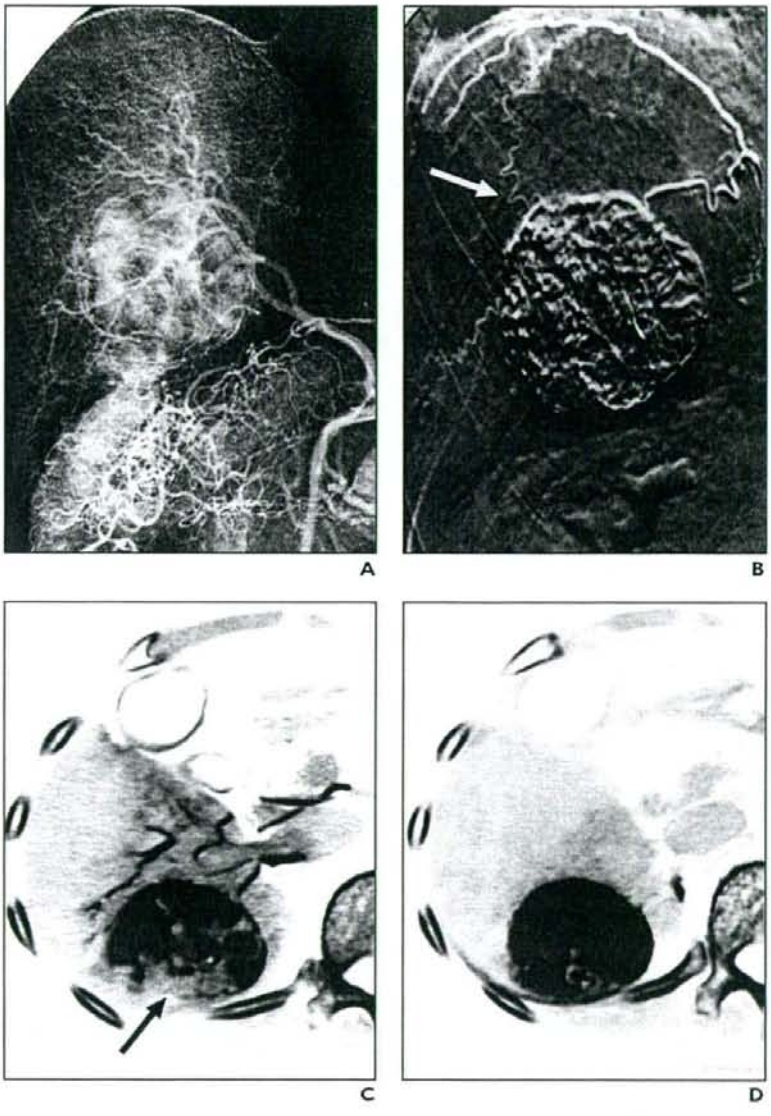

Fig. 2—64-year-old woman who underwent transcatheter arterial chemoembolization (TACE) for hepatocellular carcinoma (HCC) with feeding from the extrahepatic artery.
A and **B**, Angiography images show HCC was fed from branches of hepatic artery and from branch of right infraphrenic artery (*arrow*, **B**).
C, CT image shows that there was part of HCC that lacked retention of lipiodol after embolization of branches of hepatic artery (*arrow*).
D, CT image shows retention of lipiodol in entire HCC tumor after embolization of branch of right infraphrenic artery, and TACE procedure was completed.

fied CTA system than those examined with it ($p = 0.0012$).

The overall survival rate of patients who underwent angiography with a unified CTA system was significantly higher than that of patients who underwent angiography without it ($p < 0.0001$) (Fig. 3). When focusing on patients with stage I HCC tumor progression (single HCC with a maximum diameter of < 2 cm and without portal vein invasion), the survival rate of 172 patients who underwent pretreatment examination with a unified CTA system was significantly higher than that of 116 patients who underwent the examination without it ($p = 0.0093$) (Fig. 4). In contrast, no significant difference was found in survival rates between patients examined with a unified CTA system and those without it when focusing on patients with HCC at stages II, III, or IV, respectively (data not shown).

Characteristics and Survival Rates of Patients Who Underwent TACE With or Without a Unified CTA System

TACE was performed as a treatment for initial HCC (not a recurrence) solely with an angiography apparatus in 219 patients before July 1997 and with a unified CTA system in the other 219 patients after July 1997. The background characteristics of patients who underwent TACE with and without a unified CTA system are shown in Table 3. Patients who underwent TACE with a unified CTA system were older than those who underwent TACE without it ($p < 0.0001$). With regard to liver function, patients treated by TACE with a unified CTA system had higher serum albumin levels ($p = 0.0203$) but higher 15-minute retention of indocyanine green ($p = 0.0461$) than those treated by TACE without a unified CTA system. As for the progression of HCC, the prevalence of patients with HCC greater than 5 cm in diameter was higher in patients treated by TACE without a unified CTA system than those treated by TACE with it ($p = 0.0085$). Consequently, the prevalence of patients with stage IV HCC was higher in patients treated by TACE without a unified CTA system than those treated with it ($p = 0.0078$). Locoregional ablative therapy (one or two sessions) was performed as an additional treatment with TACE within 2 weeks after the TACE procedure in seven patients treated by TACE without a unified CTA system and in 10 patients treated by TACE with the unified CTA system. Fourteen patients who underwent TACE without a unified CTA system for treatment of initial HCC received TACE with a unified CTA system as a treatment of recurrent HCC.

The rate of local control without local recurrences was compared between patients who underwent TACE with and without a unified CTA system, excluding patients with stage IV HCC, which is far advanced and usually cannot be controlled by TACE. Local control was achieved by TACE in 84 of

TABLE 2: Study Patients Examined With and Without a Unified CT Angiography (CTA) System

Patients Characteristics (n = 1,312)	Without Unified CTA System (n = 603)	With Unified CTA System (n = 709)	p
Age (y)	65.6 ± 9.2	65.3 ± 9.4	0.6270
Sex			0.9582
M	443 (73.5)	521 (73.5)	
F	160 (26.5)	188 (26.5)	
Total bilirubin (mg/dL)	1.3 ± 1.9	1.4 ± 2.1	0.6348
Albumin (g/dL)	3.4 ± 0.6	3.3 ± 0.6	0.0079
Prothrombin time (%)	81.7 ± 19.1	82.1 ± 18.1	0.9876
Child-Pugh classification			0.9137
A	369 (61.2)	425 (59.9)	
B	181 (30.0)	221 (31.2)	
C	53 (8.8)	63 (8.9)	
Maximum tumor size (cm)			0.5104
< 2	213 (35.3)	229 (32.3)	
2–5	211 (35.0)	258 (36.4)	
> 5	179 (29.7)	222 (31.3)	
Number of tumors			0.0864
Single	311 (51.6)	332 (46.8)	
Multiple	292 (48.4)	377 (53.2)	
Portal vein invasion			0.2327
Absent	480 (79.6)	545 (76.9)	
Present	123 (20.4)	164 (23.1)	
Tumor stage[a]			0.0055
I	116 (19.2)	172 (24.2)	
II	188 (31.2)	233 (32.9)	
III	142 (23.6)	173 (24.4)	
IV	157 (26.0)	131(18.5)	

Note—Data in parentheses are percentages.
[a]TNM tumor stage according to the Liver Cancer Study Group of Japan.

173 (48.6%) patients treated by TACE without a unified CTA system and in 126 of 198 (63.6%) patients treated by TACE with a unified CTA system. The local control rate of patients who underwent TACE with a unified CTA system was significantly higher than that of patients who underwent TACE without it (p = 0.0048). The survival rate was also compared between patients who underwent TACE with and without a unified CTA system, excluding patients with stage IV HCC (Fig. 5). The survival rate of patients who underwent TACE with a unified CTA system was significantly higher than that of patients who underwent TACE without it (p = 0.0023). This difference in survival rate was maintained when excluding 17 patients who underwent locoregional ablative therapy as an additional treatment after TACE (p = 0.0094) (Fig. 6). We conducted multivariate analysis for the factors that influenced patient survival rate (Table 4). The use of a unified CTA system on TACE had an independent impact on increasing survival rates (p = 0.0387) as did Child-Pugh class and TNM tumor stage.

Discussion

The efficacy of CT during angiography—that is, CTAP and CTHA—has been shown to be beneficial for accurate evaluation of HCC [6–8]. Although one study reported a high rate of false-positive findings of HCC tumors with this method [14], this high false-positive rate could be attributed to the lack of analysis for the coronal enhancement that is observed in the late phase of CTHA in HCC [15]. The unified CTA system made it easy to perform both CTAP and CTHA without transporting a patient from the angiography

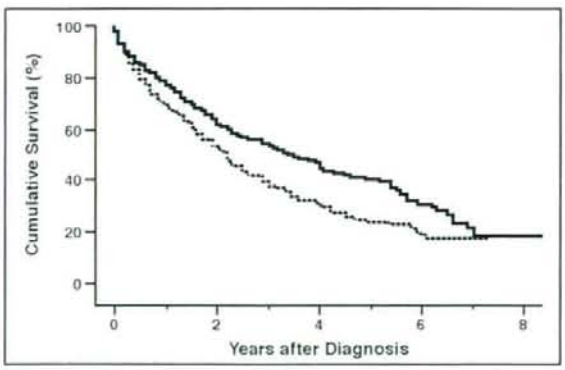

Fig. 3—Graph shows overall survival rates of patients who underwent angiography examinations with (n = 709) (*solid line*) or without (n = 603) (*dotted line*) unified CT angiography (CTA) system. Survival rate of patients who underwent angiography with unified CTA system was significantly higher than that of patients who underwent angiography without it (p < 0.0001).

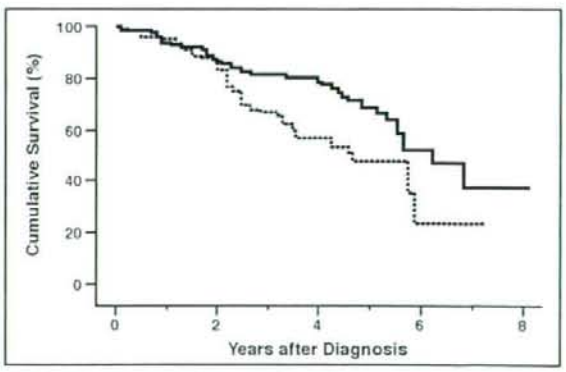

Fig. 4—Graph shows survival rates of patients with stage I hepatocellular carcinoma who underwent angiography with (n = 172) (*solid line*) or without (n = 116) (*dotted line*) unified CT angiography (CTA) system. Survival rate of patients who underwent angiography with unified CTA system was significantly higher than that of patients evaluated by angiography without it (p = 0.0093).

TABLE 3: Study Patients Who Underwent Transcatheter Arterial Chemoembolization (TACE) With and Without Unified CT Angiography (CTA) System

Patient Characteristics (n = 438)	Without Unified CTA System (n = 219)	With Unified CTA System (n = 219)	p
Age (y)	63.3 ± 8.5	68.3 ± 8.9	< 0.0001
Sex			0.0963
M	173 (79.0)	157 (71.7)	
F	46 (21.0)	62 (28.3)	
Total bilirubin (mg/dl)	1.2 ± 1.1	1.1 ± 0.7	0.3214
Albumin (g/dl)	3.2 ± 0.6	3.3 ± 0.5	0.0203
Prothrombin time (%)	81.4 ± 19.0	80.0 ± 16.3	0.4643
15-minute retention of ICG	24.2 ± 14.4	26.5 ± 13.1	0.0461
Child-Pugh classification			0.3458
A	119 (54.3)	134 (61.2)	
B	90 (41.1)	77 (35.2)	
C	10 (4.6)	8 (3.6)	
Maximum tumor size (cm)			0.0085
< 2	48 (21.9)	53 (24.2)	
2–5	92 (42.0)	116 (53.0)	
> 5	79 (36.1)	50 (22.8)	
Number of tumors			0.9203
Single	76 (34.7)	78 (35.6)	
Multiple	143 (65.3)	141 (64.4)	
Portal vein invasion			0.2843
Absent	191 (87.2)	199 (90.9)	
Present	28 (12.8)	20 (9.1)	
Tumor stage[a]			0.0078
I	16 (7.3)	24 (11.0)	
II	73 (33.3)	78 (35.6)	
III	84 (38.4)	96 (43.8)	
IV	46 (21.0)	21 (9.6)	

Note—Data in parentheses are percentages. ICG = indocyanine green.
[a]TNM tumor stage according to the Liver Cancer Study Group of Japan.

apparatus to the CT scanner and vice versa and allowed these examinations to be performed easily on all patients. Use of the unified CTA system allowed CTAP and CTHA for all patients during angiography, resulting in an increase in the accuracy of the evaluation of HCC progression.

The prevalence of patients with stage I HCC (earliest stage HCC) was higher in patients examined with a unified CTA system than in patients examined without it. The surveillance system for the early detection of HCC has greatly improved in Japan during the study period [3, 16]. This strongly contributed to the increase in the percentage of findings of stage I HCC at the time of diagnosis in patients who underwent examination with a unified CTA system because HCC was diagnosed in these patients after July 1997 when the surveillance system was improved compared with the period before July 1997.

Despite the increase in the percentage of patients with HCC of an earlier stage, the prevalence of patients with multiple tumors at diagnosis tended to be higher in patients who underwent examination with a unified CTA system than in those who underwent examination without it. This indicates that the detailed examination with CTAP and CTHA enabled the detection of minute HCC that had not been detected with conventional CT, sonography, or DSA, resulting in an increase in the number of HCC findings at diagnosis. The accurate evaluation of the progression of HCC at the time of diagnosis could contribute to the appropriate choice of treatment technique and to a decrease in the amount of HCC sites that are missed for treatment. Consequently, the overall survival rate of patients with HCC examined with a unified CTA system was significantly higher than that of patients with HCC examined without the unified CTA system. The significantly higher survival rate in patients examined with a unified CTA system was especially evident when focusing on patients with stage I HCC. This indicates that the higher survival rate in patients examined with a unified CTA system is not simply due to the increase in detection of early HCC that arose with improved surveillance during the period of July 1997 through 2003 compared with the period of 1990 through July 1997. This indicates that patients with stage I HCC examined with the unified CTA system were more strictly evaluated and, therefore, were more accurately classified as stage I HCC in comparison with the patients with stage I HCC examined without a unified CTA system. However, there were several developments in the diagnostic and treatment technology during the study period, and these factors might also have contributed to the higher survival rate of patients with HCC examined using a unified CTA system.

TACE was initially used to treat HCC by Doyon et al. [17] in 1974 and in Japan was applied to inoperable HCC using gelatin sponge particles and anticancer agents [18]. In the mid 1980s, lipiodol was newly introduced, primarily to enhance the therapeutic effect [19–23]. TACE with the injection of a mixture of an anticancer agent and lipiodol followed by embolization with gelatin sponge particles is now the mainstay treatment of choice for noncurative HCC [24–29]. Although the survival benefits of TACE have been controversial [30–33], recent randomized controlled trials showed the survival benefits of TACE compared with a control population [34, 35].

The ideal TACE for HCC should be a superselective catheterization into the feeding artery followed by an injection of the emulsion of iodized oil and anticancer agent deposited only in the targeted lesion with no washout of iodized oil. This should be achieved in only one session, and the noncancerous liver tissue should remain completely free from unnecessary embolization.

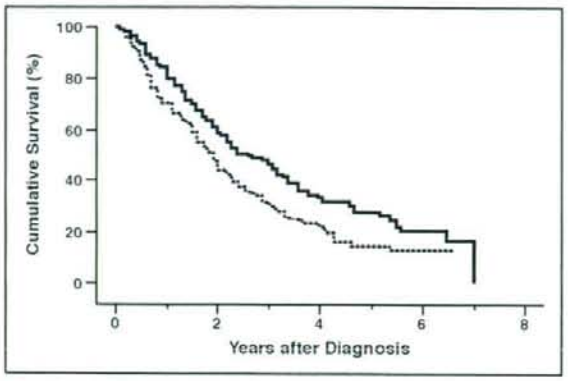

Fig. 5—Graph shows survival rates of patients, excluding those with stage IV hepatocellular carcinoma, who underwent transcatheter arterial chemoembolization (TACE) with (n = 219) (*solid line*) or without (n = 219) (*dotted line*) unified CT angiography (CTA) system. Survival rate of patients who underwent TACE with unified CTA system was significantly higher than that of patients who underwent TACE without unified CTA system (p = 0.0023).

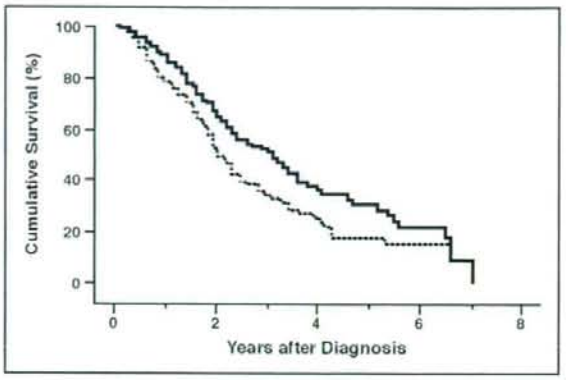

Fig. 6—Graph shows survival rates of patients who underwent only transcatheter arterial chemoembolization (TACE) with (n = 188) (*solid line*) or without (n = 167) (*dotted line*) unified CT angiography (CTA) system, excluding patients who had stage IV hepatocellular carcinoma and patients who underwent additional treatment (percutaneous ethanol injection, percutaneous microwave thermocoagulation, or radiofrequency ablation) after TACE. Survival rate of patients who underwent TACE with unified CTA system was significantly higher than that of patients who underwent TACE without unified CTA system (p = 0.0094).

TABLE 4: Multivariate Analysis for the Factors That Influenced Survival Rate of Patients Who Underwent TACE

Factor	Parameter Estimate	Standard Error	Chi-Square	Risk Ratio (95% CI)	p
Age	0.0141	0.0095	2.24	1.0142 (0.9956–1.0332)	0.1348
Sex					
M				1	
F	0.0253	0.0889	0.08	1.0256 (0.8581–1.2165)	0.7765
Child-Pugh class					
A				1	
B	0.3379	0.0767	19.09	1.4020 (1.2063–1.6294)	< 0.0001
C	0.2761	0.1633	2.50	1.3180 (0.9308–1.7762)	0.1135
Tumor stage*					
Stage I				1	
Stage II	0.1232	0.1385	0.83	1.1311 (0.8727–1.5071)	0.3631
Stage III	0.3392	0.1370	7.00	1.4038 (1.0875–1.8664)	0.0082
Use of unified CT angiography system					
No				1	
Yes	−0.1571	0.0759	4.28	0.8546 (0.7365–0.9917)	0.0387

*TNM tumor stage according to the Liver Cancer Study Group of Japan.

TACE was originally developed with the introduction of a unique carrier of anticancer agents, iodized oil, gelatin sponge particles, and a microcatheter that makes segmental [27] or subsegmental [28] TACE possible. However, without the assistance of sectional imaging such as CT at TACE, superselective catheterization into the correct feeding artery to obtain complete necrosis of the entire HCC lesion has not always been successful. As a result, inadvertent mistaken embolization has sometimes occurred. In such a mistaken embolization, the targeted lesion was not embolized. Instead, the noncancerous hepatic portion that should have been preserved was embolized, resulting in unnecessary liver damage. This kind of mistaken embolization usually is not found immediately after the TACE procedure but rather after completion of the TACE procedure at the follow-up CT. With the advent of a unified CTA system, this difficulty of performing TACE with a conventional angiography apparatus has been overcome, and several advantages have been added. Using selective arteriography of the feeding artery followed by confirmation using CT arteriography and repeating these studies after advancing the microcatheter to a more distal artery, it has become possible to achieve targeted TACE, strengthening the effect of TACE on the targeted lesion and reducing damage to the surrounding noncancerous portion. Indeed, the local control rate by TACE was significantly higher in patients who underwent TACE with a unified CTA system than in patients who underwent TACE without it.

The survival rate of patients treated by TACE with a unified CTA system was higher than that of patients treated by TACE without a unified CTA system. The technique for subsegmental and superselective TACE of hepatic arteries was established in Japan in the beginning of the 1990s [27, 28], which overlaps the entire period of the present study. Microcatheters were constantly used for TACE procedures throughout the study period. The use of farmorubicin hydrochloride as an anticancer agent, iodized oil, and gelatin sponge particles was also constant throughout the study period. These factors, therefore, could not contribute to the difference in the survival rate between patients

treated by TACE with a unified CTA system and those treated without it. The result of the multivariate analysis showed that the use of a unified CTA system independently influenced the increase in the patient survival rate as did the TNM tumor stage and Child-Pugh class. Therefore, the improvement of targeted TACE with the use of a unified CTA system contributed to the improvement in survival of patients treated with TACE.

In conclusion, the use of a unified CTA system contributed to the accurate evaluation of tumor progression in patients with HCC, resulting in the appropriate choice of treatment options and improved management of patients. In patients who underwent TACE for the treatment of HCC, a unified CTA system contributed to the increase in the patient survival rate by improving the targeted TACE by enhancing treatment efficacy and reducing mistaken embolization.

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APPENDIX 1: TNM Stage Classification of the Liver Cancer Study Group of Japan [13]

T factor	I, single; II, < 2 cm; III, no vascular involvement
T1	Fulfilling three factors
T2	Fulfilling two factors
T3	Fulfilling one factor
T4	Fulfilling no factors
Stage	
I	T1 N0 M0
II	T2 N0 M0
III	T3 N0 M0
IV-A	T4 N0 M0, or any T N1 M0
IV-B	Any T N0 or N1 M1

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Incidence of hepatocellular carcinoma in hepatitis C carriers with normal alanine aminotransferase levels☆

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***Background/Aims*: This study sought to identify the independent risk factors involved in the development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C virus (HCV) infection who have normal alanine aminotransferase (ALT) levels.**

***Methods*: A total of 519 patients with average ALT integration values less than or equal to 40 IU/L over 10 years were included. Baseline ultrasound was done in all patients and 68 patients underwent liver biopsy at the start of this study. Factors associated with the cumulative incidence of HCC were determined.**

***Results*: HCC occurred in 48 of 519 patients (9.2%). The following factors were significantly associated with the incidence of HCC: age > 65 years (adjusted hazard ratio: 2.006 [95% confidence interval: 1.078–3.733]), ALT > 20 IU/L (6.242 [1.499–25.987]), platelet count < 15.0 × 10^4/m^3 (2.675 [1.407–5.085]), total bilirubin > 1.2 mg/dL (2.798 [1.257–6.228]), ALP > 338 IU/L (2.486 [1.327–4.657]), and total albumin < 3.5 g/dl (2.707 [1.177–6.223]). The 5- and 10-year cumulative incidences of HCC were 4.4% and 26.5% in patients with ALT > 20 IU/L and platelet count < 15.0 × 10^4/m^3, respectively.**

***Conclusions*: High ALT level and low platelet count are closely associated with the development of hepatocarcinogenesis. Therefore, individuals within this group are candidates for antiviral therapy.**

Keywords: **Hepatocellular carcinoma; Hepatocarcinogenesis; Hepatitis C virus; Normal ALT; Average integration value**

1. Introduction

Hepatitis C virus (HCV) infection is widespread and often leads to chronic hepatitis, cirrhosis, or hepatocellular carcinoma (HCC). In Japan, deaths from HCC have increased annually and in the 1990's exceeded 30,000 [1], with 75–80% of HCC cases related to HCV infection [2]. HCC develops in 6–8% of patients with HCV-related cirrhosis every year in Japan [3–5]. A persistent necroinflammatory process and subsequent proliferation of hepatocytes (as observed by increased DNA synthesis) are important for the development of HCC in patients with HCV [6–10]. Thus, appropriate treatment of chronic HCV infection is needed to prevent the development of HCC.

Tarao et al. reported that maintenance of low alanine aminotransferase (ALT) levels may prevent hepatic carcinogenesis [9,10]. They reported that 27 of 33 patients (81.8%) with persistently high ALT levels (annual average ALT ⩾ 80 IU/L) developed HCC, whereas only 12 of 41 (29.3%) patients with persistently low ALT levels

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(annual average ALT < 80 IU/L) developed HCC. There was a statistically significant difference between the two groups [10].

ALT activity is the most widely used laboratory parameter in the evaluation of necroinflammatory activity in liver disease [11–13]. However, measurement of the annual mean value or simple arithmetic mean is problematic for the following reason: if the ALT level is high, the measurement interval shortens, whereas if the ALT level is low, the interval lengthens. As a result, the arithmetic mean value becomes greater and greater in patients with increased ALT levels due to the increased number of measurements being taken. For this reason, in a previous report we suggested that it would be more useful to measure the time integral of the ALT level ("integration value") [14]. The average ALT integration value was well correlated to the cumulative incidence of hepatic carcinogenesis. However, it is well known that HCC occurs in some HCV carriers with normal ALT values. There is little information about how many patients with normal ALT develop HCC. This study, therefore, sought to identify the independent risk factors associated with the development of HCC in chronically infected HCV patients with average ALT integration values less than or equal to 40 IU/L.

2. Materials and methods

2.1. Patient selection

A total of 4620 patients who tested positive for HCV visited the Department of Gastroenterology at Ogaki Municipal Hospital, Japan, between September 1995 and August 2004. After analyzing each patient's long-term prognosis, we selected 519 patients for further study who (1) had tested positive for HCV RNA for at least 6 months; (2) displayed no evidence of hepatitis B virus (HBV) infection; (3) had no other possible causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) had a follow-up period of greater than 3 years; (5) had no evidence of HCC for at least 3 years from the start of the follow-up period; (6) had no history of therapy involving interferon and/or ribavirin; (7) had ALT measurements taken more than twice in 1-year; and (8) had average ALT integration values less than or equal to 40 IU/L.

All patients had follow-up examinations at least every 6 months. During each examination, the following parameters were measured at least every 6 months: prothrombin time (PT), ALT, aspartate aminotransferase (AST), platelet count, γ-glutamyl transpeptidase (γ-GTP), total bilirubin, alkaline phosphatase (ALP), cholinesterase, total protein, albumin, and total cholesterol. ALT, AST, γ-GTP, and ALP were expressed as average integration values [14]. When we explained ALT as an example, the integration value of ALT was calculated as follows: $(y_0 + y_1) \times x_1/2 + (y_1 + y_2) \times x_2/2 + (y_2 + y_3) \times x_3/2 + (y_3 + y_4) \times x_4/2 + (y_4 + y_5) \times x_5/2 + (y_5 + y_6) \times x_6/2 + (y_6 + y_7) \times x_7/2 + (y_7 + y_8) \times x_8/2$ (Fig. 1). We calculated the area of a trapezoid with ALT value and the measurement interval and added the values. We divided the integration value of ALT by the observation period to obtain the average integration value (Fig. 2). In addition, patients were classified into two groups according to the change pattern of ALT: persistently normal ALT group and intermittently normal ALT group. The persistently normal ALT group includes patients with persistently normal ALT values less than or equal to 40 IU/L during follow-up period. The intermittently normal ALT group includes patients with temporary ALT fluctuations but the average integration value was less than or equal to 40 IU/L. We also recorded PT, platelet count, total bilirubin, cholinesterase, total protein, albumin, and total cholesterol values at the time of entry into the study. HCV genotype was determined by PCR using genotype-specific primers [15] and HCV RNA was quantified (Amplicor 2; Diagnostics K.K., Tokyo, Japan) [16].

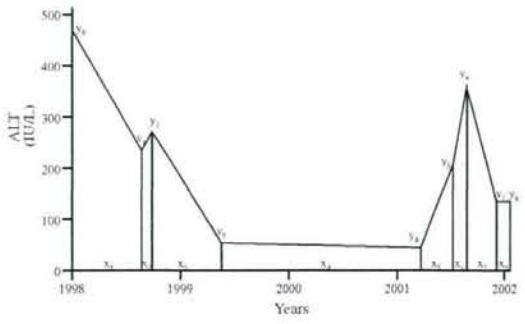

Fig. 1. Integration value of alanine aminotransferase (ALT). The integration value of ALT was calculated as follows: $(y_0 + y_1) \times x_1/2 + (y_1 + y_2) \times x_2/2 + (y_2 + y_3) \times x_3/2 + (y_3+y_4) \times x_4/2 + (y_4 + y_5) \times x_5/2 + (y_5 + y_6) \times x_6/2 + (y_6 + y_7) \times x_7/2 + (y_7 + y_8) \times x_8/2$. We divided the integration value of ALT by the observation period and expressed it as an average integration value.

Histological confirmation was obtained in 68 out of 519 patients. The degree of fibrosis was staged according to Desmet et al. as follows; F0, no fibrosis; F1, mild fibrosis; F2, moderate fibrosis; F3, severe fibrosis; F4, cirrhosis [17].

Ultrasound (US) was performed in all patients at the start of the follow-up period for the evaluation of liver fibrosis. The diagnosis of cirrhosis was performed according to typical ultrasound findings, e.g. liver surface nodularity, increased echogenicity and echotexture of the liver parenchyma, and signs of portal hypertension (splenomegaly > 120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [18,19].

To detect early-stage HCC, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), digital subtraction angiography (DSA), and/or measurement of tumor markers (i.e. AFP, *Lens culinaris* agglutinin-reactive AFP, and des-γ-carboxyprothrombin) were

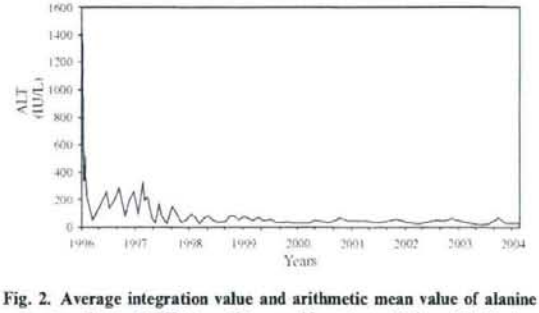

Fig. 2. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) for a 71-year-old patient with hepatitis C virus (HCV). The patient was followed up for 8 years. The number of ALT examinations was 82. The integration value of ALT was 636.8 IU/L × years. The average integration value was 76.6 IU/L, whereas the arithmetic mean value was 144.4 IU/L. This difference is due to the number of ALT measurements between a period of high ALT level and low ALT level.

performed for all patients, at least every 6 months. Blood biochemistry data used in this study were obtained over a 1-year prior to HCC development. The median follow-up period was 8.8 years (range, 3.0–13.3 years). A total of 14,347 blood examinations were performed, with the median number of examinations per patient being 22 (range, 6–158). Study of each patient ended in December 2007 or on the date of HCC identification, whichever came earlier. Diagnosis of HCC was confirmed via histologic examination (resected specimens $n = 13$ or liver biopsy $n = 7$) or via typical characteristics radiological findings such as hypervascularity at DSA or hyperattenuation at CT during hepatic arteriography [20] in addition to US, CT, and MRI ($n = 28$).

2.2. Statistical analysis

Statistical analyses were performed using the Statistical Program for Social Science (SPSS version 11.5 for Windows; SPSS Japan Inc., Tokyo, Japan). Continuous variables are expressed as median (range). The Kruskal–Wallis test was used to assess continuous variables with a skewed distribution, and the χ^2-test was used to assess categorical variables. An actuarial analysis of the cumulative incidence of hepatocarcinogenesis was performed using the Kaplan–Meier method, and differences were tested by a log-rank test. The Cox proportional hazard model and forward selection method were used to estimate the hazard ratio of HCC development associated with the following parameters: age (≤65 years or >65 years), sex (woman or man), body mass index (<25.0 kg/m^2 or ≥25.0 kg/m^2), HCV genotype (type 1 or type 2), viral concentration (≤100 KIU/mL or >100 KIU/mL), PT (≤70% or >70%), average ALT integration value (≤20 IU/L or >20 IU/L), average AST integration value (≤40 IU/L or >40 IU/L), platelet count (<15.0 × 10^4/mm^3 or ≥15.0 × 10^4/mm^3), average γ-GTP integration value (≤56 IU/L or >56 IU/L), total bilirubin (≤1.2 mg/dL or >1.2 mg/dL), average ALP integration value (≤338 IU/mL or >338 IU/mL), cholinesterase (<431 IU/mL or ≥431 IU/mL), total protein (<6.5 g/dL or ≥6.5 g/dL), albumin (<3.5 g/dL or ≥3.5 g/dL), and total cholesterol (<130 mg/dL or ≥130 mg/dL). We used the lower or upper limit of the reference values at our institute as cut-off values for PT, ALT, AST, platelet count, γ-GTP, total bilirubin, ALP, cholinesterase, total protein, albumin, and total cholesterol. Statistical significance was defined as $p < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital and performed in compliance with the Helsinki Declaration.

3. Results

3.1. Patient characteristics

HCC developed in 48 of 519 patients (9.2%) in this follow-up study. The 5- and 10-year cumulative incidences of hepatocellular carcinoma were 2.0% and 11.2%, respectively. Profiles and data from the 519 patients with normal ALT values are summarized in Table 1.

Table 1
Patient characteristics.

Age (years)	66 (18–88)
Sex (W/M)	290/229
BMI (kg/m^2)	22.4 (14.1–34.6)
HCV Genotype (1/2/unknown)	239/129/159
Viral concentration (KIU/mL)	285 (1–30,000)
Prothrombin time (%)	96.0 (21–145)
ALT (IU/L)[a]	27.4 (7.3–40.0)
Persistently normal ALT(+/−)[b]	148/371
AST (IU/L)[a]	31 (9–127)
Platelet (×10^4/mm^3)	17.2 (2.4–58.8)
γ-GTP (IU/L)[a,c]	23 (6–192)
Total bilirubin (mg/dL)	0.6 (0.3–4.7)
ALP (IU/L)[a]	247 (84–907)
Cholinesterase (IU/L)	264 (55–600)
Total protein (g/dL)	7.3 (4.5–9.2)
Albumin (g/dL)	4.1 (2.1–5.2)
Total cholesterol (mg/dL)	165 (72–290)
Fibrosis (F0/F1/F2/F3/F4)[c]	7/38/10/6/7
Cirrhosis (−/+)[a,d]	432/87
Follow up period (years)	8.8 (3.0–13.3)
Hepatocaricinogenesis (+/−)	48/471

Values are expressed as median (range). W, women; M, men; BMI, body mass index; HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, asparate aminotransferase; GTP, glutamyl transpeptidase; ALP, alkaline phosphatase.
[a] Average integration value.
[b] Persistently normal ALT values less than or equal to 40 IU/L.
[c] Staging of chronic hepatitis according to Desmet et al. [17].
[d] Cirrhosis diagnosed by ultrasound findings.

3.2. Factors associated with the incidence of hepatic carcinogenesis

Factors significantly associated with the incidence of HCC on univariate analysis are listed in Table 2. The following associations were statistically significant: age > 65 years, ALT > 20 IU/L, intermittently normal ALT, platelet count < 15.0 × 10^4/m^3, γ-GTP > 56 IU/L, total bilirubin > 1.2 mg/dL, ALP > 338 IU/L, total protein < 6.5 g/dL, albumin < 3. 5 g/dl, high fibrous stage, and presence of cirrhosis. Hepatic carcinogenesis occurred at significantly higher rates in patients with average ALT integration value greater than 20 IU/L ($n = 402$) than in patients with average ALT integration value less than or equal to 20 IU/L ($n = 117$, $p = 0.011$, Fig. 3). Hepatic carcinogenesis occurred at significantly higher rates in patients with platelet counts less than 15.0 × 10^4/mm^3 ($n = 179$) than in patients with platelet counts greater than or equal to 15.0 × 10^4/mm^3 ($n = 340$, $p < 0.001$, Fig. 4).

Factors associated with the incidence of HCC as determined by the Cox proportional hazard model and the forward selection method are listed in Table 3, and are as follows: age > 65 years, ALT > 20 IU/L, platelet count <15.0 × 10^4/m^3, total bilirubin > 1.2 mg/dL, ALP > 338 IU/L, and total albumin <3.5 g/dl were significantly associated with the incidence of HCC.

3.3. Group classification according to average ALT integration value and platelet counts

HCV carriers with normal ALT levels were divided into four groups (A: ALT ≤ 20 IU/L and platelet count ≥ 15.0 × 10^4/mm^3 [$n = 82$]; B: ALT > 20 IU/L and platelet count ≥ 15.0 × 10^4/mm^3 [$n = 258$]; C: ALT ≤ 20 IU/L and platelet count < 15.0 × 10^4/mm^3 [$n = 35$]; D: ALT > 20 IU/L and platelet count < 15.0 × 10^4/mm^3 [$n = 144$], Table 4). Age, total bilirubin, ALP, and fibrous staging in Group D were higher than in the other groups ($p < 0.001$). Group D

Table 2
Factors associated with hepatocarcinogenesis (univariate analysis).

		Hazard ratio (95% CI)	P
Age (years)	⩽ 65	1	
	> 65	2.420 (1.326–4.414)	0.004
ALT (IU/L)[a]	⩽ 20	1	
	> 20	6.263 (1.520–25.808)	0.011
AST (IU/L)[a]	⩽ 40	1	
	> 40	3.194 (1.799–7.111)	<0.001
Persistently normal ALT[b]	Presence	1	
	Absence	2.426 (1.031–5.709)	0.042
Platelets ($\times 10^4/m^3$)	⩾ 15.0	1	
	< 15.0	4.297 (2.357–7.834)	<0.001
γ-GTP (IU/L)[a]	⩽ 56	1	
	> 56	2.521 (1.368–4.645)	0.003
Total bilirubin (mg/dL)	⩽ 1.2	1	
	> 1.2	5.563 (2.832–10.927)	<0.001
ALP (IU/L)[a]	⩽ 338	1	
	> 338	3.180 (1.740–5.811)	<0.001
Total protein (g/dL)	⩾ 6.5	1	
	< 6.5	2.550 (1.005–6.466)	0.049
Albumin (g/dL)	⩾ 3.5	1	
	< 3.5	3.543 (1.756–7.150)	<0.001
Staging[c]	F0, F1	1	
	F2, F3, F4	20.339 (2.575–160.656)	0.004
Cirrhosis[d]	Absence	1	
	Presence	10.003 (5.597–17.878)	<0.001

P-values and hazard ratios were calculated by Cox proportional hazard model. ALT, alanine aminotransferase; AST, asparate aminotransferase; GTP, glutamyl transpeptidase; ALP, alkaline phosphatase.

[a] Average integration value.
[b] Persistently normal ALT values less than or equal to 40 IU/L.
[c] Staging of chronic hepatitis according to Desmet et al. [17].
[d] Cirrhosis diagnosed by ultrasound findings.

showed the highest rate of hepatic carcinogenesis, followed by Groups B and C, as compared with Group A (Fig. 5). The 5- and 10-year cumulative incidences of HCC were 4.4% and 26.5% in Group D, respectively.

3.4. Change of platelet count in patients with HCC development in Groups A and B

Table 5 shows the profile of patients in Groups A and B who developed HCC. In 12 out of 16 patients (75.0%), platelet counts decreased less than $15 \times 10^4/mm^3$ during the follow-up period (2.3 years [0.9–9.5 years]) prior to HCC development.

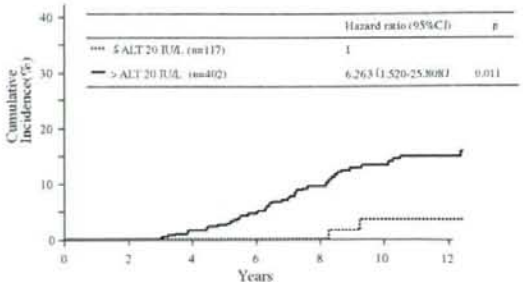

Fig. 3. Incidence of hepatocarcinogenesis as a function of average integration serum alanine aminotransferase (ALT) levels. The 5- and 10-year cumulative incidences of HCC were 0.0% and 3.6% in patients with average ALT integration value less than or equal to 20 IU/L ($n = 117$) and 2.6% and 13.3% in patients with average ALT integration value greater than 20 IU/L ($n = 402$), respectively. Hepatic carcinogenesis occurred at significantly higher rates in the latter group than in the former group ($p = 0.011$).

4. Discussion

We previously showed that increased liver inflammation, as assessed by increased ALT levels, is associated with increased risk for development of HCC in patients with HCV infection [14]. This suggests that suppression of inflammation, as assessed by maintenance of a low ALT level, could inhibit HCC development in HCV carriers. However, some patients develop HCC, even if their ALT levels are within the normal range. It is, therefore, important to identify candidates for antiviral therapy in patients with normal ALT levels.

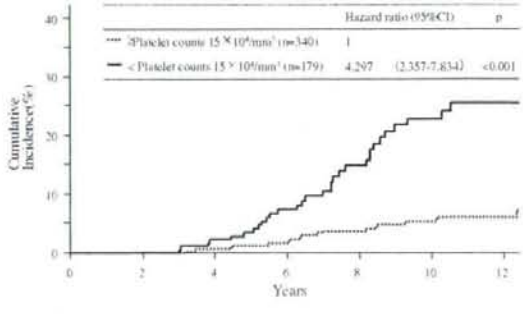

Fig. 4. Incidence of hepatocarcinogenesis as a function of serum platelet levels. The 5- and 10-year cumulative incidences of HCC were 1.2% and 5.4% in patients with platelet counts equal to or greater than $15.0 \times 10^4/mm^3$ ($n = 340$) and 2.5% and 22.9% in patients with platelet counts less than $15.0 \times 10^4/mm^3$ ($n = 179$), respectively. Hepatic carcinogenesis occurred at significantly higher rates in the latter group than in the former group ($p < 0.001$).

Table 3
Factors associated with hepatocarcinogenesis (multivariate analysis).

		Hazard ratio (95% CI)	P
Age (years)	⩽ 65	1	
	> 65	2.006 (1.078–3.733)	0.028
ALT (IU/L)[a]	⩽ 20	1	
	> 20	6.242 (1.499–25.987)	0.012
Platelets ($\times 10^4/m^3$)	⩾15.0	1	
	< 15.0	2.675 (1.407–5.085)	0.003
Total bilirubin (mg/dL)	⩽ 1.2	1	
	> 1.2	2.798 (1.257–6.228)	0.012
ALP (IU/L)[a]	⩽ 338	1	
	> 338	2.486 (1.327–4.657)	0.004
Albumin (g/dL)	⩾3.5	1	
	< 3.5	2.707 (1.177–6.223)	0.019

P-values and hazard ratios were calculated by Cox proportional hazard model. ALT, alanine aminotransferase; ALP, alkaline phosphatase.
[a] Average integration value.

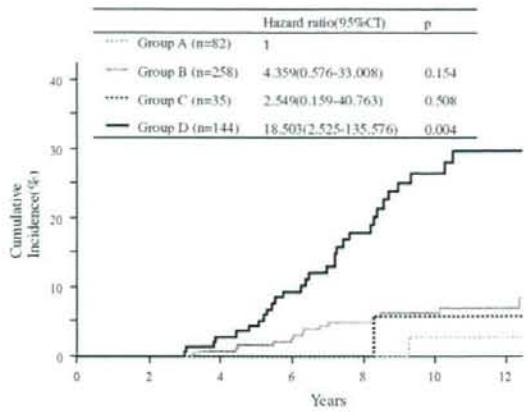

Fig. 5. Cumulative hepatocarcinogenesis as a function of platelet count and average integration serum alanine aminotransferase (ALT) levels. Patients were classified into four groups according to average ALT integration value and platelet count (A: ALT ⩽20 IU/L and platelet counts ⩾ $15.0 \times 10^4/mm^3$ [$n = 82$]; B: ALT > 20 IU/L and platelet counts ⩾ $15.0 \times 10^4/mm^3$ [$n = 258$]; C: ALT ⩽ 20 IU/L and platelet counts $<15.0 \times 10^4$ /mm^3 [$n = 35$]; D: ALT > 20 IU/L and platelet counts $< 15.0 \times 10^4/mm^3$ [$n = 144$]). The 5- and 10-year cumulative incidences of HCC were 0.0% and 2.9% in Group A, 1.6% and 6.2% in Group B, 0.0% and 5.9% in Group C, and 4.4% and 26.5% in Group D, respectively. Group D showed the highest rate of hepatocarcinogenesis compared to Groups A–C.

ALT concentration is the most commonly used variable in the assessment of liver disease [21–23]. ALT level fluctuates within individual patients. Therefore, repeated measurement of this parameter is important for accurate interpretation of the data. The arithmetic mean value of a series of measurements is often the value used for analysis; however, this value can be greatly affected by the period of time between measure-

Table 4
Baseline characteristics between 4 groups.

	Group A	Group B	Group C	Group D
ALT	⩽20 IU/L	>20IU/L	20 IU/L	>20 IU/L
Platelet	⩾$15 \times 10^4/m^3$	⩾$15 \times 10^4/m^3$	<$15 \times 10^4/m^3$	<$15 \times 10^4/m^3$
	($n = 82$)	($n = 258$)	($n = 35$)	($n = 144$)
Age (years)[a]	62 (21–87)	65 (18–87)	69 (48–88)	67 (41–87)
Sex (W/M)[b]	58/24	138/120	18/17	76/68
BMI (kg/m^2)	20.8 (15.8–26.8)	22.0 (14.1–34.6)	21.4 (17.9–33.3)	20.5 (14.3–31.1)
HCV Genotype (1/2)	28/25	140/67	6/7	65/30
ALT (IU/L)[c]	17.1 (9.3–20.0)	29.0 (20.1–40.0)	16.8 (7.3–20.0)	32.4 (20.1–40.0)
Persistently normal ALT (+/−)[a,d]	56/26	52/206	20/15	20/124
Viral concentration (KIU/mL)	82.5 (0.51–4900)	360 (0.54–30,000)	290 (1.6–1000)	270 (0.55–5000)
Platelet ($\times 10^4/m^3$)	20.7 (15.0–58.8)	19.6 (15.0–56.8)	12.5 (3.7–14.9)	10.55 (2.4–14.9)
Total bilirubin (mg/dL)[a]	0.4 (0.4–4.4)	0.5 (0.2–3.7)	0.6 (0.2–4.6)	0.7 (0.2–4.7)
ALP (IU/L)[b]	229 (114–417)	238 (84–623)	249 (147–437)	274 (158–907)
Albumin (g/mL)	4.1 (2.7–5.1)	4.2 (2.3–4.8)	4.0 (2.6–4.7)	3.9 (2.1–5.2)
Staging (F0/F1/F2/F3/F4)[b,e]	1/9/0/1/0	3/17/4/1/0	3/2/2/1/1	0/10/3/4/6
Cirrhosis(−/+)[a,f]	80/2	256/12	24/11	82/62
Hepatocaricinogenesis (+/−)[a]	1/81	15/243	1/34	31/113

P-values were calculated by Kruskal-Wallis test or χ^2-test. BMI, body mass index; HCV, hepatitis C virus; ALT, alanine aminotransferase; ALP, alkaline phosphatase.
[a] $P < 0.001$.
[b] $P < 0.05$.
[c] Average integration value.
[d] Persistently normal ALT values less than or equal to 40 IU/L.
[e] Staging of chronic hepatitis according to Desmet et al. [17].
[f] Cirrhosis diagnosed by ultrasound findings.

Table 5
Characteristics of patients that developed HCC in Groups A and B.

No.	Sex	Age (years)	Average ALT integration value (IU/L)	Platelet count at entry (10^4/mm^3)	Platelet counts decreased[a]	Duration (years)[b]
1	Male	79	23.2	16.9	Yes	0.6
2	Male	82	37.9	18.7	Yes	0.6
3	Male	69	17.2	15.1	Yes	0.6
4	Female	77	24.0	15.4	Yes	0.8
5	Female	63	39.2	15.5	Yes	2.3
6	Female	68	31.1	19.2	Yes	2.3
7	Male	67	35.6	15.7	Yes	2.3
8	Male	70	37.5	20.4	Yes	3.8
9	Male	85	25.0	16.3	Yes	3.9
10	Male	67	28.6	56.8	Yes	4.9
11	Male	70	23.8	16.7	Yes	6.7
12	Male	55	39.1	17.5	Yes	9.5
13	Male	82	25.5	23.8	No	
14	Female	73	28.9	16.1	No	
14	Female	73	28.9	16.1	No	
16	Male	73	27.5	22.9	No	

ALT; alanine aminotransferase.
[a] Platelet counts decreased under 15×10^4/mm^3.
[b] Duration from the time at entry to the date platelet counts decreased under 15×10^4/mm^3.

ments. Therefore, we used the time integral of the ALT level to determine the value for analysis. Because this determination is strongly affected by the follow-up period, we divided the average integration value by the time of follow-up. We have previously argued that the average integration value is more meaningful than the arithmetic mean value [14]. In the present study, the average integrated value of ALT increased along with the incidence of HCC. The cumulative incidence of hepatocarcinogenesis was 6.242-fold higher (1.499–25.987) in patients with average ALT integration values greater than 20 IU/L than in patients with average ALT integration value less than or equal to 20 IU/L. Kim et al. reported that the adjusted hazard ratio of mortality from liver disease for patients with ALT concentrations of 20–29 IU/L and 30–39 IU/L were 2.9 (95% confidence interval 2.4–3.5) and 9.5 (7.9–11.5) in men and 3.8 (1.9–7.7) and 6.6 (1.5–25.6) in women compared to that for patients with ALT concentrations <20 IU/L [23]. Furthermore, we evaluated the change pattern of ALT: persistently normal ALT group and intermittently normal ALT group. Although the intermittently normal ALT group is the factor significantly associated with the incidence of HCC on univariate analysis, this factor was not selected on multivariate analysis.

The present study also reveals that a low platelet count is a predictive factor for the development of HCC. Cirrhosis is an established risk factor for HCC in patients with HCV [2–10]. US produce detailed cross-sectional images of the liver and its surrounding structures. We attempted to distinguish cirrhosis patients from non-cirrhosis patients according to typical ultrasound findings [19,20]. The presence of cirrhosis diagnosed by US was strongly associated to the increased incidence of HCC on univariate analysis. Anatomical constraints and interobserver variability, however, remain limiting factors. Because of this, we excluded the factor of cirrhosis diagnosed by US from the multivariate analysis. In this study, histological confirmation was obtained in only 68 patients (13.1%). F2, F3, and F4 showed the higher incidence rate of HCC than F0 and F1 on univariate analysis. It is thought that this study had limitations because the liver histologies were not obtained in many cases. Over the past 50 years, percutaneous liver biopsy has become the primary tool for diagnosing and staging liver disease, and its techniques, indications, and contraindications have been well characterized. However, it is not practical to perform biopsies on all patients who do not receive active treatment because of the potential complications that might arise from this procedure. Furthermore, results often differ depending on the pathologist, and liver fibrosis results from liver biopsy specimens do not always reflect the fibrosis grade of the entire liver. It is likely that low platelet counts account for a large proportion of cirrhosis cases, suggesting that HCC may develop in patients with progressive or advanced liver disease. Platelet count is a useful marker for the diagnosis of cirrhosis. Lu et al. reported that the best cut-off platelet count for a diagnosis of cirrhosis is 15.0×10^4/mm^3 [24]. We adopted this cut-off level in this study.

Older age, high total bilirubin, high ALP, and low albumin were also significantly associated with incidence of HCC. Increases in conjugated bilirubin are highly specific for disease of the liver or bile ducts [25]. However, only total bilirubin was measured in this series and total

bilirubin > 1.2 mg/dL was found in only 35 cases (6.7%). ALP is found in many organs (i.e. kidney, liver, bone, ileal mucosa, and placenta) and has many isoenzymes. Measurement of other associated enzymes (such as γ-GTP) is necessary for correct evaluation of liver function [25]. Therefore, we did not use these parameters in further analyses. Albumin is the most abundant plasma protein produced by hepatocytes. The rate of albumin production is dependent on several factors, including the number of functioning hepatocytes. Plasma albumin gradually falls with progression to cirrhosis [25]. Ten of the 53 patients (18.9%) with albumin less than 3.5 g/dL developed HCC during the follow-up period. We concluded that low platelet count and hypoalbuminemia were confounding factors for identifying cirrhosis. For this study, we selected platelet count as a parameter for further analyses.

We divided patients into four groups according to the average ALT integration value and platelet count in the present study. Patients in Group D (ALT > 20 IU/L and platelet counts $<15.0 \times 10^4/mm^3$) showed the highest rate of hepatocarcinogenesis (21.5%) compared with Groups A–C. In addition, platelet counts decreased in 12 of 16 patients in Groups A and B who developed HCC. Therefore, it is important to evaluate not only ALT levels but also platelet counts in order to predict hepatic carcinogenesis precisely.

In conclusion, relatively high ALT levels and low platelet counts are closely associated with the development of hepatocarcinogenesis in patients infected with HCV. Therefore, this group is a candidate for antiviral therapy, even if their ALT values are within the current normal range.

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What can be revealed by extending the sensitivity of HBsAg detection to below the present limit?☆

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***Background/Aims*: We investigated what can be revealed by extending the sensitivity of HBsAg detection to below the present limit.**

***Methods*: We examined the sensitivity of this immunoassay in comparison with real-time PCR detection of HBV DNA using serially diluted sera from HBV carriers. Low HBsAg was measured in 210 healthy volunteers and 368 patients with non-B chronic liver diseases who were negative for HBsAg by a standard EIA method.**

***Results*: The radical immunoassay was able to detect HBsAg at a concentration of 0.025 ng/ml. Low HBsAg was positive in 6 of 210 normal volunteers (2.86%), 5 of 65 non-B, non-C cirrhosis patients (7.69%), 6 of 62 non-B, non-C hepatocellular carcinoma patients (9.68%: $p = 0.04$ vs. volunteers), 12 of 134 chronic hepatitis C patients (8.96%: $p < 0.02$ vs. volunteers), and 11 of 107 hepatocellular carcinoma patients complicated by chronic hepatitis C (10.28%: $p < 0.008$ vs. volunteers). Although no HBV DNA was positive in healthy volunteers, 9 patients with non-B chronic liver diseases were positive for HBV DNA by real-time PCR analysis.**

***Conclusions*: Increasing the sensitivity of HBsAg detection to below the present limit has revealed that infection with HBV, including occult HBV, is far more endemic than suspected previously.**

***Keywords*: Electron spin resonance; Non-B chronic liver diseases; Occult HBV; Radical immunoassay; Real-time PCR; Clinical research**

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Abbreviations: DMSO, dimethyl sulfoxide; ESR, electron spin resonance; HBV, hepatitis B virus; HBsAg, hepatitis surface antigen; HCC, hepatocellular carcinoma; HTIO, 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline-3-oxide; MOPS, 3-morpholinopropanesulfonic acid; *p*-AP, *p*-acetamidophenol; PCR, polymerase chain reaction; *S/N*, signal to noise.

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1. Introduction

About 350 million people worldwide are chronic carriers of the hepatitis B virus (HBV) [1]. The infection can cause acute and chronic liver diseases including fulminant hepatic failure, cirrhosis and hepatocellular carcinoma. Each year, acute and chronic HBV infection causes roughly one million deaths [1,2]. HBV infection occurs either vertically or horizontally. Therefore, surveillance of HBV infection in individuals is mandatory from a public health perspective, particularly in highly endemic areas. The diagnosis of chronic HBV infection is based on the persistent presence of viral envelope protein, hepatitis surface antigen (HBsAg), in the blood [3]. Recent advances in gene technology have prompted the concept of occult HBV, which is defined as HBV DNA detectable by sensitive polymerase chain reaction (PCR) among individuals negative for HBsAg [4,5]. Particularly in carriers with a low HBV load, it is essential to distinguish such carriers from donors in order to ensure safe blood transfusion [6]. Moreover, investigation of chronic infection with a low HBV load and its clinical significance is considered to make a significant contribution to prevention and treatment.

Recently, we have developed a radical immunoassay method that can detect HBsAg in serum with revolutionarily high sensitivity [7,8]. This radical immunoassay is based on measurement of peroxidase activity in peroxidase–antibody conjugates, in which a stable nitroxide radical is generated in the presence of H_2O_2, *p*-acetamidophenol (*p*-AP), and 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline-3-oxide (HTIO) [7–9]. In this method, peroxidase activity is quantified by measuring the stable nitroxide radical by electron spin resonance (ESR) spectroscopy, which yields a markedly high degree of sensitivity. To expand the availability of this immunoassay, we further developed an automated ESR analyzer for measurement of HBsAg. We therefore expect that this new analytical method will make it possible to diagnose low HBV load carriers, including occult HBV carriers, with convenience, high sensitivity and low cost, and will become a powerful examination tool for worldwide use. In the present study, we investigated what would be revealed when the detection sensitivity for HBsAg was increased to below the present detection limit. In non-B chronic liver diseases including hepatocellular carcinoma (HCC), the positivity rate for low HBsAg was significantly high and individuals with occult HBV were not rare, suggesting the involvement of occult HBV in these diseases. Here we show that low-concentration HBs antigenemia exists in two forms, i.e. low HBsAg positive with low HBV DNA, and low HBsAg positive without HBV DNA.

2. Methods

2.1. Chemical

p-AP and sodium azide were purchased from Wako Pure Chemical Industries, Osaka, Japan. HTIO was from Aldrich (Milwaukee, USA). 3-Morpholinopropanesulfonic acid (MOPS) was from Dojindo Laboratories (Kumamoto, Japan). Dimethyl sulfoxide (DMSO) was from Kanto Chemical (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

2.2. Principle of HBsAg detection by the radical immunoassay method

Fig. 1 shows the principle of the radical immunoassay. Phenoxy radicals are produced from *p*-AP by the action of horseradish peroxidase in the presence of H_2O_2. One of the hydroxylamine compounds, HTIO, is converted to stable nitroxide radicals by oxidation of phenoxy radicals. Peroxidase activities are quantified by measuring these stable nitroxide radicals with the automated ESR analyzer. The radical immunoassay method can amplify peroxidase activity by 10^6 times (Fig. 1) [9].

Beads coated with HBs-antibody, a peroxidase-labeled monoclonal antibody against HBsAg (anti-HBs peroxidase conjugate) and HBsAg-negative controls (human sera non-reactive for both HBsAg and anti-HBsAg) attached to AUSZYME II (Abbott Laboratories, N. Chicago, IL) were used. An aliquot of 240 μl serum was incubated with both 60 μl of anti-HBs peroxidase conjugate and a bead at 37 °C for 30 min. After washing the beads with 0.01% W1A (Sigma Chemical Company, St. Louis, MO, USA) solved in distilled water 11 times, they were again incubated with 200 μl of reagent (2 mM *p*-AP, 0.017 mM HTIO, DMSO and 33 mM MOPS, pH 6.5) and 100 μl of 0.001% H_2O_2 for 30 min. Then, 50 μl of 100 mM NaN_3 was added to the reaction mixture to stop the enzyme reaction (Fig. 1). All the reagents were prepared with milli-Q water.

HBsAg levels in the sera were determined by the radical immunoassay method, using an automated electron spin resonance (ESR) analyzer (Tohoku Seiki Industry, Yamagata, Japan), equipped with a pipettor, an incubator, a washer, and a reader station. The ESR spectroscopic settings for measurement were done automatically. The signal intensity of the middle-field component of the triplet nitroxide radical was measured. The result was expressed as the signal to noise (*S/N*) ratio, calculated by dividing the signal intensity of the sample (signal) by that of a paired HBsAg-negative serum sample (noise) (Fig. 1).

2.3. Confirmation of the specificity of HBsAg detection

To confirm the specificity of HBsAg detection by the radical immunoassay method, we performed an absorption test using HBsAb-coated ferrite particles (Fujirebio Diagnostics Inc., Tokyo, Japan). HBsAg panel serum (adr, genotype C, Institute of Immunology Co., Ltd., Tokyo, Japan) at a concentration of 0.1 ng/ml was incubated with the HBsAb-coated ferrite particles for 30 min at room temperature. Then, the ferrite particles were removed using a magnet, and HBsAg in the absorbed panel serum was measured by radical immunoassay. Our preliminary study showed that a concentration of 0.1 ng/ml detected by the present HBsAg panel serum was nearly equal to 0.2 IU/ml by the WHO International Standard.

2.4. Establishment of the cut-off value

The radical immunoassay was conducted in 146 healthy volunteers whose ALT level was normal and who had exhibited negative results for HBsAg (HBsAg II EIA Cobas Core, Roche Diagnostics Corp., Indianapolis, IN) and anti-HBc (Enzygnost Anti-HBc monoclonal, Boehringer Diagnostic GmbH, Germany) according to the standard ELISA. The cut-off value was established based on the distribution of the *S/N* ratio obtained from healthy volunteers [7].

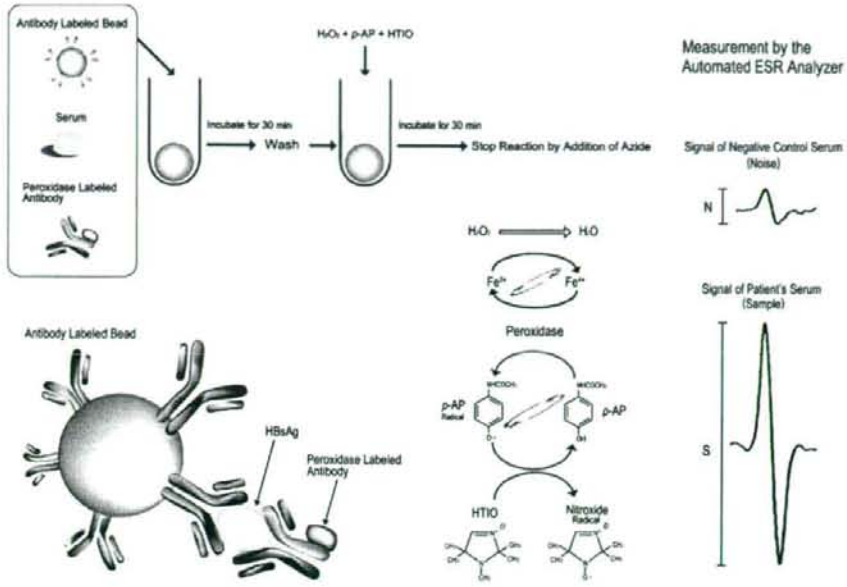

Fig. 1. Principle of the radical immunoassay method. [This figure appears in colour on the web.]

2.5. Comparison of HBsAg detection by various assay systems

To confirm the sensitivity of the radical immunoassay, we conducted the following experiments: HBsAg panel serum (adr, genotype C, Institute of Immunology Co., Ltd.) was serially diluted and then measured by EIA (HBsAg II EIA Cobas Core, Roche Diagnostics Corp.), the chemiluminescence method (Architect HBsAg, Dainabot Co., Ltd., Tokyo, Japan) and the radical immunoassay method.

2.6. Hepatitis B surface antigen and HBV DNA detection by serial dilution of sera obtained from HBV carriers

To compare the sensitivity of HBsAg detection by radical immunoassay with that of HBV DNA by real-time PCR, serial dilution tests were conducted with sera from HBV carriers. HBV DNA was measured using a real-time direct test for HBV (HBV-Direct Mag, JSR, Tokyo, Japan), which combines the use of a DNA extraction system based on magnetic beads coated with polyclonal anti-HBsAg and the real-time detection method [10,11]. The PCR primers and probe used were designed using Primer Express software (Applied Biosystems, CA, USA) and were available for eight HBV genotypes (A–H) on the basis of alignment with their sequences [10]. The detection limit of the test is $1.0 \log_{10}$ copies per ml.

2.7. Measurement of HBsAg in sera from normal volunteers and patients with non-B chronic liver diseases

We analyzed sera from an additional 210 healthy volunteers, being different from the volunteers who participated in the establishment of the cut-off value, 65 patients with cirrhosis of unknown cause (non-B, non-C cirrhosis), 62 patients with hepatocellular carcinoma of unknown cause (non-B, non-C hepatocellular carcinoma), 134 patients with HCV chronic hepatitis or cirrhosis, and 107 patients with hepatocellular carcinoma complicated by HCV chronic hepatitis or cirrhosis. All the examined cases were negative for HBsAg by ELISA (HBsAg II EIA, Cobas). The sera of patients with non-B chronic liver diseases positive for HBsAg by the radical immunoassay method were further subjected to measurement of anti-HBs (Lumipulse II HBsAb, Fujirebio Diagnostics Inc.), anti-HBc (Lumipulse II HBcAb, Fujirebio Diagnostics Inc.), and HBV DNA. All patients gave their informed consent.

2.8. Statistical analysis

Comparisons of values between two groups were performed using the Mann–Whitney *U* test. Statistical analysis of HBsAg positivity rate among the groups was conducted by Fisher's exact test. Differences at $p < 0.05$ were considered to be statistically significant. The analysis software used was the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) version 12.

3. Results

3.1. Establishment of the cut-off value

The minimum and maximum *S/N* ratios of the healthy volunteers were 0.63 and 2.15, respectively (Fig. 2A). The data from four volunteers were excluded from this analysis by the Smirnov test because of their abnormal distribution. Calculations of the logarithm of *S/N* ratios showed that in the healthy volunteers the minimum and maximum values were −0.1973 and 0.2389, respectively (Fig. 2B). The distribution of *S/N* ratios in the healthy volunteers was considered to be a normal distribution with a skewedness of 0.008 and a

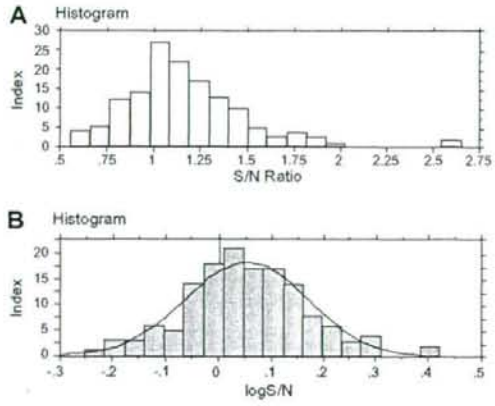

Fig. 2. Establishment of the cut-off values. (A) The minimum and maximum *S/N* ratio for 146 normal volunteers. (B) Logarithm of the *S/N* ratio of 146 normal volunteers. The distribution of *S/N* ratios in the negative control group was considered to be a normal distribution.

kurtosis of 3.249 (Fig. 2B). From these data, *S/N* ratios of <means + 2SD (*S/N* = 2.208) were considered to be negative (−), ⩾means + 4SD (*S/N* = 3.249) to be positive (+), and ⩾means + 2SD and <means + 4SD to be undeterminable (±).

3.2. Specificity and sensitivity of HBsAg detection by radical immunoassay

The absorption of HBsAg by the HBsAb-coated ferrite particles revealed a marked decrease in the ESR signal, as shown in Fig. 3. The radical immunoassay was able to detect 0.025 ng/ml HBsAg, with an undeterminable range of 0.01 ng/ml, while the lowest determinable level of EIA was 1.2 ng/ml and that of the chemiluminescence immunoassay 0.2 ng/ml (Table 1).

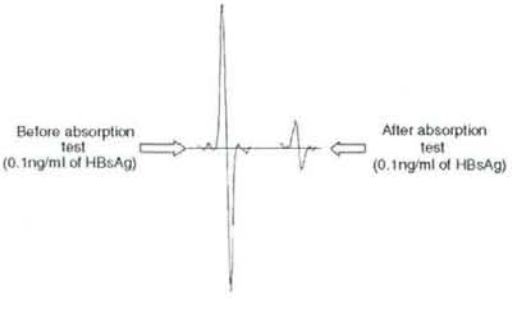

Fig. 3. Confirmation of specificity by radical immunoassay. HBsAg panel serum at a concentration of 0.1 ng/ml was incubated with HBsAb-coated ferrite particles and HBsAg was absorbed. A drastic decrease of the ESR signal was observed after absorption of HBsAg by the HBsAb-coated ferrite particles.

Table 1
Comparison of the sensitivity between EIA, CLIA, and radical immunoassay using HBsAg panel serum

HBsAg (ng/ml)	EIA	CLIA	Radical immunoassay (*S/N* ratio)
1.2	+	+	+(27.95)
0.8	−	+	+(25.64)
0.2	−	+	+(13.42)
0.1	−	−	+(9.31)
0.05	−	−	+(6.27)
0.025	−	−	+(3.70)
0.010	−	−	±(2.33)

HBsAg panel serum: adr, genotype C CLIA, chemiluminescent immunoassay.

3.3. Comparison of radical immunoassay and HBV RTD-direct test

The levels of HBV DNA and HBsAg in the diluted sera from 9 HBV carrier patients were measured simultaneously by HBV RTD-direct test and the radical immunoassay. In case those original sera were diluted to an HBV DNA concentration of 1.2–2.4 LOG IU/ml, the sera were positive for HBsAg by the radical immunoassay. When the sera were further diluted to a level of less than 1.0 LOG IU/ml HBV DNA, the diluted sera were still positive for HBsAg by the radical immunoassay (Table 2).

3.4. Examination of HBsAg by radical immunoassay in healthy volunteers and patients with non-B chronic liver diseases

Serum HBsAg was examined by the radical immunoassay method using the automatic ESR analyzer in healthy volunteers and patients with non-B, non-C liver cirrhosis and non-B, non-C hepatocellular carcinoma. As shown in Fig. 4, HBsAg was positive in 6 of the 210 volunteers, 5 of the 65 non-B, non-C liver cirrhosis patients, and 6 of the 62 non-B, non-C hepatocellular carcinoma patients ($P = 0.04$ vs. volunteers). We also examined the low level of HBsAg in the sera from HCV-positive patients. Even though HBsAg was negative by routine EIA, the radical immunoassay showed positivity for HBsAg in 12 of the 134 patients with chronic hepatitis C ($p < 0.02$ vs. volunteers) and 11 of the 107 patients with HCV-positive HCC ($p < 0.008$ vs. volunteers). The positivity rate of HBsAg in patients with non-B chronic liver diseases (34 out of 368 patients) was significantly higher than that in normal volunteers ($p < 0.005$).

3.5. Details of low HBsAg-positive patients with chronic non-B liver diseases

HBV DNA detection was performed in sera from 6 volunteers and 34 patients with chronic liver diseases

Table 2
Comparison of sensitivity between HBV RTD-direct test and radical immunoassay

Case (genotype)	Diluted sera from HBV carriers		Further dilution of the diluted sera from HBV carriers	
	HBV RTD-direct (PCR: LOG IU/ml)	Radical immunoassay (*S/N*)	HBV RTD-direct (PCR: LOG IU/ml)	Radical immunoassay (*S/N*)
1 (B)	1.8	+(35.4)	10 times Negative (less than 1)	+(5.0)
2 (C)	1.7	+(339)	10 times Negative (less than 1)	+(31.3)
3 (B)	1.3	+(12.4)	4 times Negative (less than 1)	+(4.2)
4 (B)	1.2	+(23.2)	5 times Negative (less than 1)	+(6.3)
5 (B)	1.3	+(13.3)	4 times Negative (less than 1)	+(4.3)
6 (C)	1.4	+(82.9)	30 times Negative (less than 1)	+(6.8)
7 (C)	2.4	+(461)	100 times Negative (less than 1)	+(16.4)
8 (C)	1.2	+(10.7)	3 times Negative (less than 1)	+(3.7)
9 (B)	1.2	+(9.6)	3 times Negative (less than 1)	+(3.9)

who were positive for HBsAg by radical immunoassay. No HBV DNA-positive case was found among 6 volunteers who showed low HBsAg positivity by radical immunoassay. One patient with non-B, non-C liver cirrhosis with a *S/N* ratio of 7.13, two patients with non-B, non-C HCC with *S/N* ratios of 12.2 and 9.34, three patients with chronic hepatitis C with *S/N* ratios of 11.6, 5.46 and 4.53, and three patients with HCC complicated by HCV with *S/N* ratios of 10.8, 8.86 and 5.57 were positive for HBV DNA. The *S/N* ratios for HBV DNA-positive cases tended to be higher than those for HBV DNA-negative cases (Table 3).

Two of 9 HBV DNA-positive cases were positive for anti-HBs and 6 of 25 HBV DNA-negative cases were positive for anti-HBs. Four of 9 HBV DNA-positive cases were positive for anti-HBc and 10 of 25 HBV DNA-negative cases were positive for anti-HBc. As shown in Table 3, there was no clear relationship between HBV DNA positivity, anti-HBs positivity, and anti-HBc positivity.

4. Discussion

Fig. 5 summarizes the status of low HBs antigenemia clarified by highly sensitive HBsAg measurement. In combination with radical immunoassay and real-time PCR, we found two groups of patients with low HBs antigenemia: low HBs antigenemia with HBV DNA and low HBs antigenemia without HBV DNA. The former group is considered to represent occult HBV carriers. In the latter group, it is uncertain whether there is actual infection with a low HBV load, and may simply imply a false positive. Our present study demonstrated that our radical immunoassay method is a promising new tool for screening occult HBV carriers from healthy subjects and patients with various liver diseases.

As shown in our data comparing the HBV RTD-direct test and radical immunoassay using diluted sera from HBV carriers, the radical immunoassay still showed positivity for HBsAg when the HBV RTD-

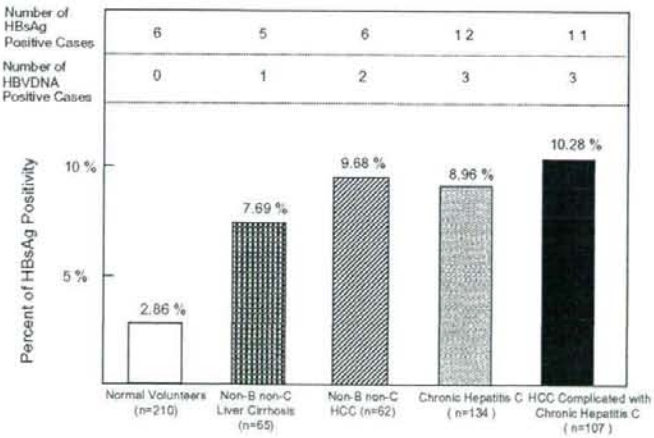

Fig. 4. Examination of HBsAg by the radical immunoassay method in normal volunteers and patients with chronic liver diseases.

Table 3
Characteristics of 34 cases

Patient	Age/sex	Diagnosis	Radical immunoassay (*S/N* ratio)	HBV DNA (LOG IU/ml)	Anti-HBs	Anti-HBc
1	58/M	Non-B, non-C liver cirrhosis	7.13	1.2	–	+
2	69/M	Non-B, non-C liver cirrhosis	4.86	–	–	–
3	57/F	Non-B, non-C liver cirrhosis	4.28	–	+	–
4	54/M	Non-B, non-C liver cirrhosis	3.72	–	–	–
5	68/F	Non-B, non-C liver cirrhosis	3.31	–	–	+
6	72/M	Non-B, non-C HCC	12.20	2.3	–	+
7	75/F	Non-B, non-C HCC	9.34	1.3	–	–
8	73/M	Non-B, non-C HCC	4.12	–	+	+
9	68/M	Non-B, non-C HCC	4.10	–	–	–
10	66/F	Non-B, non-C HCC	3.68	–	–	+
11	69/M	Non-B, non-C HCC	3.29	–	–	–
12	64/M	Chronic hepatitis C	11.60	1.6	–	–
13	69/M	Chronic hepatitis C	8.64	–	+	+
14	53/F	Chronic hepatitis C	7.42	–	–	–
15	47/F	Chronic hepatitis C	7.15	–	–	+
16	36/M	Chronic hepatitis C	5.92	–	+	–
17	53/F	Chronic hepatitis C	5.46	2.0	–	–
18	54/F	Chronic hepatitis C	5.23	–	–	+
19	64/M	Chronic hepatitis C	4.53	1.8	+	+
20	50/M	Chronic hepatitis C	4.25	–	–	–
21	59/M	Chronic hepatitis C	4.07	–	–	+
22	60/M	Chronic hepatitis C	3.81	–	+	–
23	36/F	Chronic hepatitis C	3.37	–	–	–
24	69/M	HCC with HCV	10.8	1.2	–	–
25	70/F	HCC with HCV	8.86	1.3	+	+
26	61/M	HCC with HCV	6.25	–	–	–
27	65/F	HCC with HCV	5.57	1.0	–	–
28	64/M	HCC with HCV	5.12	–	–	+
29	62/M	HCC with HCV	4.41	–	–	+
30	73/F	HCC with HCV	4.23	–	–	–
31	59/F	HCC with HCV	3.74	–	–	+
32	54/M	HCC with HCV	3.46	–	+	–
33	71/F	HCC with HCV	3.35	–	–	–
34	73/F	HCC with HCV	3.54	–	–	–

Anti-HBs positivity (+): ⩾5 mIU/ml Anti-HBc positivity (+): ⩾50% inhibition.

direct test indicated negativity for HBV DNA (less than 1.0 LOG IU/ml). In low-HBV load carriers, the proportion of HBsAg and HBV DNA concentrations in sera may differ among individuals. However, approximately 26.5% of low-HBs antigenemia patients with non-B chronic liver diseases proved to be positive for low HBV DNA, and were considered to be occult HBV carriers. In comparison with HBV DNA, which exists in an episomal or integrated pattern, HBV-associated proteins such as HBsAg fluctuate to a lesser degree [12]. Therefore, radical immunoassay for measurement of low HBsAg seems to be advantageous to HBV DNA measurement by PCR.

Occult HBV is defined as the presence of HBV DNA detectable by sensitive PCR among individuals testing negative for HBsAg [5,13–15]. There are two main reasons for occult HBV: mutations in the S gene of HBV DNA or a low level of HBV viremia [16,17]. Bréchot et al. have provided very strong evidence that most cases of occult HBV are related to very low levels of HBV (low HBV load) rather than to HBV mutants that do not express or produce aberrant HBV surface protein [17]. Full-length genome analysis has shown that multiple alterations in the HBV genome may have a synergistic effect in down-regulation of HBsAg production, making it difficult to establish a specific mutation in a particular gene [18]. Therefore, we expected that our radical immunoassay system would be able to screen the majority of occult HBV carriers conveniently because of its high sensitivity. Testing of whole samples by real-time PCR of HBV DNA may be of interest regarding the proportion of HBV DNA positivity in HBsAg-negative samples and will reveal the proportion of HBV DNA positivity in HBsAg-positive samples by the radical immunoassay method, thus validating its rationality.

In the present study, an important issue was whether individuals with low HBsAg positivity without HBV DNA were HBV carriers. We confirmed the specificity of HBsAg detection by an absorption test using diluted HBsAg panel serum and anti-HBsAb. Although we did not show the data, we also performed the absorption

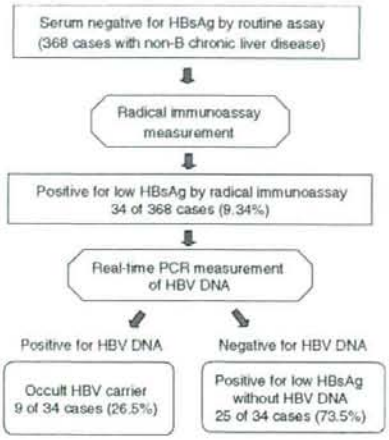

Fig. 5. Summary of low HBsAg and HBV DNA measurement in patients with non-B chronic hepatitis. We found two groups: low HBs antigenemia with HBV DNA, which was often observed in patients with non-B chronic liver diseases, and low HBs antigenemia without HBV DNA, which was predominant in healthy volunteers.

test on several low HBsAg-positive cases and confirmed its specificity. Our analytical results therefore appear to be reliable. Natural clearance of HBsAg has been reported to occur in chronic HBV carriers [19,20]. Among 34 cases with low HBsAg positivity and HBV DNA negativity, 23.5% were positive for anti-HBs and 41.2% were positive for anti-HBc. We speculate that some cases showing low HBsAg but negativity for HBV DNA might reflect the process of natural clearance of HBsAg. In the present study, we were unable to obtain data indicating that patients with low HBsAg positivity with HBV DNA negativity were HBV carriers. Further study will be required to clarify this issue.

In areas where HBV infection is highly endemic, vertical and horizontal transmissions are now a social health problem [21]. Although the prevalence of occult HBV infection is speculated to be high in these endemic areas, the PCR method cannot be used routinely for screening large numbers of samples because of the complexity of sample preparation, the expense of reagents including Taq polymerase, and the time taken for sample processing. Moreover, the sensitivity of PCR analysis depends on DNA concentration, selection of PCR primers, and assay conditions [22]. Thus, PCR analysis of occult HBV is not suitable as a universal screening method. Occult HBV carries a risk of transmission [23,24]. It is anticipated that the radical immunoassay method will become a powerful tool for worldwide prevention of vertical and horizontal transmission of occult HBV, including cases with a low virus load.

In conclusion, the radical immunoassay method has high sensitivity for HBsAg detection. This method demonstrated that a low concentration of HBsAg was present in healthy volunteers and a higher percentage of patients with non-B chronic liver diseases. The present findings clearly demonstrate that infection with HBV, including occult HBV, is far more prevalent than previously thought, as a result of increasing the sensitivity of HBsAg detection to below the present limit.

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