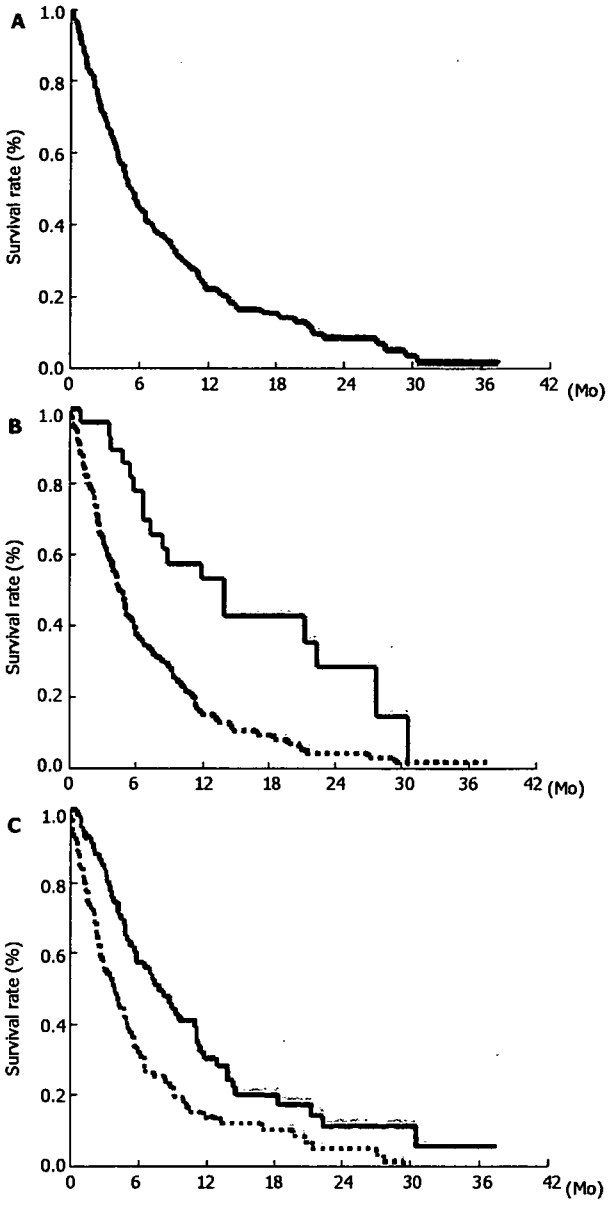

Figure 1 Survival rate of 151 HCC patients with extrahepatic metastases (**A**), intrahepatic tumor stage (**B**) [solid line: T0-T2, dashed line: T3, T4 (log-rank test: $P < 0.001$)], and after treatment of extrahepatic metastases (**C**) [solid line: treatment group, dashed line: no treatment group (log-rank test: $P < 0.001$)].

These three were all alive without recurrence of extrahepatic metastases during the observation period. In each of these 3 patients, hepatic reserve was Child-Pugh stage A, no intrahepatic HCC was not detected, and PS was 0.

TACE was performed in 8 (5%) patients (7 patients with adrenal gland metastases, and one patient with paraaortic lymph node metastases). Systemic chemotherapy was used in 39 (26%) patients. Chemotherapy included 5-fluorouracil, carboplatin, cisplatin. Twenty-five of the 39 patients had lung metastases, 10 had lymph node metastases, 2 had bone metastases, one had lung and lymph node metastases, and one had lung, adrenal gland and lymph node metastases.

Radiotherapy was performed in 36 (24%) patients.

Table 3 Univariate analysis of predictors of survival after initial diagnosis of extrahepatic metastases in 151 patients

Variable	Hazard Ratio	95% CI	*P*
PS (0 *vs* 1-4)	2.181	1.50-3.17	< 0.001
Age (≤ 65 *vs* > 65 yr)	0.988	0.97-1.0	0.18
Sex (M *vs* F)	0.889	0.57-1.38	0.601
Child Pugh stage (A *vs* B, C)	2.323	1.73-3.12	< 0.001
Intrahepatic main tumor size (≤ 50 *vs* > 50 mm)	2.321	1.52-3.54	< 0.001
Intrahepatic tumor volume (≤ 50 *vs* > 50%)	2.523	1.71-3.72	< 0.001
Intrahepatic tumor morphology (nodular *vs* non nodular)	1.506	1.04-2.18	0.03
Vp (0-2 *vs* 3, 4)	2.247	1.53-3.29	< 0.001
AFP (≤ 400 *vs* > 400 ng/mL)	1.158	0.80-1.68	0.439
DCP (≤ 1000 *vs* > 1000 mAU/mL)	1.584	1.08-2.33	0.02
Treatment (performed *vs* not performed)[1]	2.385	1.51-3.77	< 0.001
Site (lung *vs* others)[2]	1.065	0.74-1.52	0.731
Site (bone *vs* others)	1.61	1.11-2.33	0.012
Site (only lymph node *vs* others)	1.133	0.74-1.74	0.567

[1]Treatments: various treatments for extrahepatic metastases (surgical resection, TACE, systemic chemotherapy and/or radiotherapy); [2]Site: site of extrahepatic metastases.

Curative therapy was performed in 10 patients (6 patients with lymph node metastases and 4 patients with adrenal gland metastases). Palliative therapy was performed in the remaining 26 patients who had severe pain due to bone metastases. Furthermore, 9 patients with painful bone metastases were treated with RFA therapy combined with cementoplasty[21]. Nonsteroidal anti-inflammatory drugs or opioids were used in patients with bone metastases due to severe pain.

Survival data

The cumulative survival rates of the 151 HCC patients with extrahepatic metastases after initial diagnosis of extrahepatic metastases at 6, 12, 24, and 36 mo were 44.1%, 21.7%, 14.2%, and 7.1%, respectively (Figure 1A). The median survival period was 4.9 mo (range, 1-37 mo). Survival was compared among patients with intrahepatic tumor stage T0-T2 and T3, T4 (Figure 1B). The rate was significantly higher in the intrahepatic tumor stage T0-T2 groups than in the T3, T4 groups ($P < 0.001$). We investigated the determinants of survival after initial diagnosis of extrahepatic metastases. Univariate analysis identified the following 9 factors significantly influencing survival: PS, 0 ($P < 0.001$); Child-Pugh grade, A ($P < 0.001$); intrahepatic main tumor size, < 50 mm ($P < 0.001$); intrahepatic tumor volume, < 50% ($P < 0.001$); portal venous invasion, Vp 0-2 ($P < 0.001$); use of treatment for extrahepatic metastases ($P < 0.001$, Figure 1C); bone metastasis ($P = 0.012$); DCP < 1000 mAU/mL ($P = 0.02$); and nodular type intrahepatic tumor ($P = 0.03$) (Table 3). Since the variables could be mutually correlated, multivariate analysis was performed. The analysis identified the following four variables as significant and independent determinants of survival after initial diagnosis of extrahepatic metastases: PS ($P < 0.001$), portal venous invasion ($P < 0.001$), treatment of extrahepatic metastases ($P = 0.003$), and Child-Pugh grade ($P = 0.009$) (Table 4).

Table 4 Multivariate analysis of predictors of survival after initial diagnosis of extrahepatic metastases among 151 patients

Variable	Hazard ratio	95% CI	P
PS (0 *vs* 1-4)	5.576	2.431-12.152	< 0.001
Vp (0-2 *vs* 3, 4)	4.792	2.137-10.712	< 0.001
Treatment (performed *vs* not performed)	4.134	1.539-11.011	0.003
Child pugh stage (A *vs* B, C)	2.372	1.247-4.914	0.008

Causes of death

Twenty-five patients were still alive at the end of this study while 126 patients died. Of the latter group, intrahepatic tumor stages at the first diagnosis of extrahepatic metastases were T0-2 in 17 patients and T3-4 in 109 patients. One hundred and twelve (89%) patients died of intrahepatic HCC or liver failure. Fourteen (11%) patients died of extrahepatic HCC (Table 5). Eight patients died of respiratory failure due to lung metastases. Four patients died of bone metastases-related disease. Two patients died of obstructive jaundice due to portohepatic node metastasis.

Of the 4 patients who died of bone metastases-related disease, 3 died of intracranial hypertension due to skull metastasis. Another patient died of vertebra metastasis-related disease. He was 69-year old at first diagnosis of bone metastases. He suffered from complete spinal cord injury due to vertebral metastasis with gradual worsening of PS. Finally, PS changed to 4 and the patient died of aspiration-related pneumonia. The survival period after first diagnosis of extrahepatic metastases was 11.5 mo.

Among the 14 patients who died of extrahepatic HCC, 3 had chronic hepatitis, 7 had cirrhosis of Child-Pugh grade A, 3 had cirrhosis of Child-Pugh grade B, and 1 had cirrhosis of Child-Pugh grade C. All patients who died of extrahepatic HCC with the exception of that with Child-Pugh grade C had some hepatic reserve until death. Intrahepatic tumor stage at first diagnosis of extrahepatic metastases was T0 (3 patients), T1 (4 patients), T2 (1 patient), T3 (5 patients), and T4 (1 patient). All 8 patients with intrahepatic tumor stage T0-T2 were treated previously for intrahepatic HCC. Eight of 17 (47%) patients with intrahepatic tumor stage T0-T2 died of extrahepatic metastases. On the other hand, 6 of 109 (6%) patients with intrahepatic tumor stages T3 and T4 died of extrahepatic metastases. The mortality rate of patients with intrahepatic tumor stage T0-T2 was significantly higher than that of patients with intrahepatic tumor stages T3 and T4 ($P = 0.001$) (Table 6).

Table 5 Clinical profile of 14 patients who died of extrahepatic metastases during the follow-up period

Case	Presentation	Site	Intrahepatic HCC stage	Sex	Age (yr)	Child-Pugh stage	Etiology
1	R	Lung	T3	M	65	A	HCV
2	R	Lung	T4	M	35	CH	HBV
3	R	Lung	T3	M	56	A	HBV
4	R	Lung, vertebra	T0	M	40	CH	HBV
5	R	Lung, vertebra	T1	M	69	A	HBV
6	R	Lung, LN	T0	M	63	B	HBV
7	R	Lung, vertebra, nasal	T0	M	50	A	HBV
8	R	Lung	T3	M	73	A	NBNC
9	I	Skull	T1	M	57	A	HCV
10	I	Skull	T2	F	72	C	HCV
11	I	Skull	T3	M	56	B	HCV
12	A	Vertebra	T3	M	69	A	HCV
13	O	Lung, rib, LN	T1	M	74	A	HCV
14	O	Vertebra, LN	T1	M	70	B	HCV

All patients with intrahepatic tumor stage T0-T2 were treated previously for intrahepatic HCC. R: respiratory failure; CH: chronic hepatitis; LN: lymph node; NBNC: no hepatitis B virus or hepatitis C virus; I: intracranial hypertension symptom; A: aspiration-related pneumonia; O: obstructive jaundice.

Table 6 Causes of death of 126 HCC patients with extrahepatic metastases

Intrahepatic tumor stage	Intrahepatic HCC or liver failure	Extrahepatic HCC
T0-2 ($n = 17$)	53% (9/17)	47% (8/17)
T3-4 ($n = 109$)	94% (103/109)	6% (6/109)

DISCUSSION

The prognosis of HCC patients with extrahepatic metastases is unsatisfactory[16,17] and often not well known[18]. In the present study, we assessed the clinical features and prognosis of 151 consecutive HCC patients with extrahepatic metastases. The incidence of extrahepatic metastases from HCC was 15.2%. The most frequent metastatic sites were the lung, lymph nodes, bone, and adrenal gland. The cumulative survival rates of the 151 patients after the initial diagnosis of extrahepatic metastases at 6, 12, 24, and 36 mo were 44.1%, 21.7%, 14.2%, 7.1%, respectively. The median survival period was 4.9 mo (range, 1-37 mo). The mortality rate due to extrahepatic metastases from HCC was 11% (14/126).

Extrahepatic metastases have been reported to occur in 13.5%-42% of HCC patients[22-24]. In this study, the prevalence of extrahepatic metastases was 15.2%. Though we screened all HCC patients at regular intervals for intra/extra hepatic metastases, not all patients received a full metastatic follow up based on the use of several diagnostic techniques. Since the majority of HCC patients with extrahepatic metastases were asymptomatic, it is possible to miss asymptomatic metastases such as those in the lungs, distant lymph nodes, muscles and rectum.

Based on the initial diagnosis of intrahepatic HCC, Natsuizaka *et al*[16] reported that patients with advanced HCC develop extrahepatic metastases significantly more frequently than those with less advanced HCC. At the initial diagnosis of extrahepatic metastases, many HCC patients with extrahepatic metastases have been reported

to have advanced intrahepatic stage[16,22]. In our study, 123 (81%) patients with extrahepatic metastases had intrahepatic tumor stages T3 (28%) and T4 (53%), at the initial diagnosis of extrahepatic metastases, suggesting that HCC patients with advanced intrahepatic tumor stage (T3, T4) are at risk of developong extrahepatic metastases, and that such patients should be followed up carefully.

On the other hand, our study identified 28 (19%) patients with early intrahepatic tumor stage (T0-T2) at the initial diagnosis of extrahepatic HCC. Eight of the 17 (47%) patients later died of extrahepatic metastases. With regard to previous treatment, 27 of 28 patients with early intrahepatic tumor stage were treated previously for intrahepatic HCC. Considering the possibility of extrahepatic metastases, HCC patients with early intrahepatic tumor stage should be followed up carefully, particurally those who have been treated previously for intrahepatic HCC. This also includes HCC patients who have received complete resection or ablation.

In this study, the most frequent metastatic sites were the lungs, lymph nodes, bones, and adrenal glands. Other studies have reported similar findings[16,22]. HCC is thought to spread mainly *via* the hematogenous route, thus causing intra/extra hepatic metastases. Most of HCCs are hypervascular tumors. Moreover, HCC tends to invade vessels, such as portal and hepatic veins. Therefore, HCC could spread through the lung and systemic circulation via the hepatic or portal vein. This could explain why the lung is the most frequent site of metastases in HCC. Most of HCC patients with lung metastases are asymptomatic. To detect lung metastases from HCC, chest CT should be performed at regular intervals during routine metastasis follow-up.

Though there is no standard treatment for extrahepatic metastases of primary HCC, several authors have reported the use of various treatment modalities for extrahepatic m etastases[7,15,23,25-29]. Some reports have described successful treatment of extrahepatic metastases with no or few intrahepatic HCC[7,25-27]. However, only few HCC patients can undergo surgical resection of extrahepatic metastases because of hepatic reserve or intraheatic tumor stage. In this study, the prognosis of 3 patients after surgical resection of extrahepatic metastases seemed good. These 3 patients had good hepatic reserve, no intrahepatic HCC (PS = 0) and no intra/extra hepatic HCC and are expected to have good prognosis. The clinical features of HCC patients with extrahepatic metastases varied widely. All patients were not symptomatic and thus not necessary to receive treatment of extrahepatic metastases. Thus, treatment of extrahepatic metastases from primary HCC must be performed carefully taking into consideration the clinical features.

Multivariate analysis in our study identified PS, portal venous invasion, treatment for extrahepatic metastases, and Child-Pugh grade as important determinants of survival after the initial diagnosis of extrahepatic metastases. Ishii *et al*[17] reported that brain metastases, number of metastatic tumors and primary tumor status are important factors for survival. In our study, only two patients had brain metastases. With regard to the number of metastatic tumors, we might miss asymptomatic metastases. Thus,

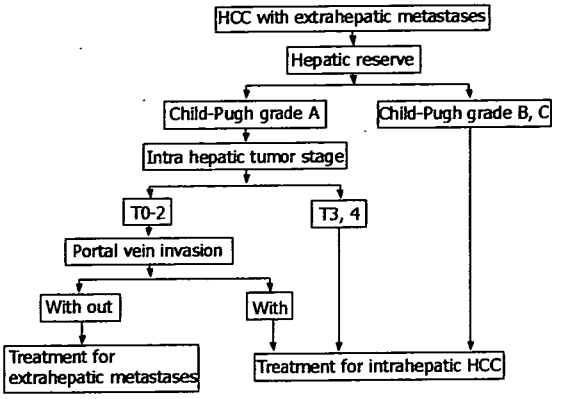

Figure 2 Initial sites to be treated.

we did not include brain metastasis and number of metastatic tumors in this multivariate analysis. Treatment of extrahepatic metastases was an important determinant of survival in our study. There might be selection bias of patients treated for extrahepatic metastases because many of them had good hepatic reserve. HCC patients with poor hepatic reserve did not receive treatment for extrahepatic metastases in this study. Regardless of such bias, treatment of extrahepatic metastases might be important for improvement of prognosis.

With regard to the cause of death, many HCC patients with extrahepatic metastases died of intrahepatic HCC or liver failure and few (11%) died of extrahepatic HCC. Of the 14 patients who died of extrahepatic metastases, 10 had good hepatic reserve and 8 had early intrahepatic tumor stage, at the initial diagnosis of extrahepatic metastases. Usually, HCC patients with good hepatic reserve, no or few intrahepatic HCCs, and those without portal venous invasion show relatively good prognosis. According to the univariate analysis of HCC patients with extrahepatic metastases, patients with early intrahepatic tumor stage have a significantly better prognosis than those with advanced intrahepatic tumor stage. In our study, the mortality rate due to extrahepatic metastases with early intrahepatic tumor stage was significantly higher than that due to those with advanced intrahepatic tumor stage. This might be explained by the differences in survival periods between these intrahepatic tumor stage groups. Extrahepatic metastases with early intrahepatic tumor stage can spread during the relatively long survival period, and few patients die of extrahepatic metastases. Extrahepatic metastasis with early intrahepatic tumor stage is a very important cause of death of HCC patients. Successful treatment of extrahepatic metastases in HCC patients with early intrahepatic tumor stage might improve the prognosis.

In conclusion, the majority of HCC patients with extrahepatic metastases should undergo treatment for intrahepatic HCC. Selected HCC patients with critical extrahepatic metastases could undergo treatment for extrahepatic metastases. However, these selected patients must have good hepatic reserve, intrahepatic tumor stage: T0-T2, and are free of portal venous invasion (Figure 2). The important sites of critical metastases from primary

HCC are the lungs, bones and the portohepatic node. Further studies are needed for the improvement of the prognosis of HCC patients with extrahepatic metastases.

REFERENCES

1 **Poon RT**, Fan ST, Lo CM, Ng IO, Liu CL, Lam CM, Wong J. Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years. *Ann Surg* 2001; **234**: 63-70

2 **Wu MC**, Shen F. Progress in research of liver surgery in China. *World J Gastroenterol* 2000; **6**: 773-776

3 **Tang Z**. Recent advances in clinical research of hepatocellular carcinoma in China. *Chin Med J (Engl)* 1995; **108**: 568-570

4 **Lo CM**, Lai EC, Fan ST, Choi TK, Wong J. Resection for extrahepatic recurrence of hepatocellular carcinoma. *Br J Surg* 1994; **81**: 1019-1021

5 **Lam CM**, Lo CM, Yuen WK, Liu CL, Fan ST. Prolonged survival in selected patients following surgical resection for pulmonary metastasis from hepatocellular carcinoma. *Br J Surg* 1998; **85**: 1198-1200

6 **Kuromatsu R**, Hirai K, Majima Y, Fujimoto T, Shimauchi Y, Tsukiyama Y, Aoki E, Saitsu H, Nakashima O, Kojiro M. A patient with hepatocellular carcinoma who underwent resection of the primary lesion 10 years ago and resection of a giant adrenal metastasis 8 and a half years later. *Gastroenterol Jpn* 1993; **28**: 312-316

7 **Inagaki Y**, Unoura M, Urabe T, Ogino H, Terasaki S, Matsushita E, Kaneko S, Morioka T, Furusawa A, Wakabayashi T. Distant metastasis of hepatocellular carcinoma after successful treatment of the primary lesion. *Hepatogastroenterology* 1993; **40**: 316-319

8 **Okazaki N**, Yoshino M, Yoshida T, Hirohashi S, Kishi K, Shimosato Y. Bone metastasis in hepatocellular carcinoma. *Cancer* 1985; **55**: 1991-1994

9 **Kay RM**, Eckardt JJ, Goldstein LI, Busuttil RW. Metastatic hepatocellular carcinoma to bone in a liver transplant patient. A case report. *Clin Orthop Relat Res* 1994; : 237-241

10 **Knight TE**, Woo AS Jr, Blaisdell JM. Hepatocellular carcinoma invasive to chest wall. *Int J Dermatol* 1992; **31**: 273-276

11 **Kim PN**, Kim IY, Lee KS. Intraperitoneal seeding from hepatoma. *Abdom Imaging* 1994; **19**: 309-312

12 **Barasch E**, Frazier OH, Silberman H, Shannon RL, Wilansky S. Left atrial metastasis from hepatocellular carcinoma: a case report. *J Am Soc Echocardiogr* 1994; **7**: 547-549

13 **Fujimoto H**, Murakami K, Nosaka K, Arimizu N. Splenic metastasis of hepatocellular carcinoma. Accumulation of Tc-99m HDP. *Clin Nucl Med* 1992; **17**: 99-100

14 **Kim HS**, Shin JW, Kim GY, Kim YM, Cha HJ, Jeong YK, Jeong ID, Bang SJ, Kim do H, Park NH. Metastasis of hepatocellular carcinoma to the small bowel manifested by intussusception. *World J Gastroenterol* 2006; **12**: 1969-1971

15 **Zeng ZC**, Tang ZY, Fan J, Zhou J, Qin LX, Ye SL, Sun HC, Wang BL, Zhang JY, Yu Y, Cheng JM, Wang XL, Guo W. Radiation therapy for adrenal gland metastases from hepatocellular carcinoma. *Jpn J Clin Oncol* 2005; **35**: 61-67

16 **Natsuizaka M**, Omura T, Akaike T, Kuwata Y, Yamazaki K, Sato T, Karino Y, Toyota J, Suga T, Asaka M. Clinical features of hepatocellular carcinoma with extrahepatic metastases. *J Gastroenterol Hepatol* 2005; **20**: 1781-1787

17 **Ishii H**, Furuse J, Kinoshita T, Konishi M, Nakagohri T, Takahashi S, Gotohda N, Nakachi K, Yoshino M. Extrahepatic spread from hepatocellular carcinoma: who are candidates for aggressive anti-cancer treatment? *Jpn J Clin Oncol* 2004; **34**: 733-739

18 **Okusaka T**, Okada S, Ishii H, Nose H, Nagahama H, Nakasuka H, Ikeda K, Yoshimori M. Prognosis of hepatocellular carcinoma patients with extrahepatic metastases. *Hepatogastroenterology* 1997; **44**: 251-257

19 **Oken MM**, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, Carbone PP. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982; **5**: 649-655

20 Liver Cancer Study Group of Japan. The general rules for the clinical and pathological study of primary liver cancer (in Japanese). 4th ed. Tokyo: Kanehara, 2000: 19

21 **Toyota N**, Naito A, Kakizawa H, Hieda M, Hirai N, Tachikake T, Kimura T, Fukuda H, Ito K. Radiofrequency ablation therapy combined with cementoplasty for painful bone metastases: initial experience. *Cardiovasc Intervent Radiol* 2005; **28**: 578-583

22 **Katyal S**, Oliver JH 3rd, Peterson MS, Ferris JV, Carr BS, Baron RL. Extrahepatic metastases of hepatocellular carcinoma. *Radiology* 2000; **216**: 698-703

23 **Shuto T**, Hirohashi K, Kubo S, Tanaka H, Yamamoto T, Higaki I, Takemura S, Kinoshita H. Treatment of adrenal metastases after hepatic resection of a hepatocellular carcinoma. *Dig Surg* 2001; **18**: 294-297

24 **Si MS**, Amersi F, Golish SR, Ortiz JA, Zaky J, Finklestein D, Busuttil RW, Imagawa DK. Prevalence of metastases in hepatocellular carcinoma: risk factors and impact on survival. *Am Surg* 2003; **69**: 879-885

25 **Nakayama H**, Takayama T, Makuuchi M, Yamasaki S, Kosuge T, Shimada K, Yamamoto J. Resection of peritoneal metastases from hepatocellular carcinoma. *Hepatogastroenterology* 1999; **46**: 1049-1052

26 **Kurachi K**, Suzuki S, Yokoi Y, Okumura T, Inaba K, Igarashi T, Takehara Y, Konno H, Baba S, Nakamura S. A 5-year survivor after resection of peritoneal metastases from pedunculated-type hepatocellular carcinoma. *J Gastroenterol* 2002; **37**: 571-574

27 **Lam CM**, Lo CM, Yuen WK, Liu CL, Fan ST. Prolonged survival in selected patients following surgical resection for pulmonary metastasis from hepatocellular carcinoma. *Br J Surg* 1998; **85**: 1198-1200

28 **Momoi H**, Shimahara Y, Terajima H, Iimuro Y, Yamamoto N, Yamamoto Y, Ikai I, Yamaoka Y. Management of adrenal metastasis from hepatocellular carcinoma. *Surg Today* 2002; **32**: 1035-1041

29 **Zeng ZC**, Tang ZY, Fan J, Qin LX, Ye SL, Zhou J, Sun HC, Wang BL, Wang JH. Consideration of role of radiotherapy for lymph node metastases in patients with HCC: retrospective analysis for prognostic factors from 125 patients. *Int J Radiat Oncol Biol Phys* 2005; **63**: 1067-1076

S- Editor Liu Y **L- Editor** Wang XL **E- Editor** Lu W

Journal of Medical Virology 79:1811–1817 (2007)

Successful Treatment of an Entecavir-Resistant Hepatitis B Virus Variant

Hiromi Yatsuji,[1,2,3] Nobuhiko Hiraga,[1,2] Nami Mori,[1,2] Tsuyoshi Hatakeyama,[1,2] Masataka Tsuge,[1,2] Michio Imamura,[1,2] Shoichi Takahashi,[1,2] Yoshifumi Fujimoto,[2] Hidenori Ochi,[2,4] Hiromi Abe,[1,4] Toshiro Maekawa,[4] Fumitaka Suzuki,[3] Hiromitsu Kumada,[3] and Kazuaki Chayama[1,2,4]*

[1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*
[2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[3]*Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan*
[4]*Laboratory for Liver Disease, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan*

Emergence of a lamivudine (LAM)-resistant hepatitis B virus (HBV) with amino acid substitutions in the YMDD motif is a well-documented problem during long-term LAM therapy. Entecavir (ETV) is a new drug approved for treatment of HBV infection with or without LAM-resistant mutants. This report describes an ETV-resistant strain of HBV, which emerged after prolonged ETV therapy in a patient who did not respond to LAM therapy. Direct sequence analysis of the ETV-resistant strain showed appearance of amino acid substitution rtS202G in the reverse transcriptase (RT) domain, together with rtL180M + M204V substitution that had developed at the emergence of LAM-resistant mutant. In vitro analysis demonstrated that the rtL180M + M204V + S202G mutant strain displayed a 200-fold and a 5-fold reduction in susceptibility to ETV compared with the wild-type and the rtL180M + M204V mutant strain, respectively. Adefovir was effective against the ETV-resistant strain both in vitro and during the clinical course. In conclusion, this study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV and confirmed that the addition of rtS202G substitution to the rtL180M + M204V mutant strain is responsible for ETV resistance and we could treat the resistant mutant successfully. ***J. Med. Virol. 79:1811–1817, 2007.*** 2007 Wiley-Liss, Inc.

KEY WORDS: HBV; rtS202G; lamivudine; adefovir; in vitro

2007 WILEY-LISS, INC.

INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma [Bruix and Llovet, 2003; Ganem and Prince, 2004]. To date, interferon and three nucleoside and nucleotide analogs (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and improve transaminase levels and liver histology [Nevens et al., 1997; Lai et al., 1998; Suzuki et al., 1999]. However, prolonged therapy results in the emergence of drug-resistant mutants.

LAM is associated with a higher rate of emergence of drug-resistant mutants than ADV or ETV, which is 24% and 70% after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels [Lai et al., 2003]. Most LAM-resistant

Abbreviations used: HBeAg,hepatitis B e antigenHBsAg,hepatitis B surface antigenHBV,hepatitis B virusORF,open reading framePCR,polymerase chain reactionRT,reverse transcriptase

Grant sponsor: Ministry of Education, Sports, Culture, and Technology; Grant sponsor: Ministry of Health, Labor and Welfare.

*Correspondence to: Kazuaki Chayama, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima-shi 734-8551, Japan.
E-mail: chayama@hiroshima-u.ac.jp

Accepted 28 June 2007
DOI 10.1002/jmv.20981
Published online in Wiley InterScience (www.interscience.wiley.com)

WILEY InterScience® DISCOVER SOMETHING GREAT

strains show amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [Allen et al., 1998; Delaney et al., 2003].

Both in vitro and clinical studies have shown recently that ADV and ETV could suppress both wild-type and LAM-resistant strains and were confirmed as salvage therapy for LAM-refractory patients [Levine et al., 2003; Sherman et al., 2006; Rapti et al., 2007]. However, a few studies have already reported the emergence of resistant mutants to these drugs.

ADV-resistant mutations are infrequent and their appearance is delayed in treatment-naïve patients; mutation occurs at 0% after 1 year and 28% after 5 years and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [Maecellin and Asselah, 2005]. On the other hand, the emergence rate of ADV-resistant mutations in LAM-resistant patients was 18% after 48 weeks of ADV monotherapy [Lee et al., 2006]. A recent study reported patients treated with combination therapy of ADV with LAM did not develop resistance to ADV for 3 years [Rapti et al., 2007].

ETV is the most novel nucleotide analogue of the three drugs and displays greater in vitro potency than LAM or ADV against wild-type HBV. ETV-resistance is reported to be rare in treatment-naïve patients [Colonno et al., 2006]. However, ETV-resistant mutants appeared at 6–9% per year in LAM-refractory patients [Tenney et al., 2004, 2007; Sherman et al., 2006].

In the present study, an ETV-resistant strain of HBV was identified after prolonged ETV therapy in a patient who did not respond to LAM therapy. To our knowledge, this is the first report that breakthrough hepatitis was induced by emergence of an ETV-resistant strain and was successfully treated with ADV. This study checked the importance of amino acid substitutions in the HBV polymerase for resistance to ETV in vitro. Furthermore, the susceptibility of the mutant strain to ADV was analyzed.

MATERIALS AND METHODS

Antiviral Compounds

LAM [( )-β-L-2′, 3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, UK). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and ETV {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingfold, CT).

Analysis of Virological Markers

Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) were determined by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using the Light Cycler (Roche, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAA-CAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Cloning of HBV-DNA and Plasmid Construction

HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. The full-length HBV-DNA was amplified using the above HBV-DNA samples by the method of Gunther et al. [1998]. Nucleotide sequence positions were numbered from the unique *Eco*RI site. The 1.4 genome lengths HBV-DNA amplified from the serum of a patient who showed ETV resistance was cloned into a plasmid vector pcDNA3 (Invitrogen, San Diego, CA). In brief, the PCR product amplified using serum from the patient was cleaved with *Bam*HI and *Apa*I (HBV positions 1,400–2,600) and cloned into pcDNA3, which was named pcDNA3-1. Similarly, the PCR product was cleaved with *Apa*I and *Bam*HI (HBV positions 2,600–3,215, 1–1,400) and cloned into pBluerscript SK+ (Stratagene, La Jolla, CA), which was named pB-1. The *Kpn*I-*Bam*HI fragment from pB-1 and *Kpn*I-*Apa*I fragment from pcDNA3-1 were cloned into pcDNA3-1. To introduce the nucleotide substitutions into the rtL180M, M204V, and S202G, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Four plasmids with/without amino acid substitutions were created and are listed in Table IV.

Cell Culture, Transfection, and Determination of IC_{50}

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C under 5% CO_2. Cells were seeded to semi-confluence in 6-well tissue culture plates. Transient transfection of the plasmids into HepG2 cell lines was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each anti-viral drug, various concentrations of LAM, ADV, and ETV were added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The medium containing the drugs was changed at days 1, 3, and 4. All experiments were performed in triplicate. GraphPad prism (GraphPad Prism Software, Inc., San Diego, CA) was used to determine the best-fit values for individual dose–response equations.

Analysis of Replicative Intermediate of HBV by Quantitation

The cells were harvested at 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH

7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000*g*. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec, and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Statistical Analysis

Data are expressed as mean SD. Group comparisons were performed using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Patient's Profile

An ETV-resistant strain of HBV was isolated from a 44-year-old Japanese woman with hepatitis B e antigen-positive chronic HBV infection (Fig. 1A). In this patient, LAM successfully reduced the HBV at the initial stage of treatment. However, viral breakthrough was observed at 11 months after the beginning of LAM therapy and the HBV viral load reached up to 7.5 log copies/ml. After 17 months of LAM, interferon was added to LAM therapy for 6 months. However, after withdrawal of IFN, the viral load and ALT rebounded. Thus, the patient was switched to 0.5 mg of ETV. This resulted in reduction of HBV-DNA and normalization of ALT. After 12 months of ETV therapy, the viral load rebounded, and following 12 more months of ETV, breakthrough hepatitis was observed. After stopping ETV, because of the inadequate effect of IFN monotherapy for one month, the patient was switched to 10 mg of ADV. This treatment reduced both the viral load and ALT level to acceptable levels (Fig. 1).

treatment		month	ALT (IU/L)	HBV-DNA (log copies/ml)
		-3	246	7.2
LAM		0	46	5.2
		5	28	3.7
		11	33	4.1
	IFN	17	72	7.5
		18	1184	5.6
		20	39	3.9
		23	34	3.4
		27	117	7.1
ETV		31	112	7.2
		39	40	2.9
		43	28	4.2
	IFN	56	140	6.8
ADV		57	313	6.8
		60	38	4
	LAM	71	24	3.3
		75	19	3.1

Fig. 1. Clinical course of a patient who developed entecavir resistant mutant.

Isolation of a Multiple Drug-Resistant Hepatitis Strain

Isolates from this patient were analyzed for substitutions in HBV reverse transcriptase (RT). Comparison of the nucleotide sequences by the direct sequence method obtained throughout the clinical course showed three amino acid substitutions in the RT domain of the polymerase (Table I). At the baseline of LAM, all three substitutions were of the wild-type by direct sequence analysis and clonal analysis (Table II). After breakthrough hepatitis induced by LAM, direct sequence analysis showed mixed type (YIDD and YVDD) mutant strain. The rtM204V mutant was detected in 65% of HBV clones and the rest were all the YIDD type. Importantly, at this point, there was no amino acid substitution at rt202. After 12 months of ETV therapy when the viral load was slightly increased, the rtL180M + M204V + S202G mutant was detected in 45% of the HBV clones, followed by decrease of the YIDD and YVDD mutants without substitution at rtS202G. Finally, after 24 months of ETV therapy, when the breakthrough hepatitis occurred, the rtL180M + M204V + S202G mutant was detected in 92% of the HBV clones and the rest were rtL180M + M204V mutants without substitution at rtS202G. Interestingly, the rtM204I + S202G strain never appeared during nucleotide therapy.

Susceptibility of Mutants to Entecavir In Vitro

To analyze the role of the rtL180M, rtG202S, and rtM204V substitutions in ETV resistance, four patient-specific strains were transfected into HepG2 cells (Table III). ETV was added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The core-associated HBV genome was extracted from cells and quantified by real-time PCR. The double amino acid substitutions rtL180M + M204V, which is related to LAM resistance, displayed a 38-fold decrease in susceptibility to ETV compared with the wild-type. Moreover, triple amino acid substitutions rtL180M + M204V + S202G, isolated from the patient

TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

	rt L180	rt S202	rt M204
(1) At the beginning of LMV	—	—	—
(2) At the beginning of ETV	L/M	—	I/V
(3) One year after ETV	M	G/S	V
(4) Two years after ETV	M	G	V

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC_{50} values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

	Relative rate (%) of clones (no. of clones/total)			
	Wild	M204I	L180M + M204V	L180M + M204V + S202G
(1) At the beginning of LMV	100 (6/6)	0	0	0
(2) At the beginning of ETV	0	35 (7/20)	65 (13/20)	0
(3) 12 months after ETV	0	14 (3/22)	41 (9/22)	45 (10/22)
(4) 24 months after ETV	0	0	8 (1/13)	92 (12/13)

LMV, lamivudine.

TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

				ETV	
	rt L180	rt M204	rt S202	IC_{50} (μM)	Resistance (fold)
Wild	—	—	—	0.00081	1
S202G	—	—	G	0.00054	0.67[a]
L180M + M204V	M	V	—	0.031	38**
L180M + M204V + S202G	M	V	G	0.16	198**

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo. Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

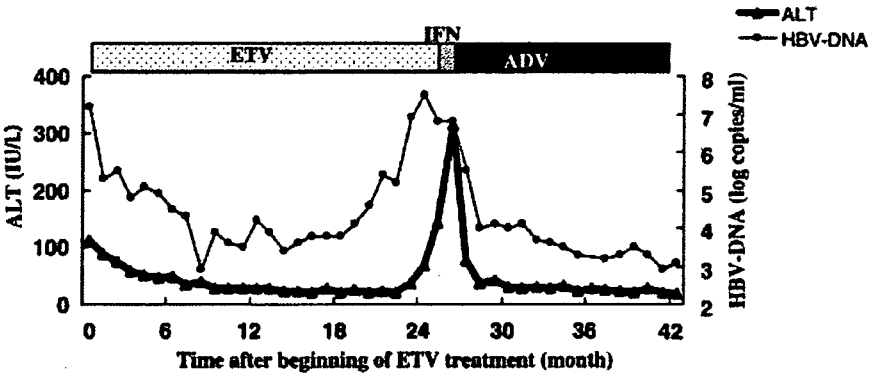

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

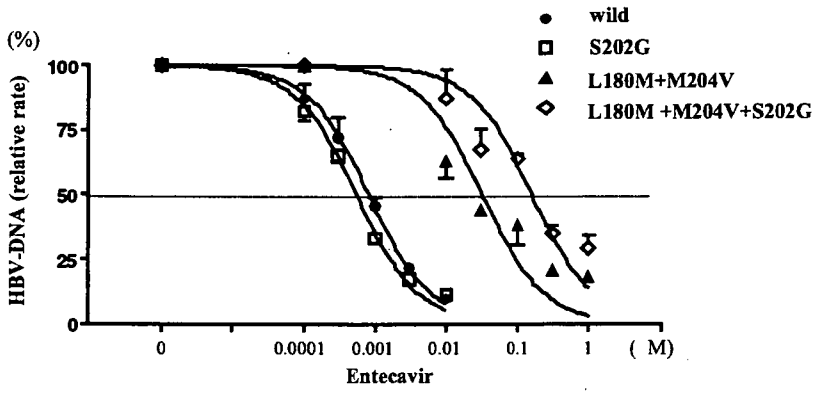

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose–response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC_{50} values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

	LAM		ADV	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
Wild	0.1	1	0.39	1
L180M + M204V	>100	>1,000**	—	—
L180M + M204V + S202G	>100	>1,000**	0.32	0.82[a]

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

ACKNOWLEDGMENTS

This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. The authors thank Kana Kunihiro, Rie Akiyama, Yoshiko Seo, Yoshiko Nakata, and Eiko Miyoshi for their excellent technical assistance. This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Education, Sports, Culture, and Technology and the Ministry of Health, Labor and Welfare.

REFERENCES

Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 27:1670–1677.

Bruix J, Llovet JM. 2003. Hepatitis B virus and hepatocellular carcinoma. J Hepatol 39:S59–S63.

Colonno RJ, Rose R, Baldick CJ, Leveine S, Pokornowski K, Yu CF, Walsh A, Fang J, Hsu M, Mazzucco C, Eggers B, Zhang S, Plym M, Klesczewski K, Tenney DJ. 2006. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology 44:1656–1665.

Delaney WE, Yang H, Westland CE, Das K, Arnold E, Gibbs CS, Miller MD, Xiong S. 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J Virol 77:11833–11841.

Ganem D, Prince AM. 2004. Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 350:1118–1129.

Gunther S, Sommer G, Von Breunig F, Iwanska A, Kalinina T, Sterneck M, Will H. 1998. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: Frequency and functional consequences of PCR-introduced mutations. J Clin Microbiol 36:531–538.

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

Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. 2003. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis 36:687–696.

Lee YS, Suh DJ, Lim YS, Jung SW, Lee HC, Chung YH, Lee YS, Yoo W, Kim SO. 2006. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. Hepatology 43:1385–1391.

Levine SD, Hernandez G, Yamanaka S, Zhang R, Rose R, Weinheimer S, Colonno RJ. 2002. Efficacies of entecavir against lamivudine-resistant hepatitis B virus replication and recombinant polymerases in vitro. Antimicrob Agents Chemother 46:2525–2532.

Maecellin P, Asselah T. 2005. Resistance to adefovir: A new challenge in the treatment of chronic hepatitis B. J Hepatol 43:920–923.

Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, De Man RA, Thomas HC. 1997. Lamivudine therapy for chronic hepatitis B: A six-month randomized dose-ranging study. Gastroenterology 113:1258–1263.

Ono SK, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, Carrilho FJ, Omata M. 2001. The polymerase L528M mutation cooperates

with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 107:449–455.

Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. 2007. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. Hepatology 45:307–313.

Sherman MC, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, Boron-Kaczmarska, A, Martin P, Goodman Z, Colonno R, Cross A, Denisky G, Kreter B, Hindes R. 2006. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. Gastroenterology 130:2039–2049.

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.

Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, Plym M, Pokornowski K, Yu CF, Angus P, Ayres A, Bartholomeusz A, Sievert W, Thompson G, Warner N, Locarnini S, Colonno RJ. 2004. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. Antimicrob. Agents Chemother. 48:3498–3507.

Tenney DJ, Rose RE, Baldick CJ, Levine SM, Pokornowski KA, Walsh AW, Yu CF, Zhang S, Mazzucco CE, Eggers B, Hsu M, Plym MJ, Poundstone P, Yang J, Colonno RJ. 2007. Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. Antimicrob Agents Chemother 51:902–911.

Villet S, Ollivet A, Pichoud C, Barraud L, Villeneuve JP, Trepo C, Zoulim F. 2007. Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. J Hepatol 46:531–538.

Yim HJ, Hussain M, Liu Y, Wong SN, Fung SK, Lok SF. 2006. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology 44:703–712.

Serum HBV RNA is a Predictor of Early Emergence of the YMDD Mutant in Patients Treated with Lamivudine

Tsuyoshi Hatakeyama,[1,2] Chiemi Noguchi,[1,2] Nobuhiko Hiraga,[1,2] Nami Mori,[1,2] Masataka Tsuge,[1,2] Michio Imamura,[1,2] Shoichi Takahashi,[1,2] Yoshiiku Kawakami,[1,2] Yoshifumi Fujimoto,[2,3] Hidenori Ochi,[2,3] Hiromi Abe,[1,2,3] Toshiro Maekawa,[3] Hiroiku Kawakami,[4] Hiromi Yatsuji,[1,2] Yasuyuki Aisaka,[5] Hiroshi Kohno,[5] Shiomi Aimitsu,[5] and Kazuaki Chayama[1,2,3]

Lamivudine (LAM) is a nucleoside analogue widely used for the treatment of chronic hepatitis B virus (HBV) infection. Emergence of resistant strains with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of reverse transcriptase is a serious problem in patients on LAM therapy. The amount of covalently closed circular DNA in the serum is reported to be higher in patients who develop YMDD mutants than in those without mutants. However, there is no useful serum marker that can predict early emergence of mutants during LAM therapy. Analysis of patients who were treated with entecavir (n = 7) and LAM (n = 36) showed some patients had high serum levels of HBV RNA. Median serum levels of HBV RNA were significantly higher in patients in whom the YMDD mutant had emerged within 1 year (n = 6, 1.688 log copies/ml) than in those in whom the YMDD mutant emerged more than 1 year after treatment (n = 12, 0.456 log copies/ml, $P = 0.0125$) or in whom the YMDD mutant never emerged (n = 18, 0.688 log copies/ml, $P = 0.039$). Our results suggest that HBV RNA is a valuable predictor of early occurrence of viral mutation during LAM therapy. (HEPATOLOGY 2007;45:1179-1186.)

The hepatitis B virus (HBV) is a member of the hepadnaviridae family. Worldwide, approximately 350 million people are estimated to be chronically infected with HBV.[1] Patients with chronic HBV infection develop chronic hepatitis, cirrhosis, and hepatocellular carcinoma, accounting for approximately 1 million deaths per year.[2] Recently, inhibitors of reverse transcriptase have been developed and widely used for patients with chronic HBV infection. Lamivudine (LAM), a cytosine nucleoside analogue, was first developed as an antiviral agent against HIV and later was used effectively against HBV because HBV also uses reverse transcriptase for replication.[3,4] Because LAM suppresses HBV replication, patients who are treated with LAM show a decreased level or disappearance of HBV DNA in serum and hepatitis B e antigen, normalization of serum alanine aminotransferase (ALT) level, and histological improvement.[5-12] However, discontinuation of therapy often leads to reactivation of HBV.[6,8,13,14] Therefore, long-term therapy is necessary for many patients with chronic HBV infection. During long-term LAM therapy, drug-resistant mutants with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif emerge, resulting in expression of HBV DNA increasing again and in worsening of hepatitis.[6,10,15-18] Moreover, some patients develop a severe flare-up of hepatitis that could lead to fatal hepatic failure. Therefore, prediction of the emergence of YMDD mutants is an important issue.

In our hunt for useful serum markers to detect the early emergence of YMDD mutants, we noticed some patients who showed a discrepancy in the expression of HBV DNA measured by the transcription-mediated amplifica-

Abbreviations: cccDNA, covalently closed circular DNA; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; PCR, polymerase chain reaction; RT, reverse transcription; TMA-HPA, transcription-mediated amplification and hybridization protection assay; YMDD, tyrosine-methionine-aspartate-aspartate.

From the [1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima-shi, Japan; [2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan; [3]Laboratory for Liver Disease, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan; [4]Kawakami Clinic, Hiroshima-shi, Japan; [5]Department of Hepatology, Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital, Hiroshima-shi, Japan.

Received September 5, 2006; accepted December 5, 2006.

Address reprints to: Professor Kazuaki Chayama, M.D., Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: chayama@hiroshima-u.ac.jp; fax: (81) 82-255-6220.

Copyright © 2007 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/hep.21581
Potential conflict of interest: Nothing to report.

Table 1. Clinical Characteristics of the 3 Groups

	Group A	Group B	Group C
Number	6	12	18
Age, median (range)	50 (37-67)	49 (31-66)	49 (27-68)
Sex (M:F)	3:3	9:3	13:5
Observation period (months)	34.5 (13-58)	38 (16-64)	34 (13-58)
Time before emergence of mutants (months)	8.5 (4-11)	19 (13-36)	
HBV DNA (LGE/ml)	7.8 ± 0.95	6.13 ± 0.84	6.64 ± 1.63
Hbe-antigen-positive	4 (66.7%)	6 (50%)	10 (55.6%)
Hbe-antibody-positive	1 (16.7%)	6 (50%)	9 (50%)
ALT (U/l)	136.1 ± 122.8	114.5 ± 104.1	129.8 ± 206.4

Group A: patients who showed early emergence of the mutants (within 1 year).
Group B: patients who developed resistance after 1 year of LAM therapy.
Group C: patients in whom mutants did not develop.

tion and hybridization protection assay (TMA-HPA) and that measured by the Amplicor HBV Monitor test. Because the former method detects both HBV DNA and HBV RNA, we thought that the difference in measurement by the 2 methods was a result of the presence of a large amount of HBV RNA.[19-21] We thus studied patients with chronic HBV infection who were being treated with LAM or entecavir (ETV) for the presence of HBV RNA. We also assumed that the presence of a large amount of HBV RNA would indicate that transcription and virus particle formation were still active in such patients. We thus assessed the value of this indicator in the prediction of the emergence of YMDD mutants during LAM therapy.

Patients and Methods

Patients. We studied 36 patients with chronic hepatitis B who were being treated with LAM from 2001 to 2006 at Hiroshima University Hospital, Kawakami Clinic, and Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital. We also analyzed 7 patients who were being treated with ETV from 2004 to 2006 at Hiroshima University Hospital. No patients showed clinical signs of cirrhosis or hepatocellular carcinoma. They were not treated with other antiviral agents, corticosteroids, or immunosuppressant drugs during LAM/ETV therapy. The LAM-treated patients were 25 men and 11 women whose median age was 52 years (range 27-68 years; Table 1). They were divided into 3 groups (groups A, B, and C) according to how long it took for YMDD mutants to appear. Group A (n = 6) was composed of patients who showed early emergence of the mutants (within 1 year); group B (n = 12) had patients who developed resistance after 1 year of LAM therapy; and group C (n = 18) was composed of patients who did not show resistance to LAM therapy. Each of the 36 patients received 100 mg of LAM daily for 4-58 months (median, 21.5 months). All patients continued LAM therapy throughout the course of the study. Patients in the ETV group were 6 men and 1 woman whose median age was 37 years (32-50 years). They received 0.01-0.5 mg of ETV daily for 21-28 months (median, 25 months), and all patients continued ETV therapy throughout the course of the study. Blood samples were obtained from patients of both groups just before commencement of antiviral therapy and every 4 weeks during therapy. Informed consent was obtained from each patient.

Quantification of HBV DNA. HBV DNA serum level was determined by using the TMA-HPA (Fujirebio Inc., Tokyo, Japan) and the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the former assay is $10^{3.7}$-$10^{8.7}$ genome equivalents (GE)/ml (3.7-8.7 LGE/ml),[22] whereas the range of the latter test was $10^{2.6}$-$10^{7.6}$ copies/ml (2.6-7.6 log copies/ml).[23] These quantitative assays of HBV DNA were performed at the Special Reference Laboratory (Tokyo, Japan).

Extraction of Nucleic Acid of HBV and Reverse Transcription. Nucleic acid was extracted from 100 μL of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL of H_2O for DNA analysis or 8.8 μL of ribonuclease-free H_2O for RNA analysis. The latter solution was reverse-transcribed by using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan). In the next step, 25 pM of random primer was added to 8.8 μL of nucleic acid extract and heated at 65°C for 5 minutes. The samples were set on ice for 5 minutes. Then 4 μL of 5× reverse transcription (RT) buffer, 2 μL of 10 mM dNTPs, 2 μL of 0.1 M dithiothreitol (DTT), 8 units of ribonuclease inhibitor, and 100 units of M-MLV reverse transcriptase were added to each sample. The reaction mixture was incubated at 30°C for 10 minutes and 42°C for 60 minutes, followed by inactivation at 99°C for 5 minutes.

Quantitative Analysis of HBV DNA by Real-Time Polymerase Chain Reaction. One microliter of DNA solution or cDNA solution was amplified by real-time polymerase chain reaction (PCR) with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. Amplification was performed in a 25-μL reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of forward primer (5′-TTTGGGGCATGGACAT-TGAC-3′, nucleotides 1893-1912), 200 nM of reverse primer (5′-GGTGAACAATGGTCCGGAGAC-3′, nucleotides 2029-2049), and 1 μL of DNA or cDNA solution. After incubation for 2 minutes at 50°C, the sample was heated for 10 minutes at 95°C for denaturing, followed by a PCR cycling program consisting of 40 2-step cycles of 15 seconds at 95°C and 60 seconds at 60°C. The lower detection limit of this assay was 10^3 copies/ml.

Confirmation of Presence of HBV RNA in Serum by RNase Digestion. To confirm the presence of HBV RNA, nucleic acid extracted from the serum samples by SMITEST (Genome Science Laboratories, Tokyo) was digested with 1 μg/μL of RNase A (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 60 minutes, digested with proteinase K (New England Biolabs Inc., Ipswich, MA) at 37°C for 60 minutes, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in water. Treated nucleic acid with or without RNase was analyzed by real-time PCR after reverse transcription with a random primer and reverse transcriptase, as already described.

Detection of YMDD Mutant. Mutations in the YMDD motif of reverse transcriptase of HBV were examined by PCR with peptide nucleic acid clamping, as described previously.[24]

Statistical Analysis. Differences between groups were examined for statistical significance using the Student t test, and correlations of parameters were examined by the Spearman's rank correlation. A difference with a P value less than 0.05 was considered statistically significant. All statistical analyses were performed with StatView version 5.0 (SAS Institute, Cary, NC).

Results

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test During ETV Therapy. High expression of HBV RNA was initially observed by measuring HBV nucleic acid with the TMA-HPA and HBV DNA with the Amplicor HBV monitor test. As shown in Fig. 1, expression of HBV nucleic acid was higher than HBV DNA during the initial 6 months of ETV therapy. We assumed that the discrepancy in the measurements by these 2 methods was a result of the large amount of HBV RNA in the serum because the TMA-HPA measures both HBV DNA and HBV RNA, whereas the Amplicor HBV monitor test detects only HBV DNA. We measured the HBV nucleic acid levels in the 7 patients who received ETV therapy 3 and 6 months after the start of therapy. The HBV nucleic acid levels of all 7 patients determined by the TMA-HPA were 10-100 times higher than those determined by the Amplicor HBV Monitor test except for 2 patients who received a small amount (0.01 mg) of ETV (Fig. 2). The small dif-

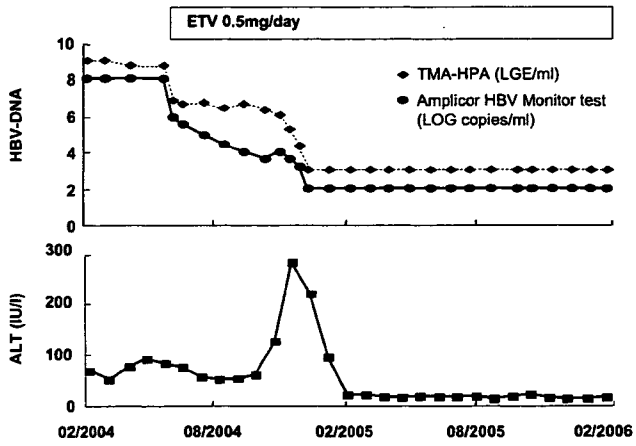

Fig. 1. Time courses of serum HBV DNA and ALT levels of patients treated with ETV. Expression of HBV nucleic acids determined by the TMA-HPA was higher than that determined by the Amplicor HBV Monitor test soon after beginning administration of ETV. The discrepancy was less marked when both measurements were low and when both were negative.

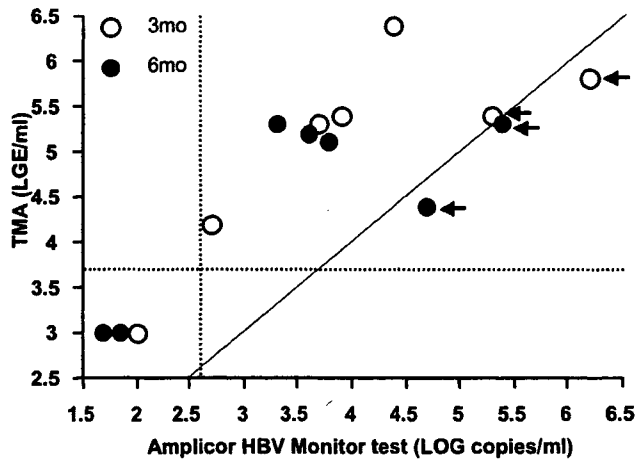

Fig. 2. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during ETV therapy. Serum samples obtained from the 2 patients who received low-dose ETV (0.01 mg) are indicated by arrows. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

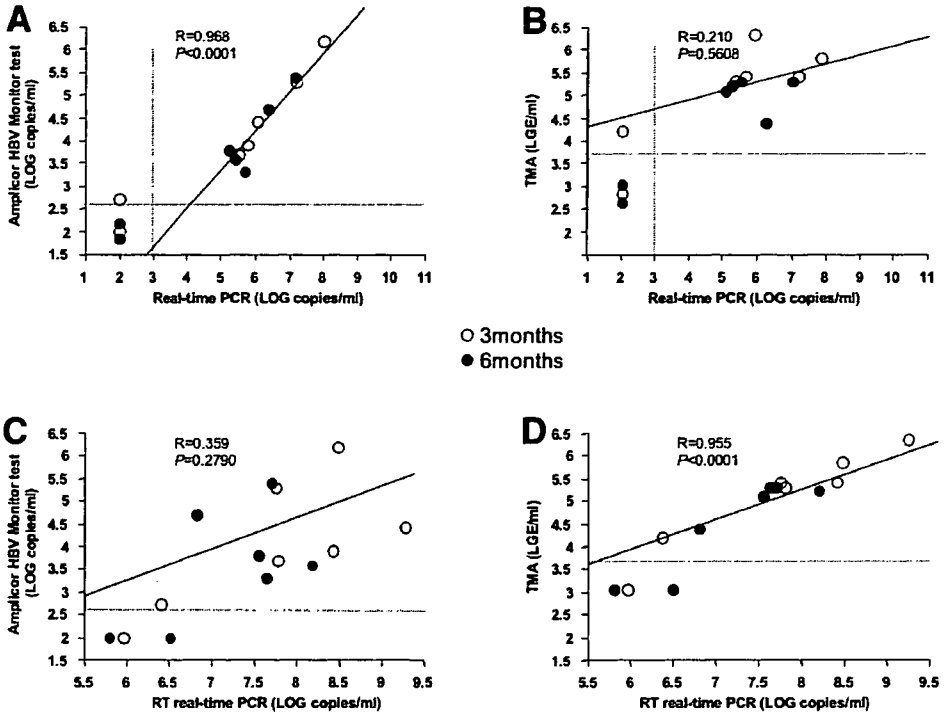

Fig. 3. Correlation between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of ETV therapy. (A) Correlation between HBV DNA level determined by Amplicor HBV Monitor test and that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA and of HBV DNA determined by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined by real-time RT-PCR. (D) Correlation of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

ference in nucleic acid level of these patients is probably a result of the small effect of the small amount of ETV.

Comparisons of HBV Nucleic Acid and DNA Values Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with ETV. We measured HBV DNA by in-house real-time PCR and HBV nucleic acid by real-time RT-PCR using serum samples obtained from the patients after 3 and 6 months of ETV therapy and compared these values with those obtained by the TMA-HPA and the Amplicor monitor test. HBV DNA determined by real-time PCR correlated well with that obtained by the Amplicor HBV Monitor test ($r = 0.968$, $P < 0.0001$; Fig. 3A), but not with HBV nucleic acid determined by the TMA-HPA ($r = 0.210$, $P = 0.5608$; Fig. 3B). Expression of HBV DNA determined by the in-house real-time PCR assay was $10^{1.5}$-10^{2} higher than that determined by the Amplicor HBV Monitor test. We confirmed the accuracy of our assay using limiting dilution and detection with nested PCR assay. When we diluted the standard samples used in our in-house assay to 1 copy/μL, we detected them by nested PCR using 1 μL of such samples. Three of the 10 (30%) samples tested positive by nested PCR. We thus conclude that our assay accurately measure the amount of HBV DNA in serum.

To examine if measurement by the TMA-HPA reflected the total amount of HBV RNA and HBV DNA in serum samples, we performed real-time RT-PCR using serum samples obtained from patients after 3 and 6 months of ETV therapy. In contrast to the values determined by real-time PCR without RT, the measurement of HBV nucleic acid determined by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test ($r = 0.359$, $P = 0.2790$; Fig. 3C), but did correlate well with that obtained with the TMA-HPA ($r = 0.955$, $P < 0.0001$; Fig. 3D). These results show that the TMA-HPA measures both HBV DNA and HBV RNA in serum. To further confirm the presence of HBV RNA, we digested 3 nucleic acid samples arbitrarily picked from serum samples obtained from patients treated by lamivudine for 3 months, by RNase A. As shown in Fig. 4, RNase treatment reduced the amount of HBV DNA detected by real-time RT-PCR to about 1% of that originally detected.

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test during LAM Therapy. We then investigated the levels of HBV DNA in serum samples obtained from 36 patients after 3 and 6 months of LAM therapy. In some patients, HBV DNA was already negative after 3 and 6 months of therapy (Fig. 5). Similar to the results obtained from patients treated with ETV, comparisons of values obtained from patients who showed measurable HBV DNA levels revealed that HBV nucleic acid levels determined by the TMA-HPA tended to be higher than those determined by the Amplicor HBV Monitor test (Fig. 4).

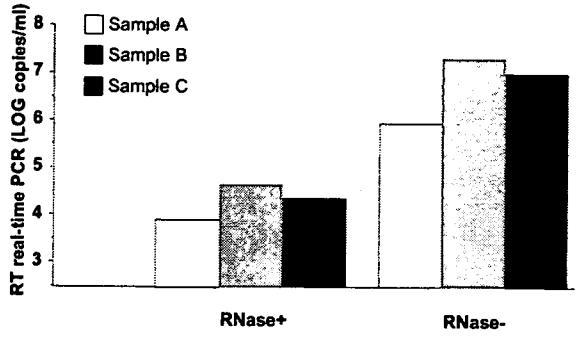

Fig. 4. Presence of HBV RNA confirmed by RNA treatment of 3 nucleic acid samples (samples A-C) obtained from patients after 3 months of LAM therapy. Extracted nucleic acid samples with or without RNase digestion were further digested by proteinase K and ethanol-precipitated after phenol/chloroform extraction. The amount of HBV DNA in each sample was then measured by real-time RT-PCR.

Comparisons of HBV Nucleic Acid Values and HBV DNA Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with LAM. We measured HBV nucleic acid and DNA levels by the same 4 methods and investigated the correlations between them after 3 and 6 months of LAM therapy (Fig. 6). HBV DNA levels determined by real-time PCR correlated better with those determined by the Amplicor HBV Monitor test (r = 0.653, P = 0.0083; Fig. 6A) than with those determined by the TMA-HPA (r = 0.456, P = 0.1173; Fig. 6B). Similarly, measurement of HBV nucleic acid by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test (Fig. 6C), but showed better correlation with that obtained by the TMA-HPA (r = 0.452, P = 0.0907, and r = 0.675, P = 0.0114, respectively; Fig. 6D). These results also showed that the TMA-HPA detects both HBV RNA and HBV DNA.

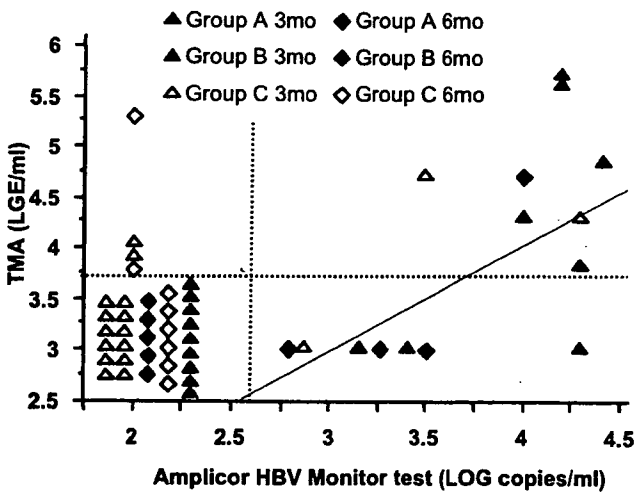

Fig. 5. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during LAM therapy. During ETV therapy the TMA-HPA showed higher expression of HBV DNA in patients regardless of the presence of the mutation than did the Amplicor HBV Monitor test. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

HBV RNA in Serum after 3 Months of LAM Therapy Is Higher in Patients Who Showed Early Emergence of YMDD Mutants. In LAM-treated patients, it was assumed that a high serum level of HBV RNA was a marker of the active transcription form of covalently closed circular DNA (cccDNA) and packaging of HBV RNA in the liver. We assumed that YMDD mutants easily emerged under such condition. We compared HBV RNA values (HBV nucleic acid determined by real-time RT-PCR minus HBV DNA determined by real-time PCR) in patients who showed early emergence of mutants (within 12 months) with those who showed late emergence of mutants (more than 12 months) and those who did not show emergence of mutants (Table 1). As shown in Fig. 7, HBV RNA levels were significantly higher in patients who showed early emergence of mutants than the other 2 groups after 3 months of LAM therapy. There was no significant difference in the amount of HBV RNA between group A (patients who showed emergence of mutants within 12 months) and the other 2 groups at the beginning of LAM therapy (data not shown).

Discussion

In this study, we addressed the discrepant measurements of HBV nucleic acid by the TMA-HPA and the Amplicor Monitor test. The presence of HBV RNA in serum samples of patients with HBV infection has been previously reported.[19-21] Because the TMA-HPA uses RNA transcription and amplification of transcripts by T7 RNA polymerase,[22] we assumed that the discrepancy was a result of the presence of HBV RNA in the serum of LAM- and ETV-treated patients. The presence of HBV RNA in a patient treated with LAM was reported previously.[21] In that report, the authors mainly analyzed truncated HBV RNA, which they assumed was transcribed from the integrated genome.[20, 21] They showed a large difference between HBV DNA and truncated HBV RNA, which did not decrease during LAM therapy. We also detected HBV DNA and HBV nucleic acid by real-time PCR and real-time RT-PCR. The values determined by these 2 methods showed less than a 1 log difference (data not shown); we assume that the effect of truncated HBV RNA in serum was only minimal in our study. As we demonstrated in this study, HBV nucleic acid measured by real-time RT-PCR correlated with that determined by the TMA-HPA. This finding suggests that the

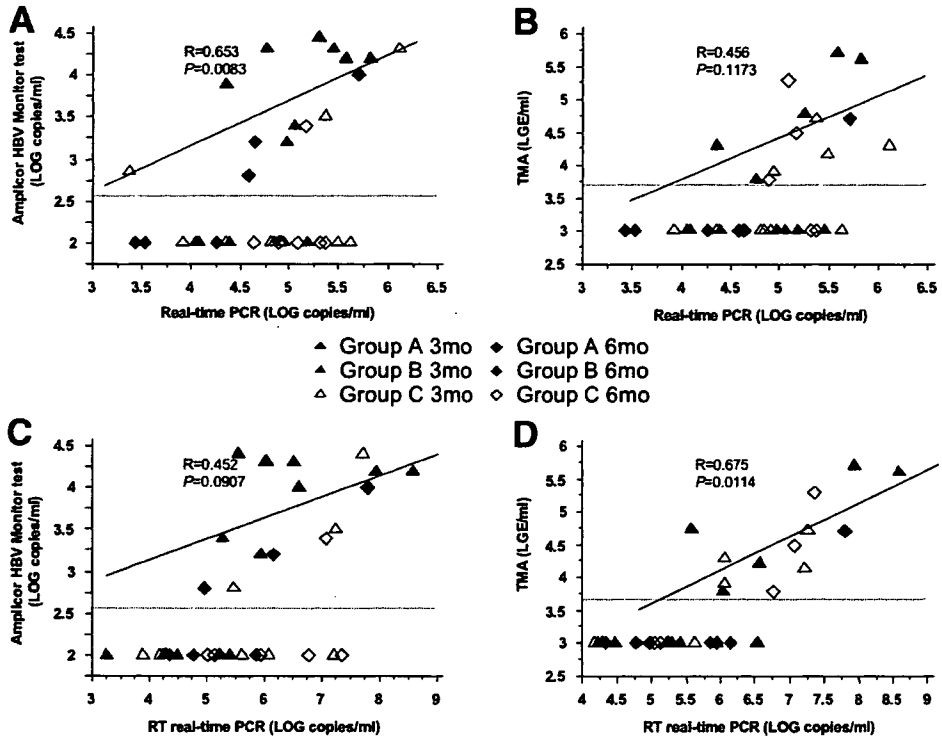

Fig. 6. Correlations between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of LAM therapy. (A) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA with HBV DNA by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined real-time RT-PCR. (D) Correlations of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

discrepancy in the values measured by the TMA-HPA and the Amplicor Monitor test is a result of the presence of HBV RNA in the serum.

We showed that a large amount of HBV RNA in the serum was produced during the early stage of ETV (Fig. 1) and LAM treatments (within 6 months). Because ETV and LAM work only on reverse transcription, it is difficult to conceive that the level of transcription from the cccDNA was altered by these drugs. Thus, the slow decrease in HBV RNA seems to reflect that a certain amount of cccDNA still existed in the liver and that the virus replication machinery was still actively operational. This is consistent with previous reports that showed that the amount of cccDNA in the liver tissues[25, 26] and in serum,[26] which correlated well with intrahepatic cccDNA,[27] reflected the effect of LAM and is a marker for cessation of therapy without viral level increasing again after stopping the therapy.

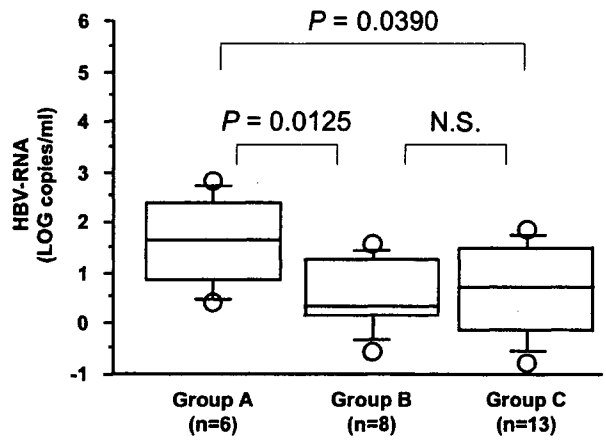

Fig. 7. Box plots of HBV RNA levels of patients in group A (patients who showed emergence of the mutants within 1 year, group B (those who developed resistance after 1 year of LAM therapy), and group C (patients who did not show resistance to LAM therapy). HBV RNA level represents the difference between HBV nucleic acid level determined by real-time RT-PCR minus HBV DNA level determined by in-house real-time PCR. Nine samples that tested negative for in-house real-time PCR were omitted from the analysis (4 samples of group B and 5 samples of group C).

Whether a large amount of HBV RNA originates from a large amount of cccDNA template in hepatocytes or from active transcription (or both) is actually unknown. However, it is assumed that the probability of developing mutants is high in patients who have large amounts of HBV RNA. We thus analyzed the amount of HBV RNA in patients treated with LAM and compared it in patients who showed early emergence of mutants and those who did not. As expected, the amount of HBV RNA in the serum was significantly higher in patients who showed early emergence of mutants than in those who showed late emergence and those who did not show emergence of mutants.

Using complex analysis, previous studies identified several factors predictive of emergence of YMDD mutants such as HBV genotype,[28] ALT level,[29, 30] HBV DNA level

before therapy,[28,30-32] degree of decline of HBV DNA level during therapy,[33,34] presence of hepatitis B e antigen,[17,29,31,32,35] presence of core promoter mutations,[36] deletion of pre-S region,[37] and HBV core–related antigen.[38] We also showed that a slow decrease in HBV nucleic acid measured by the TMA-HPA is a marker of early emergence of mutants. Our finding is important because this assay is routinely used in daily clinical practice. However, the results did not reach statistical significance, probably because of the small number of patients analyzed in our study and the low sensitivity of the assay (detection limit 3.7 log copies/ml). We assume that a sensitive measurement of HBV RNA is useful for predicting the emergence of mutants. Development of such an assay is needed for the proper treatment of patients using different nucleotide and nucleoside analogues. Mechanisms that control transcription of HBV from cccDNA deserve further investigation in order to develop more effective therapies for HBV infection.

Acknowledgment: This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. The authors thank Kana Kunihiro, Rie Akiyama, Yoshiko Seo, Yoshiko Nakata, and Kiyomi Toyota for their excellent technical assistance. This work was supported in part by grants-in-aid for scientific research and development from the Ministry of Education, Sports, Culture, and Technology and the Ministry of Health, Labour, and Welfare.

References

1. Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000;61:362-366.
2. Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 1988;61:1942-1956.
3. Chang CN, Skalski V, Zhou JH, Cheng YC. Biochemical pharmacology of (+)-and (+)- 2′,3′-dideoxy-3′-thiacytidine as anti-hepatitis B virus agents. J Biol Chem 1992;267:22414-22420.
4. Benhamou Y, Dohin E, Lunel-Fabiani F, Poynard T, Huraux JM, Katlama C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995;345:396-397.
5. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995;333:1657-1661.
6. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61-68.
7. Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, et al. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 1999;30: 743-748.
8. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-1263.
9. Dienstag JL, Schiff ER, Mitchell M, Casey DE Jr, Gitlin N, Lissoos T, et al. Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. HEPATOLOGY 1999; 30:1082-1087.
10. Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, et al. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 2000;119:172-180.
11. Suzuki Y, Arase Y, Ikeda K, Saitoh S, Tsubota A, Suzuki F, et al. Histological improvements after a three-year lamivudine therapy in patients with chronic hepatitis B in whom YMDD mutants did not or did develop. Intervirology 2003;46:164-170.
12. Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003;124:105-117.
13. Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, et al. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology 1997;113:1258-1263.
14. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. HEPATOLOGY 2000;32:803-806.
15. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. HEPATOLOGY 1996;24:711-713.
16. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA–dependent DNA polymerase confers resistance to lamivudine *in vivo*. HEPATOLOGY 1996;24:714-717.
17. Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, et al. 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 1998;27:1711-1716
18. Lau DT, Khokhar MF, Doo E, Ghany MG, Herion D, Park Y, et al. Long-term therapy of chronic hepatitis B with lamivudine. HEPATOLOGY 2000;32:828-834.
19. Köck J, Theilmann L, Galle P, Schlicht HJ. Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus. HEPATOLOGY 1996;23:405-413.
20. Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, et al. Circulating hepatitis B virus nucleic acids in chronic infection: representation of differently polyadenylated viral transcripts during progression to nonreplicative stages. Clin Cancer Res 2001;7:2005-2015
21. Zhang W, Hacker HJ, Tokus M, Bock T, Schröder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. J Med Virol 2003;71:24-30.
22. Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, et al. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 1999;37: 310-314.
23. Ranki M, Schätzl HM, Zachoval R, Uusi-Oukari M, Lehtovaara P. Quantification of hepatitis B virus DNA over a wide range from serum for studying viral replicative activity in response to treatment and in recurrent infection. HEPATOLOGY 1995;21:1492-1499.
24. Ohishi W, Chayama K. Rare quasispecies in the YMDD motif of hepatitis B virus detected by polymerase chain reaction with peptide nucleic acid clamping. Intervirology 2003;46:355-361.
25. Sung JJ, Wong ML, Bowden S, Liew CT, Hui AY, Wong VW, et al. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. Gastroenterology 2005;128: 1890-1897.
26. Yuen MF, Wong DK, Sum SS, Yuan HJ, Yuen JC, Chan AO, et al. Effect of lamivudine therapy on the serum covalently closed-circular (ccc) DNA of chronic hepatitis B infection. Am J Gastroenterol 2005;100:1099-1103.
27. Wong DK, Yuen MF, Yuan H, Sum SS, Hui CK, Hall J, et al. Quantification of covalently closed circular hepatitis B virus DNA in chronic hepatitis B patients. HEPATOLOGY 2004;40:727-737
28. Zollner B, Petersen J, Puchhammer-Stockl E, Kletzmayr J, Sterneck M, Fischer L, et al. Viral features of lamivudine resistant hepatitis B genotypes A and D. HEPATOLOGY 2004;39:42-50.
29. Nafa S, Ahmed S, Tavan D, Pichoud C, Berby F, Stuyver L, et al. Early detection of viral resistance by determination of hepatitis B virus polymerase mutations in patients treated by lamivudine for chronic hepatitis B. HEPATOLOGY 2000;32:1078-1088.

30. Yuen MF, Sablon E, Hui CK, Yuan HJ, Decreamer H, et al. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. HEPATOLOGY 2001;34:785-791.
31. Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 2003;46:182-189.
32. Sun J, Wang Z, Ma S, Zeng G, Zhou Z, Luo K, et al. Clinical and virological characteristics of lamivudine resistance in chronic hepatitis B patients: A single center experience. J Med Virol 2005;75:391-398.
33. Puchhammer-Stockl E, Mandl CW, Kletzmayr J, Holzmann H, Hofmann A, Aberle SW, et al. Monitoring the virus load can predict the emergence of drug-resistant hepatitis B virus strains in renal transplantation patients during lamivudine therapy. J Infect Dis 2000;181:2063-2066.
34. Zollner B, Schafer P, Feucht HH, Schroter M, Petersen J, Laufs R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. J Med Virol 2001;65:659-663.
35. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321.
36. Lok AS, Hussain M, Cursano C, Margotti M, Gramenzi A, Grazi GL, et al. Evolution of hepatitis B virus polymerase gene mutations in hepatitis B e antigen-negative patients receiving lamivudine therapy. HEPATOLOGY 2000;32:1145-1153.
37. Tanaka Y, Yeo AE, Orito E, Ito K, Hirashima N, Ide T, et al. Prognostic indicators of breakthrough hepatitis during lamivudine monotherapy for chronic hepatitis B virus infection. J Gastroenterol 2004;39:769-775.
38. Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, et al. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance. Liver Int 2006;26:90-96.

FEBS Letters 581 (2007) 1983–1987

Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

Nobuhiko Hiraga[a,b], Michio Imamura[a,b], Masataka Tsuge[a,b], Chiemi Noguchi[a,b], Shoichi Takahashi[a,b], Eiji Iwao[c], Yoshifumi Fujimoto[b,d], Hiromi Abe[b,d], Toshiro Maekawa[b,d], Hidenori Ochi[b,d], Chise Tateno[b,e], Katsutoshi Yoshizato[b,f], Akihito Sakai[g], Yoshio Sakai[g], Masao Honda[g], Shuichi Kaneko[g], Takaji Wakita[h], Kazuaki Chayama[a,b,d,*]

[a] *Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*
[b] *Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[c] *Pharmaceuticals Research Unit, Mitsubishi Pharma Corporation, Yokohama, Japan*
[d] *Laboratory for Liver Diseases, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan*
[e] *Yoshizato Project, CLUSTER, Prefectural Institute of Industrial Science and Technology Higashihiroshima, Japan*
[f] *Developmental Biology Laboratory and Hiroshima University 21th Century COE, Program for Advanced Radiation Casualty Medicine, Department of Biological Science, Graduate School of Science, Hiroshima University, Higashihiroshima, Japan*
[g] *Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan*
[h] *Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Japan*

Received 15 February 2007; revised 31 March 2007; accepted 5 April 2007

Available online 20 April 2007

Edited by Hans-Dieter Klenk

Abstract **We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.**
© 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

*Corresponding author. Address: Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Fax: +81 82 255 6220.
E-mail address: chayama@mba.ocn.ne.jp (K. Chayama).

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

0014-5793/$32.00 © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2007.04.021