surrounding liver tissue. Eighty tumors (75%) yielded high-quality gene-expression profiles (see the Supplementary Appendix). Using a leave-one-out cross-validation procedure and a nearest-neighbor–based algorithm, we failed to detect a significant gene-expression correlate of either tumor recurrence (P=0.22) or survival (P=0.70) (Fig. 2A in the Supplementary Appendix). Furthermore, a previously reported signature associated with survival among patients with hepatocellular carcinoma[15] was not associated with survival in our series of patients (P=0.76) (Fig. 2B in the Supplementary Appendix). This failure to identify an outcome-associated signature is unlikely to be due to a technical flaw of the formalin-fixed, paraffin-embedded DASL method, because we observed the same molecular-subclass structure in the formalin-fixed, paraffin-embedded samples as that observed in collections of frozen samples of hepatocellular carcinoma (Fig. 2B and 3B in the Supplementary Appendix). Although this result does not exclude the possibility of tumor-derived expression profiles as predictors of the outcome of hepatocellular carcinoma, the data suggest that at least in this training set, the outcome was largely related to other factors.

SURVIVAL SIGNATURE IN ADJACENT LIVER TISSUE

The lack of association between tumor-derived gene-expression profiles and survival led us to consider the pattern of recurrence of early-stage hepatocellular carcinoma. In contrast to advanced tumors, which tend to recur rapidly after resection, early-stage tumors, which are increasingly diagnosed in modern clinical practice, recur much later, generally more than 2 years after resection[9,10] (Fig. 4 in the Supplementary Appendix). This emerging pattern of late recurrence of hepatocellular carcinoma (due at least in part to the diagnosis of hepatocellular carcinoma at an early stage) has led to the notion that a late recurrence may not be an actual recurrence but rather a second primary tumor in an at-risk liver, presumably due to the carcinogenic effects of cirrhosis.[1,2,9] We therefore hypothesized that the surrounding liver tissue — not the tumor itself — might harbor a gene-expression signature associated with subsequent recurrence.

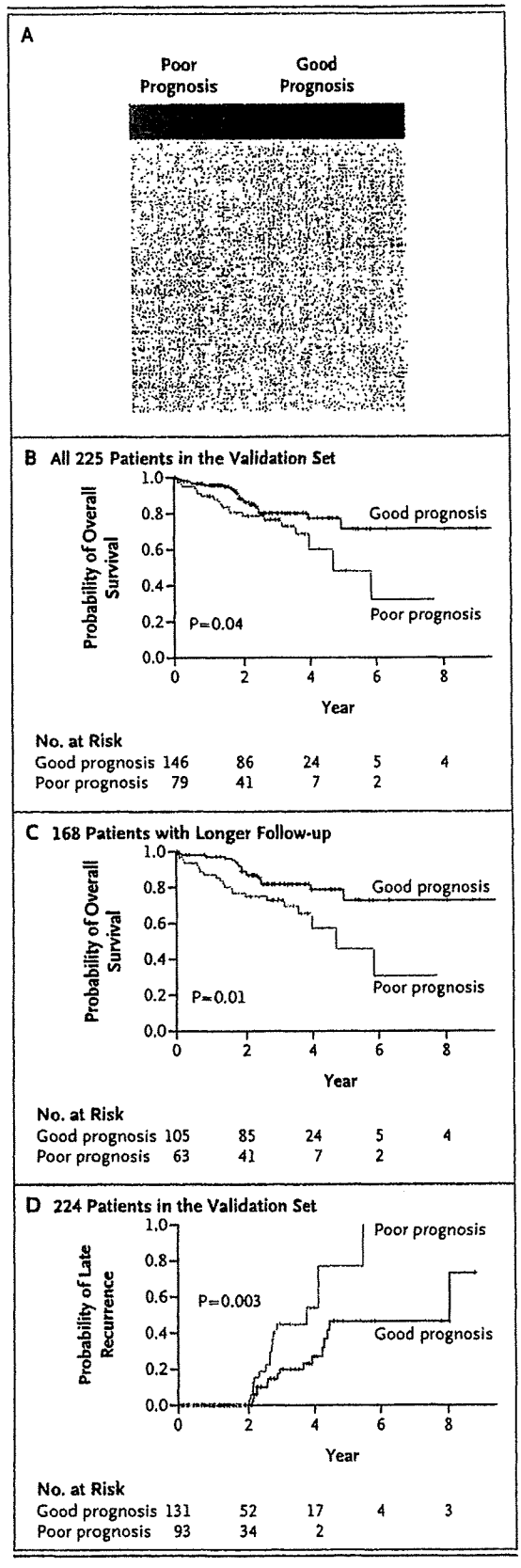
To test this hypothesis, we assessed the gene-expression profiles of the liver tissue surrounding the resected tumor in the 106 formalin-fixed, paraffin-embedded blocks that constituted the training set. Eighty-two samples (77%) yielded high-quality gene-expression profiles (see the Supplementary Appendix). Using a standard leave-one-out cross-validation procedure, we found the liver signature to be significantly correlated with survival (P=0.02) (Fig. 2A). The aggregate survival-correlated signature contained 186 genes (Fig. 2B and 2C, and Table 2 in the Supplementary Appendix) and was tested in the validation set. Using GSEA, which shows whether a defined set of genes has a significant association with a phenotype of interest, we found the good-prognosis signature to contain genes associated with normal liver function (Tables 2 and 3 in the Supplementary Appendix), including the plasma proteins C4, C5, C8, C9, and F9 and several drug-metabolizing enzymes: the alcohol dehydrogenases ADH5 and ADH6, the aldo–keto-reductases AKR1A1 and AKR1D1, the aldehyde dehydrogenase ALDH9A1, the cytochrome P450 CYP2B6, and hepatic lipase (LIPC). These findings are consistent with the association between impaired liver function and a poor outcome.[1] In addition, the poor-prognosis signature contained gene sets associated with inflammation, including those related to interferon signaling, activation of nuclear factor-κB, and signaling by tumor necrosis factor α. Histologic features of liver inflammation were not found to be associated with the outcome (Fig. 2D, and Table 4 and Fig. 5 in the Supplementary Appendix). Of particular interest, GSEA showed that the downstream targets of interleukin-6 were strongly associated with the poor-prognosis signature, which is consistent with the finding that disruption of interleukin-6 signaling protects mice from chemically induced hepatocellular carcinoma.[16]

VALIDATION OF THE LIVER-DERIVED SURVIVAL SIGNATURE

We next tested the 186-gene survival signature in an independent set of tissue samples from eligible patients at three treatment centers in the United States and Europe. Of the 234 samples in this validation set, 225 (96%) yielded gene-expression profiles of high quality (see the Supplementary Appendix). The survival signature (Fig. 3A) was associated with significant differences in survival among patients (P=0.04) (Fig. 3B), despite the modest duration of follow-up (median, 2.2 years). The separation of the survival curves was even more pronounced when, in a prespecified subgroup analysis, we limited our attention to the 168

Figure 3. **Survival Signatures and Survival Curves in the Validation Set.**

Panel A shows the expression pattern of the 186-gene survival signature. Red indicates a poor prognosis; blue indicates a good prognosis. Survival curves are shown for overall survival according to the level of expression of the 186 signature genes among all 225 patients whose tissue samples constituted the validation set (Panel B) and among the 168 patients with a longer duration of follow-up (treated no later than 2004) (Panel C). Panel D shows the probability of late recurrence according to the level of expression of the late-recurrence gene signature. Data are missing for one patient in Panel D.



patients with a longer duration of follow-up (median, 2.8 years; P=0.01) (Fig. 3C). These results support the validity of the survival signature and highlight the potential role of nontumoral liver tissue in predicting the outcome for patients with early hepatocellular carcinoma.

RECURRENCE-ASSOCIATED SIGNATURE

We performed a similar analysis using tumor recurrence as the clinical end point. A 132-gene late-recurrence signature defined in the training set was tested in the validation set. Whereas the recurrence signature did not show an association with recurrence within the first 2 years after surgery (a finding that was consistent with its development in association with late recurrence) (Fig. 6A and 6B in the Supplementary Appendix), it was significantly associated with late recurrence (P=0.003) (Fig. 3D). Not surprisingly, a nonparametric enrichment test indicated that the survival and late-recurrence signatures were closely associated (P<0.001) (Fig. 6C in the Supplementary Appendix).

MULTIVARIATE ANALYSIS

We next examined the signature in the context of the factors that are generally accepted as indicating a poor prognosis for patients with hepatocellular carcinoma (tumor multinodularity, the presence of microvascular invasion, and a high serum alpha-fetoprotein level[1,9]) in the validation set. These factors were associated with early recurrence (within 2 years after treatment) (Table 5 in the Supplementary Appendix). In contrast, multivariate analysis showed that the late-recurrence signature was the only independent prognostic variable for late recurrence (Table 2). Prespecified subgroup analyses showed that this associa-

Table 2. **Associations of Gene-Expression Signatures and Clinical Variables with Late Recurrence or Overall Survival, from Multivariate Analysis of the Validation Set.**

Variable	Hazard Ratio (95% CI)*	P Value
Late recurrence: late-recurrence signature	2.94 (1.39–6.20)	0.005
Overall survival		
All 225 patients		
Poor-prognosis signature	2.08 (1.03–4.18)	0.04
Alpha-fetoprotein >100 ng/ml	2.29 (1.14–4.61)	0.02
Vascular invasion	2.01 (1.01–3.99)	0.05
168 Patients with longer follow-up		
Poor-prognosis signature	2.56 (1.22–5.38)	0.01
Alpha-fetoprotein >100 ng/ml	2.01 (0.94–4.26)	0.07
Vascular invasion	2.20 (1.06–4.53)	0.03

* The hazard ratio was for late recurrence among patients with the late-recurrence gene signature as compared with those without the signature or for overall survival among patients with the poor-prognosis gene signature as compared with those without the signature, with alpha-fetoprotein levels of more than 100 ng per milliliter as compared with levels of 100 ng per milliliter or less, or with vascular invasion as compared with the absence thereof.

tion remained significant in both the subgroup of 168 patients with a longer period of follow-up and the subgroup of 207 patients with early-stage hepatocellular carcinomas (BCLC stage 0 or A) (Fig. 4, and Table 6 in the Supplementary Appendix). Similarly, the survival signature was independently associated with survival in multivariate analysis (Table 2), and this association persisted in the subgroup of patients with longer follow-up (Table 2).

These results indicate that clinical and histopathological factors are associated with early recurrence of hepatocellular carcinoma and that late recurrence is associated with the gene-expression signature of nontumoral liver tissue adjacent to the primary tumor. The latter finding is consistent with the notion that late recurrences are not actually recurrences but rather new primary tumors. In support of this view, we detected highly discordant patterns of gains and losses in gene-copy number (including in regions exhibiting loss of heterozygosity) between the primary and recurrent hepatocellular carcinoma tumors but did not detect such patterns in endometrial, ovarian, renal, or lymphoma tumors (Table 7 and Fig. 7 in the Supplementary Appendix). These results strongly suggest that the primary and recurrent hepatocellular carcinoma tumors arise from distinct clones.

DISCUSSION

The full potential of gene-expression profiling of cancer has been hindered in part by technical limitations — in particular, the requirement of frozen material for analysis. Although frozen tissues are increasingly being banked at tertiary care centers, the duration of clinical follow-up of these collections is usually short, and the vast majority of tumor-biopsy specimens and resections are performed outside of major research hospitals. There is therefore a need for methods that allow for the genomewide expression profiling of formalin-fixed tissue samples, which are routinely collected in the clinical setting. Such approaches have been described,[17] but their extensive validation has yet to be reported. We describe here a DASL-based method capable of profiling approximately 6000 human transcripts, and we have tested the method on more than 2000 formalin-fixed, paraffin-embedded blocks collected as long as 24 years ago. Through the assay of 6000 genes across the genome that show maximal variation in expression, this approach is expected to capture the bulk of transcriptional differences in any collection of samples. However, recent increases in array density support the analysis of all human genes on a single array (whole-genome DASL assay, Illumina).

The DASL-based discovery method that we describe here should be distinguished from candidate-gene profiling methods based on the reverse transcriptase (RT)-PCR assay, such as those used in the commercially available OncotypeDx test for determining the prognosis in patients with breast cancer.[18] Whereas standard RT-PCR methods can measure a small number of transcripts in formalin-fixed, paraffin-embedded samples, genomewide discovery studies are not feasible with the use of RT-PCR–based methods. In addition, we speculate that the use of formalin-fixed, paraffin-embedded tissue specimens will aid the transition from exploratory research to clinical implementation.

We applied the DASL profiling method to an increasingly important challenge in the care of patients with hepatocellular carcinoma. Tumors are often small at the time of diagnosis (owing to increased surveillance and advanced imaging in patients at risk), and existing prognostic factors are less informative for patients with small tumors than for those with larger tumors.

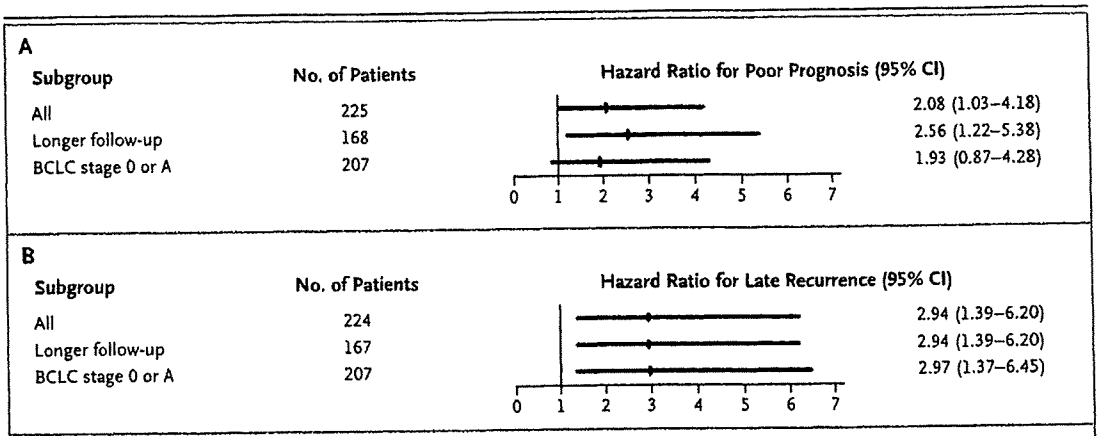


Figure 4. **Hazard Ratios for Poor Survival and Late Recurrence in Selected Subgroups of Patients in the Validation Set.** The hazard ratio was for poor survival among patients with the poor-prognosis gene signature (Panel A) or for late recurrence among patients with the late-recurrence gene signature (Panel B), as compared with those without the signature. BCLC denotes Barcelona Clinic Liver Cancer staging system, which ranks hepatocellular carcinoma in five stages, ranging from 0 (very early stage) to D (terminal stage). Data are missing for one patient in Panel B.

We did not observe a significant association between the expression profiles of the tumors themselves and the outcome for patients with surgically resected early hepatocellular carcinoma. In contrast, others have described tumor-derived prognostic signatures for hepatocellular carcinoma.[15,19] The populations of patients in those studies, however, tended to have more advanced disease. Our training set primarily exhibited a pattern of late recurrence that is typical of small tumors.[1,9] Accordingly, it is likely that early recurrence (reflecting locally invasive and incompletely resected tumor) is associated with molecular features of the primary tumor, but such features are not associated with late recurrences, which seem to result from new primary tumors arising in a damaged organ (the "field effect") rather than the proliferation of residual tumor cells derived from the original tumor.

Also supporting the concept that late recurrence of hepatocellular carcinoma represents new primary tumors in patients at risk, we found little correlation between the molecular characteristics of tumors resected at initial diagnosis and those from the same patients at the time of recurrence. In particular, the results of clonality analysis indicated that the late recurrences of hepatocellular carcinoma tended to derive from a different clone than the preceding primary tumors. In addition, the obvious measures of liver damage (e.g., the extent of cirrhosis and the Child–Pugh stage[20]) were not associated with survival in our study, given that we restricted our analysis to patients with preserved liver function.

Our findings indicate a field effect, in which environmental exposure (e.g., viral infection) leads to an increased potential for future malignant transformation. This has in general been overlooked by genomic approaches to studying cancer that have focused only on tumor cells. Our results suggest that a gene-expression signature can serve as a sensitive "readout" of the biologic state of the liver in at-risk patients. It is likely that the survival signature reflects the extent of liver damage and the presence or absence of a proinflammatory milieu, which is mediated in part by gene products involved in an inflammatory response. A heritable basis for the signature, although improbable, cannot be ruled out. Additional work is needed to fully understand the biologic basis of the signature.

Further clinical validation of the survival signature will be needed before it is introduced into clinical practice; our observation that the signature is associated with the outcome across heterogeneous populations of patients is encouraging. We envision the use of this test to identify the patients at highest risk for recurrence of hepatocellular carcinoma and to target intensive clinical follow-up or chemopreventive strategies in such patients.[21]

Supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (1R01DK076986-01, to Dr. Llovet), the National Cancer Institute (5U54 CA112962-03, to Dr. Golub), the Samuel Waxman Cancer Research Founda-

tion (to Dr. Llovet), the Spanish National Health Institute (SAF-2007-61898, to Dr. Llovet), Institució Catalana de Recerca i Estudis Avançats (to Dr. Llovet), Centro de Investigaciónes en Red de Enfermedades Hepáticas y Digestivas (to Drs. Llovet and Bruix), the Fund for Health of Spain of the Institute of Health Carlos III (PI05-0150, to Dr. Bruix), the National Institutes of Health (DK37340, to Dr. Friedman), the Italian Association for Cancer Research (to Dr. Mazzaferro), Helse Vest and Norwegian Cancer Society, Harald Andersens grant (to Dr. Salvesen), the Charles A. King Trust fellowship (to Dr. Hoshida), and Fundación Pedro Barrié de la Maza, the Sheila Sherlock Fellowship, and the National Cancer Center Fellowship (all to Dr. Villanueva).

No potential conflict of interest relevant to this article was reported.

We thank David Peck, Jun Lu, Aravind Subramanian, and Oleg Iartchouk for technical advice; Joshua Gould, Heidi Kuehn, and Barbara Hill for technical help; David Harrington for critical reading of a draft of the manuscript, and Mariko Kobayashi and Jadwiga Grabarek for general support. Prostate samples and lymphoma cell lines for pilot DASL experiments were kindly provided by Sunita Setlur, Mark Rubin, Kunihiko Takeyama, and Jeffery L. Kutok. Single-nucleotide-polymorphism profiling data for endometrial, ovarian, and renal cancers and lymphoma were provided by Rameen Beroukhim, Matthew Meyerson, Mark Rubin, Stefano Monti, and Margaret Shipp.

REFERENCES

1. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907-17.
2. Llovet JM, Bruix J. Novel advancements in the management of hepatocellular carcinoma in 2008. J Hepatol 2008;48:Suppl 1:S20-S37.
3. Ikeda K, Arase Y, Saitoh S, et al. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor: a prospective randomized study of hepatitis C virus-related liver cancer. Hepatology 2000;32:228-32.
4. Muto Y, Moriwaki H, Ninomiya M, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. N Engl J Med 1996;334:1561-7.
5. Takayama T, Sekine T, Makuuchi M, et al. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. Lancet 2000;356:802-7. [Erratum, Lancet 2000;356:1690.]
6. Lau WY, Leung TW, Ho SK, et al. Adjuvant intra-arterial iodine-131-labelled lipiodol for resectable hepatocellular carcinoma: a prospective randomised trial. Lancet 1999;353:797-801.
7. Fan JB, Yeakley JM, Bibikova M, et al. A versatile assay for high-throughput gene expression profiling on universal array matrices. Genome Res 2004;14:878-85.
8. Bibikova M, Talantov D, Chudin E, et al. Quantitative gene expression profiling in formalin-fixed, paraffin-embedded tissues using universal bead arrays. Am J Pathol 2004;165:1799-807.
9. Llovet JM, Schwartz M, Mazzaferro V. Resection and liver transplantation for hepatocellular carcinoma. Semin Liver Dis 2005;2:181-200.
10. Imamura H, Matsuyama Y, Tanaka E, et al. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol 2003;38:200-7.
11. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-50.
12. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208-36.
13. Mazzaferro V, Romito R, Schiavo M, et al. Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. Hepatology 2006;44:1543-54.
14. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. Nat Genet 2006;38:500-1.
15. Lee JS, Chu IS, Heo J, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology 2004;40:667-76.
16. Naugler WE, Sakurai T, Kim S, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007;317:121-4.
17. Coudry RA, Meireles SI, Stoyanova R, et al. Successful application of microarray technology to microdissected formalin-fixed, paraffin-embedded tissue. J Mol Diagn 2007;9:70-9.
18. Habel LA, Shak S, Jacobs MK, et al. A population-based study of tumor gene expression and risk of breast cancer death among lymph node-negative patients. Breast Cancer Res 2006;8(3):R25.
19. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 2003;9:416-23.
20. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg 1973;60:646-9.
21. Llovet, JM, Di Bisceglie AM, Bruix J, et al. Design and endpoints of clinical trials in hepatocellular carcinoma. J Natl Cancer Inst 2008;100:698-711.

INTERNAL MEDICINE

ORIGINAL ARTICLE

Natural Human Interferon β Plus Ribavirin Combination Therapy in Japanese Patients Infected with Hepatitis C Virus and a High Viral Load

Yoshio Katamura[1], Fumitaka Suzuki[1], Norio Akuta[1], Hitomi Sezaki[1], Hiromi Yatsuji[1], Norihiro Nomura[1], Yusuke Kawamura[1], Tetsuya Hosaka[1], Masahiro Kobayashi[1], Yoshiyuki Suzuki[1], Satoshi Saito[1], Yasuji Arase[1], Kenji Ikeda[1], Mariko Kobayashi[2] and Hiromitsu Kumada[1]

Abstract

Objective The aim of this pilot study was to determine the safety and efficacy of natural human interferon β (nIFNβ) plus ribavirin (RBV) in patients with chronic hepatitis C who did not respond to pegylated interferon alpha (PEG-IFN), with special emphasis on the incidence of mental disorders or refusal for fear of adverse effects.

Methods We studied 19 patients with HCV genotype 1b, 2a or 2b and a high viral load, including 8 patients with mental disorders. They were treated with nIFNβ-RBV. Eleven patients with HCV genotype 1b of these patients were treated with nIFNβ-RBV for 48 weeks (group A), and compared with 22 matched controls treated with PEG-IFN plus RBV for 48 weeks (group B). The other 8 patients with HCV genotype 2 were treated with nIFNβ-RBV for 24 weeks.

Results Six of 8 patients with mental disorders and 9 of 11 patients without mental disorders completed nIFNβ-RBV therapy; 1 patient with mental disorder dropped out due to exacerbation of depression, and 3 patients suspended the therapy due to insufficient response. The sustained virological response (SVR) was 27% (3/11) in group A and 41% (9/22) in group B ($p = 0.70$). During treatment, platelet count increased in group A but not in group B. SVR was 88% (7/8) in patients of genotype 2 and high viral load treated with nIFNβ plus RBV.

Conclusion nIFNβ-RBV therapy offers sufficient safety and efficacy for patients with mental disorders, and thus could represent an excellent second-line therapy for subpopulations that are not suitable for PEG-IFN-RBV.

Key words: hepatitis C virus, interferon β, ribavirin, depression

(Inter Med 47: 1827-1834, 2008)
(DOI: 10.2169/internalmedicine.47.1436)

Introduction

Pegylated interferon α (PEG-IFN) plus ribavirin (RBV) is the first line treatment for patients infected with hepatitis C virus (HCV) genotype 1, and high viral load, and can achieve sustained virological response (SVR) in 41-47% of these patients (1-3). However, such treatment causes adverse effects in some patients, such as mental disorders, apathy and laboratory abnormalities. Previous studies indicated that 10-16% of patients treated with PEG-IFN plus RBV for 48 weeks discontinued the therapy due to adverse effects, especially depression (1-3). Individuals with depression or previous history of interferon (IFN) α-induced mental disorders

[1]Department of Hepatology, Toranomon Hospital, Tokyo and [2]Research Institute for Hepatology, Toranomon Hospital, Tokyo
Received for publication June 21, 2008; Accepted for publication July 15, 2008
Correspondence to Dr. Fumitaka Suzuki, fumitakas@toranomon.gr.jp

are not suitable candidates for IFNα therapy (4). Moreover, several patients reject the therapy for fear of depression arising as a side effect. IFNβ, a type I IFN that binds to the same cell surface receptor as IFNα, triggers distinct biological responses and elicits distinct patterns of gene expression within the same target cells (5-8). Three forms of human IFNβ are available (9): *1)* Natural human IFNβ (nIFNβ) is produced by human fibroblasts. *2)* Recombinant human IFNβ-1a (rhIFNβ-1a) is procured by mammalian cells and is identical to nIFNβ. *3)* Recombinant human IFNβ (rhIFNβ-1b) is produced by *Escherichia coli* in which cysteine at position 17 is substituted by serine. Previous reports showed that IFNβ has sufficient tolerability (10). Other reports indicated that IFNβ is effective in HCV eradication, although it seems that IFNβ monotherapy does not result in a satisfactory outcome in patients with HCV, particularly those infected with genotype 1 and have a high viral load (11, 12). Recent randomized trials demonstrated the efficacy of rhIFNβ-1a plus RBV (13, 14). However, there is little information regarding nIFNβ plus RBV (15, 16). Furthermore, the safety of IFNβ for patients with mental disorders has not been reported.

There is evidence to suggest that monitoring of early viral kinetics is useful for earlier identification of the likelihood of response to IFN therapy (17). Correlation viral kinetics and therapeutic outcome of PEG-IFN plus RBV has been investigated (18, 19), but not that of IFNβ plus RBV.

The present pilot study included 19 patients who had not received PEG-IFN or IFNα for their mental disorders or refused PEG-IFN for fear of adverse effects. The objective of this study was to assess the safety and efficacy of nIFNβ plus RBV in Japanese patients infected with HCV genotype 1b or 2 and high viral load. In addition, we assessed viral kinetics in patients infected with HCV genotype 1b with high viral load treated with nIFNβ plus RBV.

Patients and Methods

Study population

Nineteen HCV-infected Japanese patients were enrolled in this trial between 2001 and 2006 at Toranomon Hospital, Tokyo. The enrollment criteria were HCV genotype 1b, 2a or 2b confirmed by polymerase chain reaction (PCR) ; serum HCV RNA levels >100,000 international units (IU)/mL on quantitative PCR assay (defined as "high" viral load, Amplicor HCV Monitor version 2.0, Roche Diagnostics, Tokyo, Japan) ; No treatment with corticosteroids, immunosuppressants, or antiviral agents within 6 months prior to this trial; negativity for hepatitis B surface antigen (HBsAg), as determined by radioimmunoassay; hemoglobin concentration >12.0 g/dL; neutrophil count >1,500/μL; platelet count >60,000/μL; serum creatinine <1.5 times above the upper limit of normal; and body weight between 40 and 100 kg. The exclusion criteria were liver cancer or severe liver failure; pregnant or breastfeeding women; past history of hypersensitivity reactions to IFN or ribavirin. Psychiatric exclusion criteria included preexisting severe depression and suicidal ideation and/or attempt.

Eleven patients with HCV genotype 1b treated with nIFNβ plus RBV were defined as group A. To compare the clinical efficacy of the treatment, we retrospectively selected 22 patients treated with PEG-IFN plus RBV, matched 1:2 with patients of group A for genotype, sex, age, and response to previous IFNα or IFNα plus RBV (control group; group B). Patients of group B were selected from among 407 patients of Toranomon Hospital.

Study protocol

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained from each subject. Treatment was provided for 48 weeks to HCV genotype 1b, and for 24 weeks to HCV genotype 2a or 2b, with subsequent 24-week follow-up period.

nIFNβ group: 11 patients with HCV genotype 1b (group A), were treated with nIFNβ (Feron, Toray Industries Inc., Tokyo) intravenously at a dose of 6 million units (MU) daily for 2-8 weeks, followed by three times a week for 40-46 weeks (total 48 weeks). In group A, nIFNβ was administrated daily for 2 weeks to 6 patients, for 4 weeks to 1 patient and for 8 weeks to 4 patients. Eight patients with HCV genotype 2 were treated with nIFNβ intravenously at a dose of 6 MU daily for 2-8 weeks and then three times a week for 16-22 weeks (total 24 weeks).

Control group: 22 patients with HCV genotype 1b (group B) were treated with PEG-IFNα2b (Schering-Plough, Osaka, Japan) subcutaneously at a dose of 1.5 μg/kg weekly for 48 weeks.

Each patient was treated with oral RBV (Schering-Plough) at a total dose of 600-1,000 mg twice daily for 48 weeks for HCV genotype 1b and for 24 weeks for HCV genotype 2. The dose was adjusted according to body weight (600 mg for patients weighing ≤60 kg, 800 mg for those between 60 and 80 kg, and 1,000 mg for patients weighing between 80 and 100 kg). Both nIFNβ or PEG-IFNα2b and RBV were concurrently initiated.

Serum samples were collected from the patients at 0, 2 days, and 2, 4, 12 weeks, at the end of therapy, and 24 weeks after the end of therapy. HCV RNA in serum was quantified at each point by a quantitative reverse-transcription polymerase chain reaction (PCR) assay (Cobas Amplicor HCV Monitor version 2.0 using the 10-fold dilution method, Roche) with a low detection limit of 5 KIU/mL. When HCV RNA was undetectable by quantitative PCR assay, it was assessed using qualitative detection assay (Amplicor HCV, Roche) with a low detection limit of 50 IU/mL. End-of-treatment response (ETR) was defined as no detectable serum HCV RNA at the end of treatment. SVR was defined as no detectable serum HCV RNA at 24 weeks after the end of treatment.

Table 1. Clinical Characteristics of Chronic Hepatitis C Patients with High Viral Load Treated with Combination Therapy of nIFNβ Plus Ribavirin

n	19
Age (years)*	60 (33-73)
Gender (male/female)	9/10
Leukocytes (/μL)*	4600 (2300-7500)
hemoglobin (g/dL)*	14.3 (10.5-15.9)
Platelets ($\times 10^3$/μL)*	19 (6.8-25.4)
Alanine aminotransferase (IU/L)*	60 (24-726)
Genotype (1b/2a/2b)	11/4/4
HCV-RNA (KIU/mL)*	1100 (400-5000)
Histology (F: 1/2/3/4/ND)	3/6/1/1/8
Interferon: naive/retreatment	9/10
Previous IFN therapy	
Virological response : yes/no	6/4
Monotherapy / combination therapy	9/1

*Data represent the median (range) values.

nIFNβ, natural human interferon β; IFN, interferon

Liver fibrosis classified as: F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; ND, not done.

Dose reduction of IFN and RBV

nIFNβ was reduced from 6 MU to 3 MU when neutrophil count decreased to <750/μL or platelet count to <30,000/μL. Furthermore, the dose of PEG-IFNα2b was reduced from 1.5 to 1.0-0.5 μg/kg/week if neutrophil count decreased to <750/μL or platelet count to <80,000/μL. RBV was reduced in a stepwise fashion by 200 mg/day when hemoglobin concentration decreased to 10 g/dL. Further dose reduction or discontinuation of these drugs was applied for ongoing hematological adverse effects or other unendurable adverse effects such as mental disorder, flu-like syndrome, and gastrointestinal symptoms.

Statistical analysis

Treatment outcome was analyzed on intention-to-treat basis. Mann-Whitney U test or Fisher's exact probability test was used for comparison between groups. All p values for statistical tests were two-tailed and those <0.05 were considered to denote a significant difference. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

Results

Clinical background

The nIFNβ group consisted of 19 patients; 5 (26%) patients developed depression or had a history of depression, 3 (16%) had a history of IFNα-induced depression, 7 (37%) refused PEG-IFN for fear of adverse effects such as depression, 3 (16%) were older than 65 years, and 1 (5%) suffered from severe fatigue associated with previous IFNα therapy.

In all patients who developed depression during treatment or had previous history of depression, depression was diagnosed by psychiatrists at our hospital. Table 1 shows the clinical features of 19 patients treated with nIFNβ plus RBV. Ten (53%) patients had been treated with IFN previously. Among the retreatment patients, 4 (40%) were nonresponders to previous IFN therapy. Table 2 shows the clinical features of 11 patients with HCV genotype 1 and high viral load treated with IFNβ plus RBV (case; group A) and 22 patients treated with PEG-IFN (control; group B) groups. There were no significant differences between the two groups in HCV-RNA level, fibrosis score, and laboratory pa-

Table 2. Comparison of Clinical Features of Patients with Genotype 1b and High Viral Load Treated with nIFNβ Plus Ribavirin and PEG-IFN Plus Ribavirin

	Group A (IFNβ+RBV)	Group B (PEG-IFN+RBV)	p value
n	11	22	
Age (years)*	57 (36-67)	54 (29-67)	matched
Sex (male/female)	7/4	14/8	matched
Interferon: naive/retreatment	4/7	8/14	matched
Previous IFN therapy			
Virological response: yes/no	4/3	8/6	matched
Monotherapy/IFN+RBV	6/1	12/2	matched
HCV-RNA (KIU/mL)*	1300 (530-3400)	2200 (370-4500)	0.26
Leukocytes (/μL)*	4700 (2300-6000)	4350 (2800-7100)	0.65
Hemoglobin (g/dL)*	14.7 (12.8-15.9)	14.5 (12.8-16.4)	0.88
Platelets ($\times 10^3$/μL)*	18.8 (9-24.9)	15.6 (9-25)	0.47
Alanine aminotransferase (IU/L)*	60 (40-156)	69 (28-237)	0.29
γ-glutamyl transpeptidase (IU/L)*	43 (15-106)	43 (20-244)	0.38
LDL-C (mg/dL)*	105 (52-162)	109 (50-162)	0.70
ICG-R(15) (%)*	11 (5-16)	12 (8-45)	0.052
RBV dose/body weight (mg/kg)*	11.8 (11.1-13.3)	11.1 (2.7-14)	0.063
Histology (F: 1/2/3/4/ND)	2/3/1/1/4	11/5/4/0/2	0.23

nIFNβ, natural human interferon β; PEG-IFN, pegylated interferon α;

*Data represent the median (range) values.

IFN, interferon; RBV, ribavirin; LDL-C, low density lipoprotein cholesterol

ICG-R(15), indocyanine green retention rate at 15 minutes

Liver fibrosis classified as: F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; ND, not done.

rameters shown in Table 2.

Safety profile of nIFNβ plus RBV therapy

Table 3 shows the clinical features of 8 patients (genotype 1b; n=5, genotype 2; n=3) who developed mental disorders. One patient (12.5%) received antidepressant, and 2 patients (25%) received anti-anxiety drugs at the start of the treatment. During the therapy, those 3 patients did not need additional drugs. On the other hand, 1 patient (12.5%) received anti-anxiety drugs but such treatment was discontinued due to exacerbation of depression at 32 weeks after the start of therapy, and 1 patient (12.5%) received antidepressant during the therapy. The remaining 3 patients (37.5%) did not need anti-anxiety drugs/antidepressants. Among the remaining 11 patients with no history of mental disorder, 1 patient (9%) received anti-anxiety drugs during the therapy.

nIFNβ dose reduction was necessary in 1 (5.3%) patient due to the development of neutropenia. RBV dose reduction was applied in 9 (47%) patients, due to anemia (n=8) and extensive skin eruption (n=1). Therapy was suspended in 3 (16%) patients due to insufficient response to the therapy.

In the control group (n=22), treatment was discontinued in 2 (9%) patients due to adverse effects (fatigue and depression). Eight (36%) patients required PEG-IFN dose re-

Table 3. **Clinical Features of Patients with Mental Disorders Treated with nIFNβ Plus Ribavirin**

Patient No.	Gender	Age	Genotype	Mental disorder	Prescription at start of therapy	Prescription during therapy	nIFNβ plus ribavirin	Therapy duration (W)	Virological response
1	F	60	1b	depression	none	Anti-anxiety drug	Dropped out due to depression	32	NR
2	M	46	1b	depression	antidepressant	keeping on	Dropped out due to insufficient response	24	NR
3	M	49	1b	depression	none	antidepressant	completed	48	NR
4	M	40	1b	IFN-Induced	none	none	completed	48	SVR
5	M	60	1b	IFN-Induced	none	none	completed	48	NR
6	F	57	2a	depression	antianxiety drug	keeping on	completed	24	SVR
7	F	63	2a	IFN-Induced	antianxiety drug	keeping on	completed	24	SVR
8	F	60	2b	depression	none	none	completed	24	SVR

nIFNβ, natural human interferon β; IFN, interferon; SVR, sustained viral response; NR, non-responder

Table 4. **Virological Response of Patients with Genotype 1b and High Viral Load Treated with nIFNβ Plus Ribavirin and PEG-IFN Plus Ribavirin**

	Group A (nIFNβ+RBV)	Group B (PEG-IFN+RBV)	p value
End-of-treatment response	5/11 (45%)	15/22 (68%)	0.270
Sustained virological response	3/11 (27%)	9/22 (41%)	0.703

nIFNβ, natural human interferon β; PEG-IFN, pegylated interferon α; RBV, ribavirin

duction due to fatigue (n=2), dizziness (n=1), neutropenia (n=4) and thrombocytopenia (n=1). RBV dose reduction was applied in 12 (55%) patients due to anemia. Therapy was suspended in 2 (9%) patients at 24 weeks after commencement due to insufficient response.

Efficacy of IFNβ plus RBV therapy

Table 4 shows the ETR and SVR rates of groups A and B. ETR and SVR were achieved in 45% (5/11) and 27% (3/11) patients of group A (nIFNβ plus RBV) and in 68% (15/22) and 41% (9/22) patients of group B (PEG-IFN plus RBV), respectively. Differences between groups A and B were not significant (p = 0.27 and 0.70, respectively). In patients of genotype 2 and high viral load, ETR and SVR to nIFNβ plus RBV therapy were achieved in 88% (7/8) and 88% (7/8) patients, respectively.

Profile of leukocyte count, hemoglobin concentration and platelet count

Figure 1 shows profile of leukocyte count, hemoglobin concentration, platelet count of groups A and B. These parameters were assessed at week 2, week 4, and every 4 weeks until week 48. In groups A and B, the average leukocyte count, hemoglobin concentration and platelet count at baseline were 4,540/mm^3 and 4,590/mm^3, 14.4 g/dL and 14.6 g/dL, 17.4×10^4/mm^3 and 16.1×10^4/mm^3, respectively. There were no significant differences in leukocyte count and hemoglobin concentration between groups A and B at each time point. However, the dynamics of platelet count was different between the two groups. In group A, the platelet count decreased to 15.1×10^4/mm^3 at week 2, but increased above baseline after week 4. In groups A and B, the platelet count was significantly different at week 4, 8, 12, 16, 20, 24, 40, 44 and 48, respectively.

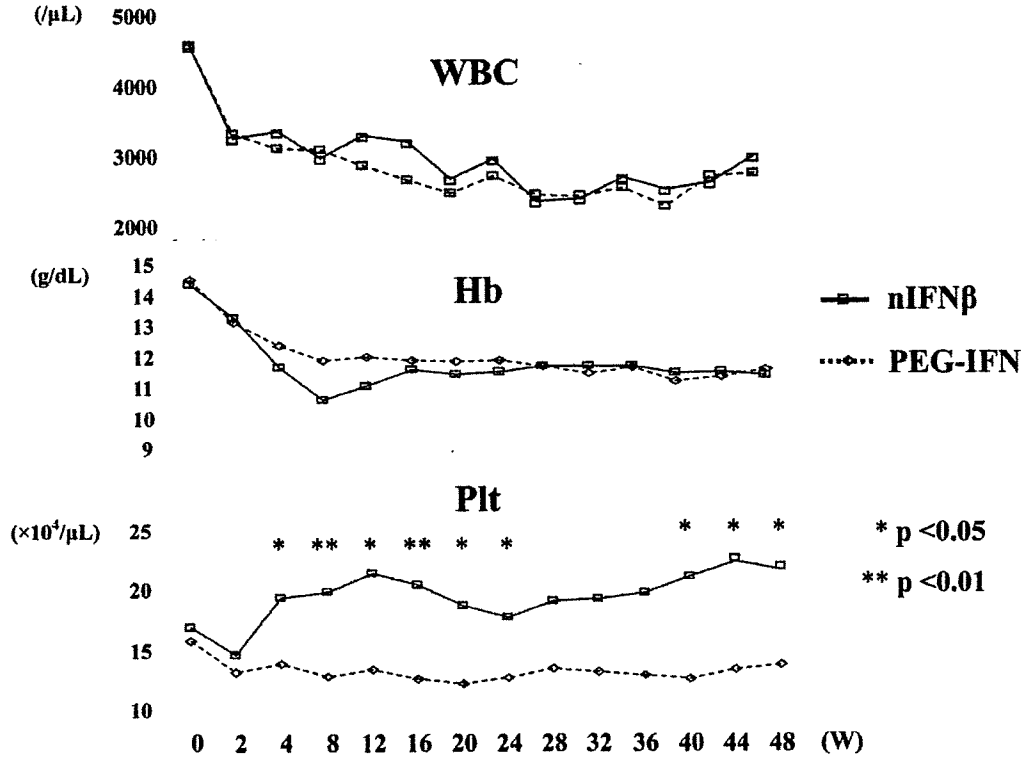


Figure 1. **Mean leukocyte count, hemoglobin concentration and platelet count during the 48 weeks of treatment of patients with chronic HCV infection, genotype 1b and high viral load. Solid lines: nIFNβ plus RBV group (group A), broken lines: PEG-IFN plus RBV group (group B). P values by Mann-Whitney U test.**

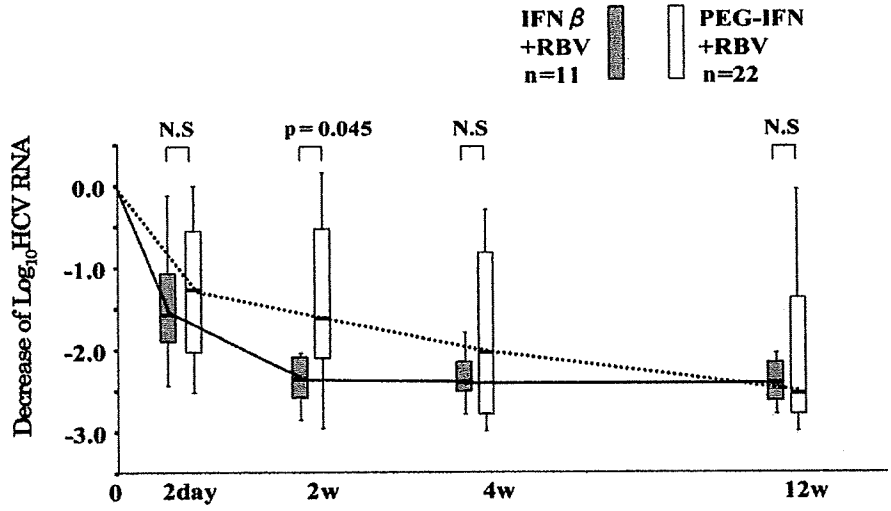


Figure 2. **Log changes in viral load from baseline during the initial 12 weeks of treatment in patients with chronic HCV infection, genotype 1b and high viral load. Gray-boxes and solid lines: nIFNβ plus RBV group (group A), light gray filled boxes and broken lines: PEG-IFN plus RBV group (group B). Bars within the boxes indicate the median value of log changes in viral load. The boxes denote the 25 to 75 percentiles, the lower and upper bars the 10 and 90 percentiles, respectively.**

Early viral kinetics

Figure 2 shows the early viral kinetics in groups A and B. Viral load was assessed at day 2, week 2, week 4 and week 12. At week 2, the median decrease in log viral load in group A was more than in group B (median, -2.34 logs vs. -1.80 logs, respectively; p = 0.045). However, the median decrease in log viral load was not significant between groups

A and B at other points.

Discussion

The frequent occurrence of psychiatric illness as a side effect of IFNα is well known; the reported incidences of IFNα- and IFNβ-induced depression range from 22 to 35% (1-3) and 10 to 21% (20-23), respectively. In general, however, IFNβ is a safe drug, but there is little or no information on its effects on patients with chronic HCV infection and depression or those with IFNα-induced depression.

In the present study, among 8 patients who developed mental disorders, only one patient stopped the therapy due to exacerbation of depression. One patient received antidepressant medication, 2 patients received anti-anxiety drugs at the start of the treatment. Moreover, during the therapy, 1 patient received anti-anxiety drugs and another one received an anti-depressant. The remaining 3 patients did not need any medications. Recently, Schaefer et al (24) reported that among 22 psychiatric patients treated with PEG-IFN plus RBV, 3 (13.6%) required antidepressants at the start of the treatment, 15 (68.2%) received antidepressants during anti-HCV therapy, and 1 (4.5%) discontinued PEG-IFN plus RBV treatment due to psychiatric disorders. The results of the above study and those of the present study indicate that the dropout rate due to psychiatric illness of patients treated with IFNβ is similar to that in patients on PEG-IFN. However, in our study, weaker anti-anxiety drugs were used during IFNβ therapy compared with the above report, suggesting that IFNβ-induced mental disorders are milder than those induced by PEG-IFN. However, the sample size of the present study is too small to make a firm conclusion and further studies are needed to specifically compare these two agents.

In the present study, the platelet count during administration of IFNβ plus RBV increased above baseline after week 4. A previous study reported that platelet count did not increase above the baseline during administration of nIFNβ monotherapy (21). In our study, at week 4, 64% (7/11) of patients were switched to 3 times a week administration. The increased platelet count after week 4 might be due to this switching and thus related to the study protocol. Interestingly, another previous study reported that platelet count increased after the time of daily administration of IFNα, but not above the baseline value (25). Such an increase of the platelet count above baseline after week 4 may be specific to only nIFNβ plus RBV combination therapy. Although the exact mechanism is not clear at this stage, nIFNβ plus RBV therapy may be useful for patients with thrombocytopenia who are not suitable for PEG-IFN plus RBV therapy. Further studies are required to clarify the mechanism of increased platelet count during nIFNβ plus RBV therapy.

The reported SVR rate of patients with genotype 1b and high viral load treated with nIFNβ monotherapy ranges from 0 to 11% (11, 12). Recent trials of rhIFNβ-1a plus RBV reported improved SVR rate. For example, the SVR reported by Chan et al (14) was 32.7% (18/55) in their patients with genotype 1b and high viral load treated with 44 μg rhIFNβ-1a (equivalent to 12 MU) 3 times weekly plus RBV for 24 weeks. Furthermore, the SVR reported by Pellicano et al (13) was 12.1% (4/33) in their patients with genotype 1 (viral titer was not mentioned) treated with 22 μg rhIFNβ-1a daily plus RBV for 24 weeks. In the present study, the SVR rate in our patients with genotype 1b and high viral load treated with 6 MU nIFNβ plus RBV for 48 weeks was 27% (3/11). This result ranked well with the above previous reports. We performed case-control study in patients treated with PEG-IFN plus RBV and the SVR rate was 41% (9/22). Although the SVR rate of the case group was lower than that of the control group, the difference was not statistically significant, suggesting that nIFNβ plus RBV combination therapy for 48 weeks is also efficacious.

The viral load of the case group decreased rapidly at week 2; the log drop was greater than that of the control group. However, after week 2, there were no differences in the rate of drop of viral load between the two groups at weeks 4 and 12. In the case group, nIFNβ was administrated daily for 2 weeks in 6 patients, for 4 weeks in 1 patient and for 8 weeks in 4 patients. These viral kinetics suggest that daily administration of nIFNβ plus RBV is more effective against HCV than PEG-IFN plus RBV, and that three times a week nIFNβ administration regimen might be less effective than PEG-IFN plus RBV. The fact that none of the patients dropped out in our study even during the 8-week daily administration of nIFNβ, suggests good tolerability of the combination therapy. Prolongation of daily administration might improve treatment outcome of nIFNβ plus RBV. However, our results, based on a small sample size, need to be confirmed in another large-scale study, including determination of the most appropriate duration of daily administration.

In conclusion, nIFNβ plus RBV therapy carries sufficient tolerability and efficacy in patients with mental disorders. nIFNβ plus RBV could be considered an efficacious and safe second-line therapy for subpopulations of patients who are not eligible for PEG-IFN plus RBV. Further studies in a larger group of will be necessary.

Acknowledgement

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

References

1. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med **347**: 975-982, 2002.
2. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon

alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. Lancet **358**: 958-965, 2001.
3. Hadziyannis SJ, Sette H Jr., Morgan TR, et al. Peginterferon-alpha 2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med **140**: 346-355, 2004.
4. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med **345**: 41-52, 2001.
5. Platanias LC, Uddin S, Colamonici OR. Tyrosine phosphorylation of the alpha and beta subunits of the type I interferon receptor. Interferon-beta selectively induces tyrosine phosphorylation of an alpha subunit-associated protein. J Biol Chem **269**: 17761-17764, 1994.
6. Pellegrini S, John J, Shearer M, Kerr IM, Stark GR. Use of a selectable marker regulated by alpha interferon to obtain mutations in the signaling pathway. Mol Cell Biol **9**: 4605-4612, 1989.
7. Colamonici OR, Pfeffer LM. Structure of the human interferon alpha receptor. Pharmacol Ther **52**: 227-233, 1991.
8. Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A **95**: 15623-15628, 1998.
9. Alam JJ. Interferon-beta treatment of human disease. Curr Opin Biotechnol **6**: 688-691, 1995.
10. Festi D, Sandri L, Mazzella G, et al. Safety of interferon beta treatment for chronic HCV hepatitis. World J Gastroenterol **10**: 12-16, 2004.
11. Kainuma M, Ogata N, Kogure T, et al. The efficacy of a herbal medicine (Mao-to) in combination with intravenous natural interferon-beta for patients with chronic hepatitis C, genotype 1b and high viral load: a pilot study. Phytomedicine **9**: 365-372, 2002.
12. Kurosaki M, Enomoto N, Murakami T, et al. Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. Hepatology **25**: 750-753, 1997.
13. Pellicano R, Craxi A, Almasio PL, et al. Interferon beta-1a alone or in combination with ribavirin: a randomized trial to compare efficacy and safety in chronic hepatitis C. World J Gastroenterol **11**: 4484-4489, 2005.
14. Chan HL, Ren H, Chow WC, Wee T. Randomized trial of interferon beta-1a with or without ribavirin in Asian patients with chronic hepatitis C. Hepatology **46**: 315-323, 2007.
15. Kakumu S, Yoshioka K, Wakita T, Ishikawa T, Takayanagi M, Higashi Y. A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology **105**: 507-512, 1993.
16. Enomoto M, Tamori A, Kawada N, et al. Interferon-beta plus ribavirin for patients with hepatitis C virus genotype 1: a randomized pilot trial. Gut **55**: 139-140, 2006.
17. Neumann AU, Lam NP, Dahari H, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science **282**: 103-107, 1998.
18. Buti M, Sanchez-Avila F, Lurie Y, et al. Viral kinetics in genotype 1 chronic hepatitis C patients during therapy with 2 different doses of peginterferon alfa-2b plus ribavirin. Hepatology **35**: 930-936, 2002.
19. Herrmann E, Lee JH, Marinos G, et al. Effect of ribavirin on hepatitis C viral kinetics in patients treated with pegylated interferon. Hepatology **37**: 1351-1358, 2003.
20. Frosi A, Sgorbati C, Bosisio Besteti M, Lodeville D, Vezzoli S, Vezzoli F. Interferon-α and -β in chronic hepatitis C: efficacy and tolerability. Clin Drug Invest **9**: 226-231, 1995.
21. Bernardinello E, Cavalletto L, Chemello L, et al. Long-term clinical outcome after β-interferon therapy in cirrhotic patients with chronic hepatitis C. Hepatogastroenterology **46**: 3216-3222, 1999.
22. Pellicano R, Palmas F, Cariti G, et al. Re-treatment with interferon-beta of patients with chronic hepatitis C virus infection. Eur J Gastroenterol Hepatol **14**: 1377-1382, 2002.
23. Habersetzer F, Boyer N, Marcellin P, et al. A pilot study of recombinant interferon beta-1a for the treatment of chronic hepatitis C. Liver **20**: 437-441, 2000.
24. Schaefer M, Hinzpeter A, Mohmand A, et al. Hepatitis C treatment in "difficult-to-treat" psychiatric patients with pegylated interferon-alpha and ribavirin: response and psychiatric side effects. Hepatology **46**: 991-998, 2007.
25. Fried MW. Side effects of therapy of hepatitis C and their management. Hepatology **36**: S237-S244, 2002.

Journal of Medical Virology 81:27–33 (2009)

Correlation Between Serum Hepatitis B Virus Core-Related Antigen and Intrahepatic Covalently Closed Circular DNA in Chronic Hepatitis B Patients

Fumitaka Suzuki,[1]* Hideo Miyakoshi,[2] Mariko Kobayashi,[3] and Hiromitsu Kumada[1]

[1]*Department of Hepatology, Toranomon Hospital, Tokyo, Japan*
[2]*Research and Development Division, Fujirebio, Inc., Tokyo, Japan*
[3]*Department of Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki, Kanagawa, Japan*

Nucleos(t)ide analogues are utilized for the treatment of chronic HBV infection, and HBe seroconversion and HBV DNA levels are commonly used as markers of viral status and as primary treatment endpoints. Recently, a new assay was prepared for the detection of serum HBV core-related antigen (HBcrAg), consisting of HBcAg, HBeAg, and p22cr, which is a precore protein from amino acid −28 to at least amino acid 150, by coding the precore/core region. In this study, we examined the correlation between serum HBcrAg concentration and viral status by the analysis of serum HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic covalently closed circular DNA (cccDNA) in 57 chronic hepatitis B patients. Intrahepatic cccDNA was detected in all 57 patients, 42 patients were HBcrAg-positive, and serum HBcrAg concentration level was closely correlated with cccDNA. Additionally, positive HBcrAg concentration level results were observed in 6 out of 13 HBsAg seroclearance patients and 20 out of 31 HBV DNA-negative patients. Moreover, the correlation between HBcrAg and cccDNA in these 31 HBV DNA-negative patients was statistically significant ($r = 0.482$, $P = 0.006$). These data suggest that serum HBcrAg concentration is well correlated with intrahepatic cccDNA level, and that the measurement of serum HBcrAg may be clinically useful for monitoring intrahepatic HBV viral status, especially in patients under treatment with nucleos(t)ide analogues. ***J. Med. Virol. 81:27–33, 2009.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HBV DNA; HBcrAg; cccDNA; HBsAg; lamivudine; entecavir

INTRODUCTION

Hepatitis B virus (HBV) is an important causative agent for liver disease such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Recently, several nucleos(t)ide analogues such as lamivudine [Dienstag et al., 1995], adefovir dipivoxil [Chin et al., 2001], and entecavir [Colonno et al., 2001] have been found to consistently produce rapid and dramatic decreases in viremia [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. For the serological monitoring of chronic hepatitis patients under treatment with nucleos(t)ide analogues, improvement of alanine transaminase level, seroconversion from HBe antigen (HBeAg)-positive to anti-HBe antibody (HBeAb)-positive, and peripheral HBV DNA concentration are used as markers in chronic active hepatitis, and both HBeAg seroconversion and HBV DNA levels below the detection limit and/or of 10^5 copies/ml are commonly used as primary treatment endpoints [Lok et al., 2004]. In addition, HBV surface antigen (HBsAg) seroclearance has been linked to a good prognosis, including improvement of liver histopathology and liver function, and prolongation of survival [Arase et al., 2006], although spontaneous HBsAg seroclearance and/or remission occurred in only a small proportion of patients during the natural history of chronic HBV infections.

However, a major problem with long-term lamivudine treatment is the potential development of drug-resistance, mainly caused by the mutation of the YMDD motif of reverse transcriptase [Chayama et al., 1998]. We previously reported the efficacy of lamivudine therapy and factors associated with the emergence of resistance in chronic HBV infection in Japan [Suzuki et al., 2003].

Grant sponsor: Ministry of Health, Labor and Welfare, Japan.

*Correspondence to: Fumitaka Suzuki, MD, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan. E-mail: fumitakas@toranomon.gr.jp

Accepted 19 August 2008
DOI 10.1002/jmv.21339
Published online in Wiley InterScience (www.interscience.wiley.com)

The measurement of the predictive serum markers of residual intrahepatic HBV DNA and/or covalently closed circular DNA (cccDNA), which is intrahepatic HBV replicated intermediate, is more important than the measurement of peripheral HBV DNA for monitoring the viral status of hepatitis patients [Sung et al., 2005]. Additionally, the amount of cccDNA in serum is reported to be higher in patients who develop YMDD mutants than in patients who do not [Yuen et al., 2005]. Recently, it was established that HBV RNA is detectable in serum and the elevation of HBV RNA is a predictor of early occurrence of viral mutation during lamivudine therapy [Rokuhara et al., 2006; Hatakeyama et al., 2007]. However, these HBV DNA, HBV RNA, or cccDNA detection assay methods remain complicated and difficult to perform. Therefore, simple methods of viral status evaluation are required for routine assays rather than for nucleic acid assays.

Recently, a new assay was performed for the detection of hepatitis B core-related antigen (HBcrAg) consisting of HBV core antigen (HBcAg), HBeAg, and 22 kDa precore protein (p22cr) coded with precore/core gene [Kimura et al., 2002, 2005]. p22cr is a precore protein from amino acid −28 to at least amino acid 150, containing an uncleaved signal sequence and lacking the C-terminal arginine-rich domain. p22cr is found in empty and HBV DNA negative virus particles; the production of empty particles is not dependent on the formation of HBV DNA [Kimura et al., 2005]. Several reports indicate that the concentration of serum HBcrAg is closely correlated with peripheral HBV DNA in untreated patients [Rokuhara et al., 2003; Tanaka et al., 2006]. Additionally, HBcrAg is considered as a prospective marker of the appearance of drug-resistant HBV mutants and of the identification of patients with low risk of HBV reactivation after discontinuation of lamivudine administration, while peripheral HBV DNA does not qualify as a prospective marker in these patients [Rokuhara et al., 2005; Shinkai et al., 2006; Tanaka et al., 2006; Matsumoto et al., 2007]. The relationship between HBcrAg and intrahepatic cccDNA levels has not yet been clarified.

In this study, we examined the correlation between HBcrAg and viral status by the analysis of HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic cccDNA in patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients and Samples

Serum samples and biopsy specimens were obtained from 57 chronic hepatitis B patients at Toranomon Hospital under informed consent. The median age of patients was 49 (range, 25–71 years). Out of 57 patients, 28 underwent nucleos(t)ide analogue administration (17 patients of lamivudine, 7 patients of both lamivudine and adefovir dipivoxil, 4 patients of entecavir), and 13 were HBsAg-negative/HBs-seroclearance patients with more than 12 months of being HBsAg-positive before HBs-seroclearance.

Routine Laboratory Tests

HBsAg, HBeAg, and HBeAb were routinely measured by the commercially available Chemiluminescent Enzyme Immunoassay (CLEIA) (Lumipulse System, Fujirebio, Inc., Tokyo, Japan).

HBcrAg Test

Serum HBcrAg was measured by CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, Fujirebio, Inc.). Briefly, 150 μl of serum was incubated with 150 μl of pretreatment solution containing 15% sodium dodecyl sulfate at 60°C for 30 min. After heat treatment, 120 μl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61, and HB114) against denatured HBcAg, HBeAg, and p22cr. After 10 min incubation at 37°C and washing, further incubation was done for 10 min at 37°C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg, and p22cr. After washing, 200 μl of substrate solution [AMPPD: 3-(2′-spiroadamantan)-4-methoxy-4- (3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) was added to the test cartridge which was then incubated for 5 min at 37°C. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg (amino acids: −10 to 183 of precore/core gene product). HBcrAg concentration was expressed as units/ml (U/ml), which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, HBcrAg value was expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analysis, HBcrAg-negative cases were calculated as 3.0 log U/ml.

HBV DNA Assay

HBV DNA in serum was measured by polymerase chain reaction (PCR) assay kit (Amplicor HBV monitor test, Roche Molecular Systems, Inc., Branchburg, NJ). Values under or over the detection range were calculated as 2.6 log copies/ml or as 7.6 log copies/ml, respectively.

Measurement of cccDNA

Liver biopsy specimens were taken and stored at −80°C before DNA extraction. HBV DNA was extracted using QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on absorbance at 260 nm. For this study, two oligonucleotide primers, cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1,424–1,444), cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1,755–1,737), and a probe cccP2 (5′-VIC-accaatttatgcctacag-MGB-3′, nucleotides 1,672–1,655), were designed using Primer Express™ software (Applied Biosystems, Foster City, CA) to flank the direct

repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4 oligonucleotide primers spanning the direct repeat region of the HBV genome allows the PCR of native viral DNA in the Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region [Mason et al., 1998]. Twenty-five microliters of the extracted DNA (0.5 μg) were detected with the sequence detector system (ABI 7900HT, Applied Biosystems) in 50 μl of a PCR mixture containing TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer, and 250 nmol of the probe. After initial activation of uracil-*N*-glycosylase at 50°C for 2 min, AmpliTaq Gold was activated at 95°C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 90 sec per cycle (SRL, Inc., Tokyo, Japan).

Statistical Analysis

The statistical analysis of the correlation data between serum HBcrAg, HBsAg, HBV DNA, and/or cccDNA was performed by SPSS software (version 14.0J, SPSS Japan Inc., Tokyo, Japan), and the statistical significance between the two sides was taken as *P*-value lower than 0.05.

RESULTS

Serological and Genetic Assay Results

We classified the 57 patients according to assay results of HBsAg, HBeAg, serum HBV DNA, intrahepatic HBV cccDNA, and serum HBcrAg. Positive results were observed with all 57 patients in the cccDNA assay, in 44 patients with the HBsAg test, in 16 patients with the HBeAg test, in 26 patients with the HBV DNA assay, and in 42 patients with the HBcrAg assay (Table I). Among the 13 patients with negative results with HBsAg, HBeAg, and HBV DNA but positive results with cccDNA assay, six patients showed HBcrAg-positive results, although the serum HBcrAg concentration value was low (mean value ± standard deviation: 3.23 ± 0.27 log U/ml) in comparison to that of the group with positive results with HBsAg, HBeAg, and HBV DNA (6.91 ± 1.06 log U/ml) tests. Among the 28 patients with HBsAg-positive but HBeAg-negative results, 20 patients were HBcrAg-positive and 10 out of these 20 patients showed negative HBV DNA assay results.

Next, assay results were analyzed according to presence/absence of nucleos(t)ide analogue treatment, HBsAg-positive/negative results, and HBeAg-positive/negative results by the combination with treated/untreated subgroups (Table II). When patients were classified into two groups, namely 28 patients treated with nucleos(t)ide analogues and 29 untreated patients, no difference was observed in average mean value of HBcrAg, HBV cccDNA, and HBV DNA (data not shown). However, since the 13 HBsAg-negative patients were clinically stable, we further analyzed the 44 HBsAg-positive patients by grouping them according to pres-

TABLE I. Summary of HBcrAg Concentration in Positive and/or Negative Patients by HBsAg, HBeAg, HBV DNA, and HBcrAg Assay

All cases		Classified by assay results with:							
		HBsAg		HBeAg		HBV DNA		HBcrAg	
Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)
All (N = 57)	4.61 (1.64)	Positive (N = 44)	5.05 (1.62)	Positive (N = 16)	6.53 (1.14)	Positive (N = 12)	6.91 (1.06)	Positive (N = 12)	6.91 (1.06)
								Negative (N = 0)	
						Negative (N = 4)	5.40 (0.38)	Positive (N = 4)	5.40 (0.38)
								Negative (N = 0)	
				Negative (N = 28)	4.20 (1.18)	Positive (N = 14)	4.29 (1.20)	Positive (N = 10)	4.81 (1.02)
								Negative (N = 4)	<3.00
						Negative (N = 14)	4.11 (1.20)	Positive (N = 10)	4.55 (1.14)
								Negative (N = 4)	<3.00
		Negative (N = 13)	3.11 (0.21)	Positive (N = 0)		Positive Negative (N = 0)		Positive Negative (N = 0)	
				Negative (N = 13)	3.11 (0.21)	Positive (N = 0)		Positive Negative (N = 0)	
						Negative (N = 13)	3.11 (0.21)	Positive (N = 6)	3.23 (0.27)
								Negative (N = 7)	<3.00

Mean value: log U/ml ± Standard deviation.

TABLE II. Classification of HBcrAg, cccDNA and HBV DNA Assay Results According to Presence/Absence of Treatment, HBeAg Test and HBsAg Test

			HBcrAg (log U/ml)		cccDNA (log copy/mg)		HBV DNA (log copy/ml)	
Item	Category	N	Mean (SD)	*P* value	Mean (SD)	*P* value	Mean (SD)	*P* value
Unclassified	All	57	4.61 (1.64)		4.25 (0.91)		3.67 (1.59)	
Treatment[a]	With	26	4.77 (1.49)	NS[c]	4.41 (0.68)	NS[c]	3.20 (1.08)	<0.001
	Without	18	5.45 (1.75)		4.54 (1.09)		5.13 (1.78)	
HBsAg	Positive							
	Total	44	5.05 (1.62)		4.46 (0.87)		3.99 (1.69)	
	Negative			<0.001		0.001		<0.001
	Total	13	3.11 (0.21)		3.52 (0.68)		2.60 (0.00)	
HBeAg[b]	Positive							
	Total	16	6.53 (1.14)		4.88 (1.06)		5.17 (1.92)	
	Negative			0.001		0.015		0.002
	Total	28	4.20 (1.18)		4.23 (0.64)		3.32 (1.09)	
	Positive							
	Treated	8	6.21 (1.09)	NS[c]	4.76 (0.95)	NS[c]	3.83 (1.64)	0.005
	Untreated	8	6.85 (1.18)		5.00 (1.21)		6.51 (1.02)	
	Negative							
	Treated	18	4.13 (1.16)	NS[c]	4.25 (0.49)	NS[c]	2.93 (0.57)	0.016
	Untreated	10	4.33 (1.26)		4.18 (0.88)		4.02 (1.45)	

[a]In the with/without treatment group, 44 HBsAg-positive patients were analyzed.
[b]HBsAg-positive 44 patients were further separated into HBeAg-positive and HBeAg–negative groups. In addition, these groups were further separated into treated and untreated groups; the mean value of each assay was calculated and a statistical analysis was done.
[c]NS: statistically not significant.

ence/absence of treatment of nucleos(t)ide analogues (Table II). HBcrAg concentration was 4.77 ± 1.49 log U/ml in 26 treated patients and 5.45 ± 1.75 log U/ml in 18 untreated patients (not statistically significant). Similar results in cccDNA levels were observed in both treated and untreated groups (4.41 ± 0.68 log copy/μg and 4.54 ± 1.09 log copy/μg, not statistically significant). In contrast, lower HBV DNA was observed in the treated group (3.20 ± 1.08 log copy/ml) as compared with the untreated group (5.13 ± 1.78 log copy/ml, $P < 0.001$).

Statistically significant results were observed under grouping according to HBsAg assay results; namely, HBcrAg was 5.05 ± 1.62 log U/ml in the HBsAg-positive group and 3.11 ± 0.21 log U/ml in the HBsAg-negative group ($P < 0.001$), HBV cccDNA was 4.46 ± 0.87 log copies/μg in the HBsAg-positive group and 3.52 ± 0.68 log copies/μg in the HBsAg-negative group ($P < 0.001$), HBV DNA was 3.99 ± 1.69 log copies/ml in the HBsAg-positive group and <2.60 log copies/ml in the HBsAg-negative group ($P < 0.001$).

Similar results were observed under grouping according to HBeAg results. In this analysis, HBsAg-negative patients were omitted because HBeAg-negative patients included both HBsAg-positive/negative patients, whereas all HBeAg-positive patients were HBsAg-positive. The mean values of HBcrAg, HBV cccDNA, and HBV DNA in the HBeAg-positive group were higher than those of the HBeAg-negative group; namely, HBcrAg concentration was 6.53 ± 1.14 log U/ml in the HBeAg-positive group and 4.20 ± 1.18 log U/ml in the HBeAg-negative group ($P < 0.001$), cccDNA was 4.88 ± 1.06 log copy/μg in the HBeAg-positive group and 4.23 ± 0.64 log copies/μg in the HBeAg-negative group ($P = 0.015$), HBV DNA was 5.17 ± 1.92 log copies/ml in the HBeAg-positive group and 3.32 ± 1.09 log copies/ml in the HBeAg-negative group ($P = 0.002$). When the HBeAg-positive group was further separated into treated and untreated groups, these three markers in eight treated patients were lower than in eight untreated patients; namely, HBcrAg (6.21 ± 1.09 log U/ml vs. 6.85 ± 1.18 log U/ml), cccDNA (4.76 ± 0.95 log copy/μg vs. 5.00 ± 1.21 log copy/μg), and HBV DNA (3.83 ± 1.64 log copy/ml vs. 6.51 ± 1.02 log copy/ml), although HBcrAg and cccDNA values between the treated and untreated groups were not statistically significant. In the HBeAg-negative group, statistically significant ($P = 0.016$) lower values of HBV DNA but not of HBcrAg and cccDNA were observed in 18 treated patients by comparison with 10 untreated patients. The HBV DNA level of 8 out of 10 untreated patients was less than 5 log copies/ml. These patients underwent a liver biopsy for progress follow-up. Therefore, there was a relatively small difference in HBV DNA level between treated and untreated patients.

Correlation Between HBcrAg, HBV DNA and/or cccDNA

The correlation between HBcrAg, HBV DNA, and/or cccDNA in all 57 patients was summarized in Figure 1. A statistically significant positive correlation was observed in all analyses, namely HBcrAg versus HBV cccDNA (Fig. 1a, $r = 0.692$, $P < 0.001$), HBcrAg versus HBV DNA (Fig. 1b, $r = 0.713$, $P < 0.001$), and HBV cccDNA versus HBV DNA (Fig. 1c, $r = 0.637$, $P < 0.001$).

Next, HBcrAg concentration in 31 HBV DNA-negative patients was measured; 20 patients showed levels greater than 3.0 log U/ml. A statistically significant correlation between HBcrAg and cccDNA in these 31 patients was observed (Fig. 2, $r = 0.482$, $P = 0.006$),

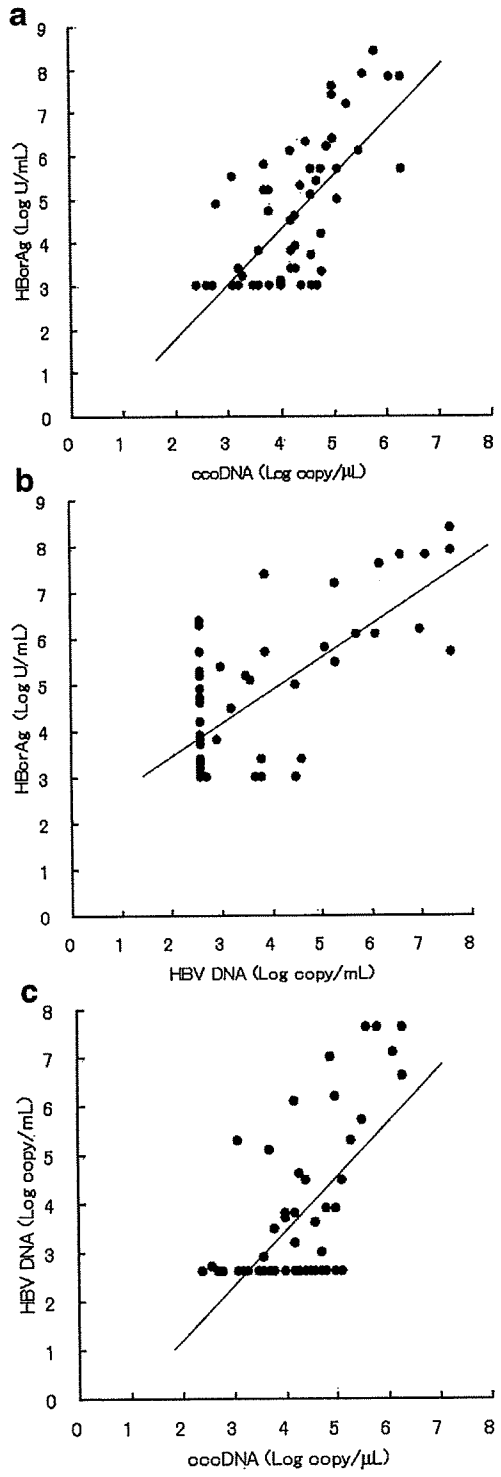


Fig. 1. Correlation between serum HBcrAg and intrahepatic HBV cccDNA in 57 patients with chronic hepatitis B (**a**: $y=1.25\times-0.69$, $r=0.692$, $P<0.001$), HBcrAg and serum HBV DNA (**b**: $y=0.74\times+1.91$, $r=0.713$, $P<0.001$), and serum HBV DNA and intrahepatic cccDNA (**c**: $y=1.11\times-1.05$, $r=0.637$, $P<0.001$). Straight lines indicate the correlation between each other.

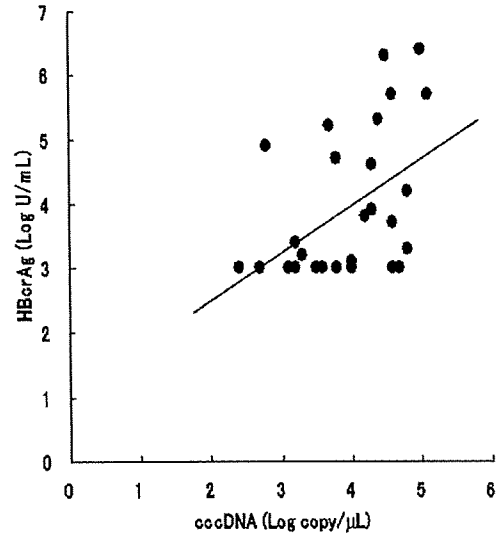


Fig. 2. Correlation between HBcrAg and cccDNA in 31 HBV DNA negative patients ($y=0.73\times+1.00$, $r=0.482$, $P=0.006$).

suggesting that HBcrAg measurement may be a useful marker in HBV DNA-negative patients as a substitute for cccDNA assay.

When 44 HBsAg positive patients were grouped according to whether they were HBeAg-positive or HBeAg-negative, HBcrAg concentration was correlated with cccDNA in both 16 HBeAg-positive patients (Fig. 3a, $r=0.687$, $P=0.003$) and 28 HBeAg-negative patients (Fig. 3a, $r=0.542$, $P=0.003$), and with HBV DNA in the HBeAg-positive (Fig. 3b, $r=0.681$, $P=0.004$) but not in the HBeAg-negative group (Fig. 3b, $r=0.311$, $P=0.107$). A positive correlation between HBV DNA and cccDNA was also observed in both the HBeAg-positive group (Fig. 3c, $r=0.588$, $P=0.017$) and the HBeAg-negative group (Fig. 3c, $r=0.442$, $P=0.018$).

DISCUSSION

Nucleos(t)ide analogues have a suppressive effect on the transcription of pregenomic RNA, and the administration of these agents can induce a rapid and dramatic decrease in peripheral HBV DNA, seroclearance of HBeAg, and remission of chronic hepatitis B [Dienstag et al., 1995, 1999; Lai et al., 1998]. However, these nucleos(t)ide analogues are unable to induce an adequate and complete elimination of HBV. Therefore, the measurement of intrahepatic HBV DNA and/or HBV cccDNA is important for monitoring the viral status of hepatitis patients [Sung et al., 2005], although these assays involve the physical stress of needle biopsy.

Several reports indicate that the level of HBcrAg, which is a complex of HBeAg, HBcAg, and p22cr coding precore/core gene [Kimura et al., 2002, 2005], reflects the natural course of viral loads in patients under treatment with nucleos(t)ide analogues, and that the reduction rate of HBcrAg is slower than that of serum

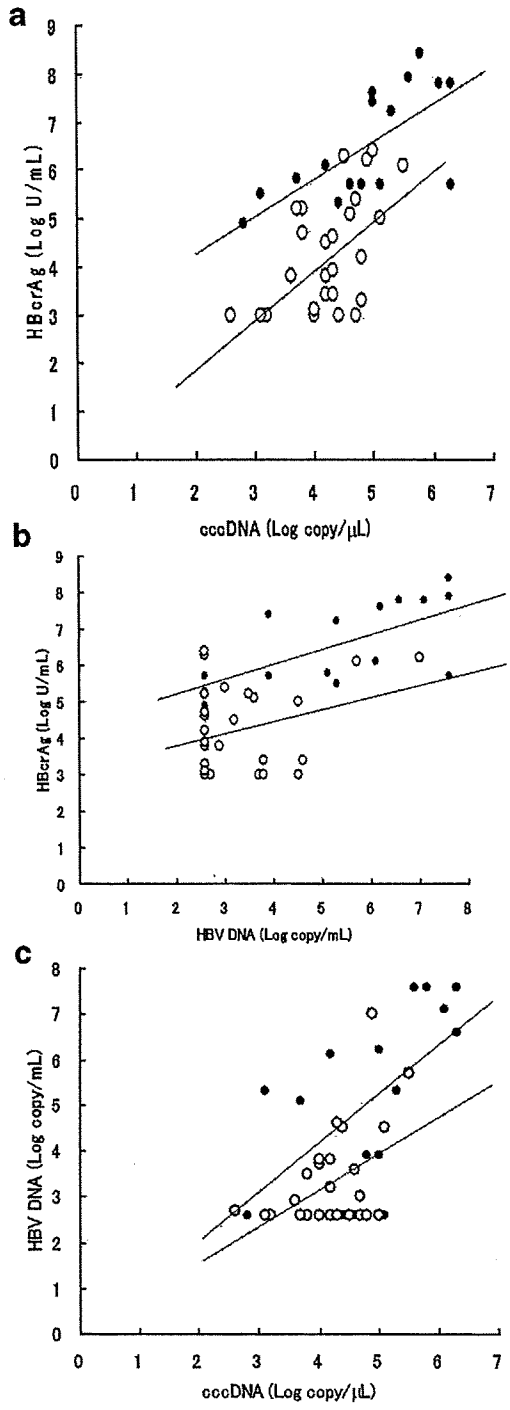


Fig. 3. Correlation between HBcrAg, cccDNA and HBV DNA in 44 HBsAg-positive with 16 HBeAg-positive and 28 HBeAg-negative patients. **a**: Correlation between HBcrAg and cccDNA (HBeAg-positive cases as closed circle; $y = 0.74 \times +2.91$, $r = 0.687$, $P = 0.003$, HBeAg-negative cases as open circle; $y = 1.00 \times -0.02$, $r = 0.542$, $P = 0.003$). **b**: Correlation between HBcrAg and serum HBV DNA (HBeAg-positive patients as closed circle; $y = 0.41 \times +4.43$, $r = 0.681$, $P = 0.004$, HBeAg-negative patients as open circle; $y = 0.34 \times +3.09$, $r = 0.311$, $P = 0.107$). **c**: Correlation between serum HBV DNA and intrahepatic cccDNA (HBeAg-positive cases as closed circle; $y = 1.07 \times -0.03$, $r = 0.588$, $P = 0.017$, HBeAg-negative patients as open circle; $y = 0.76 \times +0.12$, $r = 0.442$, $P = 0.018$).

HBV DNA [Rokuhara et al., 2003; Tanaka et al., 2006]. Similar results were observed in patients infected with genotypes B and C of HBV [Rokuhara et al., 2005]. This phenomenon may be explained by the fact that the production of HBcrAg depends on the transcription of mRNA from cccDNA, and that cccDNA still remains in high levels after treatment with these nucleos(t)ide analogues. Therefore, several reports suggest that HBcrAg may be a predicting marker for relapse after cessation of lamivudine therapy in chronic HBV infection [Shinkai et al., 2006; Matsumoto et al., 2007] and that it may also help identify patients who are at low risk of lamivudine resistance [Tanaka et al., 2006].

In this study, we analyzed the correlation between HBcrAg and several HBV markers, especially HBV cccDNA. Results indicated a good correlation of HBcrAg against serum HBV DNA and intrahepatic HBV cccDNA (Fig. 1). In addition, 20 out of 31 HBV DNA-negative patients showed more than 3.0 log U/ml in HBcrAg. All of these 20 patients were also cccDNA-positive, and there was a positive correlation between HBcrAg and cccDNA levels, although HBcrAg was negative in 11 patients (Fig. 2). The production of HBcrAg is considered to depend on the transcription of mRNA from intrahepatic cccDNA. Our data showed that serum HBcrAg may reflect intrahepatic cccDNA. Therefore, measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring chronic hepatitis B patients. Recently, the acquisition of de novo HBV-related hepatitis after liver transplantation has become an important cause of morbidity and mortality. Moreover, de novo HBV-related hepatitis has been reported in patients after hematopoietic stem cell transplantation and cytotoxic chemotherapy treatment [Dhedin et al., 1998; Hui et al., 2006]. Therefore, HBcrAg may be a useful marker of occult HBV infection in these patients.

Several reports indicate that HBsAg seroclearance confers favorable long-term outcomes in patients without hepatocellular carcinoma or decompensated liver cirrhosis [Arase et al., 2006; Kobayashi et al., 2006]. However, studies show that intrahepatic HBV DNA still remains in HBsAg seroclearance cases [Arase et al., 2006], and that 10–20% of patients have 50–100 copies/ml of serum HBV DNA for 5 and 10 years after seroclearance of HBsAg [Arase et al., 2007]. In this study, 6 out of 13 patients with HBsAg seroclearance showed HBcrAg-positive results (3.23 ± 0.27 log U/ml), and all 13 patients remained cccDNA-positive (3.52 ± 0.68 log copy/μg). These data suggest that HBV remains present for a prolonged period after HBsAg seroclearance, further studies are thus necessary to clarify the mechanism of HBcrAg production and/or the regulation of mRNA in chronic hepatitis with HBsAg seroclearance.

Meanwhile, positive correlations between HBV DNA and HBcrAg were not observed in the HBsAg-positive and HBeAg-negative group (Fig. 3b), although HBcrAg concentration was correlated with cccDNA in HBeAg-negative patients (Fig. 3a). This finding shows that

measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring patients in HBsAg-positive and HBeAg-negative groups.

In conclusion, serum HBcrAg concentration appears to be well correlated with intrahepatic cccDNA level, and the measurement of serum HBcrAg as substitute for cccDNA and/or serum HBV DNA may be clinically useful for the monitoring of intrahepatic HBV viral status.

REFERENCES

Arase Y, Ikeda K, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Hosaka T, Sezaki H, Kobayashi M, Kumada H. 2006. Long-term outcome after hepatitis B surface antigen seroclearance in patients with chronic hepatitis B. Am J Med 119:9–16.

Arase Y, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Hosaka T, Sezaki H, Sato J, Kobayashi M, Ikeda K, Kumada H. 2007. Long-term presence of HBV in the sera of chronic hepatitis B patients with HBsAg seroclearance. Intervirology 50:161–165.

Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Murashima N, Ikeda K, Kumada H. 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 27:1711–1716.

Chin R, Shaw T, Torresi J, Sozzi V, Trautwein C, Bock T, Manns M, Isom H, Furman P, Locarnini S. 2001. In vitro susceptibilities of wild-type or drug-resistant hepatitis B virus to (-)-β-D-2,6-diaminopurine dioxolane and 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil. Antimicrob Agents Chemother 45:2495–2501.

Colonno RJ, Genovesi EV, Medina I, Lamb L, Durham SK, Haung ML, Corey L, Littlejohn M, Locarnini S, Tennant BC, Rose B, Clark JM. 2001. Long-term entecavir treatment results in sustained antiviral efficacy and prolonged life span in the woodchuck model of chronic hepatitis infection. J Infect Dis 184:1236–1245.

Dhedin N, Douvin C, Kuentz M, Saint Marc MF, Reman O, Rieux C, Bernaudin F, Norol F, Cordonnier C, Bobin D, Metreau JM, Vernant JP. 1998. Reverse seroconversion of hepatitis B after allogeneic bone marrow transplantation: A retrospective study of 37 patients with pretransplant anti-HBs and anti-HBc. Transplantation 66:616–619.

Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. 1995. A preliminary trial of lamivudine for chronic hepatitis B infection. N Eng J Med 333:1657–1661.

Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HWL, Goodman Z, Crowther L, Condrey LD, Woessner M, Rubin M, Brown NA, The U.S. Lamivudine Investigator Group. 1999. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Eng J Med 341:1256–1263.

Hatakeyama T, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, Takahashi S, Kawakami Y, Fujimoto Y, Ochi H, Abe H, Maekawa T, Kawakami H, Yatsuji H, Aisaka Y, Kohno H, Aimitsu S, Chayama K. 2007. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. Hepatology 45:1179–1186.

Hui CK, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, Leung N, Luk JM, Lie AK, Kwong YL, Liang R, Lau GK. 2006. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. Gastroenterology 131:59–68.

Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. 2002. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 40:439–445.

Kimura T, Ohno N, Terada N, Rokuhara A, Matsumoto A, Yagi S, Tanaka E, Kiyosawa K, Ohno S, Maki N. 2005. Hepatitis B virus DNA-negative Dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J Biol Chem 280:21713–21719.

Kobayashi M, Suzuki F, Akuta N, Suzuki Y, Arase Y, Ikeda K, Hosaka T, Sezaki H, Kobayashi M, Iwasaki S, Sato J, Watabiki S, Miyakawa Y, Kumada H. 2006. Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotypes A, B, and C. J Med Virol 78:1276–1283.

Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF, The Asia Hepatitis Lamivudine Study Group. 1998. A one-year trial of lamivudine for chronic hepatitis B. N Eng J Med 339:61–68.

Lok ASF, McMahon BJ. 2004. Chronic hepatitis B: Update of recommendations. Hepatology 39:857–861.

Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. 1998. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 27:1736–1742.

Matsumoto A, Tanaka E, Minami M, Okanoue T, Yatsuhashi H, Nagaoka S, Suzuki F, Kobayashi M, Chayama K, Imamura M, Yotsuyanagi H, Nakaoka S, Maki N, Kawata S, Kumada H, Iino S, Kiyosawa K. 2007. Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy. Hepatol Res 37:661–666.

Rokuhara A, Tanaka E, Matsumoto A, Kimura T, Yamaura T, Orii K, Sun X, Yagi S, Maki N, Kiyosawa K. 2003. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J Viral Hepat 10:324–330.

Rokuhara A, Sun X, Tanaka E, Mimura T, Matsumoto A, Yao D, Yin L, Wang N, Maki N, Kiyosawa K. 2005. Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection. J Gastroenterol Hepatol 20:1726–1730.

Rokuhara A, Matsumoto A, Tanaka Y, Umemura T, Yoshizawa K, Kimura T, Maki N, Kiyosawas K. 2006. Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. J Gastroenterol 41:785–790.

Shinkai N, Tanaka Y, Orito E, Ito K, Ohno T, Hirashima N, Hasegawa I, Sugauchi F, Ueda R, Mizokami M. 2006. Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. Hepatol Res 36:272–276.

Sung JJY, Wong ML, Bowden S, Liew CT, Hui AY, Wong VWS, Leung NWY, Locarnini S, Chan HLY. 2005. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. Gastroenterol 128:1890–1897.

Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K. 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 30:743–748.

Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J, Takagi K, Kumada H. 2003. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 46:182–189.

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K. 2006. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance. Liver Int 26:90–96.

Yuen MF, Wong DK, Sum SS, Yuan HJ, Yuen JC, Chan AO, Wong BC, Lai CL. 2005. Effect of lamivudine therapy on the serum covalently closed circular (ccc) DNA of chronic hepatitis B infection. Am J Gastroenterol 100:1099–1103.