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-

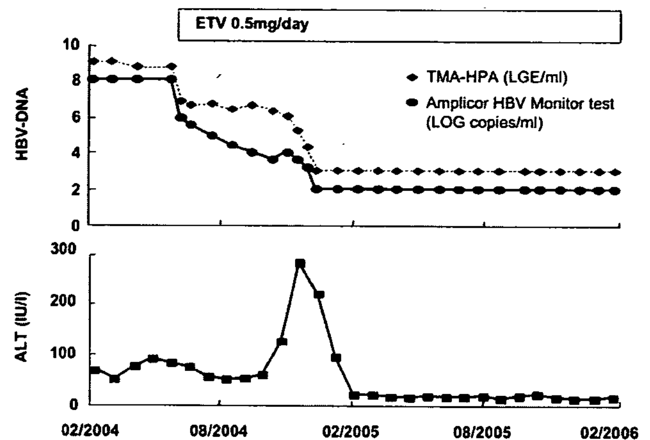

Fig. 1. Time courses of serum HBV DNA and ALT levels of patients treated with ETV. Expression of HBV nucleic acids determined by the TMA-HPA was higher than that determined by the Amplicor HBV Monitor test soon after beginning administration of ETV. The discrepancy was less marked when both measurements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

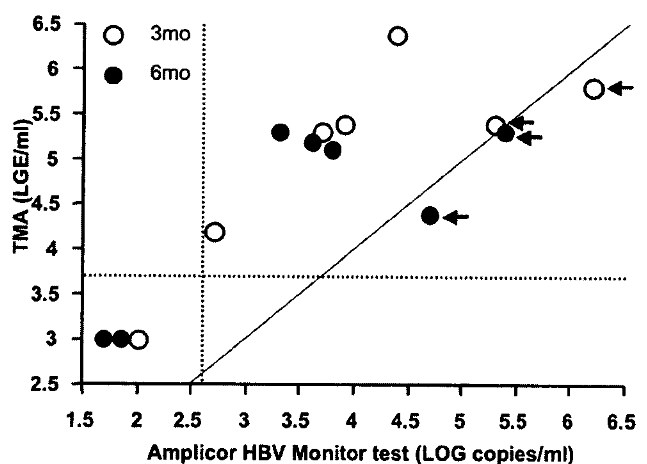

Fig. 2. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during ETV therapy. Serum samples obtained from the 2 patients who received low-dose ETV (0.01 mg) are indicated by arrows. The vertical and horizontal dotted lines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

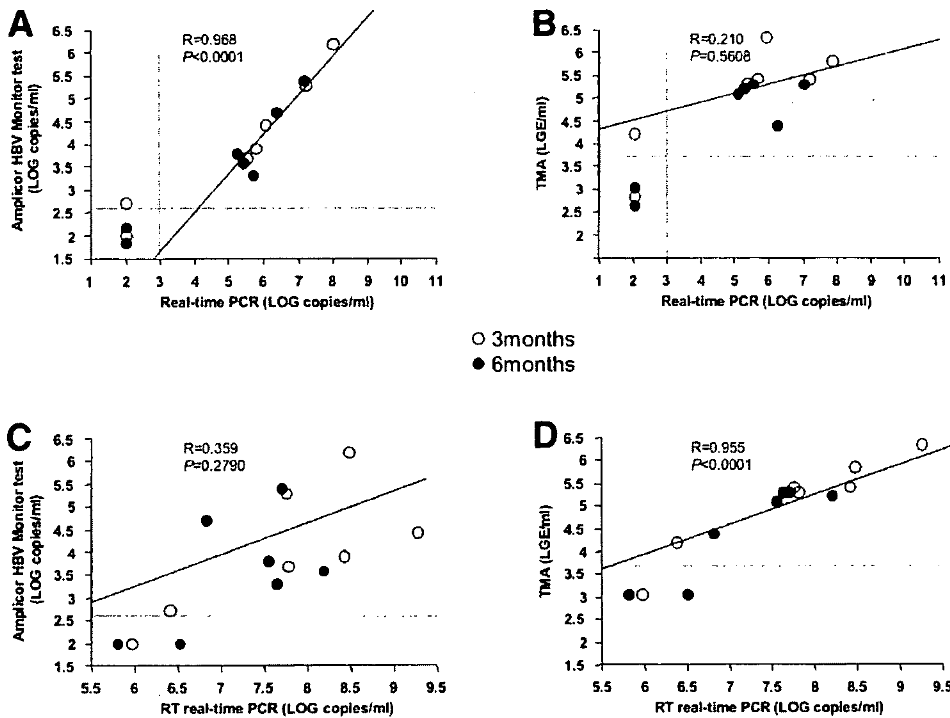

Fig. 3. Correlation between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of ETV therapy. (A) Correlation between HBV DNA level determined by Amplicor HBV Monitor test and that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA and 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).

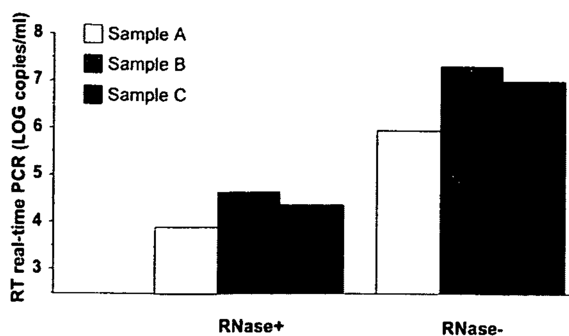

Fig. 4. Presence of HBV RNA confirmed by RNA treatment of 3 nucleic acid samples (samples A-C) obtained from patients after 3 months of LAM therapy. Extracted nucleic acid samples with or without RNase digestion were further digested by proteinase K and ethanol-precipitated after phenol/chloroform extraction. 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.

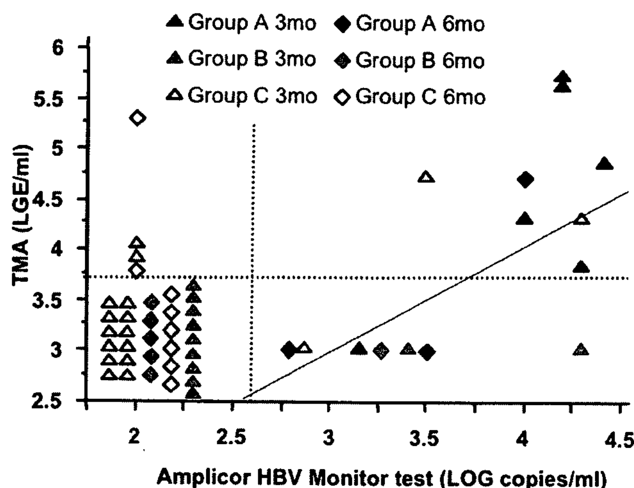

Fig. 5. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during LAM therapy. During ETV therapy the TMA-HPA showed higher expression of HBV DNA in patients regardless of the presence of the mutation than did the Amplicor 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

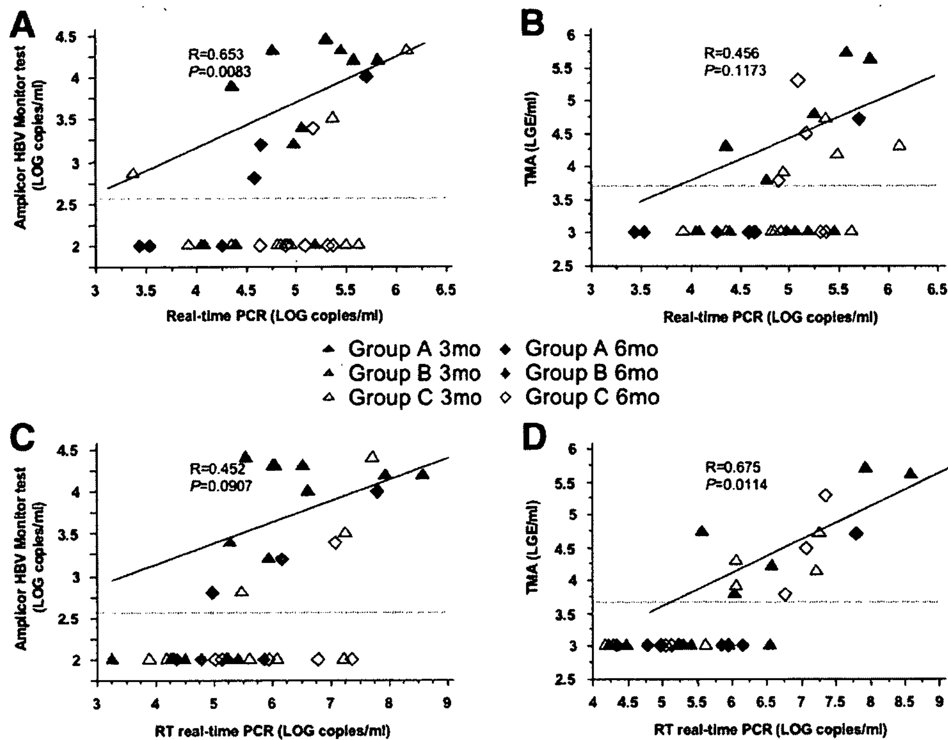

Fig. 6. Correlations between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of LAM therapy. (A) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA 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.

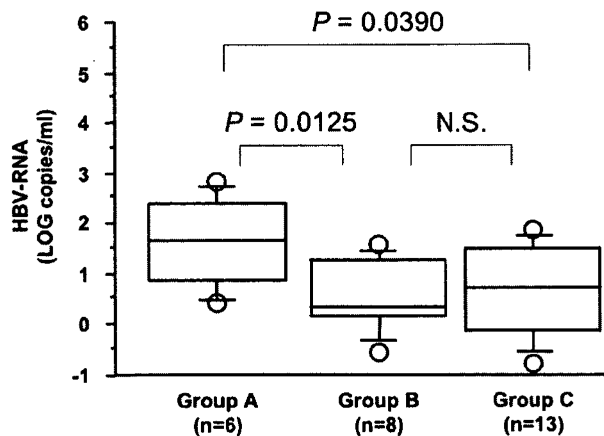

Fig. 7. Box plots of HBV RNA levels of patients in group A (patients who showed emergence of the mutants within 1 year, group B (those who developed resistance after 1 year of LAM therapy), and group C (patients who did not show resistance to LAM therapy). HBV RNA level represents the difference between 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.

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World J Gastroenterol 2007 January 21; 13(3): 414-420
World Journal of Gastroenterology ISSN 1007-9327
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RAPID COMMUNICATION

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

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Received: 2006-10-29 Accepted: 2006-12-08

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

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Key words: Hepatocellular carcinoma; Extrahepatic metastases; Prognosis; Causes of death

Uka K, Aikata H, Takaki S, Shirakawa H, Jeong SC, Yamashina K, Hiramatsu A, Kodama H, Takahashi S, Chayama K. Clinical features and prognosis of patients with extrahepatic metastases from hepatocellular carcinoma. *World J Gastroenterol* 2007; 13(3): 414-420

http://www.wjgnet.com/1007-9327/13/414.asp

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 ^{18}F-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 (≤ 50% *vs* > 50%), intrahepatic tumor morphology (nodular type *vs* non nodular type), portal venous invasion (Vp 0-2 *vs* > Vp 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).

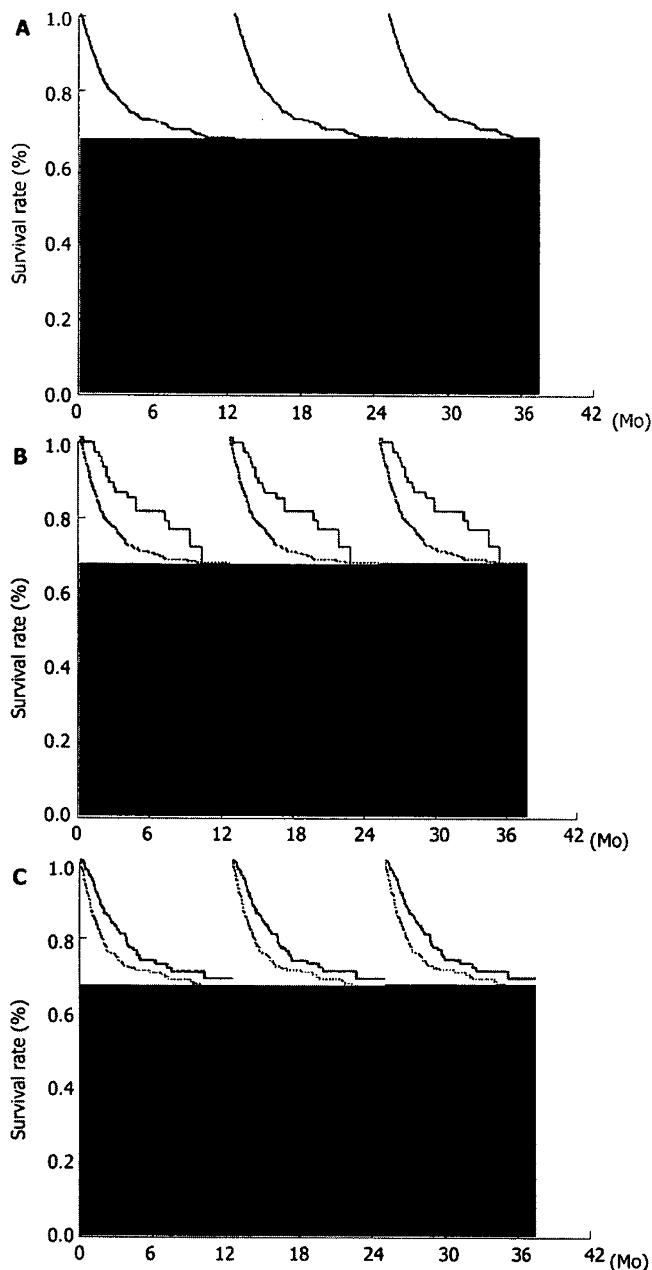

Figure 1 Survival rate of 151 HCC patients with extrahepatic metastases (A), intrahepatic tumor stage (B) [solid line: T0-T2, dashed line: T3, T4 (log-rank test: $P < 0.001$)], and after treatment of extrahepatic metastases (C) [solid line: treatment group, dashed line: no treatment group (log-rank test: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

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	*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,

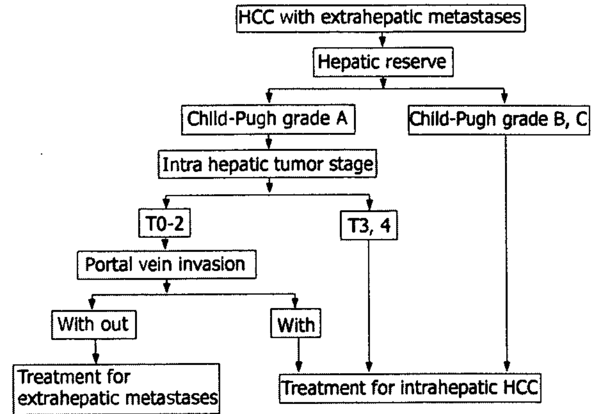

Figure 2 Initial sites to be treated.

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.

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

Res. Adv. in Microbiology 7, 2007

Validation of the use of freeze-dried sera for the diagnosis of hepatitis B and C virus infections in a longitudinal study cohort

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ABSTRACT
Use of stored serum samples has been invaluable for the collection of data for epidemiological and clinical studies related to liver diseases. We review here in this respect the usefulness of freeze-dried sera for the diagnosis of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. We compared HBV surface antigen (HBsAg), antibody to hepatitis B core antigen (anti-HBc Ab), anti-HCV antibody (anti-HCV Ab), HBV and HCV genotypes, and quantitative detection of HBV DNA and HCV RNA in frozen and freeze-dried serum samples obtained from 12 HBsAg-positive cases and 25 anti-HCV-positive cases among Adult Health Study (AHS) longitudinal cohort subjects in Hiroshima and Nagasaki. Freeze-dried sera, as well as frozen sera, were found to be useful for serological and molecular biological detection of HBV and HCV infections. Moreover, the distributions of HBV and HCV infection-positive samples within stored frozen and freeze-dried sera were found to be reliable in a nested case-control study of hepatocellular carcinoma among AHS cohort subjects. It is expected that use of freeze-dried sera, as well as frozen sera, stored for long periods may aid and abet with ongoing and future research on the etiology and pathogenesis of liver diseases.

Abbreviations: HBsAg, hepatitis B surface antigen; anti-HBc Ab, antibody to hepatitis B core antigen; anti-HCV Ab, anti-hepatitis C virus antibody; AHS, Adult Health Study; DBS, dried blood spot; RNase, ribonuclease; C_T, threshold cycle; CVs, coefficients of variation; HCC, hepatocellular carcinoma

INTRODUCTION
Molecular biological research on hepatitis B virus (HBV) and hepatitis C virus (HCV) has advanced remarkably over the last decade, resulting in the establishment of much improved methodologies for diagnosis, prevention, and treatment (1-7) of liver diseases. Several previous reports demonstrated that use of dried blood spot (DBS) samples, as well as frozen serum samples, significantly enabled essential data to be collected for epidemiological and clinical studies, as well as for the screening of subjects for liver disease (8-11).

The Atomic Bomb Casualty Commission (ABCC) and its successor, the Radiation Effects

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Research Foundation (RERF), established the Adult Health Study (AHS) longitudinal cohort in 1958. Approximately 20,000 atomic-bomb survivors and non-exposed controls comprise the cohort, and have been examined biennially in outpatient clinics in Hiroshima and Nagasaki (12). From 1969, sera obtained from the AHS participants have been stored by either freezing or freeze-drying methods, and from 1990, their samples were stored by both freezing and freeze-drying methods in Hiroshima and Nagasaki. Biochemical tests using frozen sera and freeze-dried sera showed that protein, lipid, and electrolyte levels are relatively stable in both types of sera, but that enzyme levels were more variable in the freeze-dried sera than in the frozen sera (13). Nevertheless, the freeze-drying method is considered appropriate for long-term storage, because freeze-dried sera can be stored economically at room temperatures for a long time and are therefore easier to handle than frozen sera, especially for shipping.

We have reviewed serological and molecular biological assays of HBV and HCV using sera stored by both freezing and freeze-drying methods, and have assessed the feasibility of using freeze-dried sera in liver disease research.

Preparation of frozen and freeze-dried serum samples

The utility of stored serum samples for virological assays depend on both preparative methods and storage condition. Therefore, storage methods for serum samples should be optimized for the purposes of epidemiological and clinical studies, or for simple screening. Wang et al. (14) compared the yields of PCR products for duck hepatitis B virus DNA after storage of serum dried on filters at 37, 4, –20, and –70°C, and showed that the optimal condition for storage was -70℃. Halfon et al. (15) compared HCV RNA levels after storage at room temperature, –20, and –80°C, and indicated that long-term stability was observed at –80°C, with a slight loss of about of 10% HCV RNA levels. HCV RNA is easily decomposed by ribonuclease (RNase), which exists in perspiration and saliva, and its detectability decreases by repeated freezing and thawing of sera. Halfon et al. also indicated that five freeze-thaw cycles resulted in 16% decrease of HCV RNA level (15). Therefore, it is recommended that each serum sample is dispensed into small volumes at the time of specimen procurement and stored for long-term conservation at –80°C to avoid cycles of freezing and thawing.

We used the following procedure for the preparation of frozen serum samples as previously described (16). Serum obtained from AHS participants was then dispensed into four equal volumes and aliquotted into 1.5 ml polypropylene tubes, and stored at –80°C until use. These serum samples were thawed by leaving them at room temperature for 30 minutes and then mixed well by inversion before use.

We used the following previously described (16) procedure for the preparation of freeze-dried serum samples. A 0.4-ml portion of the serum was stored in a glass tube at –80°C, and after 1 week of storage, these samples were freeze-dried in batches using a freeze-dryer (LABCONCO, Asahi Life Science Co., Tokyo, Japan), sealed automatically to maintain a vacuum, and stored at room temperature (20 to 25°C) until use. The conditions applied for freeze-drying were a gas pressure of 0.10-0.18 torr, a shelf temperature of less than 25°C, and a finishing drying time of at least 7 hours. The moisture content of freeze-dried serum samples was normally maintained at less than 0.5%. Moisture content of freeze-dried serum samples of less than 1% was necessary to retain and assay various antibodies of interest. These conditions kept the effect of temperature changes on enzyme activity to a minimum, thus served as a precautionary measure based on general principles of sample chemistry (13). These serum samples were reconstituted with the volumetric method using diethyl pyrocarbonate (DEPC)-treated MilliQ water and mixed well before use.

Serological assay

Several previous reports demonstrated the

usefulness of DBS samples for HBV surface antigen (HBsAg), antibody to hepatitis B core antigen (anti-HBc Ab), and anti- HCV antibody (anti-HCV Ab) (8, 10, 17, 18). Das et al (10) compared anti-HCV Ab and HBsAg between fresh sera and blotting paper (BP)-derived dry specimens. HBsAg tests showed excellent agreement between fresh and dry specimens. Anti-HCV Ab tests showed considerable reductions of titers in BP-derived dry specimens, although such specimens provided excellent qualitative concordance with their fresh specimens.

To assess the usefulness of freeze-dried sera for detection of HBV and HCV, we used stored serum samples obtained from 12 HBsAg-positive and 25 anti-HCV Ab-positive individuals (16) among 6,121 AHS cohort subjects who underwent hepatitis screening from 1993 through 1995 (19, 20). These serum samples had been processed and stored by both the freezing and freeze-drying methods.

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

	No. of frozen serum samples with EIA* result			Concordance
EIA result*	positive	negative	Indeterminant†	(%)
Freeze-dried serum samples				
HBsAg†				
positive	12	0		100
negative	0	25		
Anti-HBc Ab‡				
positive	24	0	0	
negative	0	8	1	92
Indeterminant	2	0	2	
Anti-HCV Ab†				
positive	24	1§		97
negative	0	12		

* HBsAg, anti-HBc Ab and anti-HCV Ab were measured by enzyme immunoassay (EIA).

† Measured values of ≥ 1.0 for HBsAg and anti-HCV Ab were defined as positive.

‡ Measured values for anti-HBc Ab of $\geq 70\%$, $< 50\%$, and $50 < \leq 70\%$ were defined as positive, negative and indeterminant, respectively.

§ Anti-HCV Ab titer is very low and HCV infection status was negative with highly sensitive qualitative PCR.

Detection of HBsAg, anti-HCV Ab, and anti-HBc Ab

In hepatitis screening from 1993 through 1995, the tests of HBsAg and anti-HCV Ab using fresh serum samples were measured by reverse passive hemagglutination (R-PHA) test kit and the second-generation PHA test kit, as described previously (19, 20). The tests of HBsAg and anti-HCV Ab using frozen and freeze-dried serum samples were measured by enzyme immunoassay (EIA) and the second-generation EIA, respectively, as described previously (16). The 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.

In our previous report (16), qualitative comparison of HBsAg and anti-HCV Ab using fresh, frozen, and freeze-dried serum samples showed excellent agreement, though the detection methods of HBsAg and anti-HCV Ab in frozen and freeze-dried serum samples were historically different from those in fresh serum samples. The results indicated that freeze-dried sera, as well as frozen sera, are interchangeable with those of fresh sera. This study also showed that freeze-dried sera are interchangeable with frozen sera in the qualitative detection of HBs Ag and anti-HCV Ab (Table 1).

Moreover, the frozen and freeze-dried serum samples were comparable in terms of the quantitative detection of HBsAg. However, in six cases there was considerable reduction an average of 35%of anti-HCV Ab titer in the freeze-dried serum samples when compared to the frozen serum samples. These six sample pairs showed all middle to low titers of anti-HCV Ab (data not shown). This finding suggests that even a minimum level enzyme activity in serum might affect anti-HCV Ab titers following prolonged storage at room temperature, even if the moisture content of freeze-dried serum samples was maintained at less than 0.5%.

In our previous report (16), we did not compare the results of the anti-HBc Ab measured by EIA in frozen and freeze-dried serum samples with those measured by PHA in fresh serum samples, because the sensitivity of the test for anti-HBc Ab is greatly affected by the methodology. Thus we compared the results for anti-HBc Ab in frozen serum samples with those in freeze-dried serum samples (Table 1). Anti-HBc Ab was measured by FIA (International Reagents Corporation, Kobe, Japan). The measured values for anti-HBc Ab of ≥ 70%, < 50%, and 50< ≤ 70% were defined as positive, negative and indeterminant, respectively. Concordance in measurement of anti-HBc Ab among frozen and freeze-dried serum samples was not complete, but was satisfactory. Frozen serum samples of two cases tested positive for anti-HBc Ab, but freeze-dried serum samples were indeterminant. On the other hand, test results of a frozen serum sample of one case proved to be indeterminant, while a freeze-dried serum sample tested negative for anti-HBc Ab. For these three cases with discrepant results, the measured values for the anti-HBc Ab specimens that yielded positive or negative result were close to being indeterminant. In addition, in these three cases the anti-HBc Ab titer was reduced by 5 to 10% in the freeze-dried serum samples compared with the frozen serum samples. As was the case for the previously mentioned reduction in titer of anti-HCV Ab, this finding also suggests that even a minimum enzyme activity in serum might affect anti-HBc Ab titers when serum samples are stored at room temperature for prolonged periods.

Anti-HBc Ab is present during the acute, convalescent, and chronic phases of HBV infection, and persists for many years (17). According to the manufacture's instruction, serum samples that had values for anti-HBc Ab of ≥ 95% were diluted two-hundred fold, and anti-HBc Ab of these diluted specimens were again measured. Measured values for anti-HBc Ab of ≥ 90% on samples diluted 200 fold were defined as high titer, and subjects that were anti-HBc Ab high titer-positive, were diagnosed as having chronic phase HBV infections. Since the measured values for anti-HBc Ab in all three cases with discrepant results were under 95%, further measurement of anti-HBc Ab by the dilution method was not required. Thus, these

indeterminant results did not seem to affect the diagnosis of chronic HBV infection.

As a whole, these findings show that freeze-dried sera, as well as frozen sera, can be useful for serological assays of HBV and HCV. We can conclude that serological assays using freeze-dried sera may be reliable for diagnosis of HBV and HCV infections. Moreover, use of more sensitive, molecular biological assays might well compensate for the loss of serological reactivity in freeze-dried serum samples.

Molecular biological assay

Several previous reports demonstrated usefulness of DBS samples for quantitative detection of HBV DNA, qualitative detection of HCV RNA, genotyping of HBV and HCV, and detection of HBV pre-core mutants and YMDD mutants (11, 21, 22). However, no report existed on usefulness of DBS samples, or dried sera, in the quantitative detection of HCV RNA. In a recent report (16), we had indicated that freeze-dried sera, as well as frozen sera, can be useful for HCV RNA quantification. In addition, it was reported recently that lyophilized standards could be used for the calibration of quantitative PCR detection assays of HCV RNA (23).

Genotyping of HBV and HCV

In recent years, HBV and HCV genotypes have been recognized as important viral factors related to pathological status, effectiveness of antiviral treatments, and prognosis of livei disease (2-5, 24). In this regard, we have tested the suitability of stored, freeze-dried sera obtained from 12 HBsAg-positive and 25 anti-HCV Ab-positive individuals for HBV and HCV genotyping. Serum DNA was extracted from either 100 µl of frozen, or reconstituted, freeze-dried serum samples using QIAamp Mini

Table 2. HBV and HCV genotype characterization in frozen and freeze-dried serum samples

	No. of frozen serum samples with PCR* results of HBV and HCV genotype			Concordance (%)
Freeze-dried serum samples				
HBV genotype	C	Undetectable		
C	9	1		92
Undetectable	0	2†		
HCV genotype	1b	2a	Undetectable	
1b	16	0	0	
2a	0	4	0	96
Undetectable	1	0	4‡	

* HBV and HCV genotypes were determined by PCR using type-specific primers.
† HBV DNA concentration was low for detecting some genotypic determinants.
‡ HCV infection status was negative with highly sensitive qualitative PCR.

Kit (Qiagen, Tokyo, Japan), according to the instructions supplied by the manufacturer. HBV genotype was determined by PCR using type-specific primers in regions of the pre-S1 through S genes, as described previously (25). Serum RNA was extracted from 100 μl of frozen or reconstituted freeze-dried serum samples using SepaGene RV-R (SankoJunyaku Co., Tokyo, Japan), according to the instructions provided by the manufacturer. The prepared RNA was reverse transcribed with random primers (6-mer) and reverse transcriptase (ReverTra Ace, TOYOBO Co., Tokyo, Japan), using the instructions supplied by the manufacturer. HCV genotype was determined by PCR using type-specific primers in the NS5 region, as described previously (26). All assays were conducted in duplicate or more.

Table 2 shows HBV and HCV genotype characterization in frozen and freeze-dried serum samples. HBV genotypes could be identified in 9 frozen and 10 freeze-dried serum samples, all of which were found to be genotype C. We could not determine the HBV genotype in 3 of the frozen and 2 of the freeze-dried serum samples, due to possible poor amplification of the low HBV DNA concentrations in these samples. HCV genotype 1b was identified in 17 frozen and 16 freeze-dried serum samples. In addition, HCV genotype 2a was identified in each of 4 frozen and 4 freeze-dried serum samples tested. We could not determine the HCV genotype in 4 frozen and 5 freeze-dried serum samples, possible due to the possibility that these samples were HCV negative, or that there was the poor amplification due to the low HCV RNA concentration.

The observed discordance presumably

Table 3. Establishment of detection limit for HCV RNA using highly sensitive qualitative PCR assay

HCV RNA (IU/ml)*	Estimated Input of HCV RNA (IU)†	Positive/Number tested	Positive Ratio %
4,700,000	47,000	8/8	100
940,000	9,400	8/8	100
188,000	1,880	8/8	100
37,600	376	8/8	100
7,520	75	8/8	100
1,504	15	8/8	100
301	3	8/8	100
100	1	8/8	100
60	0.6	4/8	50
0	0	0/8	0

* A serum sample known as HCV RNA concentration by Amplicore HCV monitor test ver. 2.0 (4700 kIU/ml, genotype 1b) was diluted with serum from an HCV –negative individual.

† Estimated input of HCV RNA in each cDNA solution was applied to the respective highly sensitive qualitative PCR assay.

was not due to differences in genotype, but rather due to difficulties in PCR amplification. Since all discordant cases were borderline, or undetectable, in the highly sensitive, qualitative PCR assays (Table 3), the discordance seemed to be attributable to variations in the detection limits of PCR, arising from the minimal amounts of DNA and RNA. Alternatively, it is possible that the HBV DNA and the HCV RNA had been degraded during prolonged storage of either the frozen or freeze-dried sera. However we can conclude that both the frozen and the freeze-dried sera may be useful for HBV and HCV genotyping.

Quantitative detection of HBV DNA and HCV RNA

In this phase of the work, we quantified and compared HBV DNA levels in HBsAg-positive frozen and freeze-dried serum samples. Quantification of HBV DNA was performed using real-time PCR and fluorescence resonance energy transfer probes and primers, that were designed for a highly conserved X region. A linear relationship in the range of 2.0×10-2.0×10^9 equivalent copies/mL was observed between threshold cycle (C_T) values and quantity of DNA copies ($r> 0.99$). In terms of reproducibility, our analyses showed the intra-assay coefficients of variation (CVs) of 2.4% and 2.5% at two known HBV DNA concentrations, whereas the inter-assay CVs were 1.4% and 2.5%. Real-time PCR detected 10 of 12 frozen and freeze-dried serum samples from HBsAg-positive subjects in the 1993-1995 hepatitis screening. The correlation between $\log_{10}$-transformed HBV DNA quantities for pairs of frozen and freeze-dried serum samples was significant ($r=0.981$, $P< 0.0001$).

In a previous report, we compared the efficiencies of HCV RNA quantification using real-time PCR in anti-HCV-positive frozen and freeze-dried serum samples (16). Real-time PCR detected HCV RNA in 18 of 25 frozen and freeze-dried serum samples from anti-HCV-positive subjects, originally identified in the 1993-1995 hepatitis screening. The correlation between $\log_{10}$-transformed HCV RNA quantities for pairs of frozen and freeze-dried serum samples was significant ($r=0.908$, $P< 0.0001$). HCV RNA is easily decomposed by RNase, and its detectability decreases by repeated freezing and thawing of sera. Therefore it was expected that the PCR products of HCV RNA would vary depending on the method and condition of storage. Nevertheless, the results of our study demonstrated that there were no remarkable differences in our capacity to detect HCV RNA, even when different methodologies and conditions of storage were employed over prolonged periods.

Comparison of HBV DNA and HCV RNA quantification in the frozen and freeze-dried sera using newly developed HBV and HCV quantitative systems showed good correlation. Therefore we can conclude that both frozen and freeze-dried sera may be useful for HBV DNA and HCV RNA quantification.

Application of stored serum samples: a nested case-control study of hepatocellular carcinoma (HCC)

We conducted a nested case-control study to investigate risk factors that contribute to the development of HCC using stored sera from the AHS longitudinal cohort subjects. We selected 224 HCC cases and 644 controls that were free of HCC, according to standard nested case-control sampling. The comparable risk set was constructed by matching on sex, age, city, time of serum storage, and method of serum storage (freezing or freeze-drying methods), and by counter matching on radiation exposure (27).

Firstly, HBsAg, anti-HBc Ab, anti-HCV Ab, and HCV RNA were measured as viral factors using available stored serum samples of 211 cases and 640 controls obtained before the HCC diagnosis. Qualitative detection of HCV RNA was performed using a thermocycler (Whatman Biometra, Goettingen, Germany) with two sets of PCR primers corresponding to the 5'-untranslated region as previously described (28). The detection limit was established by limiting dilution method. This highly sensitive