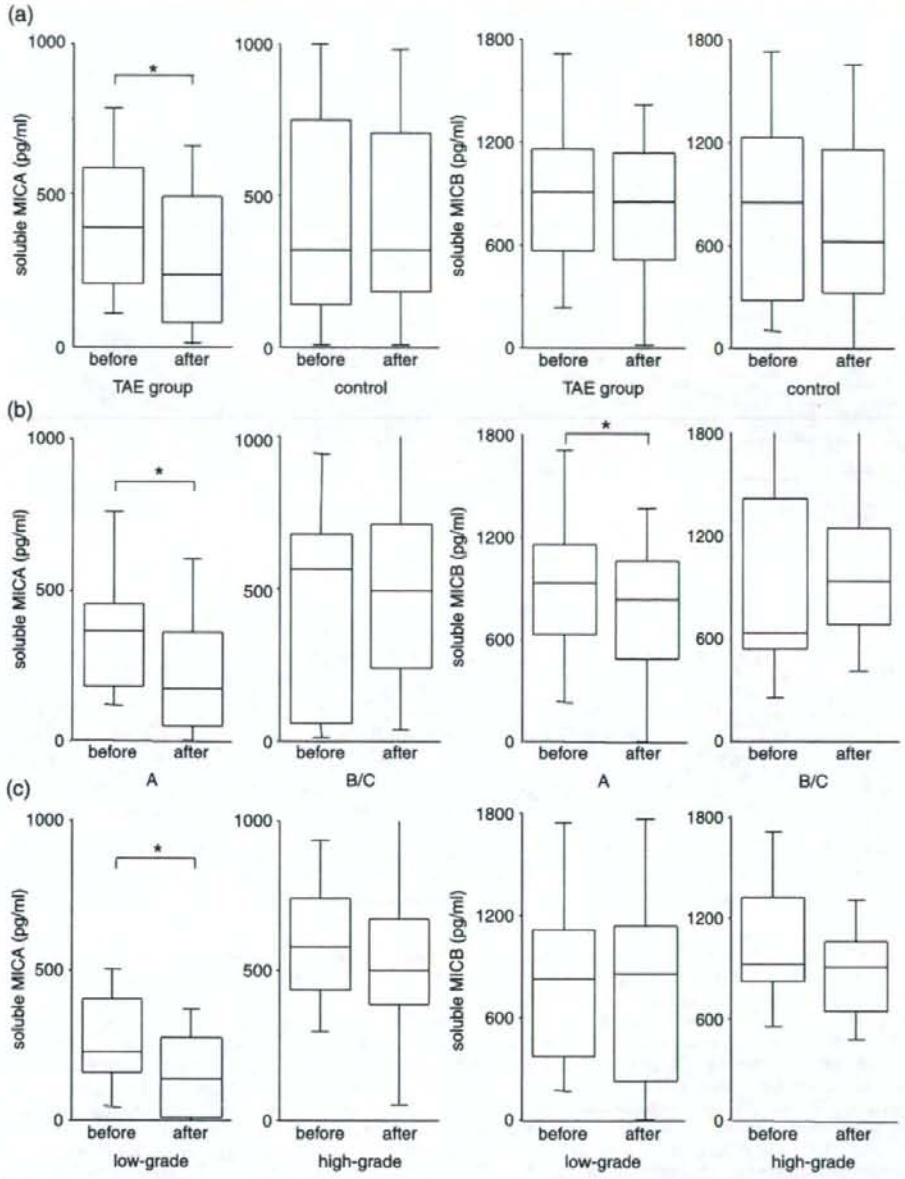

Fig. 3. Soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) during transcatheter arterial embolization (TAE) therapy. (a) Soluble MICA and soluble MICB were measured for 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as controls, with soluble MICA/B being measured twice with a 2-week interval. (b) TAE-treated patients were divide into two groups: Child–Pugh A ($n = 29$) and Child–Pugh B and C ($n = 9$). (c) TAE-treated patients were divided into two groups: low-grade hepatocellular carcinoma (HCC) ($n = 24$) and high-grade HCC ($n = 14$). $^*P < 0.05$ by paired t-test.

In the TAE-treated group, the levels of soluble MICA were decreased significantly 2 weeks after TAE therapy compared with those before TAE (Fig. 3a). In contrast, TAE did not affect the levels of soluble MICB. Neither the levels of soluble MICA nor those of soluble MICB changed during the 2-week interval in HCC patients not receiving TAE therapy. As the progression of liver disease and that of the tumor affects the levels of soluble MICA/B, TAE-treated patients were divided according to their Child–Pugh stage or tumor stage. The levels of soluble MICA decreased significantly after TAE therapy in Child–Pugh A patients but not in Child–Pugh B and C patients (Fig. 3b). Interestingly, Child–Pugh A patients showed a significant decrease even in soluble MICB levels after TAE therapy but Child–Pugh B and C patients did not. As for tumor stage, a significant decrease in

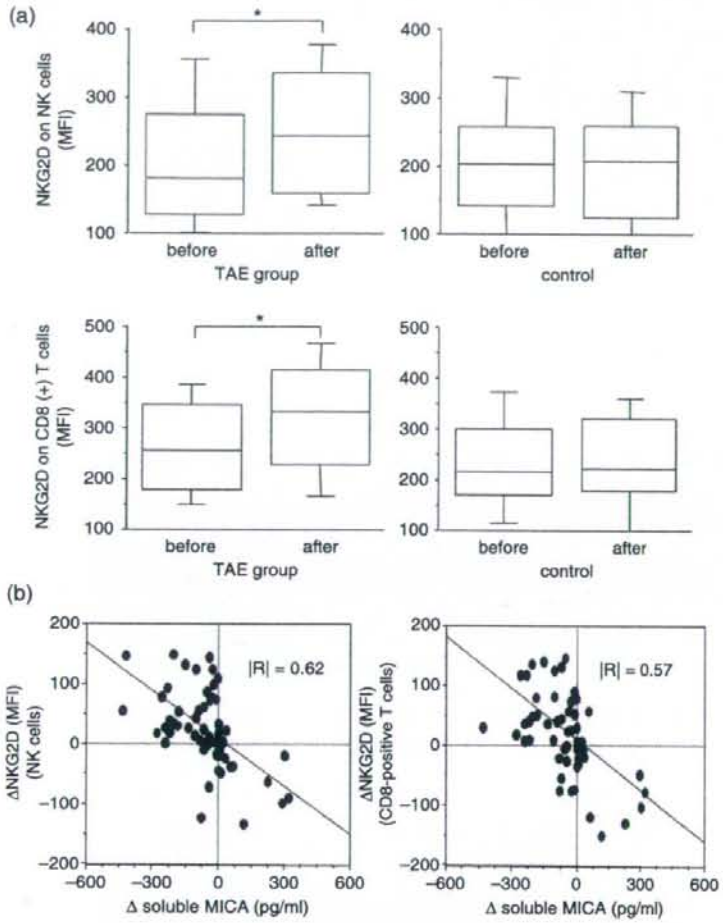

Fig. 4. Natural killer group 2, member D (NKG2D) expression during transcatheter arterial embolization (TAE) therapy. (a) NKG2D expression on natural killer (NK) cells and CD8-positive T cells. NKG2D expression on immune cells was analyzed in 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as a control by measuring NKG2D expression for 2-week interval. NKG2D expression on each cell type was evaluated by mean fluorescence intensity (MFI). $^*P < 0.05$ by paired t-test. (b) Correlation between change of soluble MICA and that of NKG2D expression on NK cells or CD8-positive T cells.

soluble MICA levels after TAE therapy was found in low-grade HCC but not in high-grade HCC (Fig. 3c). The levels of MICB did not change in the low-grade or high-grade HCC groups.

Upregulation of NKG2D expression by TAE. The number of PBMC as well as NK and T-cell subsets did not change over the 2-week interval in both the control and TAE-treated patients (data not shown). However, the levels of NKG2D expression on NK and CD8-positive T cells increased significantly upon TAE therapy, but not in the control group (Fig. 4a). To examine the involvement of soluble MICA in NKG2D expression, we analyzed the relationship of changes between soluble MICA and NKG2D expression in HCC patients. Change in soluble MICA was correlated inversely with changes in NKG2D expression on NK and CD8-positive T cells (Fig. 4b). There was no significant correlation between changes in soluble MICB and NKG2D expression (data not shown).

Discussion

In the present study, we demonstrated that soluble MICA/B increases with the progression of chronic liver disease as well as the progression of HCC. Increases in soluble MICA/B in advanced stages of tumors have been reported in some malignancies.[12] However, little is known about soluble MICA/B in the premalignant condition. Recently, Holdenrieder *et al.* examined soluble MICA/B levels in benign as well as malignant diseases from heterogeneous organs.[12,13] They found that benign diseases, such as gastrointestinal tract adenoma, pulmonary infectious disease, and gynecologic benign tumors, showed intermediate levels of soluble MICA/B between healthy controls and malignant disease. Our present findings not only agree with theirs, but also provide evidence that soluble MICA/B increases in premalignant conditions such as liver cirrhosis.

Malignant disease is known to lead frequently to the expression of MICA/B.[2] In contrast, their expression in premalignant tissues has not been fully elucidated. In the present study, MICA/B were found to be expressed in liver cirrhosis as well as HCC tissues, but not in the early stages of chronic hepatitis or in normal liver. This finding is consistent with the tendencies observed for serum-soluble MICA/B levels in chronic liver disease and HCC. Analysis of cultured cells also revealed that MICA/B expressed on hepatoma cells is released spontaneously into the culture supernatant as soluble forms, supporting the idea that MICA/B expressed in the liver may be released into the circulation. In contrast, MICA/B were not expressed on nor released from cultured non-transformed hepatocytes, which is consistent with the *in vivo* immunohistochemical finding. An issue to be resolved is the underlying mechanism by which non-transformed

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hepatocytes express and release MICA/B in pathological conditions such as liver cirrhosis. Recently, it was reported that non-transformed pulmonary epithelial cells can express MICA/B under oxidative stress-inducing conditions.[19] It was also reported that MICA/B are upregulated in non-tumor cell lines by genotoxic stress.[20] It has been speculated that oxidative and genotoxic stresses may accumulate in hepatocytes in chronic diseased liver. Thus, it is possible that those stresses may contribute to MICA/B expression in chronic diseased liver. Further study is needed to clarify this issue.

MICA/B expression in the premalignant condition raises the question of which contributes more to the production of soluble MICA/B, malignant tissues or non-malignant tissues. To address this question we analyzed the levels of soluble MICA/B in HCC patients before and after therapeutic intervention. Among treatments for HCC, TAE is a well-established technique for unresectable, advanced HCC.[16] To include HCC patients who show relatively high levels of soluble MICA/B, we chose a cohort of patients who received the TAE therapy in the present study. The data indicated that the levels of soluble MICA, but not those of soluble MICB, decreased after TAE therapy. It is not clear why soluble MICB did not change during TAE therapy. One possibility is that soluble MICB production from non-tumor livers may be relatively high compared with that of soluble MICA. In our subpopulation analysis, Child–Pugh A patients showed a significant decrease in soluble MICB levels after TAE therapy. In general, TAE therapy is more effective for Child–Pugh A patients than Child–Pugh B or C patients because the former is better able to tolerate the large dose of lipiodol emulsion and gelatin sponge that is necessary for efficient antitumor effect. Indeed, Child–Pugh A patients in our cohort showed a larger decease in α-fetoprotein levels after TAE therapy than Child–Pugh B and C patients, although the difference did not reach a significant level (our unpublished data). Thus, TAE therapy might reduce the levels of soluble MICB when it achieves substantial antitumor effect. Most importantly, the data also indicated that NKG2D expression on immune cells was clearly ameliorated with TAE therapy. Furthermore, there was an inverse correlation between a reduction in soluble MICA and upregulation of NKG2D, suggesting the link between soluble MICA and NKG2D expression in cancer patients.

It is generally speculated that soluble MICA/B produced from tumors may deactivate NKG2D-mediated immune responses.[8,9] *In vitro* experiment indicates that soluble MICA could down-regulate NKG2D expression and effector cell function. However, the regulation by soluble forms of NKG2D ligands would be more complicated *in vivo*. First, soluble forms of NKG2D ligands could be produced not only from malignant tissues but also from non-malignant tissues, as shown in the present study. Second, MHC-encoded MICA/B may not be the sole family of proteins serving as NKG2D ligands. Non-MHC-encoded UL16-binding proteins also act as NKG2D ligands and were very recently found to be cleaved proteolytically from tumor cells.[21] The present study provides evidence that soluble MICA is derived, at least in part, from HCC and regulates NKG2D expression on NK and CD8-positive T cells. Although several species of soluble NKG2D ligands may exist in the circulation, the present study suggests that soluble MICA regulates NKG2D expression directly in cancer patients.

In conclusion, soluble MICA and MICB are significantly increased in the sera of patients not only with HCC but also with chronic liver disease. Soluble MICA/B increases together with the progression of liver disease as well as the tumor. Therapeutic intervention for HCC leads to reduction of soluble MICA levels in association with upregulation of NKG2D on immune cells, offering *in vivo* evidence of soluble MICA regulating NKG2D expression. Thus, cancer therapy may have a beneficial effect on the NKG2D-mediated immune response even if some of the soluble NKG2D ligands are produced from non-cancerous premalignant tissues.

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A New Prognostic System for Hepatocellular Carcinoma Including Recurrent Cases

A Study of 861 Patients in a Single Institution

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Objective: To manage hepatocellular carcinoma (HCC) patients surviving for a long term, the treatment strategy for recurrent cancer is as important as that for the initial treatment. However, no prognostic scoring system has been available for patients with HCC recurrence. The purpose of this study was to develop a new staging system for deciding the treatment strategy not only for first-time diagnosed patients but also for recurrent patients.

Methods: A total of 861 cases diagnosed at our single institution from 1993 to 2003 were included. Overall survival was the only end point. The Cox model was used for multivariate analyses.

Results: As of August 2004, 344 cases (59%) had died. Overall median survival time was 41 months. For multivariate Cox regression analysis, independent predictive factors of survival were the number of recurrences, the Child-Pugh score, 3 nodules less than 3 cm and none of vascular invasion, and the α-fetoprotein level. A simple scoring system was thus developed, assigning scores (0/1) to the 4 covariates of the final model. Compared with the other scoring systems, the new scoring system has a greater discriminant ability.

Conclusions: We concluded that our scoring system can serve as a new prognostic system that reflects the spread of HCC, treatment response, and liver function. It should be very useful as the only method which can be applied for patients with recurrence.

Key Words: hepatocellular carcinoma, recurrence, staging system, predictive factor, cox regression analysis

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Recently, various nonsurgical treatment modalities for hepatocellular carcinoma (HCC) have been developed and surgical techniques have been also improved.[1,2] However, HCC with cirrhosis remains one of the diseases that is extremely difficult to manage, because survival in HCC is not predominantly based on the biology of the tumor, but also on the underlying hepatic function. Actually, we need consider 2 distinctive features in planning the HCC treatment from other cancers. First, even if HCC can be completely treated, the residual cirrhotic liver displays a high risk of recurrence, including new primary cancers.[3–5] Second, most options for the treatment of HCC lead to a decrease in the reserved hepatic function. In other words, they take the risk of future liver failure in return for HCC treatment. Taken together, the complexity of these factors makes HCC management difficult.

The prognosis of HCC patients is highly variable and hard to predict, which makes it difficult to effectively treat patients or to design good clinical trials. To provide guides for assessing disease severity and making therapeutic decisions, several staging or prognostic scoring systems for HCC have been proposed: the Cancer of the Liver Italian Program (CLIP) score,[6] BCLC staging,[7] and Japan Integrated Staging (JIS) scoring system,[8] which were produced on the basis of prognostic values. These staging systems can be used for assessing the prognosis of HCC patients as well as the efficiency of therapeutic modalities. Although these systems may be useful for predicting the prognosis of HCC patients at the time of the initial treatment,[9–11] there is considerable doubt about whether these systems are suitable for cases of recurrent cancer, because they cannot distinguish HCC diagnosed for the first time from recurrent HCC. In clinical practice, recurrent HCC patients are encountered 2.5 times more frequently in our institution than first-time HCC patients. Because the development of screening and follow-up programs and the improvement of radiologic techniques have facilitated the recognition of HCC at an earlier

stage,[12,13] it has become possible to repeatedly apply curative treatments.

To manage HCC patients surviving for a long term, preparing the treatment strategy for recurrent cancer becomes more important than that for initial treatment. This makes it important to predict the prognosis of recurrent patients. In other words, every time HCC is diagnosed, the prognostic value should be assessed, and then a treatment strategy should be decided. However, no attempts have been made to include prediction of the prognosis of recurrent HCC patients. The purpose of this article is to propose a new prognostic scoring system, which can be useful for deciding the treatment strategy not only for first-time diagnosed patients but also for recurrent HCC patients.

PATIENTS AND METHODS

Study Population

All (888) consecutive adult patients who were diagnosed as HCC and registered with the Division of Internal Medicine in the Osaka University Hospital between 1993 and 2003, were eligible for this study. Sixteen patients who could not be confirmed as having HCC were excluded. Three patients who underwent liver transplantation were also excluded. Eight patients who had local recurrences within 6 months were excluded because their admissions were not for the recurrent tumor but rather for the residual tumors caused by the insufficient ablation therapy. Thus, 861 patients composed the study population. The patient data were collected with both a survey of original medical records and access to the hospital information system. The patient data set was divided into 2 data sets for a split-sample validation procedure,[14] one set being retrospectively collected patients (n = 578) between September 1, 1993 and December 31, 2001, and the other being prospectively collected patients (n = 283) with the hospital database system between January 1, 2002 and December 31, 2003. The former was used as a training sample to construct a prognostic scoring system; the latter was used as a validation sample for the validation of the generated classification. HCC diagnosis was mainly established by the concomitant finding of 2 imaging techniques (n = 438), showing a nodule with arterial hypervascularization and portal hypovascularization, or by a positive imaging technique, showing hypervascularization associated with elevation of α-fetoprotein (AFP) or protein induced by vitamin K absence II (PIVKA-II) (n = 272). In addition, even if the above-mentioned features were not observed, target biopsy was performed when the findings of ultrasonography were consistent with HCC (n = 151). Details of the treatment modality showed that trans-catheter arterial chemoembolization alone or combined with percutaneous tumor ablation were mainly performed (n = 306 and 301, respectively). The number of patients treated with surgical resection, percutaneous tumor ablation alone, and best supportive care were 46, 188, and 20 respectively.

Statistical Methods

Overall survival was the only end point used in the analysis. It was defined as the time elapsed from the date of diagnosis and either the date of death related to liver disease or the date of the last follow-up information, with the final evaluation conducted on August 31, 2004. Patients lost before the last collection of follow-up information were censored at the time of their last visit. One hundred thirty-one of the 238 censored cases in the training sample were alive at the end of the period, whereas 22 patients had died from other diseases and 85 were lost to follow-up owing to change of residence (n = 21), introduction of a hospital near their residence (n = 50), and unknown reasons (n = 14). Two hundred and two of the 223 censored cases in the validation sample were alive, 3 patients died from other diseases, and 19 cases were loss to follow-up owing to the change of residence (n = 1), introduction of a hospital near their residence (n = 11), and unknown reasons (n = 7). Judging from the data at their last visit, all of the censored samples were considered to be independent of the future value of the hazard for the individual, in other words, they were noninformative censored cases. Figure 1 shows a schematic overview of investigated patients and dropouts for training and validation sample.

The following variables were used for the analysis: age and sex of the patient, date of HCC diagnosis, date of death or of last available information, viral status, the number of HCC recurrences, Child-Pugh score, the largest tumor size, tumor number, vascular invasion, AFP level, and PIVKA-II level. The cut-off levels of continuous variables were chosen on the basis of clinical meaning. For each variable, missing data were not used in the analysis if they accounted for less than 10% of the cases.

Univariate survival curves were estimated using the Kaplan-Meier method[15] and compared by means of the log-rank test.[16] The prognostic impact of the categories was assessed by means of the observed/expected ratio, as described previously.[6] Of the factors affecting patient survival in univariate analysis, baseline predictors were identified by the Akaike information criterion in a stepwise algorithm.[17] Next, a Cox proportional hazard

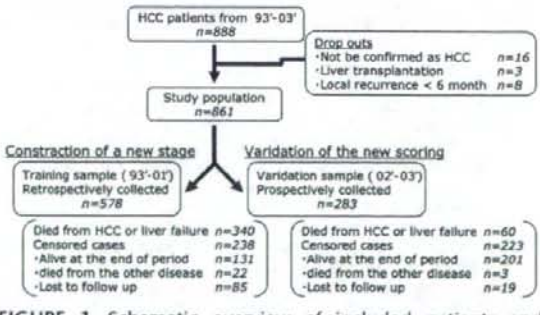

FIGURE 1. Schematic overview of included patients and dropouts for training and validation sample.

regression model was used for multivariate analyses.[18] Proportional hazard assumption was graphically assessed using plots of Log [−Log (survival time)]. Cases with missing values for one or more variables in the model were excluded from multivariate analysis. Treatment was not included in the model because the treatment choice was closely associated with the assessment of prognosis at the time of diagnosis.

Finally, the validity of the generated score was then assessed for the validation sample; a recent sample and a prospectively followed sample. The predictive accuracy of 3 models: this new score system, JIS score system, and CLIP score system was quantified by calculating the concordance index (C-index), which provides the area under the receiver operating characteristics (ROC) curve for the prediction of death at 3 years, as described previously.[19] A C-index of 0.5 indicates that outcomes are completely random, whereas a C-index of 1.0 indicates that the model is a perfect predictor.

All analyses were performed with R's software (R Foundation for Statistical Computing, Austria).[20] $P < 0.05$ was considered statistically significant in all analyses. The results were reported as a hazard ratio with 95% confidence intervals.

RESULTS

As of August 2004, 344 patients (59%) had died. The overall median survival time was 41 months (95% confidence interval, 36 to 46 mo); 1, 3, 5-year survival rates were 86%, 56%, and 35%, respectively. The baseline characteristics of the patients are given in Table 1. The first-time diagnosed HCC, shown as the number of HCC recurrences = 0 in Table 1, amounted to 295 cases, the first recurrence to 185, the second recurrence to 126, the third recurrence to 90, and more than the fourth recurrence to 165. Most cases were in the Child-Pugh A category. The baseline characteristics of the tumor are given in Table 2.

Nine variables were separately found to be associated with the outcome in univariate analysis of 11 variables (as shown in Table 3). Forward stepwise selection by Akaike information criterion was used to identify baseline predictors of 9 variables. Five variables were selected: the Child-Pugh score, the number of tumors, AFP, vascular invasion, and the number of HCC recurrences. To better reflect the treatment response, we combined 2 factors to create a single factor: we replaced "the number of tumors and vascular invasion" with "3 nodules less than 3 cm and none of vascular invasion, or not," called the tumor factor. This was done because the criterion "3 nodules less than 3 cm" reflects the possibility of complete response to ablation treatment[21] and was useful in the current clinical setting. We finally chose 4 factors for a new prognostic classification: the Child-Pugh score, tumor factor, AFP, and the number of HCC recurrences. These 4 covariates showed correlation with survival in the Cox regression analysis.

Each covariate selected by means of forward stepwise methods was divided into 2 categories to derive a simple scoring system. The cut-off levels were chosen where each estimated regression coefficient of the final Cox model was almost the same, that is, we made the relative prognostic weight of covariates the same, around 2 each (shown as in Table 4). A new scoring system was derived to assign scores (0/1) to each covariate of the final model as shown in Table 4. This classification was relatively easy to calculate by summing up each individual score of the 4 covariates. Five risk groups were constituted according to the score distribution. The survival curve of 578 patients calculated by the Kaplan-Meier method is shown in Figure 2A.

We assessed the new score system for 283 patients for the validation sample; prospectively obtained from 2002 to 2003 in Figure 2B. This result validated our scoring system and showed that it can be applied in today's clinical setting. This applicability to the present-day situation is very important, because diagnostic and

TABLE 1. Characteristics of Patients

Variables	Training Sample No. Patients	Validation Sample No. Patients
Median age, y (range)	64 (21-85)	67 (35-83)
Male (%)	425 (73.5)	192(67.8)
Cause of parenchymal disorder		
HBV/HCV/HB+HC	54/486/10	27/227/4
Alcoholic	8	10
Others	20	15
Child-Pugh score (unknown = 1)		
5-6 (A)/7-9 (B)/10-12 (C)	342/218/18	192/79/11
Number of HCC recurrence		
0/1/2/3/≥4	201/123/88/62/104	94/62/38/28/61

HBV indicates Hepatitis B virus; HCV, Hepatitis C virus.

TABLE 2. Characteristics of the Tumor

	Training Sample No. Patients	Validation Sample No. Patients
Number of tumor		
1/2/3/4/≥5	186/113/57/36/186	112/56/28/18/69
Largest size of tumor (cm)		
≤2.0/2.1-3.0/3.1-5.0/≥5.1	270/163/91/54	128/82/44/29
Vascular invasion		
Yes/no	534/44	266/17
Tumor factor [3 nodule less than 3 cm, vascular invasion (−)]		
Yes/no	285/293	159/124
AFP category (ng/mL)		
$\leq 10/10\text{-}10^2/10^2\text{-}10^3/>10^3$	137/230/129/82	65/108/70/40
PIVKA-II (mAU/mL) (unknown = 81)		
$\leq 10^2/10^2\text{-}10^3/10^3\text{-}10^4/>10^4$	327/118/64/27	110/58/20/14

TABLE 3. Univariate Analysis of Clinical Findings for Survival

Variables	No. Patients	O/E Ratio	*P*	DOF
Sex Male/female	425/153	1.11/0.73	0.00168	1
Age ≤50/50-60/60-70/≥70	37/118/291/132	0.53/0.87/1.19/0.86	0.00284	3
Etiology HCV/HBV/HB+HC/the others	486/54/10/28	1.03/0.9/1.38/0.54	0.147	3
Number of HCC recurrence 0/1/2/3/≥4	201/123/88/62/104	0.57/0.93/1.2/1.33/2.1	< 0.0001	4
Child-Pugh stage A/B/C	342/218/18	0.75/1.49/2.72	< 0.0001	2
Largest size of tumor (cm) ≤2.0/2.1-3.0/3.1-5.0/≥5.1	270/163/91/54	0.86/1.06/1.16/1.65	0.00467	3
Number of tumor 1/2/3/4/≥5	186/113/57/36/186	0.52/0.95/0.97/1.04/2.03	< 0.0001	4
Vascular invasion Yes/no	534/44	0.93/3.78	< 0.0001	1
Tumor factor [3 nodules less than 3 cm, vascular invasion (−)] Yes/no	285/293	0.67/1.53	< 0.0001	1
AFP (ng/mL) $\leq 10/10\text{-}10^2/10^2\text{-}10^3/>10^3$	137/230/129/82	0.56/0.95/1.2/2.19	< 0.0001	3
PIVKA-II (mAU/mL) $\leq 10^2/10^2\text{-}10^3/10^3\text{-}10^4/>10^4$	327/118/64/27	0.76/1.34/1.8/3.65	< 0.0001	3

DOF indicates degree of freedom; O/E ratio, observed/expected ratio; HBV, Hepatitis B virus; HCV, Hepatitis C virus.

therapeutic procedures for HCC have been improved over recent years.

Finally, the prognostic ability of the new scoring system was compared with CLIP score system and the JIS score system. Kaplan-Meier survival curves were shown in Figs. 2C, D). In addition, the predictive accuracy of 3 models was quantified by calculating a C-index, which provides the area under the ROC curve (as shown in Fig. 3). CLIP stage and JIS scoring had a C-index of 7.05 and 6.93, respectively. This new scoring system had a C-index of 7.23. Our scoring system could discriminate the survival most precisely among them.

DISCUSSION

This article revealed that the number of HCC recurrences is a prognostic factor as well as the reserved liver function and the spreading of HCC, and we have proposed a new scoring system, comprised of 4 parameters: the number of HCC recurrences, the Child-Pugh score, the tumor factor of "3 nodules less than 3 cm and none of vascular invasion," and the AFP level. Each of these parameters has so far been reported to affect patient survival. The occurrence of HCC recurrence reflects disease progression.[3–5] The Child-Pugh score is a well-recognized prognostic variable and reflects reserved liver

TABLE 4. New Scoring System

Variables	Score 0	Score 1	RR
Number of HCC recurrence (n = 578)	0 or 1 (n = 324)	≥2 (n = 254)	2.26
Child-Pugh score (n = 578)	5–7 (n = 486)	≥8 (n = 92)	2.25
Tumor factor (n = 578)	Yes (n = 285)	No (n = 293)	1.90
AFP category (ng/ml) (n = 578)	≤1000 (n = 496)	≥1001 (n = 82)	2.08

RR indicates risk ratio of Score 1 compared with Score 0, assessed by multivariate analysis.

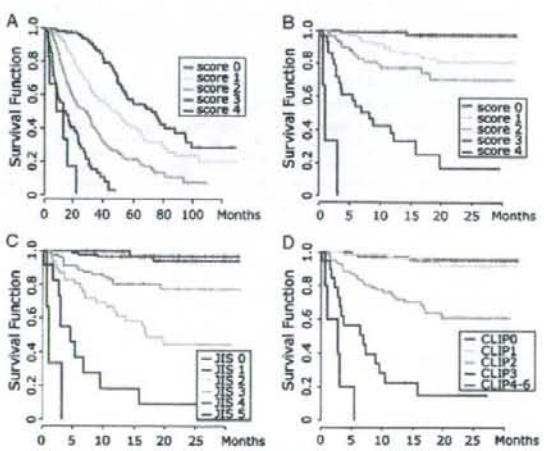

FIGURE 2. Kaplan-Meier–estimated survival curves. A, By our new scoring system in training samples. B, By our new scoring system in validation samples. C, By the CLIP score system in validation samples. D, By the JIS score system in validation samples.

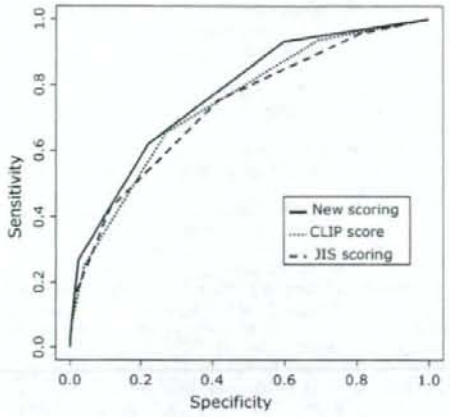

FIGURE 3. Discriminatory ability for the prediction of death at 3 years, evaluated by receiver operating characteristics curves of the new scoring, CLIP, and JIS staging systems.

function.[6,7] The criterion of 3 nodules less than 3 cm is related to the treatment response. Ablation therapy is highly effective for tumors smaller than 3 cm, achieving complete responses of around 80% to 100%.[22] The achievement of a complete and sustained response is an independent prognostic value.[23] AFP is also a well-recognized prognostic variable, and reflects the degree of cellular differentiation and the spreading of the tumor.[24] In the present study, these parameters were independent predictors of survival actually. Elevation of each parameter indicates the progression of HCC. As a result, this new scoring system reflects the spreading of HCC, the response to treatment, and the reserved liver function. In addition, our system is based on not pathologic but easily obtainable and reproducible clinical information. Therefore, this scoring system should be useful in many clinical settings.

A high possibility of recurrence is one of the major characteristics of HCC. Recurrences from either intrahepatic metastasis or de novo HCC exceed 50% at 3 years, even with hepatic resection as curative therapy.[3–5] The more the HCC recurs, the more the prognosis deteriorates because of treatment-induced liver damage and/or tumor progression. In clinical settings, it is very important to carefully follow HCC patients to detect recurrence as early as possible. More and more patients have been able to be frequently treated for recurrent HCC and prolong their survival. What is needed is a treatment strategy based on appropriate cancer staging systems for not only first-time diagnosed HCC but also for recurrent HCC. However, there has been no study reported on the prognosis of recurrent patients. Here, we first showed recurrence to be a prognostic factor with a Cox regression model, and furthermore developed a new scoring system to predict the prognosis of HCC patients including recurrent HCC patients.

What is the problem with applying the other staging systems for the recurrent cases? All of the following staging systems: the CLIP score system,[6] BCLC staging[7] and JIS scoring system[8] were derived from the analysis for first-time diagnosed HCC and were applied only at the initial treatment. Because hypothetical population is different between first-time HCC patients and all HCC patients, their baseline predictors for survival differ from the new scoring system. Indeed, the distributions of both the number of tumor and the largest size of HCC are significantly different between first-time HCC cases and all HCC patients in our cohort (data not shown). As a result, JIS system and CLIP score system may have poor stratification of survival. The goal of cancer staging is to separate patients into different groups on the basis of their predicted survival to help determine the most appropriate treatment modality. Therefore, it is unreasonable to apply their systems for recurrent HCC patients.

Although further evaluation is needed, this scoring system can be useful for conducting interventional trials. With the spread of routine screening and follow-up, the number of recurrent HCC patients can increase. More effective strategies to treat recurrent patients will be needed. In addition, a new modality of treatment will be necessary for HCC management, particularly for score 2 and 3 patients. Interventional trials may be needed to determine the most appropriate therapy for the patients in each group. This scoring system, because of good incorporation between prognosis estimation and potential treatment advances, may be useful for planning and evaluating interventional trials. It would allow us to follow a well-established treatment schedule and select the best treatment modality for each patient when managing long-term–surviving HCC patients.

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Original Article

Initial viral response is the most powerful predictor of the emergence of YMDD mutant virus in chronic hepatitis B patients treated with lamivudine

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Aim: Lamivudine (LAM) has been widely used to treat chronic hepatitis B (CHB) patients, but the emergence of a LAM-resistant virus greatly limits its therapeutic efficacy. In this study, we tried to identify factors affecting the emergence of a LAM-resistant virus in CHB patients treated with LAM.

Methods: The subjects were 190 CHB patients in continuous LAM therapy (139 males, mean age 50 years, 87 HBeAg-positive). The mean duration of follow-up was 39 months (range 12–104). The initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) at 6 months.

Results: IVR was positive in 86% of the patients. The cumulative emergence rates of LAM-resistant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years. In univariate analysis, factors contributing to the emergence of LAM-resistant virus were baseline HBV DNA > 6.5 logcopies/mL ($P = 0.0044$), HBeAg-positivity ($P = 0.0062$), IBR ($P = 0.01$) and IVR ($P < 0.0001$). The cumulative emergence rates of LAM-resistant virus in IVR-positive and -negative patients were 4% and 41% at 1 year, and 41% and 79% at 3 years. In multivariate analysis, only IVR was an independent factor affecting the emergence of LAM-resistant virus ($P < 0.0001$).

Conclusion: IVR is a useful factor for predicting the emergence of LAM-resistant virus in CHB patients treated with LAM. For IVR-negative patients, therapeutic options other than LAM monotherapy should be used because of the high incidence of the emergence of LAM-resistant virus.

Key words: chronic hepatitis B, initial viral response, lamivudine monotherapy, lamivudine-resistant virus

INTRODUCTION

MORE THAN 350 million people are chronically infected with hepatitis B virus (HBV) worldwide.[1] Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death. Nucleos(t)ide analogs are widely used to suppress HBV replication and the progression of HBV-related liver diseases. Lamivudine (LAM), the first approved nucleoside analog for chronic HBV infection, has been shown to suppress viral replication and disease activity.[2] In addition, LAM therapy has recently been reported to reduce the incidence of HCC, the risk of major complications and to improve survival.[3,4] However, the relatively high incidence of LAM resistance is a serious problem in the case of LAM therapy for chronic HBV infection. The emergence of LAM-resistant HBV is linked to the reappearance of active viral replication, followed by the worsening of liver disease.

LAM-resistant HBV is based on point mutation within the YMDD motif of the reverse transcriptase domain of

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HBV (YMDD mutation).[5,6] The emergence rates of the mutant virus have been reported to be 24% at 1 year and 70% at 4 years from the start of treatment.[7]

Recent work has shown that newly developed nucleos(t)ide analogs, such as adefovir dipivoxil (ADV) and entecavir (ETV), are also useful agents for controlling patients with chronic HBV infection.[8–11] In particular, the drug-resistant mutant virus has been reported to appear less frequently in cases of treatment with ADV and ETV than with LAM.[12,13] For this reason, LAM has been replaced by ADV and ETV for the treatment of chronic hepatitis B. However, there are still a considerable number of patients with chronic HBV infection who are already on continuous LAM therapy. Thus, further clarification is needed of what factors influence the emergence of the LAM-resistant HBV in LAM treatment for chronic HBV infection.

For a more precise evaluation, we investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus in patients with chronic hepatitis B treated with LAM.

METHODS

Patients and treatment

THIS STUDY WAS conducted at nine institutions in the Osaka area of Japan (Osaka Police Hospital, Osaka Minami Medical Center, Osaka Kouseinenkin Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda City Hospital, Osaka National Hospital, Otemae Hospital and Osaka University Hospital). The subjects were 190 consecutive patients with chronic hepatitis B who underwent continuous LAM therapy for more than 12 months. All patients tested positive for hepatitis B surface antigen (HBsAg) or had detectable levels of HBV DNA in their sera by the polymerase chain reaction (PCR)-based method (for 100 patients)[14] or the transcription-mediated amplification (TMA) method (for 90 patients).[15] Exclusion criteria were patients with antihepatitis C antibody, antihuman immunodeficiency virus antibody and other forms of liver diseases (alcoholic liver disease, drug-induced liver disease and autoimmune hepatitis). Forty-one (22%) patients had previously received interferon (IFN)-α therapy for 24 weeks.

All patients were treated with 100 mg of LAM daily. After the beginning of the therapy, liver function tests and HBV DNA were measured every other month for the first 6 months and every two months thereafter. HBeAg and anti-HBe were tested every 6 months. In 33 patients (18%), combination therapy with IFN was carried out for the initial 6 months. Three or six mega-units of natural IFN-á were administered daily for the first 2 weeks and three times a week thereafter, followed by LAM monotherapy. The mean follow-up period of the 190 patients was 39 (range 12–104) months. The LAM-resistant YMDD mutant virus was detected by the PCR-enzyme-linked minisequence (ELMA) assay[16] when the virological or biochemical breakthrough was observed. The YMDD mutant virus was found in 86 (45%) patients during follow-up. Fifty-eight of these patients underwent ADV therapy in addition to ongoing LAM treatment and were excluded from the follow-up when ADV administration began. In this study, the initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) after 6 months of therapy.

Table 1 Patient characteristics

Gender (male/female)	139/51
Age (years)	50 ± 11
Chronic hepatitis/liver cirrhosis	113/77
Hepatocellular carcinoma	14 (7%)
AST (IU/L)	122 ± 157
AST (IU/L)	177 ± 236
ALT (≤1/1–2/2–5/>5 × ULN)	22/53/65/50
Platelet (10^4/mm^3)	12.6 ± 5.1
Prothrombin time (%)	71.5 ± 16.6
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)
HBeAg (positive/negative)	87/103
Combination with interferon	33 (17%)
Duration of treatment (months)	38.9 ± 17.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ULM, upper limit normal.

The patients' clinical characteristics are shown in Table 1. There were 139 males and 51 females, ranging in age from 25 to 75 (mean 50) years. Of them, 113 (59%) patients were diagnosed as having chronic hepatitis and the remaining 77 patients (41%) as having cirrhosis according to liver histology and/or the imaging procedure. HCC was developed in 14 (7%) patients. The aspartate aminotransferase (AST) at baseline was 122 ± 157 IU/l, and the ALT at baseline was 177 ± 236 IU/L. Abnormal ALT was observed in 168 (88%) patients. Eighty-seven patients (46%) tested positive for HBeAg. The median HBV DNA at baseline was 6.5 (range 3.0 to 7.6<) logcopies/mL.

HBV testing

HBsAg, hepatitis B e antigen (HBeAg) and antihepatitis B e antibody (anti-HBe) were examined by chemiluminescent immunoassay or enzyme immunoassay.

The HBV DNA level was measured by the PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan)[14] or the TMA method (TMA-HPA; Fujirebio, Tokyo, Japan),[15] which have lower detection limits of 2.6 and 3.7 logcopies/mL, respectively. The LAM-resistant YMDD mutant virus was examined by the PCR–ELMA method.[16]

Statistical analysis

Comparisons of categorical and continuous variables between groups were done by the χ^2-test, Student's *t*-test and Mann–Whitney's *U*-test. The cumulative emergence rates of LAM-resistant virus were evaluated with the Kaplan–Meier's curve and the differences between groups were analyzed by the log–rank test. For multivariate analysis to investigate factors affecting the cumulative emergence rate of LAM-resistant virus, Cox proportional hazard regression analysis was carried out. A *P*-value of less than 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Therapeutic efficacy and the emergence of LAM-resistant mutant virus

AMONG THE 190 patients with chronic hepatitis B who underwent continuous LAM therapy, reduction of HBV DNA to less than 4 logcopies/mL was observed in 86% (163/190) at 6 months, 89% (151/170) at 1 year, 88% (83/94) at 2 years and 89% (48/54) at 3 years of the treatment. Normalization of ALT was achieved by 77% (146/190) at 6 months, 83% (141/170) at 1 year, 81% (76/94) at 2 years and 83% (45/54) at 3 years. Among the 87 HBeAg-positive patients, HBeAg was cleared in 22% (19/86) at 6 months, 26% (21/80) at 1 year, 22% (11/50) at 2 years and 43% (16/37) at 3 years. As for the virological and biochemical response at 6 months of therapy, 163 (86%) of the patients achieved IVR, whereas IBR was seen in 146 (77%) of patients.

When the various patient characteristics were compared between IVR-positive and -negative patients (Table 2), HBV DNA at baseline tended to be lower in patients showing IVR (median 6.5 [range 3.0 to 7.6<] logcopies/mL) than in those who did not show IVR (median 7.3 [range 4.3 to 7.6<] logcopies/mL) ($P < 0.0001$). IVR-negative patients had higher HBeAg positivity at baseline than IVR-positive patients (81% vs 40%, $P = 0.01$). As for the emergence of LAM-resistant mutant virus during follow-up, it was detected more frequently in IVR-negative patients (21/27, 78%) than in IVR-positive patients (65/163, 40%) ($P = 0.002$).

Among the 190 patients examined in this study, the emergence of LAM-resistant YMDD mutant virus occurred in 86 (45%) patients during follow-up. The cumulative probabilities of the emergence of the YMDD mutant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years.

Factors affecting the emergence of LAM-resistant mutant virus

Factors affecting the cumulative probability of the emergence of the YMDD mutant virus were investigated using

Table 2 Comparison of patient characteristics between IVR-positive and -negative patients

	IVR ($n = 163$)	Non-IVR ($n = 27$)	*P*-value
Gender (male/female)	118/45	21/6	NS
Age (years)	50 ± 11	48 ± 12	NS
Chronic hepatitis/liver cirrhosis	91/72	22/5	NS
Hepatocellular carcinoma	13 (8.0%)	1 (4%)	NS
AST (IU/L)	131 ± 167	69 ± 34	NS
ALT (IU/L)	190 ± 252	100 ± 55	NS
ALT (≤1/1–2/2–5/>5 × ULN)	21/43/52/47	1/10/13/3	NS
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)	7.3 (4.3–7.6<)	<0.0001
HBeAg (positive/negative)	65/98	22/5	0.01
Combination with interferon	27 (17%)	6 (22%)	NS
Emergence of LAM-resistant viruses	65 (40%)	21 (78%)	0.002
Duration of treatment (months)	39.2 ± 17.2	37.3 ± 19.1	NS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IVR, initial viral response; LAM, lamivudine; NS, not significant; ULN, upper limit normal.

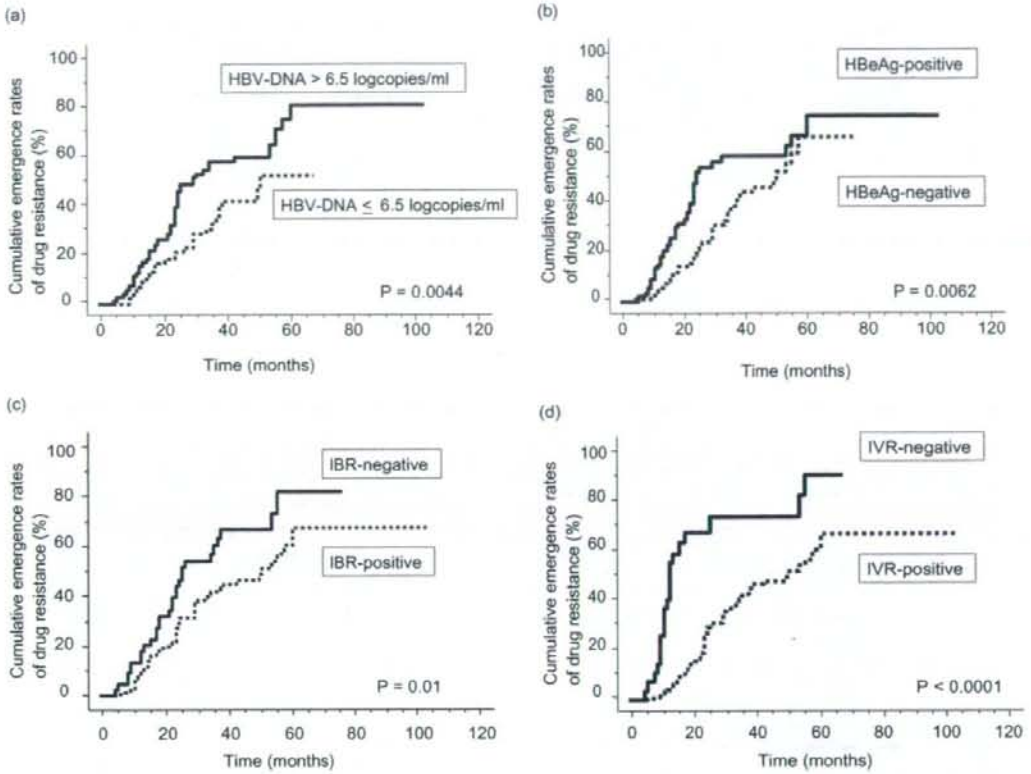

Figure 1 Cumulative emergence rate of lamivudine (LAM)-resistant virus in patients with chronic hepatitis B virus (HBV) infection treated with LAM according to: (a) HBV DNA at baseline; (b) hepatitis B e antigen (HBeAg) status; (c) the presence or absence of initial biochemical response (IBR); and (d) the presence or absence of initial viral response (IVR).

both univariate and multivariate analyses. Nine baseline and on-treatment factors – gender, age, liver disease (chronic hepatitis or cirrhosis), ALT at baseline, HBeAg positivity, HBV DNA at baseline, combination therapy with IFN-α, presence of IBR and presence of IVR – were examined. The cumulative emergence of LAM-resistant virus was significantly higher in patients with baseline HBV DNA > 6.5 logcopies/mL than in those with HBV DNA ≤ 6.5 logcopies/mL ($P = 0.0044$) (Fig. 1a). HBeAg-positive patients revealed a significantly higher emergence rate of the LAM-resistant virus than HBeAg-negative patients ($P = 0.0062$) (Fig. 1b). A significant difference was also seen in the cumulative emergence of the YMDD mutant virus between IBR-positive and -negative patients ($P = 0.01$) (Fig. 1c). Furthermore, the cumulative emergence of LAM-resistant mutant virus was much higher in the IVR-negative patients than in the IVR-positive patients ($P < 0.0001$) (Fig. 1d). The cumulative emergence rates of LAM-resistant virus in the IVR-positive and -negative patients were 4% and 41% at 1 year, 25% and 69% at 2 years, and 41% and 79% at 3 years, respectively. Gender, age, liver disease, ALT at baseline and combination therapy of IFN-α did not show a significant relation with the emergence of the YMDD mutant virus. When factors influencing the higher cumulative emergence of LAM-resistant virus were searched for by multivariate analysis, only the absence of IVR was selected as a significant independent factor ($P < 0.001$) (Table 3), with high HBV DNA, HBeAg positivity and the absence of IBR not being selected.

Table 3 Factors associate with emergence of LAM-resistant virus determined by multivariate analysis

	Hazard ratio	95% confidence interval	*P*-value
Gender			
0: male	1	0.497–1.455	0.55
1: female	1.176		
Age			
0: ≤50	1	0.640–1.700	0.87
1: >50	0.959		
Chronic hepatitis/liver cirrhosis			
0: CH	1	0.656–1.740	0.79
1: LC	0.935		
Pretreatment ALT (IU/L)			
0: ≤200	1	0.605–1.818	0.87
1: >200	0.953		
HBV DNA (logcopies/mL)			
0: ≤6.5	1	0.394–1.125	0.13
1: >6.5	1.502		
HBeAg			
0: negative	1	0.499–1.337	0.42
1: positive	1.225		
Combination therapy with interferon			
0: no	1	0.410–1.303	0.29
1: yes	1.368		
IBR			
0: positive	1	0.483–1.312	0.37
1: negative	1.256		
IVR			
0: positive	1	0.159–0.536	<0.001
1: negative	3.425		

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IBR, initial biochemical response; IVR, initial viral response; LAM, lamivudine.

DISCUSSION

IN LAM THERAPY for patients with chronic HBV infection, the emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. To resolve this, detailed studies are needed to identify factors related to the emergence of the YMDD mutant virus. To date, a few investigators have suggested male gender, advanced age, high baseline ALT, the presence of severe acute exacerbation of the liver disease, high baseline HBV DNA and HBeAg-positivity as possible predictors of the emergence of LAM-resistant virus.[7,17,18] Lower body surface area was also reported as a significant factor for virological and biochemical therapeutic effect.[19] In the present study, we studied 190 patients with chronic hepatitis B treated with LAM and investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus. Univariate analysis revealed that two baseline factors, high HBV DNA and HBeAg positivity, had a relation to the high incidence of the YMDD mutant virus, which is consistent with previous reports.[7,17,18] In addition, two on-treatment factors, IBR and IVR, were found to be correlated with the emergence of LAM resistance. Patients who did not show IVR had a 3.4-fold higher incidence of the emergence of the YMDD mutant virus than those who did show IVR. This agrees with a previous report that the HBV DNA level after 6 months of therapy may be a determinant for subsequent occurrence of a LAM-resistant mutant virus.[20] Multivariate analysis showed that only the absence of IVR was a significant factor contributing to the emergence of LAM-resistant virus. Baseline HBV DNA and HBeAg status were not selected as significant factors by multivariate analysis probably because of the tendency for higher HBV DNA and high frequency of HBeAg positivity in IVR-negative patients compared with IVR-positive patients. It is particularly interesting that the absence of IVR, rather than other baseline and on-treatment factors, was a powerful independent pre-

dictor for the emergence of the YMDD mutant virus in LAM therapy for chronic HBV infection. This means that IVR of an on-treatment factor is very important for good therapeutic effect and the stage for the next therapeutic strategy can thus be set in a new light with this information.

Our results showed that approximately one-seventh of the patients with chronic hepatitis B treated with LAM did not achieve IVR. In the non-IVR patients, the antiviral therapeutic regimen should be amended due to the frequent emergence of LAM-resistant virus. Recently, new nucleos(t)ide analogs have become available for the treatment of chronic HBV infection. ETV has been reported to be more effective for the reduction of HBV DNA and the less frequently induced drug-resistant mutant virus than LAM in "naïve" patients with chronic hepatitis B who had not previously received nucleos(t)ide analog therapy.[10,11] ETV was also effective in patients with chronic HBV infection showing LAM resistance,[21] but the emergence rate of the ETV-resistant virus was considerably higher in LAM-resistant patients than in naïve patients.[13,22] This is because the ETV-resistant HBV strain is established by LAM-resistant YMDD mutation plus additional mutation(s) at the amino acid position(s) 184, 202 and/or 250 within the reverse transcriptase domain of HBV.[22] According to these findings, switching from LAM to ETV may be useful for treating patients who do not achieve IVR on LAM administration. This should be done before the emergence of LAM-resistant YMDD mutant virus so as not to reduce the therapeutic efficacy of ETV. In clinical practice, there are still a number of patients who have already been on continuous LAM therapy, although the current first choice drug for patients with chronic HBV infection is ETV. In our opinion, foregoing patients without IVR or YMDD mutant viruses should be switched from LAM to ETV. The therapeutic efficacy of switching from LAM to ETV in non-IVR patients should be assessed by further study with a larger number of patients.

ADV and tenofovir disoproxil fumarate (TDF) have also been shown to exert antiviral efficacy in patients with chronic HBV infection with less frequent occurrence of drug-resistant mutant virus compared to LAM.[23] In addition, unlike the case of ETV, both ADV and TDF are known to be effective in LAM-refractory patients with chronic hepatitis B, as well as naïve patients.[23] Using ADV and TDF may be helpful for the treatment of non-IVR patients, especially after the establishment of LAM-resistant mutant virus.

In conclusion, our findings indicate that IVR may be a useful factor for predicting the emergence of LAM-resistant mutant virus in patients with chronic HBV infection treated with LAM. For patients who do not achieve IVR, therapeutic options other than LAM monotherapy should be promptly implemented because of the high incidence of the subsequent emergence of the YMDD mutant virus.

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Case Report

Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection

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The efficacy of entecavir for patients with hepatitis B virus/human immunodeficiency virus coinfection has not been fully elucidated. Here we examined a patient coinfected with both viruses in whom entecavir-resistant hepatitis B virus appeared. The 60-year-old Japanese male with the coinfection received antiretroviral therapy including lamivudine. The therapy initially suppressed replication of both viruses, followed by reactivation of the hepatitis B virus alone by 2 years of therapy. He subsequently received entecavir therapy in addition to the antiretroviral regimen. After entecavir administration, the hepatitis B virus DNA level was slightly reduced, but then increased after 6 months of entecavir therapy. In the sequencing analysis of hepatitis B virus, no drug resistance-associated amino acid substitutions were observed in the reverse transcriptase (rt) domain before antiretroviral therapy. The lamivudine-resistant amino acid substitutions at rt173, rt180 and rt204 were detected before entecavir administration, and further the entecavir-resistant rt202 substitution was observed after 6 months of entecavir therapy. The full-length hepatitis B sequences showed that the viral strain derived from the patient belonged to genotype H. In summary, this report describes a patient with hepatitis B virus/human immunodeficiency virus coinfection who received entecavir therapy in addition to an antiretroviral regimen and showed the early emergence of entecavir-resistance hepatitis B virus. In entecavir therapy for patients infected with both viruses, great care should be taken with respect to the emergence of entecavir-resistant hepatitis B virus, especially in patients with pre-existing lamivudine-resistant virus.

Key words: coinfection, drug-resistant hepatitis B virus, entecavir, hepatitis B virus, human immunodeficiency virus, lamivudine

INTRODUCTION

CHRONIC CARRIERS OF hepatitis B virus (HBV) number more than 350 million worldwide.[1] Chronic HBV infection is seen in approximately 10% of human immunodeficiency virus (HIV)-infected patients,[2] and coinfection with HBV and HIV is a serious health problem due to the shared mode of transmission. Since the prognosis of HIV-infected patients can be dramatically improved by highly active antiretroviral therapy (HAART), one of the major causes of mortality in HIV-infected patients is chronic liver disease due to HBV infection.[3]

Lamivudine (LAM, also abbreviated to 3TC), one of the antiretroviral drugs, has also been used for the reduction of HBV replication and improvement of HBV-related liver diseases.[4,5] However, the anti-HBV effect of LAM is hampered by the emergence of LAM-resistant mutant virus in cases of HBV monoinfection and HBV/

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HIV coinfection.[6,7] The LAM-resistant HBV strain is based on point mutation occurring within the reverse transcriptase (rt) domain of the polymerase gene. A methionine-to-valine/isoleucine amino acid substitution at rt204 (rtM204V/I) is known to confer LAM resistance.[8,9] A leucine-to-methionine substitution at rt180 (rtL180M) and a valine-to-leucine substitution at rt173 (rtV173L) have also been shown to appear in association with LAM resistance.[8,10,11] The emergence rate of LAM-resistant virus in patients coinfected with HBV and HIV has been reported to be approximately 50% after 2 years of therapy.[9]

Recently, entecavir (ETV) has been reported to be superior to LAM for the suppression of viral replication and disease activity in patients with HBV monoinfection who had not received previous treatment with other anti-HBV drugs (naïve patients).[12,13] ETV has also been shown to be effective in HBV-infected patients who had been treated with LAM and showed LAM resistance.[14] It has been demonstrated that ETV resistance occurs based with amino acid substitution(s) at rt184, rt202 and/or rt250, together with the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus after 3 years of therapy has been reported to be less than 1% in naïve patients and 15% in LAM-resistant patients with chronic HBV monoinfection.[16] However, the anti-HBV efficacy of ETV for HBV/HIV coinfection has not been fully clarified.

In this study, we examined a patient with concomitant HBV/HIV infection who underwent HAART including LAM, and showed the appearance of LAM-resistant HBV. Subsequent ETV administration did not lead to an adequate reduction of the HBV replicative level, followed by the early emergence of the ETV-resistant virus. We investigated the serial change in the drug resistance-associated mutation status within the rt domain of the HBV polymerase gene, as well as full-length nucleotide sequences of the ETV-resistant HBV strain derived from the patient.

CASE REPORT

Patient and serum sampling

A 60-YEAR-OLD JAPANESE heterosexual male first visited to the National Hospital Organization Osaka National Hospital in December 2001 due to a positive result from an HIV antibody (anti-HIV) test in voluntary HIV screening. From his anamnestic record, he had been admitted with type B acute hepatitis to another hospital 3 years earlier. Anti-HIV had been negative at that time. On his first visit, the anti-HIV positivity was confirmed by Western blot analysis. Antibodies to HIV-1 proteins, gp160, gp110/120, p68, p52, gp41, p40 and p34 were positive. As for antibodies to HIV-2 proteins, only an antibody to p68 was positive. According to these, he was judged to be infected with HIV-1. The HIV-RNA level was $10^{4.3}$ copies/mL, and the CD4+T cell counts were 275/mm^3 (normal range, >300/mm^3). He tested positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), and negative for antibody to HBsAg (anti-HBs) and antibody to HBeAg (anti-HBe). The HBV-DNA level was $>10^{7.6}$ copies/mL, and the alanine aminotransferase (ALT) level was 106 IU/L. The patient was free of HIV-related symptoms and had no opportunistic infectious diseases. HAART with LAM (300 mg/day), zidovudine (AZT) (600 mg/day) and efavirenz (EFV) (600 mg/day) was started in April 2002. AZT and EFV were then substituted for sanilvudine (d4T) (60 mg/day) and avacavir (ABC) (600 mg/day) in July 2002 because of anemia and dizziness. By July 2002, HIV-RNA decreased to below the detection limit ($<10^{1.7}$ copies/mL), whereas the CD4+ T cell counts tended to rise up to >500/mm^3. In August 2006, fosamprenavir (FPV) (2400 mg/day) was commenced in place of d4T due to peripheral nerve palsy. Suppression of HIV-RNA below the detection limit continued at the end of follow-up, irrespective of repeated alterations in the therapeutic regimen of HAART. As for HBV status, HBV-DNA declined to $10^{3.9}$ copies/mL in April 2003 but increased again to $>10^{7.6}$ copies/mL in May 2005. To control HBV replication, ETV (0.5 mg/day) was added in October 2006. After the ETV administration, HBV-DNA slightly decreased from $>10^{7.6}$ to $10^{6.2}$ copies/mL in January 2007 but rose to $10^{7.2}$ copies/mL 3 months later. ALT remained abnormal and HBeAg continued to be positive throughout the follow-up period. The clinical course of the patient is summarized in Figure 1a.

For the nucleotide sequencing of HBV-DNA, the serum samples were obtained in December 2001 (before HAART), August 2006 (before ETV administration), and April 2007 (after 6 months of ETV therapy). These serum sampling points were designated as P1, P2 and P3 (see Fig. 1a). Serum samples were stored at −80°C until use. Informed consent was obtained from the patient.

Virus markers and nucleotide sequencing

HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HIV were tested by chemiluminescent immunoassay. A

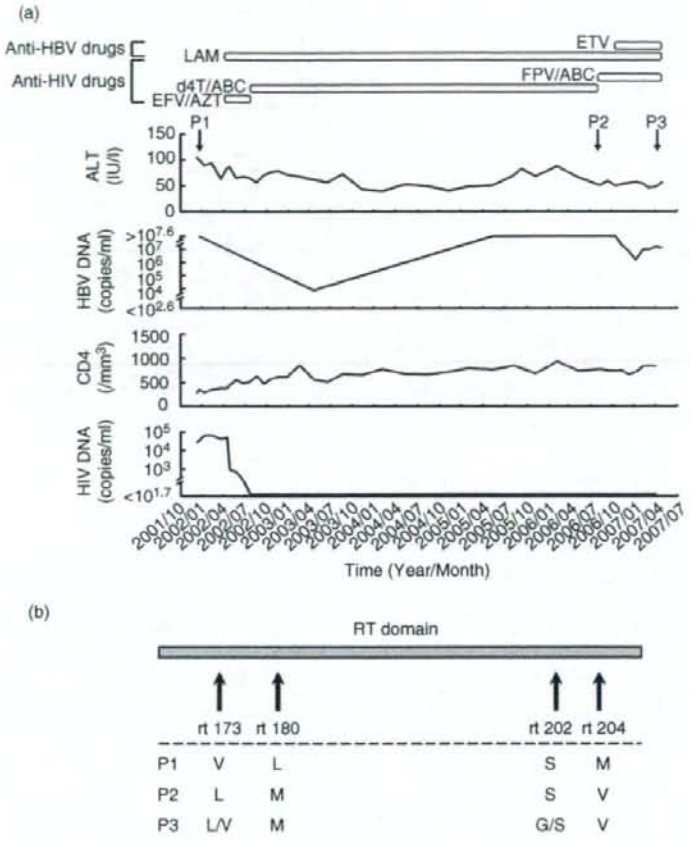

Figure 1 (a) Patient clinical course and serum sampling points. P1, P2 and P3 are the points at which serum samples were obtained. P1 was taken in December 2001 (before HAART), P2 in August 2006 (before ETV administration) and P3 in April 2007 (after 6 months of ETV therapy). ABC, avacavir; ALT, alanine aminotransferase; AZT, zidovudine; d4T, sanilvudine; EFV, efavirenz; ETV, entecavir; FPV, fosamprenavir; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LAM, lamivudine. (b) Serial change in the status of drug resistance-associated amino acid substitutions.

confirmatory anti-HIV-1/2 testing was carried out by Western blot analysis. Serum HBV-DNA was detected by means of a PCR assay (Amplicor HB monitor; Roche Diagnostics, Basel, Switzerland) with a lower detection limit of $10^{2.6}$ (=400) copies/mL. Plasma HIV-RNA was quantified by a PCR assay (Amplicor HIV-1 monitor; Roche) whose lower detection limit was $10^{1.7}$ (=50) copies/mL.

The nucleotide sequences of HBV-DNA were determined by a method based on nested PCR and direct sequencing, as described elsewhere.[17] In this study, primers BF5-2 (5′-TCC TCA GGC CAT GCA GTG GA-3′, nt 3201-20) and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195-76) were also used. Nucleotide sequences of the entire rt domain in the polymerase gene were examined in HBV strains derived from the P1 and P2 serum samples (GenBank accession nos. AB353765 and AB353766), whereas the full-length HBV-DNA was determined in the strain derived from the P3 serum sample (GenBank accession no. AB353764). The full-length HBV strain obtained in this study (designated as HBDI03), the seven representative HBV strains of genotypes A–G and the eight previously isolated HBV strains of genotype H were aligned, and the phylogenetic tree was constructed. These analyses were done at the homepage of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Results of sequencing analysis of HBV

The serial change in the nucleotide sequences in the rt domain of the HBV polymerase gene was first examined

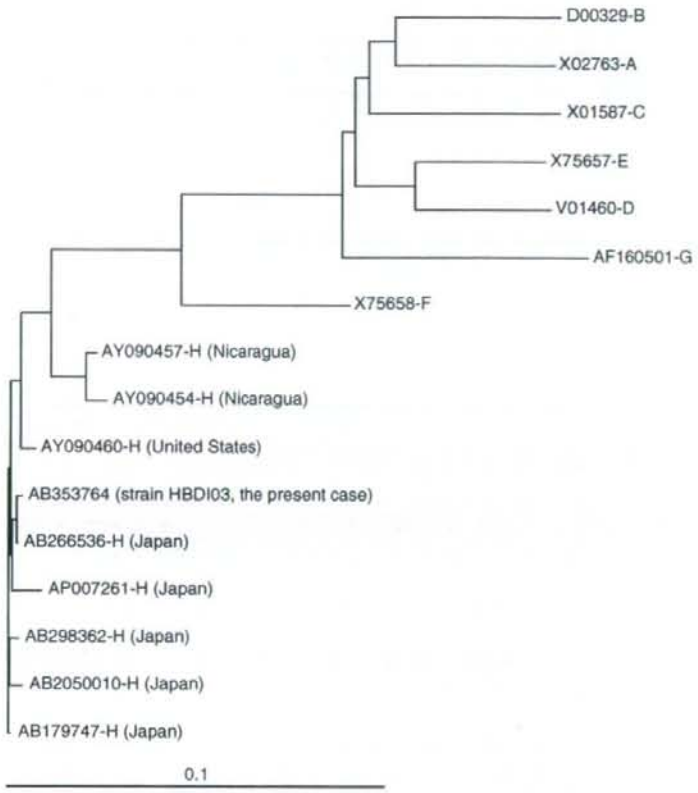

Figure 2 Phylogenetic tree analysis including the HBV strain HBDI03 obtained in this study, the seven representative HBV strains of genotypes A–G, and the eight previously isolated HBV strains of genotype H.

using serum samples obtained at P1–P3 (Fig. 1b). At point P1, no drug resistance-associated mutations were found in the rt domain, but three LAM resistance-associated substitutions, rtM204V, rtL180M and rtV173L, emerged at point P2. A serine-to-glycine substitution at rt202 (rtS202G), which has been shown to be one of the ETV resistance-associated substitutions,[15] was further observed at point P3, although rtS202G and rtV173L substitutions occurred incompletely. No other amino acid substitutions were seen in the rt domain of the HBV polymerase gene from point P1 to P3. Thus, in the patient with HBV/HIV coinfection, the emergence of the drug resistance-associated amino acid substitutions revealed a close relationship with the poor anti-HBV efficacy of LAM and ETV.

Next, the full-length nucleotide sequences of HBV were determined from the P3 serum sample of the patient with HBV/HIV coinfection showing ETV resistance. The full-length HBV strain HBDI03 comprised a total of 3215 nucleotide lengths. The phylogenetic tree was depicted using the HBV strain HBDI03, the seven representative HBV strains of genotypes A–G and the eight previously identified genotype H HBV strains. As shown in Figure 2, the HBV strain HBDI03 obtained in this study was classified as genotype H. When the nucleotide sequences of the strain HBDI03 were compared with the eight reported genotype H HBV strains, the strain HBDI03 showed a 97.2–99.8% identity with these strains. The unique amino acid substitutions in the strain HBDI03 were further investigated in comparison with these eight genotype H HBV strains. As shown in Table 1, four drug resistance-associated substitutions within the rt domain were observed, as described above. The two amino acid substitutions in the S gene were also caused by the same mutations of the drug resistance-associated rtV173L and rtM204V