

Fig. 4. Infectivity of HIV-1 virions produced from HepG2 and Huh7 cell lines. (a) Wild-type and mutant viruses lacking Vif protein produced from the two cell lines were examined for infectivity as described in Methods. The relative infectivity of the wild-type is shown. (b) Effect of APOBEC3G (A3G) expression on infectivity. HIV-1 virions produced by Huh7 cells co-transfected with the indicated number of APOBEC3G expression plasmid were used for measurement of infectivity.

similar to that of the wild-type virus (Fig. 4a). Transient expression experiments showed that the expression of APOBEC3G in Huh7 cell lines reduced infectivity of wild-type HIV-1 produced in these cell lines in a dose-dependent manner (Fig. 4b). Infectivity of Vif-deficient HIV-1 was reduced to almost undetectable levels (Fig. 4b). Thus, APOBEC3G effectively suppressed the production of infectious HIV in these cell lines.

Both IFN- α and - γ induce APOBEC3G mRNA expression and hypermutation of HBV genomes and reduce replication of HBV

We treated HepG2 cell lines stably transfected with 1.4 genome length construct HBV (Tsuge *et al.*, 2005) with either IFN- α or - γ to examine their influence on the expression of APOBEC3G mRNA and G to A hypermutation of HBV genomes. Chronological studies showed that the core-associated HBV DNA in the stably HBV-producing cell line gradually decreased until 36 h after IFN- α treatment (Fig. 5a). Expression levels of APOBEC3G mRNA, but not those of APOBEC3F, increased in this cell line at 12 h after the IFN treatment (Fig. 5a). Hypermutated genomes in this cell line increased with time until 36 h after IFN- α

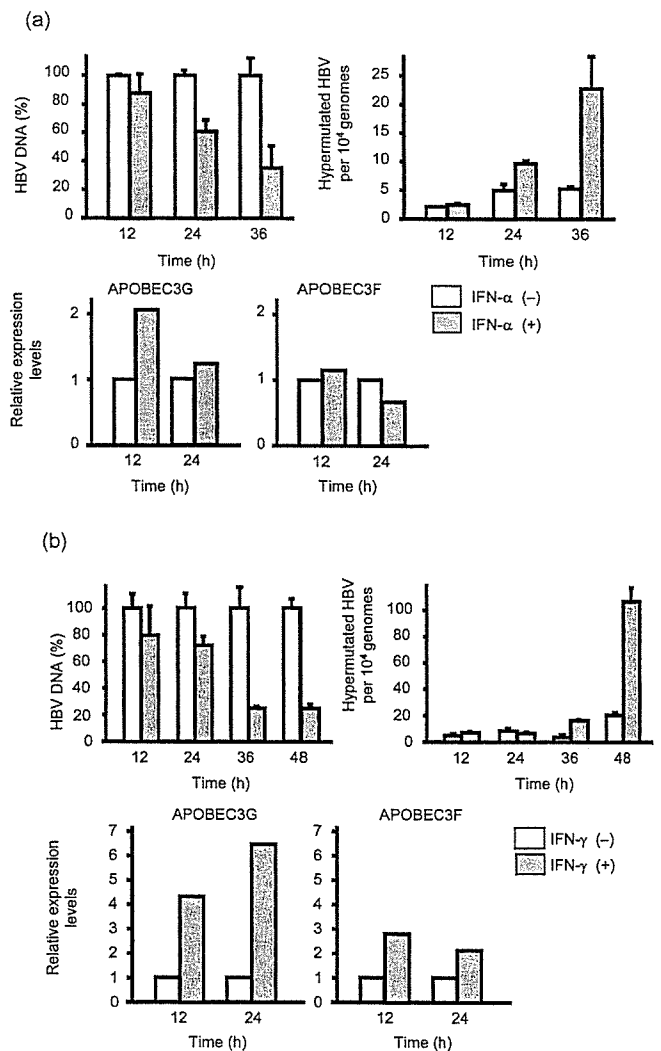


Fig. 5. Effects of IFN- α and - γ on HBV-producing cells. (a) The IFN- α -treated and -untreated HBV-producing T23 cell line was harvested at the indicated time after IFN treatment and examined for the number of core-associated HBV DNA, the number of hypermutated genome and mRNAs of APOBEC3G and APOBEC3F. (b) IFN- γ -treated and -untreated HBV-producing T23 cell line was examined as described in (a). Results are means \pm SD values of three independent experiments.

treatment. Similarly, the core-associated HBV DNA decreased gradually to about 20% of the levels in untreated cells after IFN- γ treatment (Fig. 5b). The increase in APOBEC3G mRNA expression was more prominent after IFN- γ than after IFN- α treatment. The level of APOBEC3F mRNA was also about double that of untreated cells. G to A hypermutation of HBV genomes increased markedly with time after IFN- γ treatment (Fig. 5b).

We further examined the effect of IFN on reduction of HBV replication and induction of hypermutation by comparing the effects of different doses of IFN- α and - γ . Both IFN- α

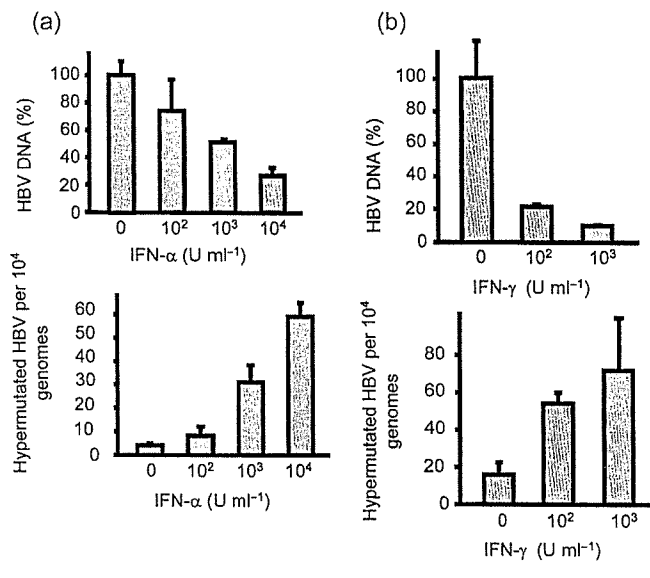


Fig. 6. Dose-dependent reduction of HBV replication and hypermutation of genomic sequences. HBV-producing cell line T23 was harvested after (a) IFN- α and (b) IFN- γ treatment for 72 h. The number of core-associated HBV DNA and the number of hypermutated genomes were measured. Results are means \pm SD values of three independent experiments.

and - γ treatment decreased core-associated HBV DNA in a dose-dependent manner (Fig. 6). Hypermutation of HBV genomes also increased with higher doses of IFN (Fig. 6).

Expression of APOBEC3G increases hypermutation of the HBV genome

To confirm that the increase in hypermutation of the HBV genome is induced by the effect of APOBEC3G, we performed expression experiments of APOBEC3G and its deaminase function-deficient mutants into HepG2 cell lines and measured the number of hypermutated HBV genomes. Transient expression experiments showed that the number of HBV DNA was decreased by co-transfection of APOBEC3G in HepG2 cells (Fig. 7a). 3D-PCR and detection with HA-yellow agarose gel electrophoresis showed the presence of heavily hypermutated genomes (Fig. 7b). No amplification was observed at the 81 °C denaturation temperature (data not shown). Quantitative analysis showed an about 334-fold increase in hypermutated genomes compared with mock-transfected control cells (Fig. 7c). However, the proportion of hypermutated genomes was 9.68 % (968 in 10^4 genomes).

To confirm the effect of APOBEC3G on HBV hypermutation, we transfected wild-type and inactive mutants of APOBEC3G (Fig. 8a, b) into Huh7 cells. Wild-type APOBEC3G effectively induced hypermutation of HBV genomes and reduced the replication of HBV. In contrast, insufficient deaminase activity in the E67Q mutant induced less hypermutation of HBV genomes than in the wild-type. No increase in hypermutation was observed in cell lines transfected with deamination-defective E259Q and E67Q/E259Q mutants, although the number of HBV replication was reduced in these cells (Fig. 8a). We observed similar reduction in HBV replication by transient transfection of APOBEC3F. Induction of hypermutation by APOBEC3F was less efficient than by wild-type and the E67Q mutant of

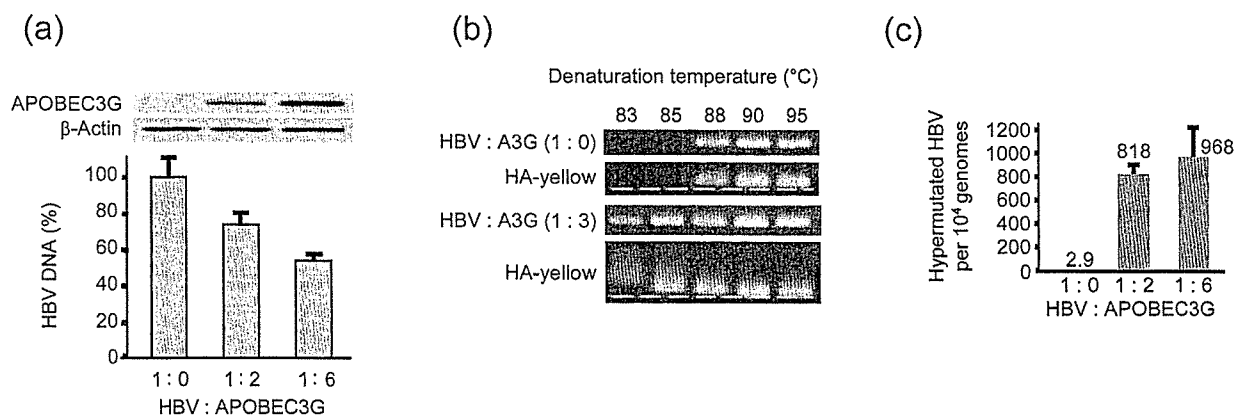


Fig. 7. Effects of APOBEC3G expression on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with pcDNA3/HA-A3G into HepG2 cells. At 72 h after transfection, the cells were harvested. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti- β -actin antibody. (b) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. Hypermutated genomes in the presence or absence of APOBEC3G-HA were amplified by 3D-PCR. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (c) Quantification analysis of hypermutated genomes by real-time 3D-PCR. Results are means \pm SD values of three independent experiments.

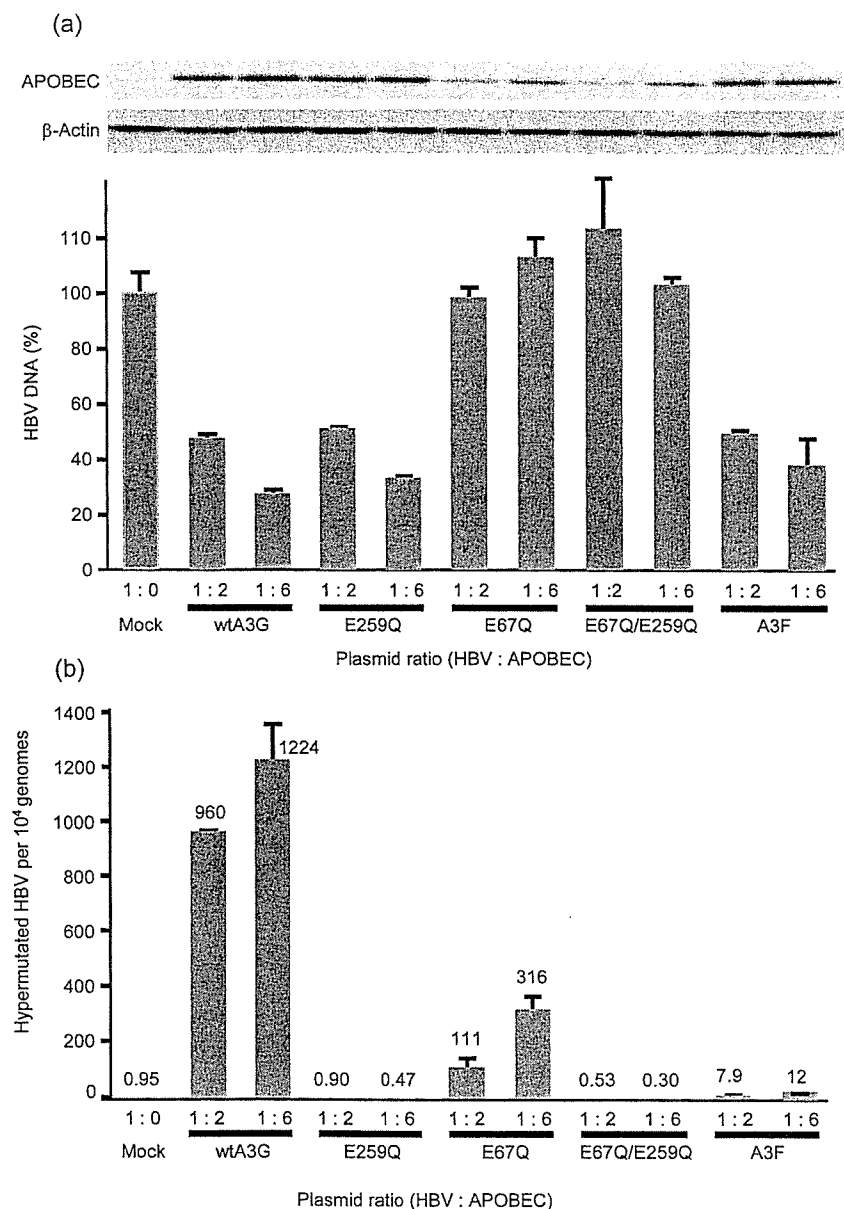


Fig. 8. Effect of APOBEC proteins on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with wild-type, enzymically impaired APOBEC3G mutants (E67Q, E259Q, E67Q/E259Q) and APOBEC3F into Huh7 cells (plasmid ratio HBV:APOBEC=1:2 or 1:6). The cells were harvested at 96 h after transfection. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti- β -actin antibody. (b) Quantification of hypermutated genomes by real-time 3D-PCR. Results are means \pm SD values of three independent experiments.

APOBEC3G. These results suggest that hypermutation of HBV contributes very little to reduce the number of replicative intermediate.

DISCUSSION

Induction of G to A hypermutation in HIV has been reported as part of host innate immunity against virus infection (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003; Sheehy *et al.*, 2002). We and others have reported the presence of hypermutated genomes of HBV in serum samples of chronically infected patients and in HepG2 cell lines (Gunther *et al.*, 1997; Suspene *et al.*, 2005a; Noguchi *et al.*, 2005; Rosler *et al.*, 2004). Hypermutation of HBV was induced in hepatocytes

(Noguchi *et al.*, 2005), and expression of APOBEC proteins in liver cell-derived cell lines increased hypermutation (Suspene *et al.*, 2005b; Rosler *et al.*, 2004). However, the estimated number of hypermutated genomes in chronically infected patients is very low (Noguchi *et al.*, 2005; Suspene *et al.*, 2005b). The reason for the partial hypermutation of HBV remains an enigma. It might be due to the low expression levels of APOBEC proteins in liver cells (Jarmuz *et al.*, 2002). Alternatively, rapid packaging of pregenome RNA into capsid might prevent access of APOBEC3G to the first strand DNA. Furthermore, rapid degradation of edited HBV genomes by uracil DNA glycosylase in liver cells might also explain the low number of hypermutated genomes.

The mechanism that controls the activities of APOBEC proteins to cause hypermutation has not been analysed until

recently. Tanaka *et al.* (2006) reported that IFN- α increases the expression levels of APOBEC3G mRNA. They reported the presence of ISRE elements in the promoter region of APOBEC3G and that the promoter was activated by IFN- α . However, they did not examine the occurrence of G to A hypermutation in their experiments. Moreover, Peng *et al.* (2006) showed that IFN- α and - γ cooperatively induce APOBEC3G expression and that the inhibition of HIV production by a small number of IFN is cancelled by a small interfering RNA (siRNA) against APOBEC3G. More recently, Bonvin *et al.* (2006) demonstrated that IFN- α induces transcription of APOBEC proteins. They showed that IFN treatment increased APOBEC3B, -3C, -3F and -3G mRNAs, particularly when they used primary cultured hepatocytes. They also reported that they were able to detect hypermutated genomes after transfection of APOBEC3 plasmids, but did not measure the direct effect of IFN on G to A hypermutation.

These studies did not analyse quantitatively the increase in hypermutation of viral genomes. The studies that analysed the expression of APOBEC protein and reduction of HBV DNA also did not analyse quantitatively the number of hypermutated genome (Suspeña *et al.*, 2005a; Noguchi *et al.*, 2005; Turelli *et al.*, 2004a, b; Rosler *et al.*, 2005). In the present study, we developed a method that accurately measures the level of hypermutation using real-time PCR. It is often difficult to design a primer set and a probe to detect G to A hypermutation because they are located in a region with many G residues, but the primer and probe sequences should not contain any. It is thus possible that we did not see any C to T substitution because we did not design a primer-probe set to detect this substitution. We also tried to select such a primer-probe set applicable for all genotypes of HBV, but were able to select only one suitable for genotype C.

Using this method, we demonstrated that both IFN- α and - γ increased G to A hypermutation of the HBV genome. Although the expression levels of APOBEC3G increased after IFN treatment, we did not observe an apparent shift of preferred dinucleotide sequence of APOBEC proteins from 3F to 3G. This is probably because the increase in APOBEC3G is only slight (Fig. 5).

The exact mechanism by which IFNs activate the transcription of APOBEC3G is unknown. Furthermore, what kind of sensor(s) detects HBV infection and how the signal is communicated for the production of IFNs and subsequent induction of effector molecules have not been analysed yet. Although the importance of the IFN system in eliminating HBV and its possible mechanism have been reported (Wieland *et al.*, 2004a, b, 2005), further studies are needed to fully describe the mechanism of action of IFNs including the activation of APOBEC3G.

We also demonstrated that the number of hypermutated genomes increased with the expression of APOBEC3G and APOBEC3F (Fig. 8), but not in deaminase-inactive mutants, as demonstrated previously in HIV studies

(Shindo *et al.*, 2003; Newman *et al.*, 2005). However, these mutants also reduced the replication of HBV almost to the wild-type level. This suggests that the contribution of hypermutation of HBV to the reduction of virus replication is only minimal and supports the previous report that showed that APOBEC3G reduced the replication of HBV through inhibition of packaging of the pregenome (Turelli *et al.*, 2004a). However, the effect of hypermutation on infectivity of the virus should be investigated further. The effects of APOBEC proteins, including other family members, especially under physiological conditions, should also be examined further. Whether any HBV protein inhibits deamination of the genomic DNA awaits further investigation. Furthermore, the mechanism that enables HBV to cause chronic infection, especially escape from innate antiviral immunity, should also be clarified in order to control chronic HBV infection and reduce HBV-related morbidity.

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RAPID COMMUNICATION

Clinical features and prognosis of patients with extrahepatic metastases from hepatocellular carcinoma

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tumor stage (T0-T2), and are free of portal venous invasion may improve survival.

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Abstract

AIM: To assess the clinical features and prognosis of 151 patients with extrahepatic metastases from primary hepatocellular carcinoma (HCC), and describe the treatment strategy for such patients.

METHODS: After the diagnosis of HCC, all 995 consecutive HCC patients were followed up at regular intervals and 151 (15.2%) patients were found to have extrahepatic metastases at the initial diagnosis of primary HCC or developed such tumors during the follow-up period. We assessed their clinical features, prognosis, and treatment strategies.

RESULTS: The most frequent site of extrahepatic metastases was the lungs (47%), followed by lymph nodes (45%), bones (37%), and adrenal glands (12%). The cumulative survival rates 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 time was 4.9 mo (range, 0-37 mo). Fourteen patients (11%) died of extrahepatic HCC, others died of primary HCC or liver failure.

CONCLUSION: The prognosis of HCC patients with extrahepatic metastases is poor. With regard to the cause of death, many patients would die of intrahepatic HCC and few of extrahepatic metastases. Although most of HCC patients with extrahepatic metastases should undergo treatment for the primary HCC mainly, treatment of extrahepatic metastases in selected HCC patients who have good hepatic reserve, intrahepatic

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant tumor with frequent intrahepatic metastasis. The prognosis of HCC patients has improved because of progress in therapeutic procedures, such as surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), and transcatheter arterial chemoembolization (TACE)^[1-3]. Moreover, progress in diagnostic modalities, such as ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and digital subtraction angiography (AG) has led to a better detection of patients with early and small HCC or asymptomatic extrahepatic metastases.

The above improvements in survival and diagnostic modalities have resulted in increased detection of extrahepatic metastases from primary HCC and further increases are anticipated in the future. Several groups have investigated extrahepatic metastases from HCC, but many of such cases were in autopsy cases, in a small number of cases or case reports^[4-15]. At present, the prognosis of patients with extrahepatic metastases from primary HCC is poor^[16,17]. In this regard, there is only little information about the causes of death of such patients^[18], and there is no consensus on the treatment strategy for extrahepatic metastases from HCC. For example, what treatment strategy should be used to treat intrahepatic HCC or extrahepatic metastases? Among patients with extrahepatic metastases from primary HCC, which patients should be treated? To our knowledge, there are no reports that

deal directly with these questions. In this relatively large study, we retrospectively assessed the clinical features and prognosis of 151 patients with extrahepatic metastases from primary HCCs, and described the treatment strategy for such patients.

MATERIALS AND METHODS

Patients

From June 1990 to December 2005, 995 consecutive patients with HCC were admitted to our hospital. Among these patients, 880 were initially diagnosed with HCC in our hospital while the others were treated previously for HCC in other hospitals. Extrahepatic metastases from primary HCC were detected in 151 (15.2%) of 995 patients. None of the patients was treated for extrahepatic metastases. All the 151 HCC patients with extrahepatic metastases (117 men and 34 women, median age: 64 years, range: 21-82 years) were enrolled in the present study.

Table 1 summarizes the clinical profile of the 151 patients at the initial diagnosis of extrahepatic metastases. These 151 patients were divided into groups A and B. Group A was consisted of 68 patients presented with extrahepatic metastases together with primary HCC at the initial diagnosis of HCC, group B was composed of 83 patients who received treatment for intrahepatic HCC, and developed extrahepatic metastases during the follow-up period. Among them, 37 (25%) patients were treated previously for primary HCC in other hospitals, 90 patients were of performance status (PS) of 0, 43 patients of 1, 9 patients of 2, 6 patients of 3, and 3 patients of 4^[19]. The etiology of the background liver disease was hepatitis B virus (HBV) in 33 patients, hepatitis C virus (HCV) in 89 patients, HBV and HCV in 5 patients, and non-B non-C in 24 patients. The hepatic reserve was Child-Pugh grade A in 88 patients, grade B in 48 patients, and grade C in 15 patients. We evaluated the primary tumor stage according to the Liver Cancer Study Group of Japan criteria^[20], based on the following three conditions (T factor): solitary, < 2 cm in diameter, and no vessel invasion. T1 was defined as fulfilling the three conditions, T2 as fulfilling two of the three conditions, T3 as fulfilling one of the three conditions, T4 as fulfilling none of the three conditions. The primary HCC tumor stage at the first diagnosis of extrahepatic metastases was T0 (no intrahepatic HCC) in 11 (7%) patients, T1 in 4 (3%) patients, T2 in 13 (9%) patients, T3 in 43 (28%) patients, and T4 in 80 (53%) patients. Twenty seven of 28 patients with intrahepatic tumor stage T0-T2 were treated previously for intrahepatic HCC. The median size of the main intrahepatic primary tumor was 48 mm (range, 0-160 mm). Intrahepatic tumor morphology was nodular type in 83 (55%) patients, non-nodular type in 57 (38%) patients, and no intrahepatic HCC in 11 (7%) patients. Table 1 lists the sites of extrahepatic metastases at enrollment. Among the 151 patients with extrahepatic metastases, the sites of metastases were the lungs in 63 patients, lymph nodes in 60 patients, bones in 51 patients, adrenal glands in 16 patients and other locations (e.g., peritoneum, pancreas and nasal passages). In some patients, two or more distant metastatic tumors were found in one or more organs.

Table 1 Clinical profile of 151 HCC patients with extrahepatic metastases at the initial diagnosis of extrahepatic metastases

Age (yr)	64 (21-82)
Sex (male/female)	117/34
Etiology (HBV/HCV/HBV + HCV/others)	33/89/5/24
PS (0/1/2/3/4)	90/43/9/6/3
Intrahepatic tumor stage (T0/1/2/3/4)	11/4/13/43/80
Intrahepatic main tumor size (mm)	48 (0-160)
Intrahepatic tumor volume (< 50% / ≥ 50%)	103/48
Intrahepatic tumor morphology (nodular type/non nodular type/no intrahepatic HCC)	83/57/11
Grade of portal vein invasion (Vp 0/1/2/3/4)	74/0/26/28/23
Child-Pugh grade (A/B/C)	88/48/15
AFP (ng/mL)	741.8 (< 5-861 600)
DCP (mAU/mL)	1300 (< 10-391 400)
Site of extrahepatic metastases, n (%)	
Lung	63 (42)
Lymph nodes	60 (40)
Bone	51 (34)
Adrenal	16 (11)
Peritoneum	1 (0.7)
Pancreas	1 (0.7)
Nasal passages	1 (0.7)

Data are expressed as medians and ranges unless indicated otherwise. HBV: hepatitis B virus; HCV: hepatitis C virus; PS: Eastern Cooperative Oncology Group performance status; T0: no intrahepatic HCC; Portal invasion assessed Vp1: tumor thrombus in a third or more of the peripheral branches; Vp2: in the second branch; Vp3: in the first branch; Vp4: in the trunk; AFP: alpha-fetoprotein; DCP: Des-γ-carboxy prothrombin.

Hepatocellular carcinoma

A definitive diagnosis of HCC was based on the finding of typical hypervascular radiological features or histopathological examination of needle biopsy specimen. HCC was also assessed by US, CT, and/or AG. Furthermore, CT was obtained during arterial portography and computerized tomographic hepatic arteriography. Further assessment of HCC was conducted by measuring α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP).

Extrahepatic metastases were diagnosed by CT, MRI, bone scintigraphy, X-ray, and/or positron emission tomography (PET) with ¹⁸F-fluorodeoxyglucose (FDG), or diagnosed by histopathological examination of surgically resected specimen or biopsy. When we suspected extrahepatic metastases with HCC, we always ruled out other malignancies (such as gastric cancer, colon cancer and lung cancer) by several imaging modalities, serological tumor markers and/or pathological examination.

Follow-up

All the 151 HCC patients with extrahepatic metastases were followed up during the observation period and no one was lost to follow-up. The median follow-up period was 4.9 mo (range, 1-37 mo). After the diagnosis of HCC, all patients were screened at regular intervals for the development of intra/extra hepatic metastases by clinical examination, AFP, DCP, and/or various imaging modalities. Serological tumor markers were measured once every month. US, CT or MRI was performed once every three to six months.

Statistical analysis and ethical considerations

Differences between groups were examined for statistical significance using the Mann-Whitney test (*U*-test) and χ^2 test where appropriate. Cumulative survival rate was assessed by the Kaplan-Meier life-table method and the differences were evaluated by the log rank test. The following 15 potential predictors were assessed in this study: PS (0 vs 1-4), age (≤ 65 vs > 65 years), sex (M vs F), Child-Pugh stage (A vs B, C), intrahepatic tumor stage (T0-T2 vs T3, T4), main intrahepatic tumor size (≤ 50 vs > 50 mm), intrahepatic tumor volume ($\leq 50\%$ vs $> 50\%$), intrahepatic tumor morphology (nodular type vs non nodular type), portal venous invasion (Vp 0-2 vs $> 3, 4$), AFP (≤ 400 ng/mL vs > 400 ng/mL), DCP (≤ 1000 mAU/mL vs > 1000 mAU/mL), site of extrahepatic metastases (lung vs others, bone vs others, only lymph node vs others), and treatment for extrahepatic metastases (performed vs not performed). All factors that were at least marginally associated with the survival after diagnosis of extrahepatic metastases ($P < 0.05$) were entered into a multivariate analysis. The hazard ratio and 95% confidence interval (95% CI) were calculated to assess the relative risk confidence. All analyses described above were performed using the SPSS program (version 11.0, SPSS Inc., Chicago, IL).

The study protocol was approved by the Human Ethics Review Committee of Graduate School of Biomedical Sciences, Hiroshima University and a signed consent form was obtained from each patient.

RESULTS

Site of extrahepatic metastases

Table 2 lists the sites of extrahepatic metastases identified throughout the follow-up period. The most frequent site of metastases that were identified throughout the follow-up period was the lung ($n = 71$ patients, 47%), followed by lymph nodes ($n = 68$ patients, 45%), bone ($n = 56$ patients, 37%), and adrenal glands ($n = 18$ patients, 12%). Brain metastases were identified in 2 (1%) patients. One (0.7%) patient each had metastases in the peritoneum, pancreas, nasal passages, muscle, skin, diaphragm, and colon. Autopsy was performed in 14 cases with metastases. Despite the detection of extrahepatic metastases in these 14 patients before autopsy, additional extrahepatic metastases were detected on postmortem examination (lymph nodes, diaphragm, and colon). At the first diagnosis of extrahepatic metastases, 109 (72%) patients had single-organ metastases, while the others had multiple organ metastases.

Among the 71 patients with lung metastases, 23 patients had bilateral lung metastases, 14 had additional extrapulmonary site of metastatic disease. The size of pulmonary nodules ranged from 9 to 30 mm at initial diagnosis of extrahepatic HCC. Few patients had symptoms (cough, dyspnea, and pleural effusion) related to lung metastases, and 8 patients who had severe symptoms died subsequently of respiratory failure. The median survival period of these 8 patients was 4.3 mo (range, 2.5-14.4 mo).

Table 2 Sites of extrahepatic HCC detected throughout the entire follow-up period

Site	Patients ($n = 151$), n (%)
Lung	71 (47)
Lymph nodes	68 (45)
Bone	56 (37)
Adrenal	18 (12)
Brain	2 (1)
Peritoneum	1 (0.7)
Pancreas	1 (0.7)
Nasal	1 (0.7)
Muscle	1 (0.7)
Skin	1 (0.7)
Diaphragm	1 (0.7)
Colon	1 (0.7)

Among the 68 patients with lymph node metastases, metastases were identified in 64 regional lymph nodes. The most common site was in the paraaortic nodes (31/64), followed by portohepatic nodes (21/64), periceliac nodes (6/64) and peripancreatic nodes (6/64). The majority of patients with regional lymph nodes metastases were asymptomatic, but few regional lymph nodes (portohepatic nodes) caused obstructive jaundice. Distant nodal metastases were found at 17 sites. The most common site was the mediastinum nodes (10/17), followed by subclavicular nodes (3/17), iliac nodes (2/17), cardiophrenic node (1/17), and retrocrural node (1/17). All distant lymph node metastases were not associated with clinical symptoms in this study.

Fifteen of 56 patients with bone metastases had multiple bone metastases at the initial diagnosis of bone metastases. The total number of bone metastatic sites was 88. The most frequent site was the vertebra (63/88; cervical vertebrae = 9, thoracic vertebrae = 38, and lumbar vertebrae = 16), followed by the ribs (8/88). Bone metastases were diagnosed by CT, MRI, bone scintigraphy, and/or PET with FDG.

Of the 18 patients with adrenal gland metastases, 13 had right adrenal gland metastases, 4 had left adrenal gland metastases and only one patient had bilateral metastases. These metastases were not associated with symptoms.

Treatments of extrahepatic metastases

All patients with Child-Pugh grade other than C or PS other than 2-4 were treated for intrahepatic HCC, and many of them were continuously treated after the diagnosis of extrahepatic metastases. On the other hand, HCC patients with Child-Pugh grade C or PS of 2-4 received supportive care. Forty-nine (32%) of 151 patients were treated for extrahepatic metastases by surgical resection, TACE, systemic chemotherapy, and/or radiotherapy. The 49 patients had extrahepatic metastases that were considered to worsen prognosis.

Surgical resection was performed in three (2%) patients (with regional lymph node, adrenal gland and lung metastases). The survival periods after surgical resection of extrahepatic metastases were 7 mo (in patients with lymph node metastases), 23 mo (in patients adrenal gland metastases), and 37 mo (in patients with lung metastases).

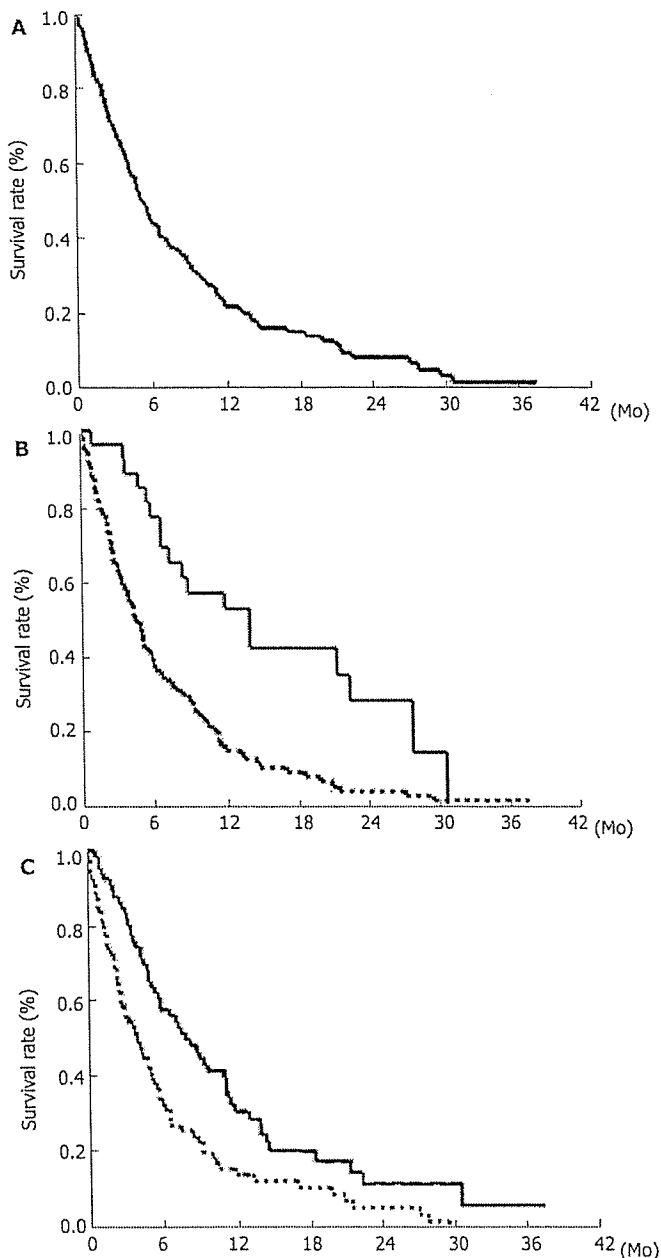


Figure 1 Survival rate of 151 HCC patients with extrahepatic metastases (A), intrahepatic tumor stage (B) [solid line: T0-T2, dashed line: T3, T4 (log-rank test: $P < 0.001$)], and after treatment of extrahepatic metastases (C) [solid line: treatment group, dashed line: no treatment group (log-rank test: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

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	<i>P</i>
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

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

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

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)

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, Natsuzaka *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 developing 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, particularly 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 metastases^[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 intrahepatic 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,

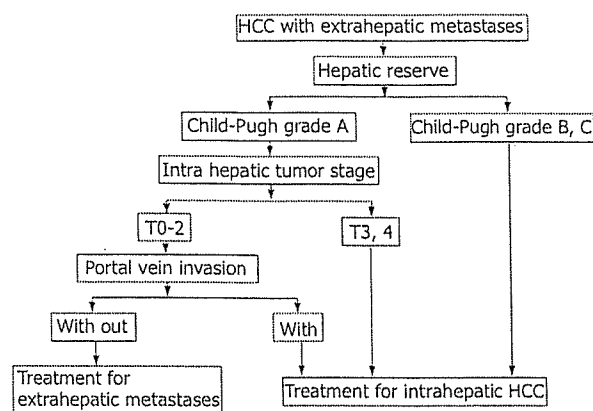


Figure 2 Initial sites to be treated.

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.

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Feasibility of Freeze-Dried Sera for Serological and Molecular Biological Detection of Hepatitis B and C Viruses[†]

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We compared hepatitis B virus (HBV) surface antigen, anti-hepatitis C virus (HCV) antibody, and HCV RNA quantification in frozen and freeze-dried serum samples to assess the usefulness of freeze-dried sera for detection of HBV and HCV. The results indicated that freeze-dried sera as well as frozen sera can be useful for serological and molecular biological analyses of HBV and HCV.

Freeze-dried sera are generally used as standards for hematochemical tests. In frozen and freeze-dried sera, protein, lipid, and electrolyte levels remain relatively stable, but enzyme levels show a greater degree of variation in freeze-dried sera than frozen sera (6). Nevertheless, freeze-dried sera can be stored at room temperature for a long time and are therefore easier to handle than frozen sera.

For the study of hepatitis B virus (HBV), dried blood spot (DBS) samples have been used for detecting hepatitis B virus surface antigen (HBsAg) and antibody to hepatitis B core antigen (2, 8). Recently, DBS samples allowed the development of a simple, sensitive, and appropriate test for quantifying HBV DNA and studying HBV genetic variants (5). As for hepatitis C virus (HCV), dried sera are used for the test of anti-HCV antibody (Ab) (2), and DBS samples allowed the development of a simple, sensitive, and reliable test for detection and genotyping of HCV RNA (1, 7). However, there is no report on their usefulness in HCV RNA quantification. We conducted serological and molecular biological tests to detect HBV and HCV using frozen and freeze-dried serum samples to determine the feasibility of freeze-dried sera.

The Atomic Bomb Casualty Commission established the Adult Health Study (AHS) longitudinal cohort in 1958; since then, the Atomic Bomb Casualty Commission and its successor, the Radiation Effects Research Foundation (RERF), have examined about 20,000 atomic-bomb survivors and controls biennially in outpatient clinics in Hiroshima and Nagasaki. We selected at random 12 consecutive HBsAg-positive and 25 consecutive anti-HCV Ab-positive individuals among 6,121 AHS longitudinal cohort subjects who underwent hepatitis screening from 1993 through 1995. Their serum samples were stored by both freezing and freeze-drying methods.

First, the procedure used for the preparation of frozen serum samples was as follows: Blood obtained from the AHS subjects was kept at room temperature for 20 min. Serum was

then divided into four equal parts and stored in 1.5-ml polypropylene tubes at -80°C until use. These samples were thawed by leaving them at room temperature for 30 min and mixed well by inversion before use. Second, the procedure used for the preparation for freeze-dried serum samples was as follows. A 0.4-ml portion of the serum was separated as mentioned above and stored in a glass tube at -80°C . After 1 week of storage, the samples were freeze-dried using a freeze-dryer, sealed, and stored at room temperature (20 to 25°C) until use. These samples were reconstituted by the volumetric method using diethyl pyrocarbonate-treated Milli-Q water and mixed well before use.

The tests for HBsAg and anti-HCV Ab using fresh serum samples in hepatitis screening from 1993 through 1995 were described previously (3, 4). In screening tests, an anti-HCV Ab titer of $\geq 2^{12}$ was defined as a high titer. In the present study, HBsAg and anti-HCV Ab were measured by enzyme immunoassay (EIA) (International Reagents Corporation, Kobe, Japan) and second-generation EIA (International Reagents Corporation), respectively. Measured values of ≥ 1.0 for HBsAg and anti-HCV Ab were defined as positive. An anti-HCV Ab titer of ≥ 50 was defined as a high titer.

Serum RNA was extracted from 100 μl of frozen or reconstituted freeze-dried serum samples using SepaGene RV-R (SankoJunyaku Co., Tokyo, Japan). The prepared RNA was reverse transcribed with random primers (6-mer) and reverse transcriptase (ReverTra Ace; Toyobo Co., Tokyo, Japan). HCV RNA was quantitated by real-time PCR using fluorescence resonance energy transfer probes. Primers and probes were designed within a highly conserved 5' untranslated region (UTR) and also targeted homologous regions of genotypes 1a, 1b, 2a, and 2b. The oligonucleotide sequences of the primers were as follows: HCVNC2, 5'-CCTGTGAGGA ACTACTGT C-3', and HCVNC1, 5'-CAACTACTCGGCTAGCAGTC-3'. The hybridization probes were as follows. Probe NCJ-LC (5'-GAACCGGTGAGTACACCGGAAT) was labeled at the 5' end with the fluorophore Red 640 and phosphorylated at the 3' end. Another probe, NCJ-FL (5'-GGGAGAGCCATAGT GGTCTGC), was labeled with fluorescein isothiocyanate at the 3' end. PCR was performed in a total volume of 20 μl ,

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containing 5 mM MgCl₂, 6 pmol of NCJ-LC, 4 pmol of NCJ-FL, 10 pmol of the two PCR primers, 2 μl of LightCycler-FastStart DNA Master hybridization probe mix (Roche Diagnostics Co.), and 1 μl of synthesized cDNA solution. The PCR cycling program consisted of an initial denaturing step at 95°C for 10 min and 50 amplification cycles of 95°C for 15 s, 55°C for 6 s, and 72°C for 10 s. Once the threshold was chosen, the point at which the amplification plot crossed the threshold was defined as the threshold cycle (C_T). The calculated C_T value is predictive of the quantity of target RNA copies. The standard curve was calculated using serially diluted plasmids containing nucleotide sequences of the HCV 5' UTR, to obtain control fragments for determination of HCV copy numbers. All assays were conducted in duplicate.

The positive-negative results of HBsAg in frozen and freeze-dried serum samples were consistent with results using fresh serum samples. The concordance in measurement of anti-HCV Ab among fresh, frozen, and freeze-dried serum samples was not complete but was satisfactory. Both frozen and freeze-dried serum samples of one case tested negative for anti-HCV Ab, despite testing positive in the 1993-1995 hepatitis screening. One freeze-dried serum sample of another case tested positive for anti-HCV Ab, despite testing negative in the 1993-1995 screening (Table 1). For these two patients with discrepant results, the specimen yielding a positive result contained only low titers of anti-HCV Ab; subsequent testing for HCV RNA by quantitative or qualitative PCR was negative in both cases (data not shown). Furthermore, 86% (18/21) of the fresh serum samples yielding high anti-HCV Ab titers by passive hemagglutination also yielded high anti-HCV Ab titers on subsequent testing of both frozen and freeze-dried serum samples by EIA; frozen and freeze-dried samples from the remain-

TABLE 1. Comparison of detection of HBsAg and anti-HCV Ab in frozen, freeze-dried, and fresh serum samples

Substance tested, sample type, and EIA result ^a	No. of fresh serum samples with PHA ^b result		Concordance (%)
	Positive	Negative	
HBsAg			
Frozen			
Positive	12	0	100
Negative	0	25	
Freeze-dried			
Positive	12	0	100
Negative	0	25	
Anti-HCV Ab			
Frozen			
Positive	24	0	97
Negative	1 ^c	12	
Freeze-dried			
Positive	24	1 ^c	95
Negative	1 ^c	11	

^a HBsAg was measured by EIA; anti-HCV Ab was measured by second-generation EIA.

^b HBsAg was measured with a reverse passive hemagglutination (PHA) test kit; anti-HCV Ab was measured with a second-generation PHA test kit.

^c HCV infection status was negative with quantitative or highly sensitive qualitative PCR.

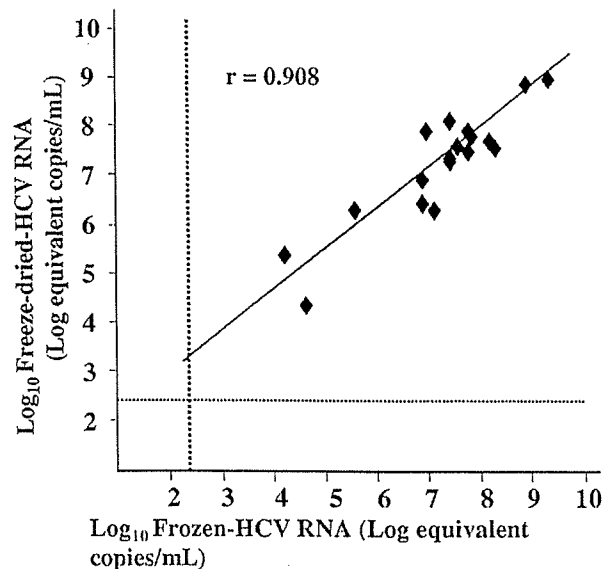


FIG. 1. Correlation of HCV RNA levels in frozen and freeze-dried serum samples. The correlation between log₁₀-transformed HCV RNA quantities for pairs of frozen and freeze-dried serum samples from 18 cases analyzed by real-time PCR was significant ($r = 0.908$, $P < 0.0001$).

ing three cases produced low-positive results. These results might be due to decay of the antibody during storage or differences in criteria for high antibody titer between the previous and present kits. However, the results for high anti-HCV Ab titers were completely consistent in frozen and freeze-dried serum samples.

A linear relationship in the range of 2.0×10^2 to 2.0×10^9 equivalent copies/ml was observed between C_T values and quantity of RNA copies ($r > 0.99$) (data not shown). Real-time PCR detected HCV RNA in 18 of 25 frozen and freeze-dried serum samples from anti-HCV-positive cases in the 1993-1995 hepatitis screening. The correlation between HCV RNA concentration in frozen and freeze-dried serum samples was significant ($r = 0.908$, $P < 0.0001$) (Fig. 1).

The intra-assay variability was determined by assaying two frozen serum samples containing HCV RNA of genotype 1b and 2a (respectively, 9×10^5 and 1.3×10^6 copies/ml) 10 times in a single day, and the respective coefficients of variation (CVs) were 6.2% and 2.9%. The respective interassay CVs calculated by assaying each of these serum samples once a day for 10 days were 3.6% and 4.3%.

On the whole, results for fresh, frozen, and freeze-dried serum samples for HBsAg and anti-HCV Ab demonstrated very good agreement, indicating that these methods and storage conditions are appropriate for serological assays of HBV and HCV. Furthermore, results of a newly developed highly sensitive and high-range HCV RNA quantitative assay for frozen and freeze-dried serum samples showed good correlation. We expected that the PCR products of HCV RNA would vary depending on storage method and conditions; however, the results showed no marked differences during 10 years of storage. The use of sera of AHS subjects stored from 1969 can further advance the study of the evolution of HBV/HCV as well as the natural history of viral liver diseases.

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Emergence of a Novel Lamivudine-Resistant Hepatitis B Virus Variant with a Substitution Outside the YMDD Motif[∇]

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Lamivudine is a major drug approved for treatment of chronic hepatitis B virus (HBV) infection. Emergence of drug-resistant mutants with amino acid substitutions in the YMDD motif is a well-documented problem during long-term lamivudine therapy. Here we report a novel lamivudine-resistant strain of HBV with an intact YMDD motif, which included an amino acid substitution, rtA181T, in the reverse transcriptase (RT) domain of HBV polymerase. The substitution also induced a unique amino acid substitution (W172L) in the overlapping hepatitis B surface (HBs) protein. The YMDD mutant strains were not detected even by using the sensitive peptide nucleic acid-mediated PCR clamping method. The detected nucleotide substitution was accompanied by the emergence of an additional nucleotide substitution that induced amino acid change (S331C) in the spacer domain. The rtA181T mutant strain displayed a threefold decrease in susceptibility to lamivudine in *in vitro* experiments in comparison with the wild type. *In vivo* analysis using human hepatocyte-chimeric mice confirmed the resistance of this mutant strain to lamivudine. We developed a method to detect this novel rtA181T mutation and a previously reported rtA181T mutation with the HBs stop codon using restriction fragment length polymorphism PCR and identified one patient with the latter pattern among 40 patients with lamivudine resistance. In conclusion, although the incidence is not high, we have to be careful regarding the emergence of lamivudine-resistant mutant strains with intact YMDD motif.

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma (4, 12, 33). To date, interferon and three nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, and entecavir) have been approved by the United States Food and Drug Administration for the treatment of chronic HBV infection. Lamivudine, an oral cytosine nucleoside analogue, potently inhibits HBV replication by interfering with RNA-dependent DNA polymerase (10, 16, 22). Lamivudine therapy suppresses HBV replication in most patients and improves transaminase levels and liver histology (16, 22, 25, 30). However, prolonged therapy results in the emergence of drug-resistant mutants in 24% and 70% of patients after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels (18).

Most lamivudine-resistant 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 (1, 9). *In vitro* analyses have confirmed that the rtL180M mutation augments the level of lamivudine resistance and enhances viral replication, while the rtV173L mutation enhances only viral replication (9, 23). On the other hand, only a few uncommon mutations associated with lamivudine resistance have been reported so far (3, 7, 24, 34). In the C domain of HBV polymerase, rtM204S and rtD205N were detected in patients with lamivudine resistance (3, 7). In the B domain, rtL180C and rtA181T were associated with lamivudine resistance (7, 24, 34). Yeh et al. (34) reported the emergence of rtA181T mutants in 4 of 23 patients who received long-term lamivudine therapy. The mutant appeared concomitantly with or after emergence of YMDD motif mutants and persisted thereafter. The nucleotide substitution in the FLLA motif resulted in early termination of the overlapping HBs gene transcription by creating a stop codon (TGG to TGA). Yeh et al. (34) demonstrated that the mutation reduced the

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susceptibility to lamivudine *in vitro*. They also detected such mutations in virus from a patient with leukemia and speculated that truncated HBs gene might be related to the development of leukemia (7).

Analyzing nucleotide and amino acid sequences of HBV in patients who developed a breakthrough, we identified a novel mutant that showed nucleotide substitutions in the B domain of the reverse transcriptase. The G residues of nucleotides 669 and 670 were mutated to T and A, respectively, and associated with the amino acid substitution rtA181T. The substitutions also induced the amino acid substitution W172L in the overlapping HBs protein. Since the nucleotide substitution was associated with nucleotide and amino acid substitutions in the putative spacer region of the polymerase, we checked the importance of these substitutions for resistance to lamivudine *in vitro*. We also analyzed the resistance of this new strain *in vivo* using a human hepatocyte-chimeric mouse (27, 31). Furthermore, we analyzed the susceptibility of the mutant strain to adefovir and entecavir. When used alone or in combination with lamivudine, these drugs are known to be effective against wild-type as well as lamivudine-resistant HBV (2, 5, 14, 17, 32). Infrequent emergence of resistance compared with lamivudine resistance has been reported for both of these two drugs (2, 5). We also developed a detection system to identify the novel and previously reported (7, 34) nucleotide substitutions to study the incidence of such mutations.

MATERIALS AND METHODS

Antiviral compounds. Lamivudine [(−)-β-L-2',3'-dideoxy-3'-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, United Kingdom). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and entecavir {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 (Wallingford, CT).

Analysis of virological markers. Hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and antibody against HBeAg (anti-HBe) were quantified by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using a Light Cycler (Roche, Mannheim, Germany). The primers used for amplification were 5'-TTGGGCATGGACA TTGAC-3' and 5'-GGTGAACAATGTTCCGGAGAC-3'. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 6 s. 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 each serum sample by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 µl H₂O. Full-length HBV DNA was amplified using the above HBV DNA samples by the method of Gunther et al. (13). Nucleotide sequence positions were numbered from the unique EcoRI site. The 1.4-genome-length HBV DNA amplified from the serum of a patient who showed lamivudine resistance was cloned into plasmid vector pTRE (Takara Bio, Tokyo, Japan) (patient strain). In brief, the PCR product amplified using serum from the patient was cleaved with BamHI and ApaI (HBV positions 1400 to 2600) and cloned into pcDNA3 (Invitrogen, San Diego, CA), and the resulting construct was named pcDNA3-1. Similarly, the PCR product was cleaved with ApaI and BamHI (HBV positions 2600 to 3215 and 1 to 1400) and cloned into pBlueScript SK+ (Stratagene, La Jolla, CA), and the resulting construct was named pB-1. The KpnI-BamHI fragment from pB-1 and the KpnI-ApaI fragment from pcDNA3-1 were cloned into pcDNA3-1. Finally, the plasmids were cleaved with HindIII and NotI within the multicloning site and cloned into plasmid vector pTRE. As a laboratory strain, we employed a plasmid containing a 1.4-genome-length wild-type genotype C HBV (wild-type strain; GenBank accession number AB206816) (31). To introduce the nucleotide substitutions into the S331C/rtA181T patient and wild-type strains, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene).

TABLE 1. *In vitro* susceptibility of the S331/rtA181 mutant to lamivudine^a

Strain		S331/rtA181 mutation	Lamivudine IC ₅₀ (µM)	Resistance (fold)
Source	Type			
Patient	WT	-/-	0.19 ± 0.01	1
	S331C	C/-	0.23 ± 0.01	1.2*
	rtA181T	-/T	0.58 ± 0.08	3**
	S331C/rtA181T	C/T	0.57 ± 0.06	3**
Laboratory	WT	-/-	0.23 ± 0.04	1
	S331C	C/-	0.3 ± 0.05	1.3*
	rtA181T	-/T	0.88 ± 0.2	3.9**
	S331C/rtA181T	C/T	0.98 ± 0.12	4.3**

^a Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** *P* < 0.001 compared to the wild type.

The eight plasmids with and without amino acid substitutions in the spacer and reverse transcriptase domain are listed in Table 1.

Cell culture, transfection, and determination of IC₅₀. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in 5% CO₂. Cells were seeded to semiconfluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 cells was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC₅₀) for each antiviral drug, various concentrations of lamivudine, adefovir, and entecavir were added after 24 h to the culture plate containing the cells, and cells were harvested after 5 days. The medium containing the drugs was changed on days 1, 3, and 4. A plasmid encoding β-galactosidase (β-Gal) was cotransfected to adjust the transfection efficiency. The β-Gal enzyme assay was performed with a β-Gal enzyme assay system (Promega, Madison, WI). All experiments were performed in triplicate. GraphPad Prism software (GraphPad Software, Inc.) was used to determine the best-fit values for individual dose-response equations.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation. The cells were harvested at 3 or 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% [vol/vol] NP-40) followed by centrifugation for 2 min at 15,000 × *g*. The core-associated HBV genome was immunoprecipitated by mouse anticore monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after sodium dodecyl sulfate-proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with SYBR green using a 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 s, annealing at 58°C for 5 s and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Evaluation of effects of antiviral drugs on mutant strains using human hepatocyte-chimeric mice. Human hepatocyte-chimeric mice were generated and used in the drug evaluation studies as described previously (27, 31). Briefly, human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and develop liver failure. The transplanted cells were characterized in terms of *in vivo* growth potential and function. The human hepatocytes progressively repopulated the murine host liver and were susceptible to cultured-cell-line-produced HBV. All animal protocols were performed in accordance with the guidelines of the local committee for animal experimentation. The mice were inoculated with 50 µl of serum samples containing wild-type and newly identified drug-resistant strains. Serum samples obtained from mice were stored at -80°C before further analyses. After stable high-level HBV viremia was confirmed, the mice were administered food containing 30 mg of lamivudine/kg of body weight/day. The nucleotide sequences of wild-type and mutant strains were confirmed by sequencing analysis.

Detection of rtA181T mutants by PCR with restriction fragment length polymorphism (RFLP). HBV DNA extracted from serum samples were amplified by

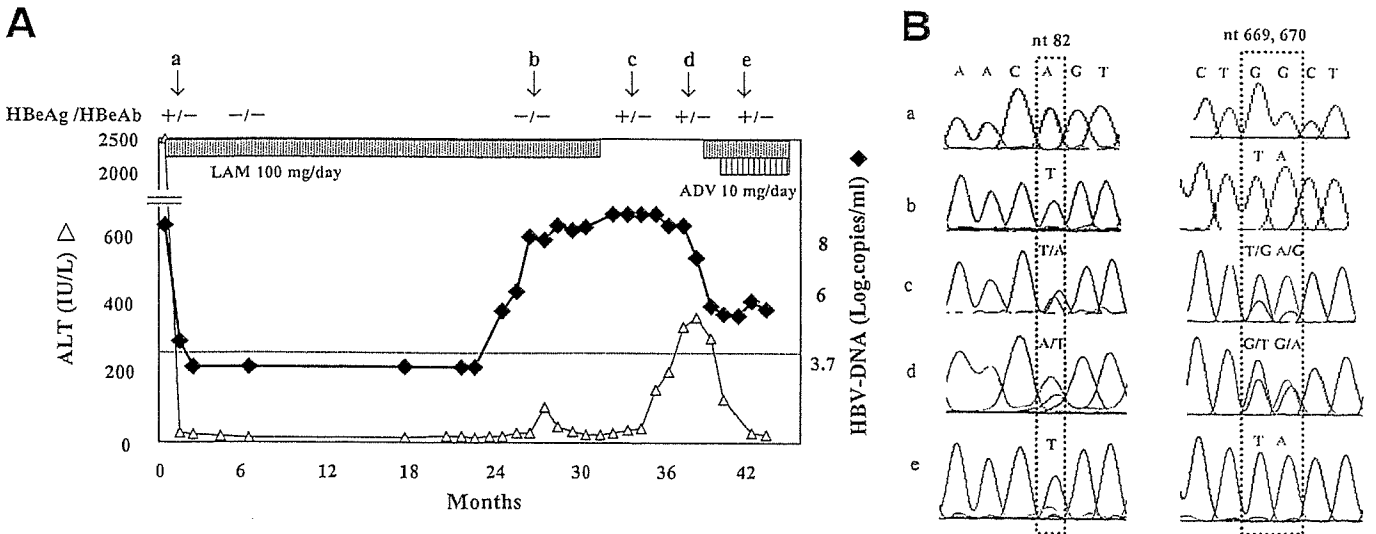


FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis B virus by direct sequencing. Time points of serum sampling (see panel A) were as follows: (a) just before lamivudine treatment, (b) after breakthrough, (c) after cessation of lamivudine treatment, (d) just before readministration of lamivudine, and (e) during adefovir and lamivudine therapy. Note that the wild type reappeared during the cessation of therapy (c and d), but it disappeared after readministration of the drug (e).

PCR using the primers 5'-GCCCGTTTGTCTCTACTTCCA-3' and 5'-ACCACTGAACAATGGCACTAGTAAGCTGA-3'. The reverse primer was designed to introduce an *EspI* site (GTCAGC) into only wild-type sequences. The PCR was performed in a total volume of 25 μ l, consisting of a reaction buffer (100 mmol/liter Tris-HCl [pH 8.3], 50 mmol/liter KCl, and 15 mmol/liter MgCl₂), 0.2 mmol/liter of each deoxynucleoside triphosphate, 1 μ l of the DNA solution, 10 pmol of each primer and 1 U of *Taq* DNA polymerase (Gene Taq; Wako Pure Chemicals, Tokyo, Japan) with 0.2 μ g of anti-*Taq* high (Toyobo Co., Osaka,

Japan). The amplification conditions included an initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 1 min, annealing of primer at 58°C for 1 min, extension at 72°C for 2 min), and final extension at 72°C for 7 min. Two μ l of PCR products was digested with 5 U of *EspI* and subjected to electrophoresis in a 3.5% agarose gel.

Statistical analysis. Data are expressed as means \pm standard deviations (SD). Group comparisons were performed using the Student *t* test. A *P* value of less than 0.05 was considered statistically significant.

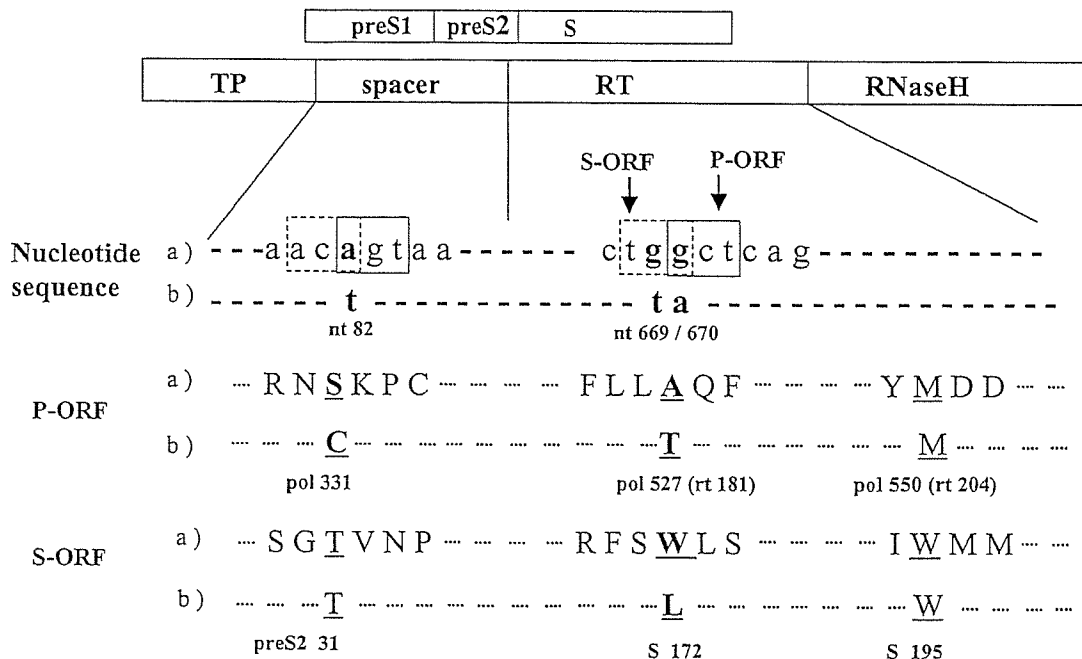


FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were compared. See Fig. 1A for time points of serum sampling. Nucleotide sequence numbers are those of typical HBV (e.g., accession no. AB206816 [31]), which starts from a unique *EcoRI* site.

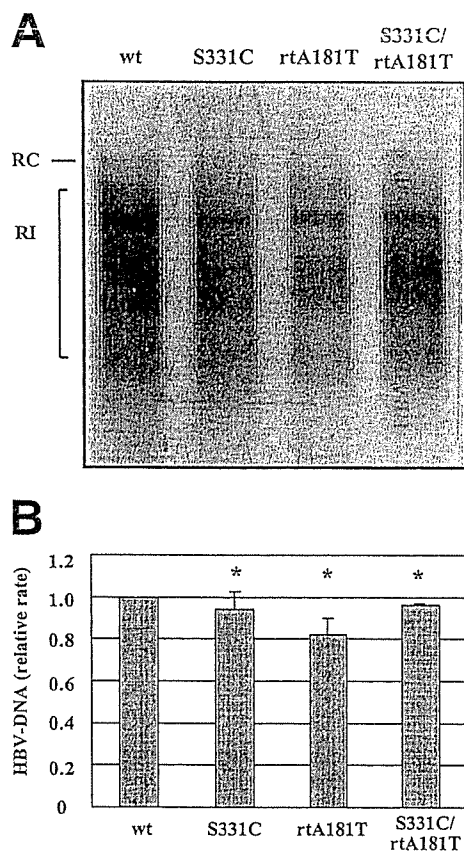


FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated from HepG2 cells at 3 days after transfection. The positions of relaxed circular DNA (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core-associated intermediates of HBV. Experiments were performed in triplicate. Values are relative to those of the wild type and are expressed as means \pm SD. *, not significant compared to the wild type.

RESULTS

Isolation of a novel lamivudine-resistant strain with an intact YMDD motif. The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV level at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD and YVDD mutants were not detected even with a peptide nucleic acid-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison by the direct sequence method of nucleotide sequences obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (polS331C) and reverse transcriptase

(polA527T or rtA181T) domains of the polymerase (Fig. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough, and eleven of them showed the above amino acid substitutions. Only one clone showed the wild-type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued, and this strain outcompeted the wild-type strain upon administration of the drug (Fig. 1B). These results prompted us to study the significance of each of these mutations.

Effect of substitutions on HBV replication. To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4-genome-length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient's wild-type strain, the replication capacities of the S331C, rtA181T, and S331C/rtA181T mutants were not different (94%, 82%, and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild-type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro. To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4; Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in either patient or laboratory strains. In contrast, an amino acid substitution in the polymerase (rtA181T) induced resistance that was 3.0 and 3.9 times greater than that of patient and laboratory strains ($P < 0.001$), respectively. The presence of both of these amino acid changes induced 3.0 and 4.3 times greater resistance in each of the above strains. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the HBs antigen produced to culture supernatant was different between the two strains (52.5 ± 8.2 and 4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte-chimeric mice. To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte-chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high-level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only 1 of 12 clones obtained from the inoculum was wild type, while the remaining 11 clones were rtA181T mutants with an intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had the

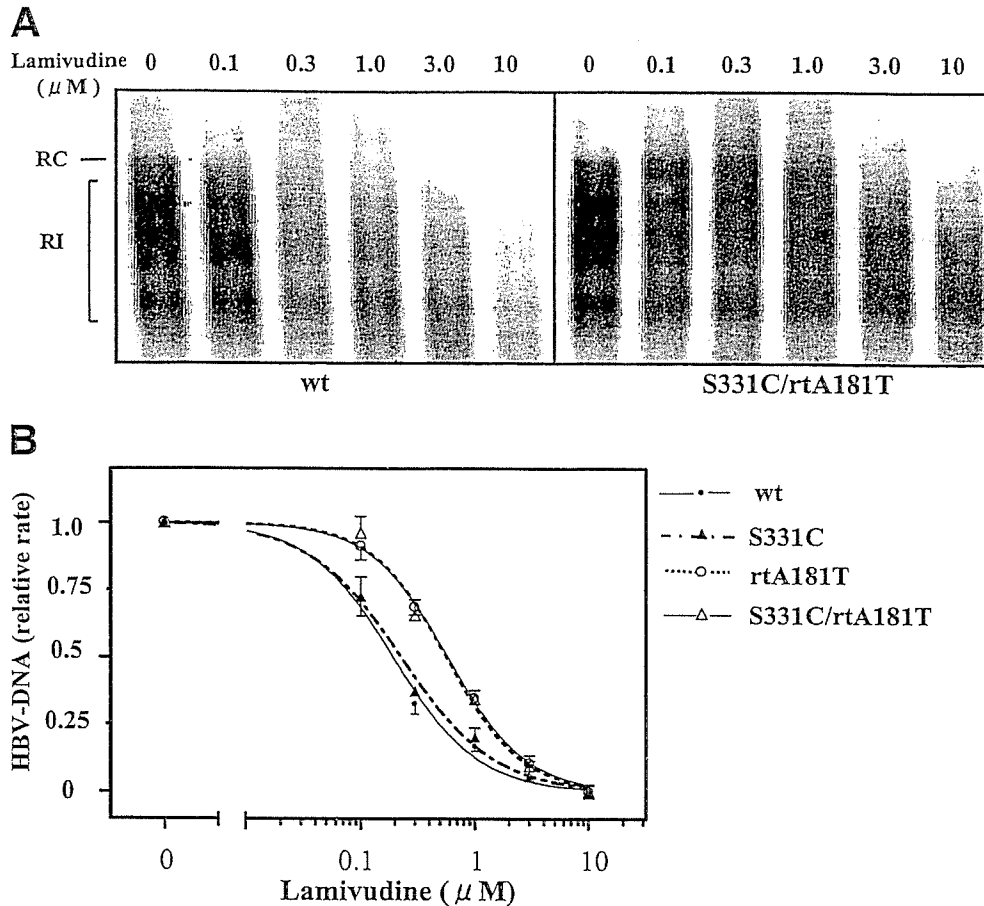


FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative results for the wild type (wt) and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediate (RI) forms of HBV DNA are indicated. (B) Dose-response curves of the four HBV strains against lamivudine. The curves were used to estimate the lamivudine IC_{50} s for each HBV strains. Values are relative to no-lamivudine controls for each strain. Experiments were performed in triplicate. Values are expressed as means \pm SD.

rtA181T mutation (data not shown). Two other mice were inoculated with wild-type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high-level viremia (8.3 and 9.3 log copies/ml, respec-

tively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After 6 weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copy/ml in the mutant ($P < 0.001$) (Fig. 5).

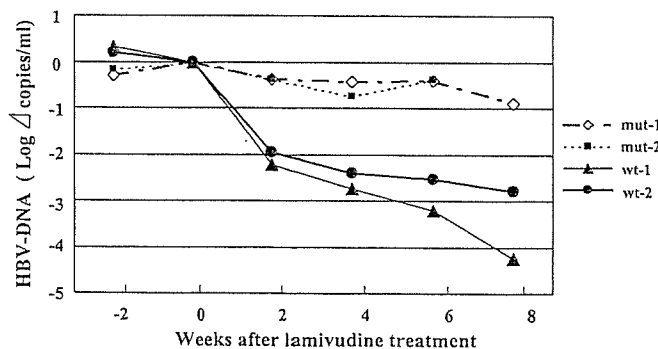


FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

Susceptibility of mutants to adefovir and entecavir in vitro. We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using a transient-transfection assay with HepG2 cells. The IC_{50} s of these drugs for the mutant strain and wild type were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine. In this study, we developed a RFLP PCR method to detect the rtA181T mutants, by which we were able to detect mutant strains even when they were mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal to or more than 1 log) during lamivudine therapy. We found that only one of these patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the