatment, received another course of lamivudine monotherapy at the same dose after a median stop (no treatment) period of 0.61 years (range, 0.15–1.8 years). The median period of retreatment was 1.1 years (range, 0.14–2.7 years). Six of these patients were HBeAg-positive, and the remaining 2 were HBeAg-negative at the commencement of retreatment. Five of 8 (62.5%) patients successfully showed normalization of ALT level and disappearance of HBV-DNA after retreatment; of whom 2 were HBeAg-negative (100%) and 3 were HBeAg-positive (50%). The other 3 patients, who did not show normalization of ALT, were HBeAg-positive, and especially 2 patients showed HBeAg reversion. Furthermore, in 2 of the latter 3 nonresponders, lamivudine therapy was terminated following the emergence of YMDD motif mutant, and both developed severe biochemical relapse (a rise in ALT level to ≥ 300 IU/l, accompanied by the elevation of total bilirubin level to ≥ 2.0 mg/dl) during retreatment. In particular, one of them developed severe relapse despite HBeAg seronegative conversion and was HBV DNA undetectable for one year (fig. 2). In summary, 5 of 6 patients (83.3%) without YMDD motif mutant at discontinuation could achieve ALT normalization again with retreatment, but 2 of 2 patients with YMDD motif mutant developed severe biochemical relapse during retreatment. Hence, retreatment with lamivudine monotherapy was effective, but tended to be not very effective for HBeAg-positive patients retreated after the emergence of YMDD motif mutant.

## Discussion

Previous studies reported that the estimated half-life of hepatocytes infected with HBV was 10–100 days, suggesting that prolonged administration of lamivudine for a period longer than one year might be needed to clear HBV in the liver by turning over most of cccDNA-containing

hepatocytes [24, 25]. However, a recent report by Ryu et al. [26] showed that HBV DNA and HBeAg reappeared in 31 and 16% of their patients, respectively at 2 years after the termination of lamivudine, even when HBV DNA and HBeAg had been persistently negative for 2 years or more. Based on these findings, they suggested that long-term additional administration of lamivudine might enhance the durability of lamivudine-induced HBeAg seroconversion [26]. Our results of the discontinuous group also indicated higher cumulative biochemical relapse rates of 64.8 and 69.2% at 1 and 2 years after discontinuation, similar to the Korean report (relapse rates, 37.5 and 49.2% at 1 and 2 years) [27], although this might be due to the criteria used for the definition of the discontinuous group, regardless of HBeAg seroconversion and inclusion of subjects who were HBeAg-negative at the start of the treatment.

With regard to long-term treatment, while continued disease suppression, or even HBeAg seroconversion, still occurred in some patients, in others, breakthrough hepatitis associated with the appearance of YMDD mutant may occur. Severe breakthrough hepatitis has been reported despite the continuation of lamivudine [28–32], even though previous studies showed that YMDD mutants are less replication-competent compared with the wild-type, and are associated with lower HBV DNA levels compared with pretreatment HBV DNA levels [4, 5, 33–37]. We have recently reported that 3-year lamivudine therapy induced histopathological improvement regardless of the appearance of YMDD mutants, associated with DNA breakthrough and breakthrough hepatitis, and suggested the benefit of long-term treatment [38].

In our study based on patients matched for age, sex, and observation period, the cumulative virological and biochemical relapse rates were compared according to the continuation or not of lamivudine monotherapy. Our results showed that the relapse rates in the discontinuous group emerged significantly earlier than the continuous group. Furthermore, the peak levels of serum ALT and bilirubin and the ratios of peak to pretreatment levels were not significantly different between the continuation and discontinuation groups, regardless of the emergence of YMDD motif mutant. To our knowledge, this is the first report based on matched patients' backgrounds that compares the virological and biochemical relapse rates according to continuation or discontinuation of lamivudine monotherapy.

One limitation of our study is the small number of patients, the use of various treatment periods, and differences in the discontinuation criteria regardless of HBeAg seroconversion in the discontinuous group. Large-scale prospective studies of each group should be conducted in the future to confirm these findings.

Previous studies showed that HBeAg-positivity, old age, high pretreatment viral loads, and the presence of PC mutant at the start of the treatment might affect the biochemical relapse after treatment [39–41]. Our study based on multivariate analysis-evaluated various aspects of clinicopathological characteristics at the termination of treatment, and identified HBeAg-positivity, liver cirrhosis, and detectable BCP mutant virus as independent significant determinants of early biochemical relapse. Mutations in BCP, increase viral replication and enhance disease activity [42, 43], and are also associated with HBV genotype C and a longer duration of infection (including the higher age, and more advanced liver disease) [44]. These results suggest that the presence of BCP mutant and liver cirrhosis might indicate the more active state of disease, and might be the responsible factors of an early relapse. In our study, the majority of patients of the discontinuous group were Japanese patients infected with HBV genotype C and were positive for a family history of HBV infection (namely, genotype C patients with the longer duration of infection), and thus the presence of BCP mutant together with genotype C and a longer duration of infection might explain the higher viral replication and biochemical relapse after treatment in endemic areas of HBV genotype C infection, such as Japan and Korea, where most HBV infection is considered to be transmitted vertically [27]. To our knowledge, this is the first report of early post-treatment biochemical relapse based on characteristics at discontinuation of lamivudine monotherapy. Previous reports in the United States indicated that viral suppression was maintained after the termination of treatment [45]. The discrepancy between the USA reports and our results are probably due to the differences in HBV genotypes, duration of infection, and follow-up period after the termination of treatment. Further studies of a large group of patients are required to clarify whether the patients' characteristics including HBV genotype and duration of infection affect the early virological and biochemical relapse after the termination of lamivudine monotherapy.

Reinstitution of lamivudine monotherapy is usually effective in controlling exacerbations in patients who have not experienced breakthrough and may result in subsequent HBeAg seroconversion [39], but the benefits of retreatment are usually transient in patients with breakthrough since YMDD mutant rapidly reappears (often within weeks) when lamivudine is resumed [46, 47]

because of possible persistence of YMDD mutant over long periods after the cessation of therapy [48]. In the present study, 83.3% of patients without YMDD motif mutant at discontinuation achieved ALT normalization again with retreatment, but all (100%) patients with YMDD motif mutant developed severe biochemical relapse during retreatment. These results suggest that care should be exercised in the management of patients in whom lamivudine is first discontinued then used again, especially those who show the emergence of YMDD motif mutants.

In conclusion, the present study indicates that the discontinuation of lamivudine monotherapy for Japanese genotype C-dominant hepatitis B should be followed carefully for virological and biochemical relapses. Further prospective studies are necessary to determine the true risk of post-treatment relapse by discontinuation and breakthrough hepatitis by continuation of long-term treatment. However, it should be stated here that it would be difficult to perform such studies based on ethical grounds. Interferon therapy and new nucleotide analogs (for example, adefovir dipivoxil and entecavir) have been recently shown to be effective in patients with YMDD mutants induced by long-term lamivudine administration [49–52]. Thus, new combination therapies of antiviral drugs or alternative drugs are expected to appear in the future.

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# Favorable Efficacy of Long-Term Lamivudine Therapy in Patients With Chronic Hepatitis B: An 8-Year Follow-Up Study

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The long-term efficacy of lamivudine therapy in patients with hepatitis B virus (HBV) infection is still not clear. In this study, 20 non-cirrhotic Japanese patients infected with HBV received lamivudine therapy for more than 1 year and were followed for a median period of 8.5 years (range, 6.7–8.7 years). The rates of HBe antigen (HbeAg) negative, HBV-DNA undetectable, and alanine aminotransferase (ALT) normal level at the start of lamivudine were 55%, 25%, and 20% and 85%, 80%, and were 80%, respectively, at the last visit, including patients who received additional treatment. The values at the last visit tended to and were significantly higher than those at the start. The values improved at the last visit regardless of the emergence of YMDD motif mutant and continuation of lamivudine. YMDD mutant and biochemical relapse with mutant virus (breakthrough hepatitis) appeared in 65% and 45% during follow-up, respectively, but severe breakthrough hepatitis occurred in only 5%. Furthermore, 80% of patients who received additional treatment for breakthrough hepatitis, regardless of continuation of lamivudine, were ALT normal level at the last visit, in contrast to 25% untreated. HBsAg clearance occurred in two patients of the discontinuous lamivudine group with non-vertical transmission, who were relatively young. One was infected with HBV genotype C with breakthrough hepatitis and the other had no YMDD mutant and was infected with genotype D, a rare type in Japan. None developed cirrhosis or hepatocellular carcinoma (HCC) during follow-up. Our results suggest that long-term lamivudine therapy improves long-term prognosis, especially when additional treatment for breakthrough hepatitis is used. ***J. Med. Virol. 75:491–498, 2005.*** © 2005 Wiley-Liss, Inc.

**KEY WORDS:** YMDD motif mutant; HBV genotype; breakthrough hepatitis; HBsAg clearance; hepatocellular carcinoma

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## INTRODUCTION

Lamivudine, an oral cytosine nucleoside analog clinically used for the treatment of chronic hepatitis B virus (HBV) infection, potently inhibits HBV replication by interfering with HBV reverse transcriptase activity [Doong et al., 1991; Dienstag et al., 1995; Nevens et al., 1997; Lai et al., 1998], and results in marked decrease of HBV-DNA and alanine aminotransferase (ALT) levels, seroconversion of HBe antigen (HBeAg) to anti-HBe (HBeAb), and histopathological improvement [Lai et al., 1998; Dienstag et al., 1999; Suzuki et al., 1999; Liaw et al., 2000; Schalm et al., 2000; Leung et al., 2001; Akuta et al., 2003a]. However, lamivudine-resistant HBV strains (YMDD motif mutant) have been reported in long-term lamivudine therapy, and the emergence of such mutant virus results in re-elevation of HBV-DNA (DNA breakthrough) and ALT (breakthrough hepatitis) [Tipples et al., 1996; Bartholomew et al., 1997; Lai et al., 1998; Dienstag et al., 1999; Liaw et al., 2000; Schalm et al., 2000; Leung et al., 2001; Yuen et al., 2001; Akuta et al., 2003a,b].

The optimal duration of lamivudine therapy for HBV-infected patients is still controversial for two main reasons; drug resistance and sustainability of the response to treatment. In particular, there is a need to evaluate short-term treatment with respect to post-treatment safety and the sustainability of the response

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to such treatment, and long-term treatment with regard to breakthrough hepatitis [Balzarini et al., 1996; Ling et al., 1996; Naoumov et al., 1996; Tipples et al., 1996; Bartholomew et al., 1997; Honkoop et al., 1997; Schalm, 1997; Allen et al., 1998]. Furthermore, the long-term prognosis of lamivudine-treated patients also remains unknown. Recently, Lok et al. [2003] reported the long-term safety and efficacy of continuous lamivudine treatment based on a median follow-up period of 4 years. However, the efficacy of long-term lamivudine treatment based on a longer follow-up period of more than 5 years is still not clear, especially when associated with or without continuation of lamivudine and with or without the emergence of YMDD mutant.

The present study included 20 consecutive non-cirrhotic Japanese patients with chronic hepatitis B, in whom more than 8 years had elapsed since the induction of lamivudine monotherapy. The aims of the present study were the following: (1) To evaluate the long-term efficacy and safety of more than 1-year lamivudine treatment in Japanese patients with genotype C-dominant hepatitis B; (2) to evaluate HBeAg status, HBV-DNA levels, and ALT levels according to continuation or discontinuation of lamivudine, and according to the emergence or not of YMDD motif mutant; and (3) to evaluate the efficacy of any additional treatment for breakthrough hepatitis.

## MATERIALS AND METHODS

### Patients

We studied 20 consecutive Japanese adult patients with chronic hepatitis B who agreed to enter a long-term lamivudine trial between September 1995 and July 1996 at the Department of Gastroenterology of Toranomon Hospital [Chayama et al., 1998]. The entry criteria included a positive test for HBV-DNA by dot hybridization and elevated ALT levels (greater than twice the upper limit of normal value [50 IU/L]) within 3 months before the start of therapy. A liver needle biopsy was performed in all patients just before the start of the trial, which confirmed the presence of chronic hepatitis. Individuals, who had hepatocellular carcinoma (HCC), apparent cirrhosis, or signs of hepatic decompensation, were excluded from the study. Also excluded were patients positive for serum markers of hepatitis C virus and human immunodeficiency virus. None of the participating patients received immunosuppressive or antiviral therapy at least 6 months before lamivudine therapy, and none had been previously treated with any nucleoside analogs. Each patient was treated with a single oral dose of 100 mg of lamivudine every day.

The patient characteristics at the start of lamivudine monotherapy are summarized in Table I. The follow-up period represented the time from the start of lamivudine monotherapy until the last visit. The median period of follow-up was 8.5 years (range, 6.7–8.7 years). During follow-up, 6 patients discontinued lamivudine treatment at their own request, and the other 14 patients continued until the last visit. The median period of lamivudine treatment was 1.4 years (range, 1.0–5.6 years) for the discontinuation group, and 8.5 years (range, 6.7–8.7 years) for the continuous patients. As a whole, the median period of lamivudine treatment was 8.4 years (range, 1.0–8.7 years). In some patients, lamivudine therapy was supplemented with other additional treatments.

TABLE I. Patient Characteristics at the Start of Lamivudine Monotherapy

| | |
|---|---|
| Number | 20 |
| Sex (male/female) | 16/4 |
| Age (year)[a] | 44 (25–65) |
| HBeAg (no. of positive) | 9 (45.0%) |
| HBV-DNA (gEq/ml)[a] | $3.2 \times 10^7$ ($7.0 \times 10^5 - 3.2 \times 10^9$) |
| HBV genotype (no. of B/C/D/F) | 3/15/1/1 |
| Histology (no. of F1/2)[b] | 12/8 |
| Family history of liver disease[c] | 13 (65.0%) |
| T-Bill (mg/dl)[a] | 0.8 (0.2–1.7) |
| AST (IU/l)[a] | 59 (24–247) |
| ALT (IU/l)[a] | 100 (11–371) |
| Albumin (g/dl)[a] | 4.1 (3.4–4.7) |
| Cholinesterase (ΔpH)[a] | 1.0 (0.8–1.3) |

T-Bill, total bilirubin; AST, aspartate transferase; ALT, alanine transferase.
[a]Data are expressed as median (range).
[b]Scoring according to the system of Desmet et al. [1994].
[c]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

### Methods

As indicators of lower activity of hepatitis, the rates of HBeAg negative, HBV-DNA undetectable (undetectable DNA levels by branched DNA signal amplification technology), and normal ALT level, were evaluated at two points; the start of lamivudine and last visit. The rates at the last visit were evaluated by including those patients who received additional treatment. Furthermore, the cumulative appearance rates of YMDD mutant by polymerase chain reaction (PCR)-based methods and breakthrough hepatitis (ALT becoming abnormal after a period of ALT normalization, accompanied by the emergence of YMDD mutant and re-elevation of HBV-DNA levels) were also evaluated. Clinical assessment and laboratory tests were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. All patients underwent abdominal ultrasonography every 6 months at least to exclude the development of cirrhosis and HCC.

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for laboratory data including ALT levels, HBV-DNA levels, HBsAg, HBeAg/eAb, and YMDD motif mutant at various time periods. The serum samples were stored in aliquots at −80°C until use. HBsAg and HBeAg/eAb were determined by radioimmunoassay

(Abbott Diagnostics, Chicago, IL). HBV-DNA was measured by the branched DNA signal amplification technology (Chiron Corp., Emeryville, CA), and the results were expressed as genomic equivalents per milliliter (gEq/ml). The lower limit of the assay is $7.0 \times 10^5$ gEq/ml. HBV genotype and subgroups of genotype B were determined using the previously reported method [Okamoto et al., 1988; Usuda et al., 1999; Sugauchi et al., 2002]. Antibody against HCV was detected with chemiluminescent enzyme immunoassay (Lumipulse® II Ortho HCV, Ortho Diagnostic Japan, Tokyo). The YMDD motif mutant was detected using sensitive PCR-restriction fragment length polymorphism (PCR-RFLP) [Chayama et al., 1998].

Liver biopsy specimens were obtained percutaneously or at laparoscopy using a modified Vim Silverman needle of 2-mm internal diameter (Tohoku University style, Kakinuma Factory, Tokyo). Each specimen was scored according to the system of Desmet et al. [1994].

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from every patient. The study was approved by the Local Ethics Committee of Toranomon Hospital.

### Statistical Analysis

Fisher's exact probability test was used to compare the rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level between the start point of lamivudine and the last visit. The cumulative appearance rates of YMDD mutant and breakthrough hepatitis during lamivudine treatment were calculated using the Kaplan–Meier technique, and were evaluated from the start point of lamivudine until the last visit or discontinuation. Statistical comparisons were performed using the SPSS software (SPSS, Inc., Chicago, IL). All $P$-values of less than 0.05 by the two-tailed test were considered significant.

## RESULTS

### Efficacy Measures, Lamivudine Resistance, and Safety For the Whole Group

The clinical course of 20 patients is shown in Figure 1. For the whole group, the rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level were 55% (11/20), 25% (5/20; HBV-DNA levels of five patients were detectable directly before treatment, but undetectable at the start by chance), and 20% (4/20) at the start of lamivudine treatment, respectively. Five patients received additional treatment for breakthrough hepatitis before or until the last visit. The rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level were 85% (17/20), 80% (16/20), and 80% (16/20) at the last visit, respectively. The rates of HBeAg negative ($P = 0.082$), HBV-DNA undetectable ($P = 0.0012$), and ALT normal level ($P = 0.00036$) at the last visit tended to be or were significantly higher than those at the start of lamivudine treatment.

YMDD mutant was not detected in any of the pretreatment serum samples. For the whole group, YMDD motif mutant and breakthrough hepatitis appeared in 65% (13/20) and 45% (9/20) during lamivudine monotherapy, respectively. Additional treatment was provided to 55.6% (5/9) of patients who developed breakthrough hepatitis, while the other 15 patients received lamivudine monotherapy until the last visit or the time of discontinuation. The cumulative appearance rates of YMDD mutant and breakthrough hepatitis were 70.2% and 46.4% at the end of 5 years; 70.2% and 52.3% at the end of 8 years, respectively (Fig. 2). None developed severe adverse events during long-term lamivudine treatment, apart from the development of breakthrough hepatitis.

### Clinical Course of 13 Patients With YMDD Motif Mutant

During lamivudine monotherapy, 69.2% (9/13) patients with emergence of YMDD mutant developed breakthrough hepatitis. In these patients, seven continued lamivudine therapy after the development to breakthrough hepatitis, and additional therapy was provided to three of the seven patients (two patients received interferon [IFN], and one received glycyrrhizin [Stronger Neo-Minophagen C®]). The remaining two patients discontinued lamivudine therapy after the development of breakthrough hepatitis, and instead received IFN therapy. At the last visit, ALT normal level and DNA undetectable were detected in 55.5% (5/9) and 66.7% (6/9) of patients who developed breakthrough hepatitis, respectively. Especially, 80% (4/5) of patients who received suitable additional treatment for breakthrough hepatitis, irrespective of lamivudine therapy, were ALT normal level at the last visit in contrast to 25% (only 1/4 patients) of patients untreated for breakthrough hepatitis.

In patients with emergence of YMDD mutant, 30.8% (4/13) did not develop breakthrough hepatitis. At the last visit, 100% (all 4 patients) and 75.0% (3/4) of patients, who did not develop breakthrough hepatitis, were ALT normal level and DNA undetectable, respectively, irrespective of continuous lamivudine therapy.

### Clinical Courses of Seven Patients Without YMDD Motif Mutant

All seven patients free of YMDD mutant were HBeAg negative, DNA undetectable, and ALT normal level at the last visit, irrespective of continuous lamivudine therapy. Three patients who transiently discontinued the treatment showed ALT relapse after the cessation of lamivudine therapy, but their ALT levels became stable spontaneously without any additional treatment.

### Efficacy Measures According to Patients' Background

The rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level at the two points of the start of lamivudine and the last visit are shown in Figure 3,

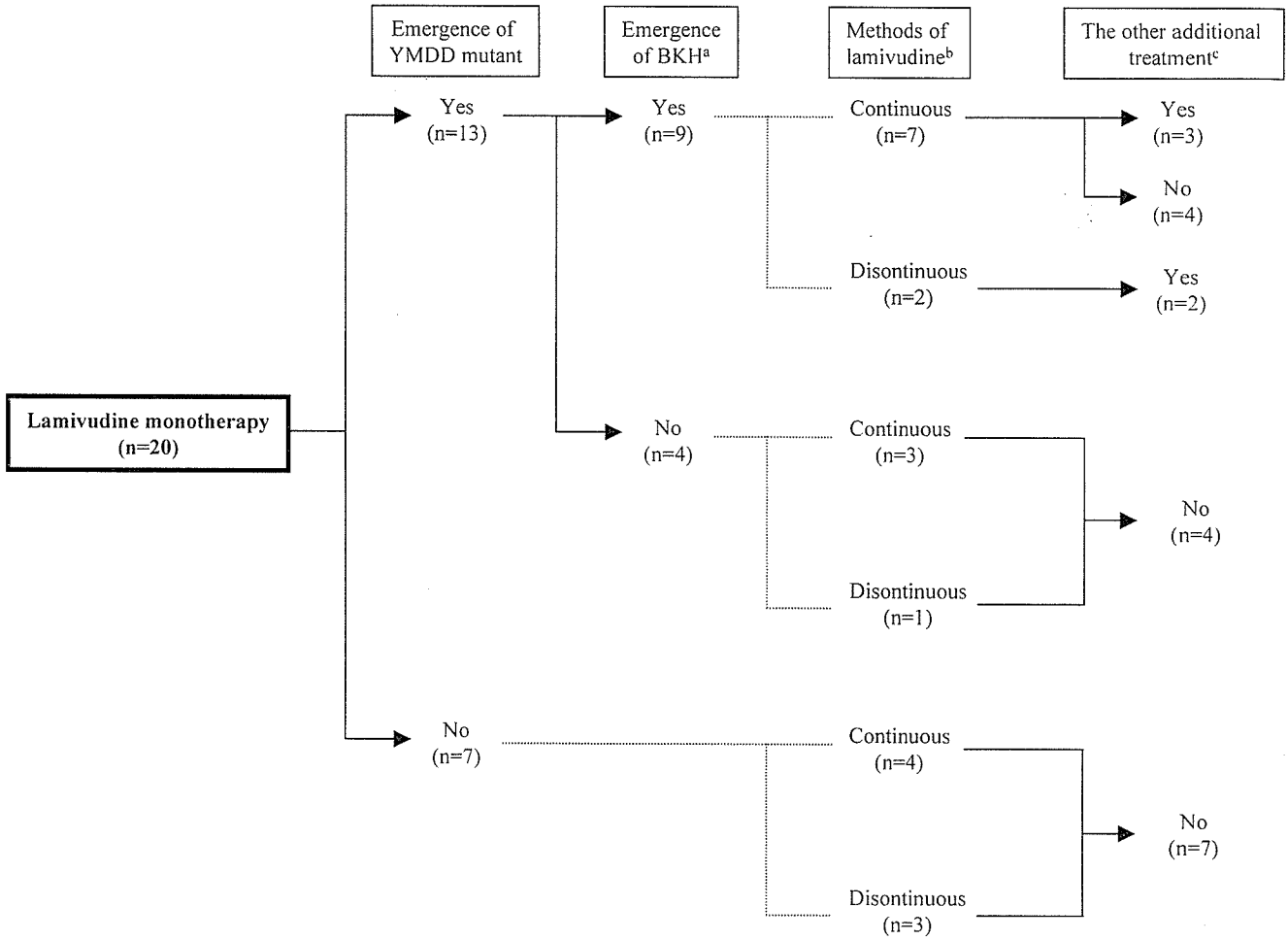


Fig. 1. Clinical course of 20 patients. YMDD motif mutant and breakthrough hepatitis (ALT becoming abnormal after a period of ALT normalization, accompanied by emergence of YMDD mutant and re-elevation of HBV-NA levels) appeared in 65% and 45% during lamivudine monotherapy, respectively. [a]BKH, breakthrough hepatitis. [b,c]The methods of lamivudine treatment and the other additional treatment for BKH were decided at patients' own requests. For the whole group, 30% discontinued lamivudine treatment during follow-up. Additional treatment was provided to 55.6% of patients who developed breakthrough hepatitis.

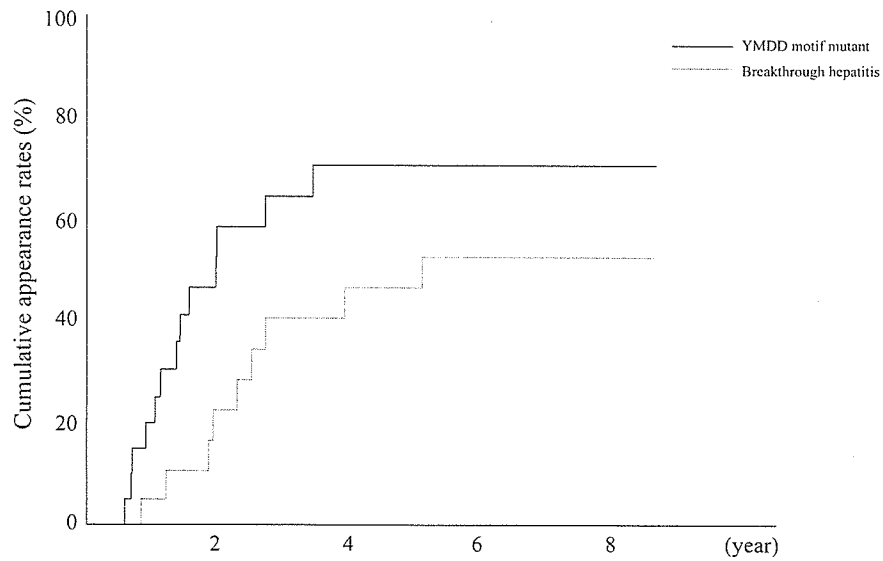


Fig. 2. Cumulative appearance rates of YMDD motif mutant and breakthrough hepatitis throughout follow-up.

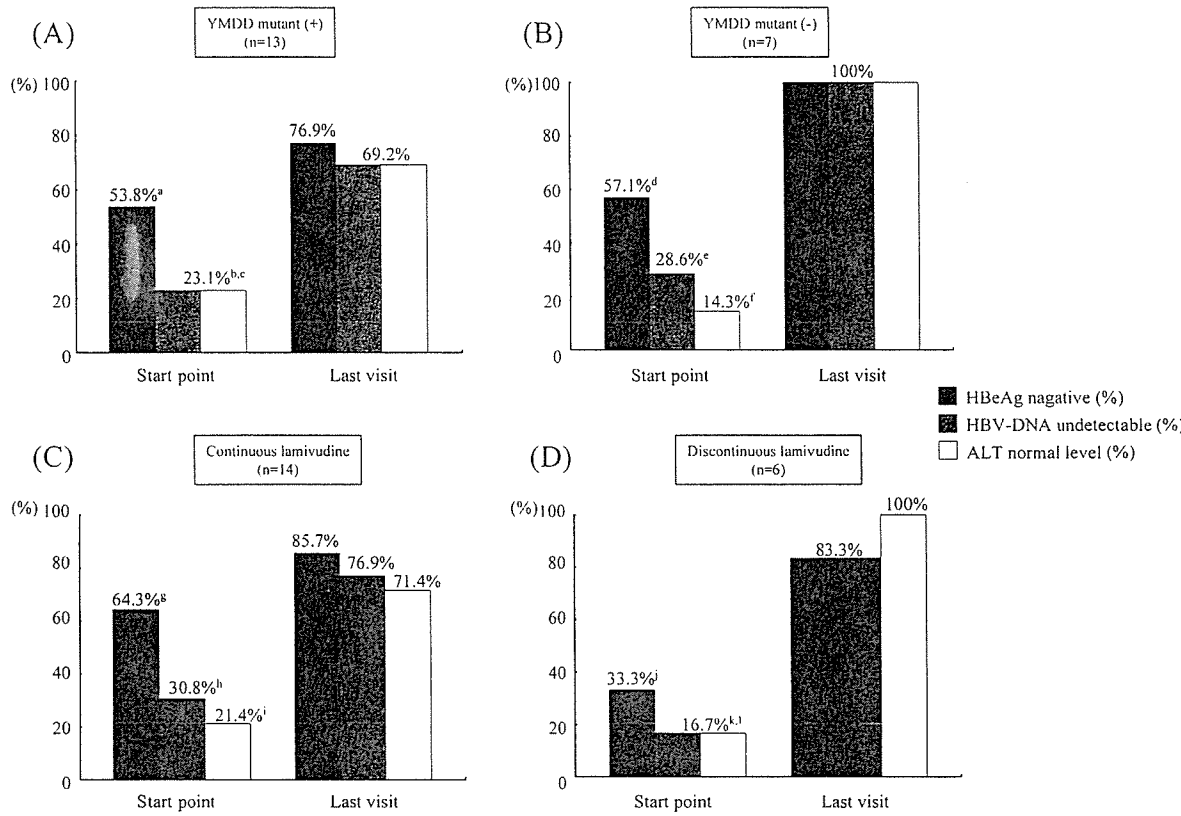


Fig. 3. The rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level at the start of lamivudine treatment and last visit. The results at the last visit were evaluated by including five patients who received additional treatment. [a,d,g,j]Not significant, compared with HBeAg negative rates at each last visit. Fisher's exact probability test. [b]$P = 0.047$, [e]$P = 0.021$, [h]$P = 0.047$, [k]$P = 0.080$, compared with HBV-DNA undetectable rates at each last visit by Fisher's exact probability test. [c]$P = 0.047$, [f]$P = 0.0047$, [i]$P = 0.021$, [l]$P = 0.015$, compared with ALT normal rates at each last visit by Fisher's exact probability test.

according to the emergence of YMDD mutant and lamivudine therapy. The rates of HBV-DNA undetectable and ALT normal level at the last visit tended to be or were significantly higher than those at the start, regardless of the emergence of YMDD mutant and continuous lamivudine therapy. The rate of HBeAg negative at the last visit was also higher than at the start, even when there was no significance based on the relatively small numbers of HBeAg-positive patients at the start of lamivudine therapy.

In five patients who received additional treatment for breakthrough hepatitis, the rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level were 20 (1/5), 0 (0/5), and 0% (0/5) at the start of lamivudine; and 60 (3/5), 60 (3/5), and 80% (4/5) at the last visit, respectively. The rates of ALT normal level at the last visit were significantly higher than at the start ($P = 0.048$). The rates of HBeAg negative and HBV-DNA undetectable at the last visit were also higher than at the start, though statistically insignificant due to the small number of patients.

### HBsAg Clearance With Lamivudine Therapy

HBsAg clearance by radioimmunoassay was noted in 10% (2 males of 20 patients) of the whole group during follow-up, and they showed undetectable HBV-DNA levels using a sensitive quantitative PCR assay (Amplicor HBV Monitor Test, Roche Molecular Systems, Inc., NJ). The lower limit of this assay is 2.6 log copies/ml. The characteristics of these two male patients who could achieve HBsAg clearance by radioimmunoassay are shown in Table II. Furthermore, HBsAg clearance occurred in 33.3% (2/6) cases of the discontinuation group and none of the continuous (0/14). The discontinuation group tended to achieve higher rates of HBsAg clearance than the continuous group ($P = 0.079$).

One patient with HBsAg clearance was infected with HBV genotype C (HBV/C), the major type in Japan, and was a relatively young adult under 40 years of age who developed HBV by non-vertical transmission (i.e., patients whose mothers did not suffer from chronic HBV infection). He developed breakthrough hepatitis, especially according to continuous YVDD type [Akuta et al., 2003c] of YMDD motif mutant, and developed HBsAg clearance at about 5 years after the cessation of long-term lamivudine treatment followed by IFN treatment for breakthrough hepatitis.

The other patient with HBsAg clearance was infected with HBV/D; a rare type in Japan, and the infection was considered not to be transmitted vertically. The YMDD motif mutant did not emerge during long-term

TABLE II. Characteristics of Two Patients Who Could Achieve HBsAg Clearance*

| Patient | Age (year) | Sex | Genotype | Histology | Etiology of HBV infection[a] | Emergence of YMDD infection[a] | Emergence of BKH[c] | Treatment for BKH | Methods of lamivudine | Duration of lamivudine (year) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 38 | M | C | F2 | Non-vertical | YVDD[b] | + | Interferon | Discontinuous | 1.0 |
| 2 | 46 | M | D | F1 | Non-vertical | — | — | — | Discontinuous | 1.1 |

*HBsAg clearance were determined by radioimmunoassay.
[a]Etiology of HBV infection in both patients was not vertical transmission from mother to infant.
[b]YMDD mutant type was continuous YVDD type, which tended to be detected in the relatively younger patients [Akuta et al., 2003c].
[c]BKH, breakthrough hepatitis.

lamivudine treatment, and HBsAg clearance at about 3 years after the cessation of lamivudine treatment was noted together with transient post-treatment ALT relapse.

In conclusion, HBsAg clearance occurred in two male patients with non-vertical transmission infection, who discontinued lamivudine therapy. One patient was a relatively young adult infected with HBV/C and developed breakthrough hepatitis and the other patient was infected with HBV/D but without YMDD mutant.

### Development of Severe Breakthrough Hepatitis

Severe breakthrough hepatitis was defined as a rise in ALT level to ≥300 IU/L, accompanied by elevation of total bilirubin level to ≥3.0 mg/dl and coagulopathy with plasma prothrombin time of <75% of control activity. Severe breakthrough hepatitis occurred in only 5% of all patients (1/20), and in 7.7% of patients with the emergence of YMDD mutant (1/13). The patient was a male infected with HBV/C and developed severe breakthrough hepatitis at about 3 years after the start of lamivudine treatment, according to the mixed type of YMDD mutant [Akuta et al., 2003c]. He was suitably provided with combination therapy of lamivudine + IFN, which resulted in the stabilization of hepatitis. At the last visit, he was HBeAg negative and ALT normal level.

### Development of Cirrhosis and HCC

None of the patients developed cirrhosis or HCC during follow-up and at end-point.

## DISCUSSION

To our knowledge, this report provides the results of the longest follow-up study (median follow-up period of 8.5 years) of patients with chronic hepatitis B on the longest lamivudine treatment (median treatment period of 8.4 years). In our study, the rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level at the last visit for the whole group tended to be and were significantly higher than those at the start of lamivudine. In particular, we could achieve very high rates (80% and 80%) of HBV-DNA undetectable and ALT normal level at the last visit, respectively, by including patients who received additional treatment for breakthrough hepatitis. Furthermore, these patients showed similar improvement at the last visit, irrespective of emergence of YMDD mutant and lamivudine therapy. In the present study of Japanese dominant HBV/C, the cumulative appearance rates of YMDD mutant and breakthrough hepatitis were also approximately similar to previous reports based on the shorter follow-up periods than this study [Lai et al., 1998; Liaw et al., 2000; Leung et al., 2001; Lok et al., 2003], and severe breakthrough hepatitis with icteric flare-up occurred in only 5% (only one infected with HBV/C). Furthermore, 80% of patients, who received suitable additional treatment for breakthrough hepatitis, were ALT normal level at the last visit. Hence, our results indicate that long-term lamivudine treatment also induces long-term favorable prognosis and is safe in patients with breakthrough hepatitis if suitable additional treatments are provided, to say nothing of the favorable prognosis in patients without YMDD mutant and breakthrough hepatitis. We agree with Lok et al. [2003] who provided data in support of the benefits of long-term lamivudine treatment [Akuta et al., 2004].

With regard to the treatment of breakthrough hepatitis, IFN and new nucleotide analogs (e.g., adefovir dipivoxil and entecavir) are reported to be effective in patients with YMDD mutant [Perrillo et al., 2000; Tassopoulos et al., 2001; Peters et al., 2002; Suzuki et al., 2002]. Especially, a recent report suggested that breakthrough hepatitis should be avoided, and that adefovir dipivoxil should be introduced when YMDD mutant is first detected or at the first sign of worsening hepatitis [Lok, 2004]. We agree with the recommendation of this report that other additional treatment for breakthrough hepatitis should be introduced earlier to achieve a more safe response to long-term lamivudine.

The benefits of continuous lamivudine after the emergence of YMDD mutant are still unclear. Previous studies showed that YMDD mutants are less replication-competent compared with the wild-type, and are associated with lower HBV-DNA levels compared with pretreatment HBV-DNA levels [Fu and Cheng, 1998; Lai et al., 1998; Melegari et al., 1998; Dienstag et al., 1999; Ling and Harrison, 1999; Ono-Nita et al., 1999; Leung, 2000]. We had reported that 3-year lamivudine therapy induced histopathological improvement regardless of the appearance of YMDD mutants, associated with breakthrough hepatitis, and suggested the benefit of long-term treatment [Suzuki et al., 2003]. In contrast, a recent study compared continuous lamivudine group with discontinuous group, and showed that there might be no benefit to continue treatment after emergence of

YMDD mutant based on comparison of the hepatitis flare rates, decompensation rates, HBeAg seroconversion rates, and HBV-DNA levels at a relatively short follow-up period [Liaw et al., 2004]. In the present study, we could not conclude whether it is beneficial to continue lamivudine therapy after emergence of YMDD mutant because we did not include a control group to compare between the continuous and discontinuous therapy, although patients with breakthrough hepatitis showed a favorable clinical course in response to additional treatment.

Recently, Liaw et al. [2003] reported that lamivudine treatment might suppress the disease progression and development of HCC in advanced chronic hepatitis B based on a median treatment duration of about 3 years. Ikeda et al. [2003] indicated that persistently low HBV-DNA might save patients from hepatocarcinogenesis in HBV-related cirrhosis, and our study also showed that the suppression of HBV-DNA levels with lamivudine also seems to suppress HCC. In our study of non-cirrhotic patients, the rate of HBV-DNA undetectable at the last visit was very high (80%) when we included five patients who received additional treatment for breakthrough hepatitis. This result supports the favorable prognosis of patients treated with long-term lamivudine who do not develop cirrhosis and HCC during follow-up. Furthermore, excluding cirrhotic patients as major risk factors for HCC might lead to a more favorable prognosis.

HBsAg clearance rates based on long-term lamivudine treatment remain inadequately defined at present. Previous reports from the United States indicated that the rate of HBsAg clearance was 23% in patients who discontinued lamivudine after HBeAg seroconversion and followed for up to 3 years [Dienstag et al., 2003]. Our results also indicated that HBsAg clearance occurred in only 10% of the whole group at a median follow-up of 8.5 years, but in contrast was noted in 33.3% of discontinuation patients and 0% in the continuous-treatment group. Thus, the discontinuation patients tended to achieve a higher rate of HBsAg clearance than continuous patients, and this result suggests that various factors, like virological rebound and/or immunological response after discontinuation of lamivudine, might affect HBsAg clearance. However, in this study of a small number of discontinuation patients, we could not conclude that HBsAg clearance could be achieved in the lamivudine discontinuation group, and further prospective studies should be performed. Interestingly, one patient, who could achieve HBsAg clearance regardless of breakthrough hepatitis, was a relatively young adult infected with HBV/C through non-vertical transmission; and the other patient infected with HBV/D, a rare type in Japan, was considered have acquired the disease through no-vertical transmission. These results suggest that these patients might have achieved HBsAg clearance with lamivudine based on the relatively short duration of HBV infection in comparison to vertical transmission and/or the different HBV genotype. To our knowledge, this is the first report that investigated the characteristics of HBsAg clearance during long-term lamivudine treatment. Further studies of a large group of patients are required to clarify whether the HBV genotype, duration of HBV infection, and method of lamivudine therapy influence HBsAg clearance following long-term lamivudine treatment.

In conclusion, the present study of Japanese patients with genotype C-dominant hepatitis B indicates that long-term lamivudine treatment is safe and induces long-term favorable prognosis, especially when a suitable additional treatment is used for breakthrough hepatitis. Further prospective studies are necessary to determine whether long-term lamivudine treatment improves long-term prognosis, including clearance of HBsAg and suppression of hepatocarcinogenesis.

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ORIGINAL ARTICLE

# Long-term follow-up of interferon monotherapy in 454 consecutive naive patients infected with hepatitis C virus: Multi-course interferon therapy may reduce the risk of hepatocellular carcinoma and increase survival

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**Abstract**

***Objective.*** The long-term effects of multi-course interferon (IFN) monotherapy in patients infected with hepatitis C virus (HCV) are still unclear. ***Material and methods.*** To evaluate the effects of multi-course IFN on hepatocarcinogenesis and survival, a follow-up study was conducted comprising 454 consecutively recruited non-cirrhotic naive patients infected with HCV, who had received IFN monotherapy between 1987 and 1992. The median follow-up was 11.3 years. ***Results.*** A sustained response (SR) after the first IFN was achieved by 152 patients (33.5%) (Group A). Of 302 patients (66.5%) with non-SR after the first IFN, 130 patients (28.6%) did not receive additional IFN (Group B), and the remaining 172 patients (37.9%) received multi-course IFN monotherapy (Group C). With regard to hepatocarcinogenesis and survival rates for liver-related deaths, Groups A and C both showed significantly better long-term clinical outcome than Group B ($p < 0.001$; log-rank test). Three independent factors were identified by multivariate analyses (fibrosis stage 3, Group B, and age $\geq 50$) for all patients and two factors (fibrosis stage 3 and age $\geq 50$) for Group C associated with hepatocarcinogenesis. With regard to hepatocarcinogenesis rates according to the mean alanine aminotransferase (ALAT) levels during the IFN-free period in Group C, significantly higher rates were noted in patients with ALAT levels above 1.5 × the upper normal limit (17.6%) than those below the limit (0%) ($p < 0.05$). ***Conclusions.*** Multi-course IFN monotherapy reduces the risk of hepatocarcinogenesis and increases survival, and low ALAT levels during the IFN-free period are associated with lower hepatocarcinogenesis rates in multi-course IFN.

**Key Words:** *HCV, hepatocellular carcinoma, interferon monotherapy, liver-related death, multi-course, survival analysis*

## Introduction

Hepatitis C virus (HCV) usually causes chronic infection, which can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1–5]. In patients with HCV chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [6,7]. Recently, several studies based on single-course IFN monotherapy also showed that in patients receiving IFN therapy there was a reduced risk of development of HCC and liver-related death in comparison with untreated patients, especially in responders to the treatment [1,8–12]. However, it is still a matter of controversy whether or not the effect of IFN therapy is beneficial [13–21].

The response to IFN therapy varies among different HCV genotypes [22,23]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b, while about 25% are genotype 2a. Sustained response (SR) to IFN monotherapy is as low as 10 to 20% in genotype 1b infection [24–27]. We also encounter IFN-resistant patients infected with genotype 2a, even

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though SR is more than 60% in this group [28,29]. We previously reported that a second course of IFN monotherapy for IFN-resistant cases could increase the SR rates in patients who could not attain SR after the first course [30]. Thus, we reported an increase in the viral clearance rates as one of the short-term benefits of two or more courses (herein called multi-course) of IFN monotherapy. However, the long-term benefits of multi-course IFN monotherapy are still unclear, whether associated with SR or not.

The present study included 454 consecutive naive cases with chronic hepatitis C, in whom more than 10 years had elapsed since the induction of IFN monotherapy. The aims of the study were: 1) to evaluate the long-term efficacy of multi-course IFN therapy on hepatocarcinogenesis and survival, examined by analysis of the outcomes of single- and multi-courses of IFN; 2) to analyze the predictive factors associated with hepatocarcinogenesis, if any, in patients who received multi-course IFN therapy.

## Material and methods

### *Patients*

Among 573 consecutively recruited HCV-infected patients, in whom IFN monotherapy was induced between February 1987 and August 1992 at Toranomon Hospital, 454 were selected for the present study based on the following criteria: 1) patients naive to IFN monotherapy; 2) patients with chronic hepatitis, without cirrhosis or HCC, as confirmed by biopsy examination within 6 months of enrolment; 3) patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, Calif., USA), and positive for HCV RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor; Roche Diagnostic Systems, Calif., USA); 4) patients free of co-infection with human immunodeficiency virus; 5) patients not treated with antiviral or immunosuppressive agents within 6 months of enrolment; 6) lifetime cumulative alcohol intake $<500$ kg (mild to moderate alcohol intake); 7) patients free of other types of hepatitis, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease; 8) patients without or with well-controlled diabetes; and 9) patients who consented to participate in the study.

With regard to the clinical features of 454 patients at the start of the first course of IFN monotherapy, there were 322 men and 132 women, aged 15–70 years with a median age of 48 years. The numbers of patients with fibrosis stages 1, 2, and 3 were 260, 160, and 34 patients, respectively. HCV genotypes were 1b in 313 patients and non-1b in 125, and the genotype in the remaining 16 patients was not determined. The median alanine aminotransferase (ALAT) level was 142 IU/l (range, 24–636 IU/l), and the median platelet count was $17.4 \times 10^4/\mu l$ (range, $7.4 \times 10^4$–$39.2 \times 10^4/\mu l$). The median viremia level was 2.7 Meq/ml (range, $<0.5$–67.1 Meq/ml). The median follow-up time was 11.3 years (range, 0.1–16.3 years).

Furthermore, at the first course of IFN monotherapy, 327 patients (72.0%) received IFN-α alone, 119 patients (26.2%) received IFN-β alone, while the remaining 8 patients (1.8%) received a combination of IFN-α and IFN-β. A median IFN dose per day of 6 million units (MU, range; 1–10 MU) was administered. As a whole, a median total dose of IFN of 526 MU (range; 10–3696 MU) was administered during a median period of 23.9 weeks (range; 0.6–205.4 weeks). Patients mainly received IFN monotherapy, including initial aggressive induction therapy (every day within 8 weeks, followed by three times per week).

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

### *Methods*

The primary measure of efficacy of treatment was SR, defined as negative HCV RNA by qualitative analysis with PCR and normalization of transaminase levels (aspartate aminotransferase (ASAT), 11–38 IU/l; ALAT, 6–50 IU/l) at 24 weeks after cessation of IFN therapy. Patients who achieved SR after the first course of IFN monotherapy were classified as Group A. Patients who did not attain SR after the first course of IFN monotherapy were classified into two groups; based on whether they received other courses of IFN monotherapy or not. Patients who did not receive further courses of IFN monotherapy, because of concerns about adverse effects, lack of time for treatment, physicians' recommendation based on the emergence of depression and cardiopulmonary disease during and after the first course of IFN, or the lower levels of ALAT, were classified as Group B. Patients who received two or more courses of IFN monotherapy were classified as Group C.

### *Laboratory investigations*

Blood samples were frozen at $-80^{\circ}C$ within 4 h of collection and were not thawed until used for testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region [31]. In all cases, HCV-RNA

viremia levels were measured by branched DNA assay version 2.0 (Chiron Corp) at commencement of therapy using frozen samples, and the results were expressed as $10^6$ genomic equivalents per milliliter (Meq/ml). The lower limit of the assay was 0.5 Meq/ml. Samples with undetectable levels using this quantitative assay ( $<0.5$ Meq/ml) were also evaluated by HCV-RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor, Roche Diagnostic Systems) during and after therapy especially, and the results were expressed as positive or negative. The lower limit of the assay was 100 copies/ml.

*Liver histopathological examination*

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo) fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained 6 or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system devised by Desmet et al. [32].

*Follow-up*

Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by means of careful interviews and medical examinations at least once every month. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month before, during, and after treatment, and were also analyzed for ALAT levels and HCV-RNA levels at various time-points.

Follow-up time represented the time from the start of the first course of IFN treatment until death, or until the last visit. During this time, we especially evaluated liver-related death, which included HCC, cholangiocellular carcinoma, liver failure, or esophageal variceal bleeding.

*Diagnosis of hepatocellular carcinoma*

Patients were examined for HCC by abdominal ultrasonography every 3 to 6 months. If HCC was suspected based on ultrasonographic results, additional procedures such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy, if necessary, were used to confirm the diagnosis.

*Statistical analysis*

The $\chi^2$ test, Fisher's exact probability test, and the Mann-Whitney U-test were used to compare the background characteristics between groups. Multiple comparisons were examined using the Bonferroni test. The cumulative hepatocarcinogenesis and survival rates were calculated using the Kaplan-Meier technique, differences between survival curves were tested using the log-rank test. Statistical analyses of hepatocarcinogenesis and survival periods according to groups were calculated using the period from start of the first course of IFN monotherapy. The stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis. We also calculated the odds ratios and 95% confidence intervals (95% CI). Potential predictive factors associated with hepatocarcinogenesis included the following 10 variables: age, gender, histological stage, HCV genotype, viremia level, serum ALAT, platelet count, total IFN dose, total IFN duration, and treatment group. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that reached statistical significance ($p<0.05$) or marginal significance ($p<0.10$) on univariate analysis were tested by the multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using SPSS software (SPSS Inc., Chicago, Ill., USA). All $p$-values of less than 0.05 by the two-tailed test were considered significant.

## Results

*Efficacy of IFN monotherapy*

SR was achieved by 152 patients (33.5%) after the first course of IFN monotherapy (Group A). After the first course of IFN, 130 (28.6%) out of 302 (66.5%) non-SR patients did not receive a second course of IFN monotherapy (Group B), while the remaining 172 (37.9%) received two or more courses of IFN monotherapy (Group C). Of the 172 patients in Group C, 103 patients received two courses of IFN (30 of whom achieved SR), 51 patients received three courses (8 of whom achieved SR), 16 patients received four courses (4 of whom who achieved SR), and 2 patients received six courses (none achieved SR). Thus, 42 patients of Group C attained SR after multi-courses of IFN monotherapy.

In Groups A and B, the median total duration of IFN was 24.4 weeks (range, 4.0–205.4 weeks) and 23.4 (range, 2.9–90.0). The median total dose of IFN was 531 MU (range, 43–3696 MU) and 495 (range, 41–877). In the first, second, third, fourth, fifth, and sixth courses of IFN monotherapy in Group C, the median total durations of IFN were 23.9 weeks (range, 0.6–149.4 weeks), 24.0 (range, 1.3–313.7), 26.3 (range, 3.1–255.0), 29.6 (range, 3.9–86.3), 34.2 (range, 23.6–44.7), and 47.7 weeks (range, 25.3–70.1), respectively. In the first, second, third, fourth, fifth, and sixth courses of IFN monotherapy in Group C, the median total doses of IFN were 519 MU (range, 10–2399 MU), 573 (range, 29–4005), 525 (range, 28–3477), 566 (range, 81–1286), 555 (range, 402–708), and 690 (range, 180–1200), respectively. The median cumulative total durations and cumulative total doses, which represented the cumulative total duration and total dose of every course of every patient of Group C, were 58.5 weeks (range, 8.4–474.4 weeks) and 1380 MU (range, 340–5240 MU), respectively. The median periods free of IFN in Group C were 3.6 years (range, 0.1–7.7 years). Finally, the median dose of IFN per week in Groups A, B, and C were 21.8 MU/week (range, 6.7–42.0), 22.0 (range, 4.5–42.0), and 22.1 (range, 3.7–44.6), respectively.

*Clinical features of patients of Groups A, B, and C*

The clinical features of patients in Groups A, B, and C at the start of the first IFN monotherapy are summarized in Table I. The ages of patients of Group B were significantly higher than those of Group A ($p=0.001$; Bonferroni test) and Group C ($p=0.013$; Bonferroni test). Viremia levels and frequencies of genotype 1b in Group A were significantly lower than those in Group B ($p<0.0001$; Bonferroni test) and Group C ($p<0.0001$; Bonferroni test). Fibrosis stage of Group A was significantly milder than those of Group B ($p=0.005$; Bonferroni test) and Group C ($p=0.004$; Bonferroni test). There were no other significant differences in clinical features at the start of IFN therapy among the three groups.

*Cumulative hepatocarcinogenesis rates in Groups A, B, and C*

During the follow-up, 2 patients (1.3%), 23 (17.7%), and 19 (11.0%) developed HCC in Groups A, B, and C, respectively. In Groups A, B, and C, the cumulative hepatocarcinogenesis rates were 1.1, 15.0, 0.7% at the end of 5 years; and 2.2, 26.0, 9.0% at the end of 10 years, respectively. The rates were significantly different among the three groups ($p<0.0001$; log-rank test) (Figure 1). In particular, the rates in Group B were significantly higher than those in Group C ($p<0.0001$; log-rank test) and Group A ($p<0.0001$; log-rank test), and the rates in Group C were significantly higher than those in Group A ($p=0.0030$; log-rank test).

*Cumulative survival rates for overall death and liver-related death in Groups A, B, and C*

During the follow-up period, 2 patients (1.3%), 13 patients (10.0%), and 8 patients (4.7%) died in Groups A, B, and C, respectively. In Groups A, B, and C, the cumulative survival rates for overall death were 100, 95.0, and 100% at the end of 5 years; and 98.0, 78.0, and 96.0% at the end of 10 years, respectively. The rates were significantly different among the three groups ($p<0.0001$; log-rank test). Especially, the rate in Group B was significantly lower than those in Group C ($p=0.0003$; log-rank test) and Group A ($p<0.0001$; log-rank test). However, the rate in Group C was not significantly lower than that in Group A (no significance; log-rank test).

During the follow-up period, 0 patients (0%), 10 patients (7.7%), and 6 patients (3.5%) died of liver-

Table I. Patient characteristics at the start of first course of interferon monotherapy.

| | Group A ($n=152$) | Group B ($n=130$) | Group C ($n=172$) |
|---|---|---|---|
| Sex (male/female) | 113/39 | 81/49 | 128/44 |
| Age (year)[a] | 47 (15–64)[a] | 52 (23–70) | 47 (22–67)[c] |
| Viremia level (Meq/ml)[a] | 1.4 (<0.5–45.0) | 5.3 (<0.5–67.0)[d] | 4.8 (<0.5–57.0)[e] |
| HCV genotype (1b/non 1b*/ND) | 65/75/12 | 112/15/3[f] | 136/35/1[g] |
| Fibrosis stage (F1/F2/F3) | 103/45/4 | 62/60/8[h] | 95/55/22[i] |
| ASAT (IU/l)[a] | 81 (16–374) | 74 (22–398) | 75 (24–400) |
| ALAT (IU/l)[a] | 151 (24–546) | 120 (32–636) | 140 (26–594) |
| Platelet count ($\times 10^4/\mu l$)[a] | 18.7 (9.7–31.0) | 17.1 (9.7–39.2) | 16.5 (7.4–34.5) |

[a](Median). [b]$p=0.001$, [c]$p=0.013$, compared with Group B by Bonferroni test. [d]$p<0.0001$, [e]$p<0.0001$.
[f]$p<0.0001$, [g]$p<0.0001$, [h]$p=0.005$, [i]$p=0.004$, compared with Group A by Bonferroni test.
*Non 1b of Group A (2a/2b/3b; 58/15/2), non 1b of Group B (2a/2b; 8/7), non 1b of Group C (2a/2b; 24/11).
Abbreviations: HCV = hepatitis C virus; ASAT = aspartate aminotransferase; ALAT = alanine aminotransferase.