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HEPATOLOGY

Significance of hepatitis B virus DNA clearance and early prediction of hepatocellular carcinogenesis in patients with cirrhosis undergoing interferon therapy: Long-term follow up of a pilot study

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Abstract

Background and Aim: Because the anti-carcinogenic effect and mechanism of interferon (IFN) in patients with hepatitis B virus (HBV)-related cirrhosis have not been elucidated, quantitative analysis of HBV-DNA concentration was carried out sequentially.

Method: Of 60 consecutive patients with cirrhosis who began IFN therapy between 1986 and 1990, 57 patients were completely observed for the appearance of hepatocellular carcinoma (HCC). All patients underwent intermittent administration of IFN for a median period of 18 months. HBV-DNA was quantified using transcription mediated amplification and hybridization protection assay. A HBV-DNA count <3.7 log-genome equivalent (LGE)/mL (equivalent to $10^{3.7}$ or 5000 copies/mL) was considered to be a negative value.

Results: Of 25 patients who had HBV-DNA loss after IFN therapy, nine lost HBV-DNA during the therapy and 16 lost HBV-DNA after cessation of the therapy. The other nine patients showed a transient loss of HBV-DNA, and the remaining 23 retained persistently positive HBV-DNA during and after therapy. Although HCC developed in two (8.0%) of the 25 patients with HBV-DNA loss, carcinogenesis was found in 11 (34.4%) of 32 patients without HBV-DNA loss (Fisher's exact test, $P = 0.026$). In the two exceptional patients, HCC was detected at 1.2 and 3.6 years after loss of HBV-DNA, respectively. When the HBV-DNA concentration decreased by 2 LGE/mL (decrease to 1/100) at 6 months after initiation of interferon, HBV-DNA became negative eventually in 15 (60.0%) of 25 patients.

Conclusion: A significant decrease or loss of serum HBV-DNA prevents development of HCC, and sequential analysis of HBV-DNA could be very useful in both the prediction and the early detection of small HCC.

Key words: cancer prevention, carcinogenesis, DNA, hepatitis B virus, hepatocellular carcinoma, interferon, liver cirrhosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of death in many parts of sub-Saharan Africa and Asia.[1,2] It is also one of the most common neoplasms in Japan. Abundant epidemiological and molecular biological evidence shows that the hepatitis B virus (HBV) is an important factor in the development of HCC,[3–6] but the precise role of HBV-DNA viruses in the oncogenesis of HCC is still unknown. Although increasing evidence indicates that the HBV plays an important role in the development of HCC, particularly after the discovery of integrated forms of HBV,[7,8] current serological and virological markers are still insufficient for establishing this relationship. Because a really curative therapy is not available for HCC at present, the accurate prediction

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and early detection of HBV-related HCC is essential in the current situation. Needless to say, a cohort of patients with HBV-related cirrhosis has a significantly high risk for the development of HCC,[6,9] but the degree of risk of carcinogenesis in an individual patient cannot be predicted as yet. Hepatocellular carcinogenesis in patients with HBV infection may be associated with persistence of aminotransferase, concentration of HBV-DNA, or merely the severity of the liver disease.

Interferon (IFN) has been reported to be effective in patients with HBV-related chronic hepatitis, which, on early control studies,[10–12] decreases serum HBV-DNA concentration and improves biochemical data and subsequently suppresses disease progression to cirrhosis.[13,14] Although the various effects of IFN in HBV infection have been well investigated from the virological, biochemical, and medico-economical viewpoints,[15–17] the influence of IFN on the long-term outcome for liver cirrhosis and on hepatocellular carcinogenesis is still controversial.[18–23] In order to clarify the mechanism of the anticarcinogenic activity of IFN, if any, we analyzed HBV-DNA concentration serially in a cohort of 60 patients with cirrhosis.

The purposes of this study are: (i) to elucidate the relation of hepatocellular carcinogenesis to longitudinal clinical courses of consecutive cirrhotic patients with IFN therapy; and (ii) to investigate a prediction of cancer preventative activity by early HBV-DNA elimination.

METHODS

Patients

Of 189 patients who were diagnosed as having HBV-related cirrhosis using peritoneoscopy and/or liver biopsy from 1983 to 1990 in our hospital, a total of 60 patients underwent IFN therapy from 1986 to 1990. Because three patients were lost to follow up, the remaining 57 patients (95.0%) were analyzed for virological outcome, carcinogenesis, and eventual prognosis: the reason for the dropout from the observation in the three patients was simply relocating house.

Table 1 shows the demography and laboratory data of the consecutive 57 patients who began IFN therapy from 1986 to 1990. There were 45 men and 12 women, with an age range from 19 to 60 years and a median of 41 years. Median values of bilirubin and albumin were 0.9 mg/dL and 4.1 g/dL, respectively. All the patients had a high HBV-DNA concentration of 3.7 log-genome equivalent (LGE)/mL or more at the time of IFN therapy.

Interferon treatment

IFN-α was administered in 35 patients (61.4%) and IFN-β in the remaining 22 patients (38.6%). The daily quantity of IFN was three million units in 22 (38.6%) and six million units in 35 (61.4%), twice a week administration was carried out in 54 (94.7%) and three times a week administration in three (5.3%). All patients received intermittent IFN therapy for a median of 18 months (range, 2–132 months), but the duration of the IFN therapy was arbitrary in this pilot study. Although the daily dose of IFN and the duration of the therapy varied in this study, 52 (91.2%) of the 57 patients received IFN for 6 months or longer.

Table 1 Demography and laboratory data of 57 patients with hepatitis B virus-related cirrhosis undergoing interferon therapy

Demography	
Men : women	45:12
Age (median, range)	41 (19–60)
Decompensated cirrhosis	3 (5.3%)
Past alcohol consumption of 500 kg or more	3 (5.3%)
Laboratory data (median, range)	
Bilirubin (mg/dL)	0.9 (0.4–2.6)
Albumin (g/dL)	4.1 (3.0–4.9)
Aspartic transaminase (IU/L)	65 (16–404)
Alanine transaminase (IU/L)	74(12–586)
Platelet count ($\times 10^3$/mm^3)	125 (68–332)
Antibodies to hepatitis C virus positive	0
Hepatitis B e antigen positive	41 (71.9%)
Hepatitis B virus DNA (LGE/mL)	7.2 (3.9–> 8.7)
Observation period (year)	13.6 (6.5–16.1)

LGE/mL, log-genome equivalent, expressed as 10^n copy/mL.

Follow up of patients and diagnosis of HCC

Follow up of the patients was made on a monthly basis after diagnosis of liver cirrhosis using monitoring virological, hematological, and biochemical data, including α-fetoprotein. All results for these laboratory tests, including HBV markers, were obtained throughout the observation period in each patient. Patients were classified into four groups according to patterns of serial concentration of HBV-DNA: type A, disappearance of HBV-DNA during and after IFN therapy; type B, loss of HBV-DNA after cessation of IFN administration; type C, transient loss of HBV-DNA only during IFN administration; type D, persistently positive HBV-DNA during and after the therapy. Clinical courses of alanine aminotransferase (ALT) fluctuation were also classified into four groups according to normalization of the ALT value.

Imaging diagnosis was made two or more times per year for each patient using computed tomography (CT), ultrasonography (US) or magnetic resonance imaging (MRI). HCC was diagnosed using typical hypervascular characteristics on angiography in addition to certain features of CT, US and MRI. Pathological confirmation of surgically resected specimens was carried out in six (46.2%) of 13 patients with HCC development.

Assays of HBV markers

Serum hepatitis B surface antigen was measured using radioimmunoassay (Dainabot, Tokyo, Japan) and reversed passive hemagglutination (Institute of Immunology, Tokyo, Japan) using commercial assay kits. hepatitis B e antigen (HBeAg) and antibody to HBeAg were determined using ELISA (Institute of Immunology) with commercial kits. Anti-hepatitis C virus antibody (third-generation anti-HCV) was assessed using ELISA kits (Dainabot).

HBV-DNA was assayed using frozen sera stored at −80°C, and quantified using transcription-mediated amplification and hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan), as described by Kamisango *et al.*[24] A HBV-DNA value of <3.7 LGE/mL (equivalent to $10^{3.7}$ copies/mL or 5000 copies/mL) was considered to be a low value. For all serial sera from the diagnosis of cirrhosis to the end of the observation period in each patient, the DNA quantification was simultaneously carried out using identical measurement kits.

Statistical analysis

Standard statistical measures and procedures were used. The Mann–Whitney *U*-test and χ^2 tests were employed for the examination of background characteristics between the groups with and without HBV-DNA elimination. Fisher's exact test was also used to analyze the relation of HBV markers to carcinogenesis. Rates of cumulative HBV-DNA disappearance, carcinogenesis and survival were calculated using Kaplan–Meier analysis,[25] and the differences between the analyzed groups were assessed using a log-rank test. A *P*-value of <0.05 using a two-tailed test was considered to be significant. Data analysis was carried out using the computer program SPSS version 11.[26]

RESULTS

HBV-DNA in clinical courses

HBV-DNA was positive in all patients at the initiation of IFN therapy (3.9–8.7 LGE/mL). HBV-DNA became negative (<3.7 LGE/mL) in 25 of 57 patients (43.9%) during the observation period, with a median of 13.6 years. The remaining 32 patients did not show a sustained negative HBV-DNA after the therapy, although nine patients did show transient negative values for a limited period during the therapy.

Clinical courses of HBV-DNA were classified into the four categories mentioned above. Nine patients (15.8%) lost HBV-DNA during and after IFN therapy (type A), 16 patients (28.1%) lost HBV-DNA after cessation of the therapy (type B). The other nine patients (15.8%) showed a transient loss of HBV-DNA (type C), and the remaining 23 (40.4%) retained persistently positive HBV-DNA (type D).

The cumulative rate of HBV-DNA disappearance was calculated using Kaplan–Meier analysis (Fig. 1).

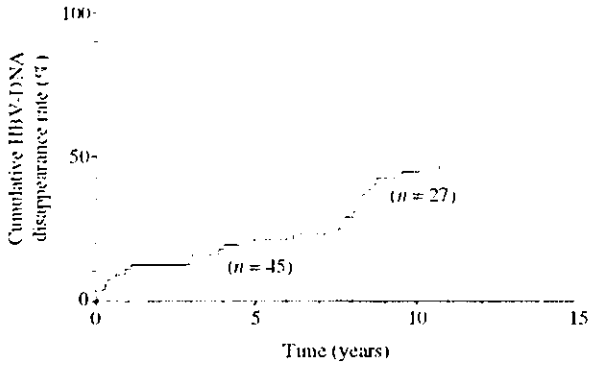

Figure 1 Cumulative hepatitis B virus (HBV)-DNA disappearance rate in the 57 cirrhotic patients with interferon therapy.

DNA became negative in 10.5% at the end of the first year after initiation of IFN therapy, in 12.3% at the third year, 21.0% at the fifth year, 43.7% at the tenth year, and 46.7% at the fifteenth year, respectively.

Hepatocellular carcinogenesis and serial concentration of HBV-DNA

A total of 13 patients developed HCC during the observation period.

The relationship between carcinogenesis and serial concentration of HBV-DNA was analyzed (Fig. 2). None of the nine patients in the type A group developed HCC. Two (12.5%) of 16 patients in the type B group developed HCC: HCC were detected 1.2 years after the disappearance of HBV-DNA in one patient, and 3.6 years after the disappearance of HBV-DNA in the other patient. Three (33.3%) of nine patients in the type C group showed carcinogenesis, and eight (34.8%) of 23 patients in the type D group developed HCC during the observation. Hepatocellular carcinogenesis was significantly associated with persistent positive HBV-DNA after initiation of IFN (2/25 *vs* 11/32; $P = 0.019$ using the $\chi 2$ test, $P = 0.026$ using Fisher's exact test).

Cumulative carcinogenesis rates were analyzed according to the ultimate course of the serial assay of HBV-DNA (Fig. 3). Fifth-year hepatocellular carcinogenesis rates were 0% in patients with HBV-DNA loss, and 9.4% in patients without HBV-DNA elimination; 10-year rates were 8.0% and 22.5%; and 15-year rates were 8% and 44.0%, respectively. The carcinogenesis rate in patients with HBV-DNA elimination was significantly lower than in those without DNA elimination ($P = 0.011$, using a log-rank test).

Hepatocellular carcinogenesis and HBeAg and aminotransferase

The relationship between carcinogenesis and HBeAg positivity during the clinical course was assessed.

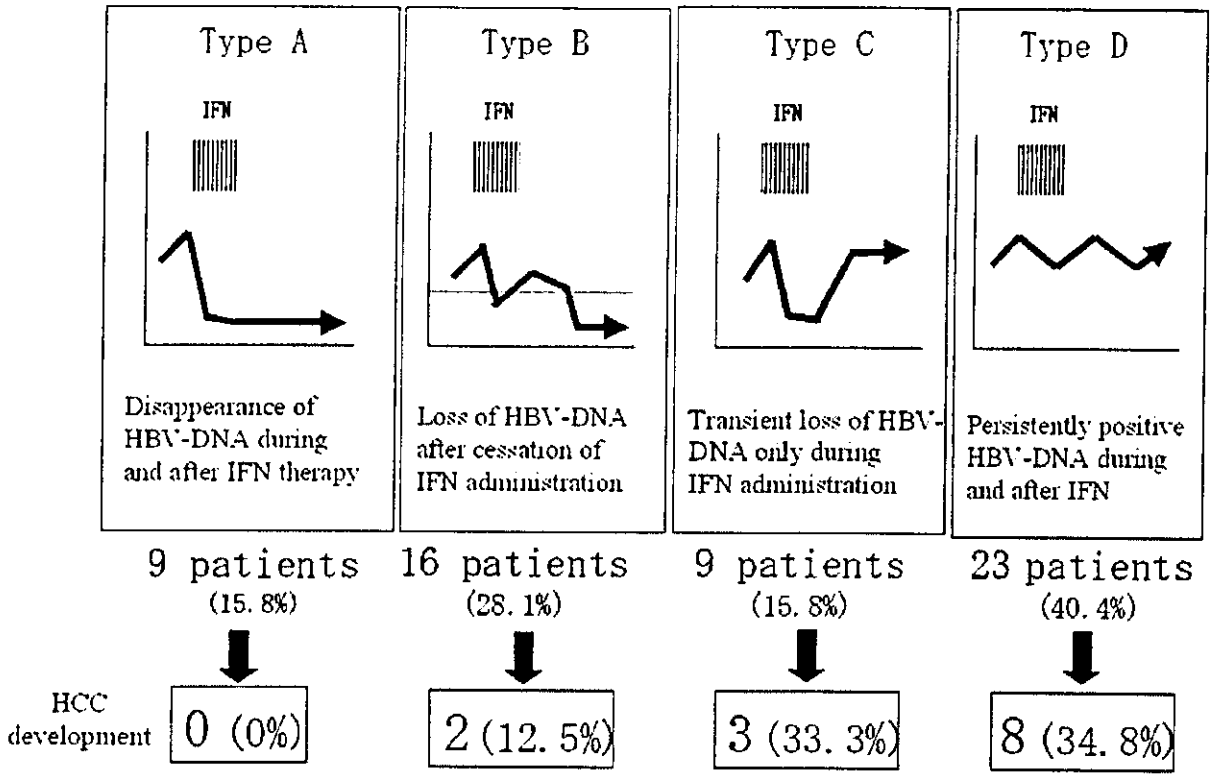

Figure 2 Relation between types of serial hepatitis B virus (HBV)-DNA concentration and carcinogenesis. HCC, hepatocellular carcinoma; IFN, interferon.

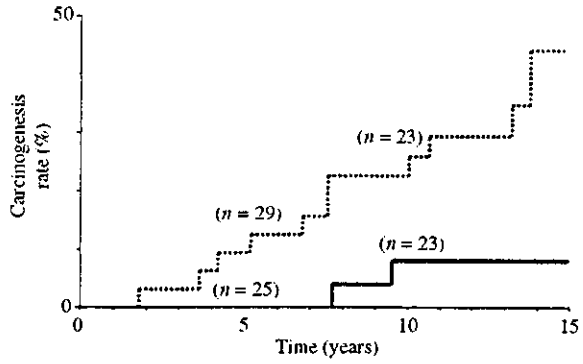

Figure 3 Cumulative hepatocellular carcinogenesis rates in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus (HBV)-DNA clearance.

HBeAg was positive in 41 patients (71.9%) and negative in 16 (28.1%) at the initiation of IFN therapy. Twenty-eight (68.3%) of the 41 patients showed continuous loss of HBeAg after IFN therapy. HCC developed in four (25.0%) of the 16 patients without HBeAg from the beginning, four (14.3%) of the 28 patients with HBeAg clearance, and five (38.5%) of 13 patients with persistent HBeAg positivity. HBeAg clearance did not significantly decrease the incidence of carcinogenesis risk ($P = 0.12$ using the χ^2 test with Yates' correction).

The relationship between carcinogenesis and a longitudinal course of ALT after IFN therapy was also analyzed. Four (18.2%) of 22 patients with normalization of ALT after IFN therapy developed HCC; nine (25.8%) of 35 patients with persistently abnormal ALT levels developed HCC. The serial values of ALT were not significantly associated with carcinogenesis risk ($P = 0.075$ using the χ^2 test with Yates' correction).

The cumulative HBeAg disappearance rate, HBV-DNA disappearance rate, and ALT normalization rate were calculated in those patients with positive HBeAg at the beginning of IFN treatment (Fig. 4). The HBeAg disappearance rate and DNA disappearance rates were 55.4% and 14.6% at the end of the fifth year, and 55.4% and 40.1% at the tenth year, respectively. The ALT normalization rate at the fifth year was 25.4% and the tenth year rate was 41.2%. Although the incidence of virological and biochemical improvement gradually increased after therapy, the rates evidently differed between virological and biochemical responses.

Influence of the length of interferon therapy on HBV-DNA loss

The influence of the length of the therapy on virological response was assessed.

Although 25 (43.8%) of 57 patients cleared HBV-DNA on overall analysis, 21 (46.6%) of 45 patients who received IFN for more than 6 months and 20 (50%) of 40 patients who received IFN for more than 12 months lost HBV-DNA. Similarly, the HBV-DNA disappearance rate slightly increased correlating with the length of IFN administration: 55.5% in patients who were treated for more than 18 months, 56.0% with more than 24 months' treatment, 64.7% in more than 36 months' treatment, 58.3% in more than 48 months' treatment, and 71.4% in more than 60 months' treatment (Fig. 5). The longer the IFN therapy was carried out, the higher the rate of HBV-DNA disappearance.

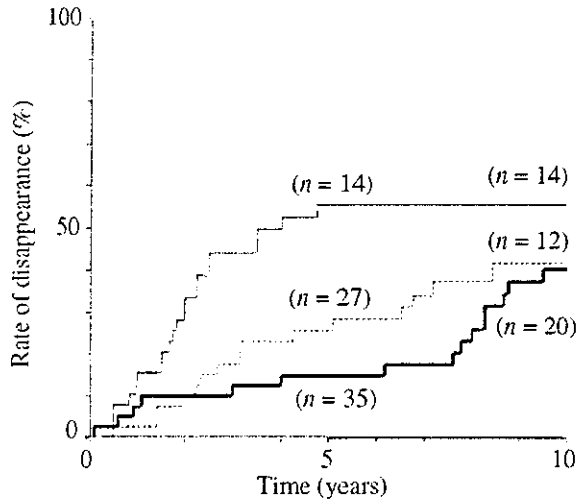

Figure 4 Cumulative (—) hepatitis B e antigen (HBeAg) disappearance rate, (—) hepatitis B virus (HBV)-DNA disappearance rate, and (---) alanine transaminase normalization rate in 41 patients with positive HBeAg at the initiation of interferon therapy.

Prediction of future HBV-DNA elimination

We assessed the relation between an early HBV-DNA response and a future HBV-DNA loss. When the HBV-DNA concentration decreased by ≥2 LGE/mL (decrease to 1/100) during the first 6 months, 15 (60.0%) of 25 patients eventually lost HBV-DNA. In contrast, when the HBV-DNA decrease was <2 LGE/mL during the period, HBV-DNA loss was found in 10 (31.3%) of 32 patients ($P = 0.036$, χ^2 test). Similarly, future HBV-DNA loss was estimated from a decrease in concentration of HBV-DNA at the end of 12 months: HBV-DNA eventually became negative in 15 (62.5%) of 24 patients with a larger DNA decrease of ≥2 LGE/mL at the end of 12 months, eventual DNA loss was found in only 10 (30.3%) of 33 patients with a smaller DNA decrease by <2 LGE/mL. The 12-month decrease of HBV-DNA was significantly associated with future DNA loss ($P = 0.030$, χ^2 test).

The early response of HBV-DNA and the length of IFN therapy were analyzed together for the prediction of eventual HBV-DNA loss. Of 25 patients with a HBV-DNA decrease of ≥2 LGE/mL during the initial 6 months, two (33.3%) of six patients with short IFN therapy of ≤6 months showed a HBV-DNA loss, but 13 (68.4%) of 19 patients with long-term IFN therapy of >6 months lost HBV-DNA. Of 32 patients with a HBV-DNA decrease of <2 LGE/mL in the first 6 months, one (20.0%) of five patients with short IFN therapy showed HBV-DNA loss, but nine (33.3%) of 27 patients with long-term IFN administration lost HBV-DNA. Therefore, according to the early HBV-DNA response and the duration of the therapy, the rate of sustained HBV-DNA decrease to <3.7 LGE/mL varied, with a range of 20.0–68.4%.

Prognosis after IFN therapy

A total of eight patients (14.0%) died in the period of observation: six from development of HCC and the

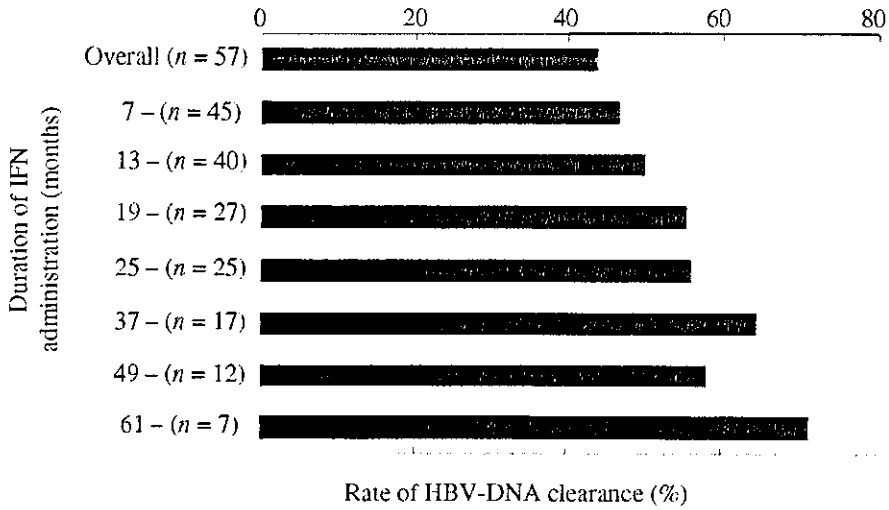

Figure 5 Influence of the length of interferon (IFN) therapy on hepatitis B virus (HBV)-DNA clearance.

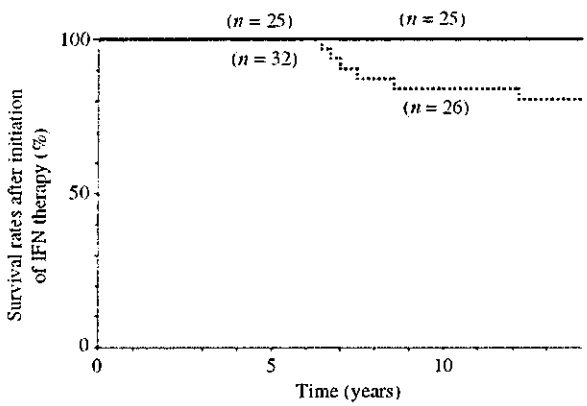

Figure 6 Cumulative survival rates after the initiation of interferon (IFN) therapy in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus DNA clearance.

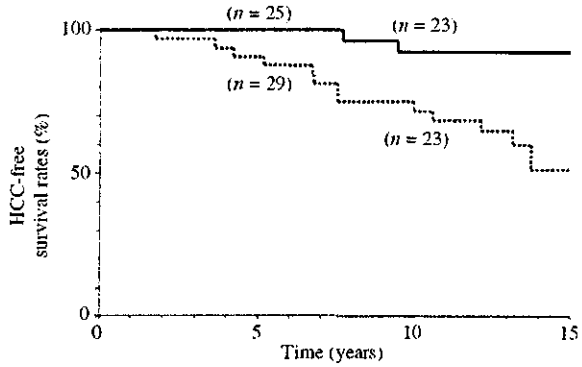

Figure 7 Hepatocellular carcinoma (HCC)-free survival rates in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus DNA clearance.

other two from liver failure due to aggravation of cirrhosis.

Of 13 patients with HCC development, two patients with HBV-DNA loss have not shown any tumor recurrence after surgical resection, and both patients are alive at the end of the observation. In contrast, nine (81.8%) of 11 patients with persistently high HBV-DNA developed HCC recurrence after therapy, and six (54.5%) of the patients died during the observation period. All six patients died from the development of HCC and none from aggravation of cirrhosis or extrahepatic disease.

Of 44 patients without HCC development until the end of the observation period, none of 23 patients with HBV-DNA loss died, but two (9.5%) of 21 patients with persistently positive HBV-DNA have died from liver failure.

Survival rates were compared between those patients with and without HBV-DNA loss (Fig. 6). Fifth-year survival rates in patients with and without HBV-DNA loss were 100% and 100%, seventh year rates were 100% and 90.5%, tenth year rates were 100% and 84.1%, and twelfth year rates were 100% and 80.6%, respectively. The cumulative survival rate in patients with HBV-DNA loss was significantly higher than that in patients without HBV-DNA clearance ($P = 0.0030$, log-rank test).

The HCC-free survival rates were also assessed in the two patient groups (Fig. 7). Fifth-year HCC-free survival rates in patients with and without HBV-DNA loss were 100% and 90.6%, seventh year rates were 100% and 81.3%, tenth year rates were 92% and 74.8%, and fifteenth year rates were 92% and 51.2%, respectively. The HCC-free survival rate in patients with HBV-DNA loss was significantly higher than that in patients without HBV-DNA clearance ($P = 0.0036$, log-rank test).

DISCUSSION

Until recently, several authors mentioned the anti-carcinogenic activity of IFN in patients with HBV-related cirrhosis. Oon[18] and Ikeda *et al.*[21] have shown that IFN significantly decreased carcinogenesis in patients undergoing IFN therapy with a relative risk of 0.03 and 0.39, respectively. Lin *et al.* also demonstrated an anti-tumor activity of IFN, with a relative risk of 0.11 in a randomized controlled trial for patients with chronic hepatitis and cirrhosis.[23] Mazzella *et al.*,[19] Fattovich *et al.*[20] and the International Interferon-alpha Hepatocellular Carcinoma Study Group in Europe[22] demonstrated a low relative risk for carcinogenesis in patients with IFN therapy, but none could show a statistically significant difference. Aside from the slightly inconsistent results after IFN therapy for cirrhosis, we tried to elucidate the relationship between virological response and HCC development, using a cohort of consecutive patients with cirrhosis who underwent IFN therapy more than 10 years ago. Considering that the disease activity and carcinogenic potency can change significantly in the course of HBV-related liver disease, a longitudinal analysis was carried out for the study of the clinical process and the mechanism of anti-tumor activity of IFN in HBV-positive cirrhosis patients.

In this clinical study, sequential trends of HBV concentration were significantly associated with hepatocellular carcinogenesis, as was found in natural clinical courses of patients without IFN.[27] Although only two of 25 patients who developed HCC showed a disappearance of HBV-DNA during or after IFN therapy, 11 of 32 patients who showed carcinogenesis could not eliminate HBV-DNA using treatment with IFN ($P = 0.019$). A point in common found in the two exceptional patients with HCC development after elimination of HBV-DNA was that the HCC were detected immediately after a significant decrease in the HBV-DNA level after using IFN in the clinical courses: 1.2 years and 3.6 years after in each patient. We can reasonably consider that the discovered HCC in the patients already existed at an indiscernible size at the time of HBV-DNA elimination, and that the minimal HCC automatically grew gradually for the following few years after the decrease in HBV-DNA levels occurred. Even including these two patients with HCC development, the risk of hepatocellular carcinogenesis was significantly associ-

ated with the persistence of a high HBV-DNA concentration. Hepatocellular carcinogenesis was assessed using serial HBV-DNA assay with a cut-off value of 3.7 LGE/mL (or $10^{3.7}$ copy/mL) in this study. Although a detailed analysis of HBV-DNA concentration with a more sensitive measurement may demonstrate a better correlation with the carcinogenesis rate than the present study, setting the HBV-DNA concentration at this cut-off value was significantly valuable in the prediction for HCC appearance.

The mechanism of anticarcinogenic activity of IFN was regarded as an anti-necroinflammatory process through suppression of HBV-DNA concentration from these results. This study dealt with the relationship between carcinogenesis and HBV-DNA principally, but clinical courses of aminotransferase were also significantly related to the HCC development. Aminotransferase values were less valuable than HBV-DNA levels in the prediction of HCC development in the natural clinical course of HBV-cirrhosis,[27,28] and aminotransferase values were also less associated with the future rate of carcinogenesis in patients undergoing IFN therapy.

Although the mere use of IFN does not guarantee a decrease in the rate of carcinogenesis in patients with HBV-related cirrhosis, a serial course of HBV-DNA concentration was significantly correlated with future HCC development during and after treatment. The value of cancer prediction was much higher from the assay of HBV-DNA than that of HBe antigen. Indeed the cut-off values of HBV-DNA concentration seemed to be discretionary; the advantage in clinical practice was marked and conspicuous. When more sensitive ways of measuring HBV-DNA concentration were applied to the analysis, hepatocellular carcinogenesis could be more successfully predicted.

In conclusion, persistence of a high concentration of HBV-DNA was significantly associated with hepatocellular carcinogenesis in cirrhotic patients with IFN therapy, and its sequential analysis would be useful in the early detection of HCC. IFN therapy is recommended to be continued as long as possible until HBV-DNA loss occurs in HBV-cirrhosis patients, from the viewpoint of cancer prevention. Further studies with a greater number of patients are required to confirm the relationship, and future studies should be aimed at defining the role and basic mechanisms by which the carcinogenesis rate was suppressed by IFN in the cohort.

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p16 Promoter Hypermethylation in Human Hepatocellular Carcinoma with or without Hepatitis Virus Infection

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Key Words
Hepatitis virus · Hepatocellular carcinoma · Methylation · *p16* · *p53*

Abstract
Background: Epigenetic alteration through methylation is one of the most important steps in carcinogenesis. However, the relation between hepatitis virus infection and epigenetic alterations is poorly understood. ***Methods:*** Sixteen patients without hepatitis B virus (HBV) and hepatitis C virus (HCV) and 35 patients with HBV or HCV who underwent liver resection for hepatocellular carcinoma (HCC) were studied. Mutation of *p53* was detected by direct sequencing. Methylation status of *p16* was evaluated in tumor and noncancerous liver tissues by methylation-specific polymerase chain reaction. ***Results:*** In HCC without HBV and HCV, *p53* mutations were detected in 5 (31%) of 16 HCCs. Methylation of *p16* promoter was detected in 2 (25%) of 8 moderately differentiated HCCs, 6 (75%) of 8 poorly differentiated HCCs, and none of 16 noncancerous tissue specimens. In HCC with HBV or HCV, *p53* mutations were detected in 8 (23%) of 35 HCCs. Methylation of *p16* promoter was detected in 2 (100%) of 2 well-differentiated HCCs, 13 (76%) of 17 moderately differentiated HCCs, 12 (75%) of 16 poorly differentiated HCCs, and 9 (26%) of 35 noncancerous liver tissue specimens. ***Conclusions:*** Our results suggest that hepatitis viruses might induce methylation of *p16* promoter in liver with chronic inflammation, before appearance of HCC.

Introduction

Hepatocellular carcinoma (HCC), one of the most prevalent malignancies in the world, is associated with chronic liver damage, such as infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). Basic research has suggested that hepatitis B X protein or hepatitis C core protein interacts with p53 or p21, both of which are cell-cycle modulators [1–3]. Overexpression of hepatitis B X or HCV core protein induces liver tumors in transgenic mice [4, 5]. In human liver, *p53* mutations are found in approximately 50% of advanced HCCs, irrespective of the presence of viral infection [6–8]. Cell-cycle disruption is one of the most important steps in human hepatocarcinogenesis. Recent studies show that epigenetic changes through methylation occur in the early stage of HCC [9,

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10]. Aberrant hypermethylation of CpG islands, CpG-dinucleotide-rich areas located mainly in the promoter regions of many genes, has been implicated in the transcriptional silencing of tumor suppressor genes in cancer [11–13]. In particular, *p16*, a cyclin-dependent kinase inhibitor, has been associated with promoter hypermethylation and decreased protein expression in cancers, including HCC [14].

In Japan, nearly all patients with HCC have been infected with hepatitis virus. Molecular changes in HCC have not been reported to differ between the presence and absence of hepatitis virus. However, delineation of differences between HCC with viral infection and HCC without viral infection may provide new insights into the mechanism of hepatocarcinogenesis. In this study, we investigated methylation status of the *p16* promoter gene and *p53* mutations in HCCs with and those without hepatitis virus infection. Also, we examined *p16* promoter methylation status in noncancerous liver.

Subjects and Methods

Samples

Fifty-one HCC samples were obtained during surgery at Osaka City University School of Medicine. One portion of each specimen was frozen in liquid nitrogen immediately after resection and stored at –80° until analysis (table 1).

Total RNA and DNA were extracted from this portion by conventional methods. Sixteen samples were not infected with HBV or HCV (mean age 61.9 years; 9 men and 7 women; 2 autoimmune hepatitis, 1 primary biliary cirrhosis, 3 excessive alcohol drinkers, 7 habitual alcohol drinkers, and 3 unknown etiology for liver disease).

Thirty-five HCCs were infected with HBV or HCV (mean age 60.0 years; 33 men and 2 women; 9 HBV-positive, 25 HCV-positive, and 1 positive for both viruses).

The samples were histopathologically examined and classified as well-differentiated HCC (n = 2), moderately differentiated HCC (n = 25), and poorly differentiated HCC (n = 24). The noncancerous tissues were classified as cirrhosis (n = 19) and noncirrhosis (n = 16). This study was performed in accordance with the Helsinki Declaration of 1975 (1983 revision) and was approved by the ethics committee of Osaka City University Medical School.

Methylation-Specific Polymerase Chain Reaction

Bisulfite modification of genomic DNA was performed as described by Herman et al. [15]. Briefly, 1 μg of DNA was denatured with NaOH, and 10 m*M* hydroquinone and 3 *M* sodium-bisulfite were added. The sample was incubated at 50° for 16 h. Modified DNA was purified with the use of Wizard DNA purification resin, followed by ethanol precipitation. DNA methylation patterns were determined by chemical modification of the unmethylated cytosines to uracil and subsequent polymerase chain reaction (PCR) using primers specific for either methylated or modified unmethylated DNA. Primer sequences were 5′-TTATTAGAGGGTGGGGCG-GATCGC-3′ (upper primer) and 5′-CAACCCCAAACCACAACCA-TAA-3′ (lower primer) for unmethylated *p16* and 5′-TTATTAGA-GGGTGGGGCGGATCGC-3′ (upper primer) and 5′-GACCCCG-AACCGCGACCGTAA-3′ (lower primer) for methylated *p16* [16]. The PCR amplification of modified DNA samples consisted of 1 cycle of 95° for 10 min; 35 cycles of 95° for 45 s, 53° for 45 s, and 72° 45 s; and 1 cycle of 72° for 2 min. Ten microliters of each PCR product was loaded directly onto nondenaturing 5% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet illumination.

Table 1. Main clinicopathologic features

Male/female	42/9
Mean age, years	60.6
HBsAg (+)	9
Anti-HCV (+)	25
HBsAg (+) and anti-HCV (+)	1
Anti-HCV (–) and HBsAg (–)	16
Tumor differentiation	
Well/moderately/poorly	2/25/24
Tumor diameter (<3 cm/>3 cm)	31/20
Vascular invasion	17 (33%)
Intrahepatic metastasis	15 (29%)

Semiquantitative Reverse-Transcription PCR Analysis

To investigate *p16* mRNA expression, we performed reverse-transcription PCR (RT-PCR) with the RNA of tumors and noncancerous lesion. Briefly, 1 μg of RNA was used as a template to generate complementary DNA by random hexamers and reverse transcriptase. The complementary DNA was used for PCR amplification. Primer sequences were 5′-CCACCCCGCTTTCGTAGTTTT-3′ (upper primer) and 5′-TGCGAGGCTCGCAAGAAAT-3′ (lower primer) for *p16* and 5′-CCTCGCCTTTGCCGATCC-3′ (upper primer) and 5′-GGATCTTCATGAGGTAGTCAGTC-3′ (lower primer) for β-actin. The PCR amplification consisted of 1 cycle of 95° for 12 min; 35 cycles of 95° for 30 s, 51° for 1 min, and 72° for 30 s; and 1 cycle of 72° for 3 min *(p16)*; 1 cycle of 94° for 3 min; 33 cycles of 95° for 30 s, 60° for 1 min, and 72° for 30 s, and 1 cycle of 72° for 3 min (β-actin). Ten microliters of each PCR product was loaded directly onto nondenaturing 5% polyacrylamide gels and the gels were stained with SYBR Greene (BioWhittaker Molecular Applications, Rockland, USA) according to the manufacturer's protocol. The intensity of the bands was quantified by densitometry.

Detection of p53 *Mutations*

We directly sequenced exon 5–8 of the *p53* genes, in which 98% of *p53* mutations had occurred, in the 51 tumors. One hundred nanograms of genomic DNA was subjected to 35 PCR cycles (94, 55, and 72° for 0.5, 0.5, and 1 min, respectively) with rTaq DNA polymerase. Double-stranded DNA was sequenced using dideoxy chain termination technique. Gel electrophoresis and DNA sequencing were performed with a DNA sequencing system.

Statistical Analysis

The factors in the two groups were examined by χ^2 test.

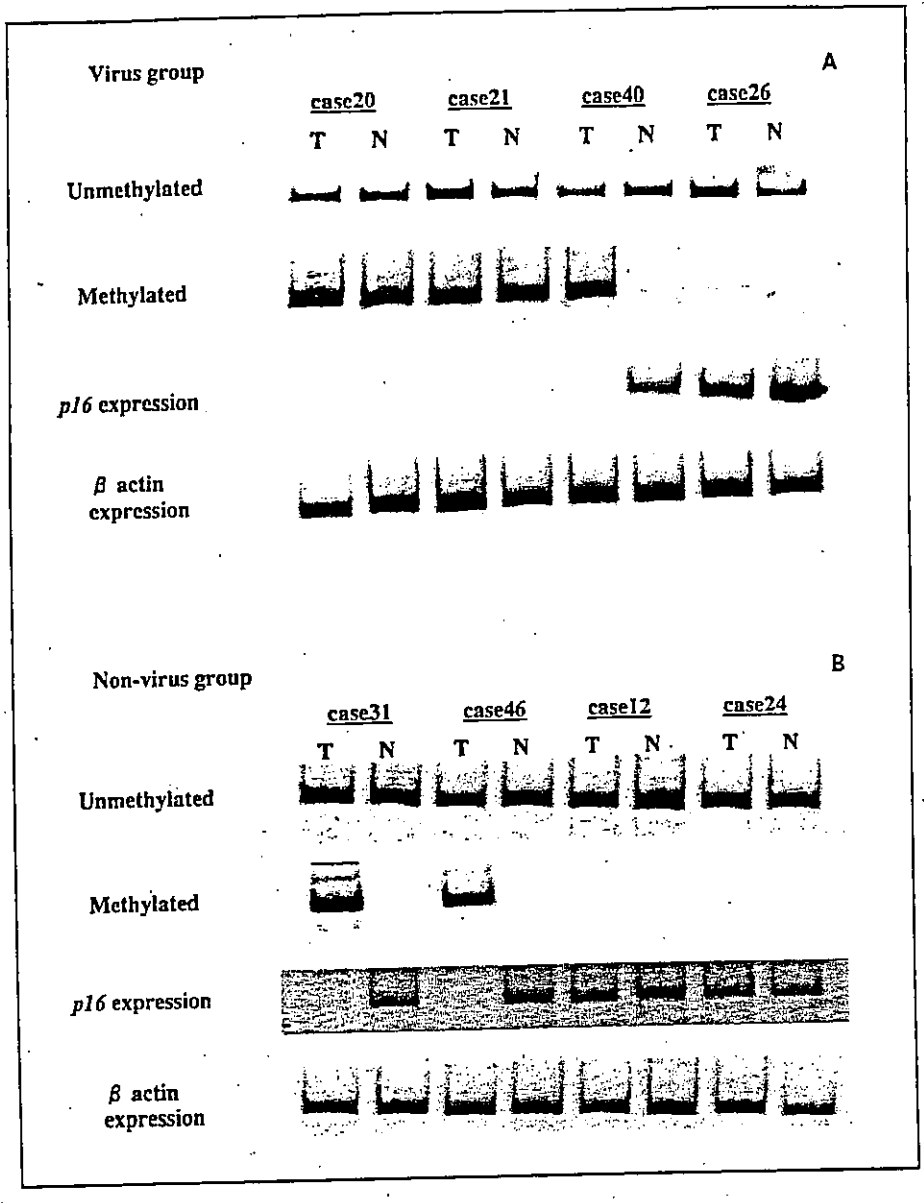

Fig. 1. Correlation between *p16* methylation and gene expression. Representative methylation-specific polymerase chain reaction results are expressed as unmethylated *p16*-specific bands or methylated *p16*-specific bands. Shown are the results of RT-PCR studies for *p16* and β-actin (used as an integrity control). Each results of HCC and noncancerous liver tissue in a case with virus infection (**A**) and without virus infection (**B**). T = HCC tumor tissue; N = noncancerous liver tissue.

Results

Methylation of p16 *Promoter and Expression of* p16 *Gene* (fig. 1, table 2)

Methylation of *p16* promoter was detected in 35 (69%) of 51 HCCs and 9 (18%) of 51 noncancerous tissue samples. *p16* expression was not detected in 31 (61%) of 51 HCCs and 9 (18%) of 51 noncancerous tissue samples. *p16* was not expressed in 29(83%) of 35 HCCs and 7 (78%) of 9 noncancerous tissue samples with methylation of *p16* promoter. In the other samples with *p16* methylation, expression of *p16* was low, compared to that of samples without methylation.

Methylation of *p16* promoter was detected in 8 (50%) of 16 HCCs without hepatitis virus infection. In brief, *p16* methylation was detected in 2 (25%) of 8 moderately differentiated HCCs and 6 (75%) of 8 poorly differentiated HCCs in this group (fig. 2). Methylation of *p16* promoter was not detected in any of 16 noncancerous tissue samples without hepatitis virus infection. Methylation of *p16* promoter was detected in 27 (77%) of 35 HCCs with HBV or HCV. In brief, *p16* methylation was detected in 5 (56%) of 9 HCCs with HBV, 21 (84%) of 25 HCCs with HCV, and 1 HCC with both viruses. According to histopathological classification, methylation of *p16* promoter was detected in 2 (100%) of 2 well-differentiated HCCs, 13

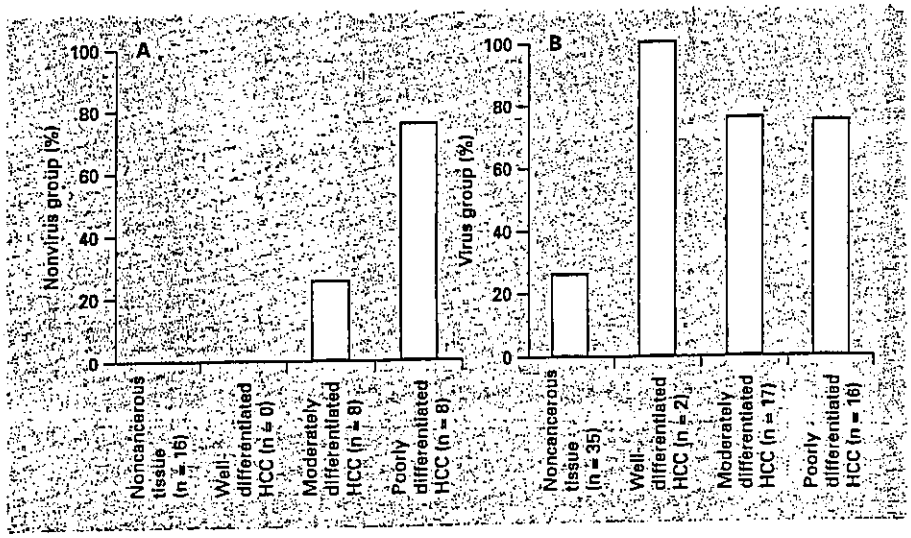

Fig. 2. Frequency of methylation studies in cases with (**A**) and without (**B**) virus infections.

Table 2. Methylation of *p16* promoter

	Nonvirus group (n = 16)	Virus group (n = 35)
Noncancerous tissue		
Noncirrhosis	0/11 (0%)	3/21 (14%)
Cirrhosis	0/5 (0%)	6/14 (43%)
HCC	8/16 (50%)	27/35 (77%)
Vascular invasion		
Positive	3/4 (75%)	8/13 (62%)
Negative	5/12 (42%)	19/22 (86%)
Intrahepatic metastasis		
Positive	1/4 (25%)	6/11 (55%)
Negative	7/12 (58%)	21/24 (88%)
p53 mutation		
Positive	1/5 (20%)	5/8 (63%)
Negative	7/11 (64%)	22/27 (81%)

Table 3. *p53* mutations in HCC

	p53 mutation	
	codon	amino acid change
Nonvirus group (n = 16)	155 ACC to AAC	Thr to Asn
HBsAg (–) and anti-HCV (–)	221 GAG to GAA	Glu to Gul
	184 GAT to TAT	Asp to Thr
	159 GCC to AGC	Ala to Ser
	225 TCT to TTT	Ser to Phe
Virus group (n = 35)	132 AAG to TTG	Lys to Leu
HBsAg (+) or anti-HCV (+)	133 ATG to TTG	Met to Leu
	272 GAG to GTG	Glu to Val
	189 GCC to GTC	Ala to Val
	220 TAT to TGT	Tyr to Cys
	123 TAT to TTC	Tyr to Phe
	275 TGT to TAT	Cys to Tyr
	292 insertion	

(76%) of 17 moderately differentiated HCCs, and 12 (75%) of 16 poorly differentiated HCCs (fig. 2). Methylation of *p16* promoter was detected in 9 (26%) of 35 noncancerous tissue samples with viral infection. In brief, *p16* methylation was detected in 3 (33%) of 9 HCCs with HBV and 6 (24%) of 25 HCCs with HCV. According to histopathological classification, methylation of *p16* promoter was detected in 6 (43%) of 14 regions of hepatic cirrhosis and 3 (14%) of 21 regions of chronic hepatitis (p = 0.581).

There was no significant difference in methylation of *p16* promoter between HCC without hepatitis virus infection and HCC with it (p = 0.525). On the other hand, the frequency of methylation of *p16* promoter was significantly higher in noncancerous tissue with hepatitis virus infection than in tissue without such infection (p = 0.025).

Mutation of p53

p53 mutations were detected in 5 (31%) of 16 HCCs without HBV and HCV (table 3). Among HCCs with HBV or HCV, *p53* mutation was detected in 8 (23%) of 35

HCCs (table 2). There is no significant relation between *p53* mutation and hepatitis virus infection. We contrasted *p53* mutation with methylation of *p16* promoter. Methylation of *p16* promoter was detected in 6 (46%) of 13 HCCs with *p53* mutation and 29 (76%) of 38 HCCs without *p53* mutation ($p = 0.043$).

Discussion

In this study, we showed that *p16* promoter was methylated in HCC, irrespective of the presence or absence of viral infection. Previous studies have documented methylation of *p16* promoter in many kinds of cancers, including lung cancer, prostate cancer, osteosarcoma, and HCC. Recently, Roncalli et al. [16] reported that cell-cycle regulator genes are frequently inactivated by methylation in HCC. Methylation of *p16* has been detected by Matsuda et al. [14] (82.7%), Liew et al. [17] (62.5%), Kaneto et al. [18] (72.7%), and Roncalli et al. [16] (82%). None of these reports described the relation between hepatitis virus infection and methylation. We found that *p16* gene promoter was methylated in 77% of HCCs with hepatitis virus infection and 50% of HCCs without such infection. In this study, no significant difference in *p16* methylation was found between the HBV-positive group and HCV-positive group. Zhong et al. [19] suggested that GSTP1 inactivation by hypermethylation is associated with HBV-related HCC. Shen et al. [20] reported that environmental exposures might induce gene methylation in HCC. There were some reports that viral infection might induce DNA methylation. For example, in Epstein-Barr virus-infected cells, methylation of DNA was demonstrated and in adenovirus-infected tumor, host DNA was frequently methylated [21, 22]. We believe that methylation status of gene promoter may be influenced by etiology, including hepatitis viral infection. The relation between hepatitis virus infection and gene methylation thus requires further study.

Recent reports suggest that gene methylation has already occurred in precancerous lesions in many types of organs [23]. Roncalli et al. [16] reported that one of the cell-cycle-related genes was frequently inactivated by methylation in hepatic cirrhosis. Kaneto et al. [18] detected methylation of *p16* in liver cirrhosis and chronic hepatitis associated with HBV or HCV infection. This group of investigators found no gene methylation in primary biliary cirrhosis, autoimmune hepatitis, drug-induced liver disease, fatty liver, or normal liver tissue in patients whose livers were negative for hepatitis virus infection. They suspected that epigenetic alterations in liver might be related to hepatitis virus infections. In our study, *p16* methylation was also detected in noncancerous liver tissue with hepatitis virus infection but not in liver tissue without hepatitis virus infection. However, in the patients without viral infection, *p16* methylation was detected in 25% of moderately differentiated HCCs and 75% of poorly differentiated HCCs. These results suggest that methylation is an early event in hepatocarcinogenesis in patients with hepatitis virus infection and is related to less differentiation of HCC in patients without virus infection.

Mutation of *p53* is one of the most common molecular alterations in human cancers. It is well known that *p53* mutation is not an early event in hepatocarcinogenesis. In this study, the detection rate of *p53* mutations in HCC without hepatitis virus was similar to that in HCC with hepatitis virus. These results suggest that *p53* mutation may be a common pathway in hepatocarcinogenesis, both in hepatitis-virus-positive and hepatitis-virus-negative patients. In this study, the frequency of methylation of *p16* promoter was significantly higher in HCC without *p53* mutation than in HCC with such mutation. This result suggests that intragenic and epigenetic alterations occur independently in HCC and support the notion that different mechanisms can lead to hepatocarcinogenesis.

In conclusion, our results suggest that *p53* mutations occur in both HCC without hepatitis virus and HCC with hepatitis virus. Methylation of *p16* promoter may be related to less differentiation of HCC without hepatitis virus. Hepatocarcinogenesis may progress through different pathways, depending on the presence or absence of viral infection.

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APPLIED NUTRITIONAL INVESTIGATION

Problems in Serum Albumin Measurement and Clinical Significance of Albumin Microheterogeneity in Cirrhotics

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OBJECTIVES: To clarify problems with the determination of serum albumin levels, the definition of hypoalbuminemia, and the implications of microheterogeneity of albumin, serum albumin was measured by using dye-binding methods and the authentic method (immunoassay) in patients with liver cirrhosis and healthy subjects.

METHODS: We enrolled 103 patients with liver cirrhosis and 36 healthy subjects. Serum albumin levels were analyzed by immunoassay and the bromcresol green and bromcresol purple methods. Oxidized albumin and glycoalbumin were determined by high-performance liquid chromatography.

RESULTS: In cirrhotic patients, serum albumin levels measured by the bromcresol green method was about 0.2 g/dL higher than that by immunoassay. Serum albumin levels measured by the bromcresol purple method also was higher in cirrhotic patients than those measured by immunoassay and varied widely. In addition, extensive variation was found across serum albumin levels determined by the bromcresol green method at individual institutions (five university hospitals) and those determined by immunoassay at a contract laboratory. The percentages of oxidized albumin and glycoalbumin within total serum albumin increased with progression of liver disease. Further, an increase in oxidized albumin led to an increase in the albumin level as measured by the bromcresol purple method.

CONCLUSION: These results show that adequate assessment of the pathophysiology and prognosis of patients with liver cirrhosis and the efficacy of treatment is not possible with dye-binding methods for determination of serum albumin. Further, the conventional definition of hypoalbuminemia as a serum albumin level of 3.5 g/dL or lower should be reconsidered, and the clinical implications of qualitative changes in albumin should be investigated in consideration of the microheterogeneity of albumin, such as oxidized albumin and glycoalbumin. *Nutrition* 2004;20:351–357. ©Elsevier Inc. 2004

KEY WORDS: serum protein, liver disease, oxidized albumin, glycoalbumin, advanced glycation end products

INTRODUCTION

Over the years, serum albumin has been commonly measured as a laboratory test to identify malnutrition[1–3] and hepatic function disorder.[4] When branched-chain amino acid (BCAA) preparations are used to improve hypoalbuminemia in cirrhotic patients,[5–8] it is important to repeatedly measure the albumin level to detect slight changes. Thus, it is necessary to measure serum albumin levels more accurately and to establish a global standard for the determination of serum albumin with minimal differences between institutions.

Currently, serum albumin is determined in Japan by using bromcresol green (BCG), which is used at 91% of levels used at medical institutions (reference value, 3.8–5.3 g/dL), and bromcresol purple (BCP), which is used at 8% of levels used at medical institutions (reference value, 3.6–5.1 g/dL). However, a problem with these two methods is the large coefficient of variation across institutions (4.5–17.6%).[9]

Therefore, a serum albumin level in a patient diagnosed with hypoalbuminemia at one institution may be considered to be normal at another institution, resulting in delay of treatment to improve the malnutrition status. The classification system developed by Child and Trucotte[10] often is used to assess the severity of cirrhosis, in which serum albumin is included as one of five parameters (3.5 g/dL is used as a reference value for classification). In cirrhotic patients, a serum albumin level of 3.5 g/dL has been used for diagnosis of hypoalbuminemia. In addition, this albumin level often has been used for pathophysiologic evaluation and to determine when to start treatment. It has been reported that improvement of hypoalbuminemia by treatment with BCAA supplementation and the effect on the prognosis depend on when treatment is started and that the efficacy of BCAA decreases as the patient's albumin level decreases.[11]

In Child's classification, serum albumin is measured by a color reaction that is performed with 2-(4-hydroxyazobenzene)benzoic acid.[11] Because this method shows poor specificity for albumin,

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38 y ago the reference value of serum albumin was 4.6 to 6.7 g/dL, which is considerably higher than that used at present. Therefore, the reference value of 3.5 g/dL used 38 y ago is considered to have very different clinical implications from the serum albumin level of 3.5 g/dL obtained by using current, more specific methods. However, even today, the serum albumin level of 3.5 g/dL in Child's classification is being used worldwide regardless of the analytical method.

Albumin used to be considered a homogeneous component as compared with other serum proteins. However, recent advances in protein chemistry and molecular biology have shown that albumin has microheterogeneity because oxidized and reduced forms of albumin[12,13] and glycoalbumin[14] have been identified. The biological and clinical implications of these molecular species remain unclear. In the current clinical scene, physicians are interested only in whether the serum albumin level is high or low. In the future, in patients with various diseases showing quantitative changes in albumin level, qualitative analysis also should be performed to clarify the kinetics of molecular species and various functions of albumin and for diagnosis, pathophysiologic analysis, and disease management.

We performed a multicenter study in patients with liver cirrhosis to investigate differences in serum albumin levels as a function of the analytical method and the institution. We also investigated changes in the levels of oxidized albumin (non-mercaptalbumin), reduced albumin (mercaptalbumin), and glycoalbumin to clarify problems with the analytical methods of serum albumin and the implications of albumin microheterogeneity.

MATERIALS AND METHODS

Subjects

Subjects were selected from among patients who visited five institutions specializing in hepatic disease during the 3 mo from January 15 to March 31, 2000. Subjects included 103 patients with cirrhosis due to any cause (inpatients and outpatients), 75 y or younger, in whom the serum albumin level was 2.2 to 4.9 g/dL by the BCG method; control subjects were 36 healthy subjects in whom the serum albumin level was considered to be within the normal range (3.6–4.9 g/dL by immunoassay; Table I). Diagnosis of cirrhosis was based on the histologic findings of liver biopsy, except for patients who were diagnosed based on blood biochemistry or imaging of the liver. Cirrhosis attributable to hepatitis C was the most frequent (68.9%), followed by hepatitis B (9.7%). Patients with alcoholic cirrhosis comprised 11.6%. Child's classification showed 49.5% for Child A, 27.2% for Child B, and 14.6% for Child C. Hepatocellular carcinoma that was not considered to affect the pathophysiology of cirrhosis was observed in 43 patients (41.7%). Complications were defined as esophageal or gastric varices, ascites, jaundice, and hepatic encephalopathy. When at least two of these four conditions were present concurrently, the patient was documented as "with complications." Patients with diabetes mellitus were identified at the time of blood sampling by asking whether oral hypoglycemic agents or insulin were used. BCAA preparations (enteral nutrition or amino acid preparations) were being administered to 48 patients (46.6%), but only 12 patients (11.7%) were receiving human albumin fluids. Informed consent was obtained in writing or orally from all patients enrolled in this study.

Blood samples were collected from the cubital vein under fasting conditions early in the morning. Immediately after blood collection, sera were separated and divided into samples for in-house testing and for testing at SRL Medisearch Inc. (SRL; Tokyo, Japan; two 0.5-mL tubes and one 2.8-mL tube). Samples for in-house testing were tested immediately by the BCG method at each institution. The following BCG kits and reference standards were used: institution A—Cica Auto ALB (Kanto Kagaku Company, Tokyo, Japan), ANA Serum ALB-TP Standard Solution (bovine; Daiichi Pure Chemical Company, Ltd., Tokyo, Japan); institution B: ALB Reagent A, ALB Standard Solution (human; International Reagent Corp., Kobe, Japan); institution C: Clinimate, ANA Serum ALB-TP Standard Solution (bovine; Daiichi Pure Chemical Company); institution D: Wako Alb Kit, Protein Standard Serum (human; Wako Pure Chemical Industries, Ltd., Tokyo, Japan); and institution E: Clinimate, ANA Serum ALB-TP Standard Solution (bovine; Daiichi Pure Chemical Company). The reference (normal) ranges for serum albumin were 4.3 to 5.4 g/dL at institution A, 3.9 to 4.9 g/dL at institution B, 3.5 to 5.0 g/dL at institution C, 4.1 to 5.0 g/dL at institution D, and 4.2 to 5.3 g/dL at institution E (thus, the lower limit of the reference range measured by the BCG method varied widely from 3.5 to 4.3 g/dL). All serum samples for testing by SRL were transferred from the individual institutions to a laboratory in Tokyo and stored in a freezer at −80°C. After all serum samples were received (samples were stored in the freezer for 3 mo at the longest), SRL thawed the frozen samples and measured albumin levels as described below.

TABLE I.

BASELINE CHARACTERISTICS OF PATIENTS

	Healthy Controls	Patients with liver cirrhosis
Patients, *n*	36	103
Male/female	22/14	60/43
Age (y)*	55.4 ± 10.1	63.7 ± 8.8
Etiology		
HCV related		71
HBV related		10
Alcohol		12
Other/unknown		6/4
Child class		
A		51
B		28
C		15
Unknown		9
Hepatocellular carcinoma		43
None		60
Complications†		31
None		72
Diabetes		18
None		85
n Patients with serum creatinine levels >1.0 mg/dL/*n* patients determined‡		1/52

* Mean ± standard error.
† Patients with at least two of the following complications: esophageal or gastric varices, ascites, jaundice, and hepatic encephalopathy.
‡ Levels were measured 2 wk before and after albumin determination.
HBV, hepatitis B virus; HCV, hepatitis C virus

Methods

The methods for albumin determination used at SRL were immunoassay (nephelometric immunoassay: N Antiserum to Human Albumin and N Protein Standard SL, Dade Behring Marburg GmbH, Marburg, Germany; reference concentration, 3.9–4.9 g/dL),[15] which was the reference method, the BCG method (Wako Alb Kit and Protein Standard Serum, human; Wako Pure Chemical Industries, Ltd.; reference concentration, 4.0–5.0 g/dL),[16] and the

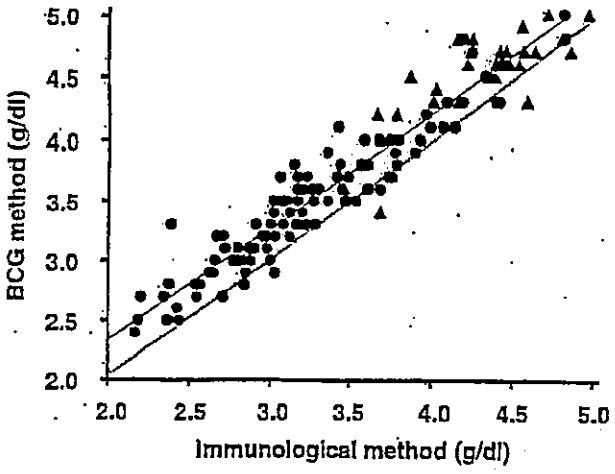

Fig. 1. Correlation of serum albumin levels measured by the BCG method at individual institutions and by immunoassay at the contract laboratory. Serum albumin levels as measured by the BCG method were higher in the measurement range than were those measured by immunoassay. A serum albumin level of 3.5 g/dL when measured by immunoassay corresponded to approximately 3.7 g/dL when measured by the BCG method. BCG, bromcresol green (3,3',5,5'-tetra-bromo-*m*-cresolsulfonphthalein); circles, patients with liver cirrhosis (n = 103); triangles, healthy controls (n = 36); solid line, regression equation: $y = 0.93x + 0.47$ ($R^2 = 0.925$); dotted line, assuming that the same measured value is obtained with both methods at a correlation coefficient of 1.0 (regression equation: $y = x$).

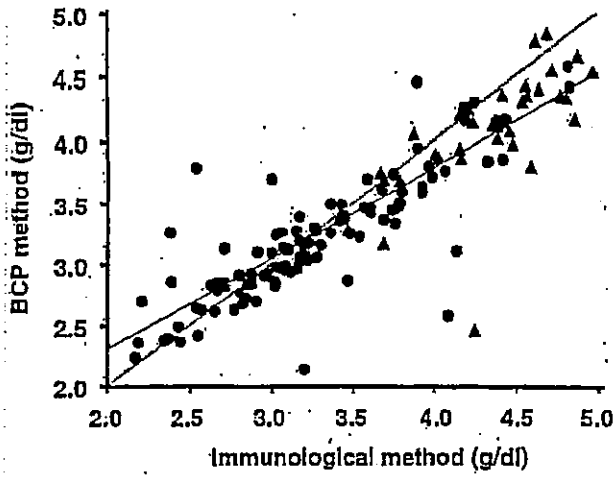

Fig. 2. Correlation of serum albumin levels measured by the BCP method at individual institutions and by immunoassay at the contract laboratory. In most patients with hypoalbuminemia, serum albumin levels as measured by the BCP method were higher than those measured by immunoassay. In normal control subjects in whom the serum albumin levels were within the normal range, serum albumin levels measured by the BCP method were lower than those measured by immunoassay. BCP, bromcresol purple (5,5'-dibromo-*o*-cresolsulfonphthalein); circles, patients with liver cirrhosis (n = 103); triangles, healthy controls (n = 36); solid line, regression equation: $y = 0.74x + 0.83$ ($R^2 = 0.667$); dotted line, assuming that the regression equation is $y = x$ at a correlation coefficient of 1.0. The regression lines with confidence intervals for healthy controls and cirrhotic patients are $y = 0.77x+0.71$ ($R^2 = 0.46$) and $y = 0.71x + 0.94$ ($R^2 = 0.52$), respectively.

BCP method, which is highly specific for albumin (Eiken EA Test ALB, TP-ALB Reference Standard for Autoanalyzer, and Eiken Standard Solution, human; Eiken Chemical Company, Ltd., Tokyo, Japan; reference concentration not specified).[17] Oxidized albumin and glycoalbumin were determined by high-performance liquid chromatography.[18,19] The percentage of each molecular species in the total serum albumin level measured by immunoassay was calculated as follows:

oxidized albumin percentage (%)

= oxidized albumin/(oxidized albumin + reduced albumin)

× 100

glycoalbumin percentage (%) = glycoalbumin/(glycoalbumin

+ non-glycoalbumin) × 100

The correlation of albumin levels between analytical methods and the correlation between the oxidized albumin percentage and the glycoalbumin percentage were tested by simple regression analysis by calculating a linear regression curve. Comparison of the mean values between two groups was performed with Student's t test, with $P = 0.05$ indicating statistical significance. Comparison of the mean values across three groups was performed by analysis of variance followed by Bonferroni's multiple test (Statview 4.54.0.0, 1996, Abacus Concepts, Inc., Berkeley, CA, USA), with $P = 0.05$ considered statistically significant.

RESULTS

Figure 1 shows the correlation between the serum albumin levels measured at the contract laboratory by immunoassay (the reference method for determination of serum albumin) and the BCG method (the most commonly used method in Japan). The serum albumin level measured by BCG ranged from 2.2 to 4.9 g/dL and tended to show higher values than the level measured by immunoassay. Figure 2 shows the correlation between serum albumin levels measured by immunoassay and the BCP method. In cirrhotic patients, the values yielded by the BCP method also were generally higher than the immunoassay values. In the control subjects, the BCP values were lower than the immunoassay values. The differences between the two methods were particularly larger in the cirrhotic patients. In general, values measured by the BCP method showed greater variation than those by the BCG method.

Using 48 serum samples (3 to 13 samples per institution) whose albumin levels measured by immunoassay ranged from 3.0 to 3.5 g/dL without significant differences in the mean values at the individual institutions, the difference between the BCG data at the individual institutions and the immunoassay data at the contract laboratory was calculated (Figure 3). At some institutions the mean value by the BCG method was higher (sites B, D, and E) or lower (sites A and C) than that by immunoassay. The coefficient of variation among institutions was 15.7%. This result suggests that a serum sample containing albumin at a level of 3.5 g/dL when measured by immunoassay may show a range of 3.3 to 3.9 g/dL when measured by the BCG method.

The possibility of a relation between serum albumin level and development of complications was investigated. Of 17 patients in whom the serum albumin level was higher than 3.8 g/dL when measured by immunoassay at the contract laboratory and by the BCG method at the individual institutions, nine patients (52.9%) had no complications. In contrast, of 83 patients in whom the serum albumin level measured by both methods was lower than 3.8 g/dL, 16 patients (19.3%) had no complications. There was a significant difference in the incidence of complications ($P < 0.01$; data not shown).

The percentage of oxidized albumin in the total serum albumin level measured by immunoassay was plotted against the serum albumin levels measured by immunoassay (Figure 4). The oxidized albumin percentage increased as the serum albumin level decreased ($P < 0.001$). Plotting of the oxidized albumin percentage versus the Child class (Figure 5) demonstrated that oxidized

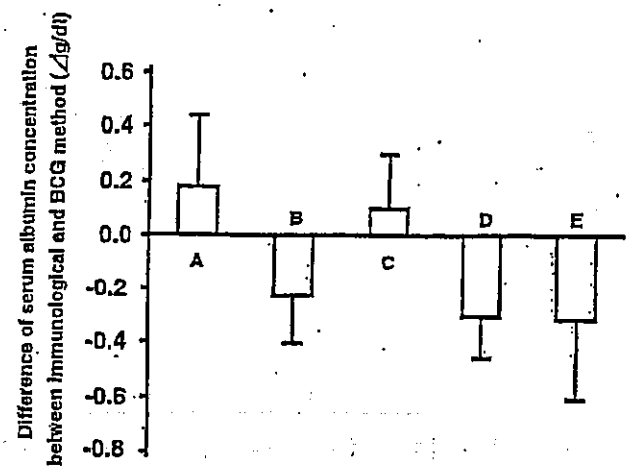

Fig. 3. Comparison of serum albumin levels in cirrhotic patients measured by the BCG method at five institutions. The serum samples were 48 samples whose serum albumin levels were 3.0 to 3.5 g/dL when measured by immunoassay at the contract laboratory. There were no significant differences in mean values across institutions. Differences (Δ g/dL) between values measured by the BCG method at individual institutions and those measured by immunoassay at the contract laboratory were calculated, and the mean ± standard deviation was plotted ($P < 0.05$). Site A ($n = 8$) versus site B ($n = 11$); site B versus site C ($n = 13$); site A versus site D ($n = 13$); site A versus site E ($n = 3$); site C ($n = 13$) versus site D ($n = 13$); site C versus site E ($n = 3$). At some institutions, mean serum albumin levels by the BCG method were 0.1 to 0.2 g/dL higher or 0.2 to 0.4 g/dL lower than those measured by immunoassay. BCG, bromcresol green (3,3',5,5'-tetra-bromo-*m*-cresolsulfonphthalein).

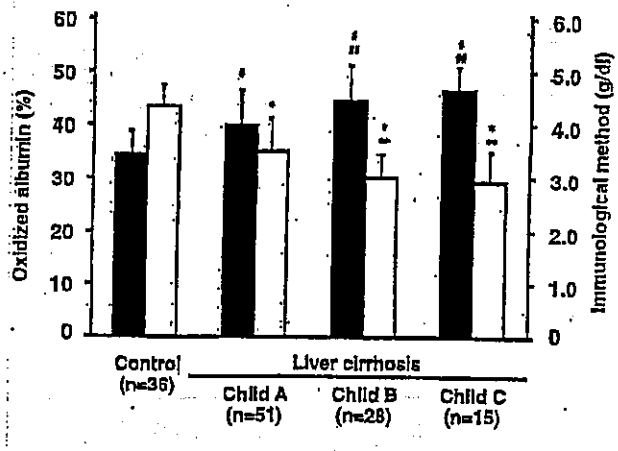

Fig. 5. Oxidized albumin percentage (solid bars) and serum albumin level (open bars) measured by immunoassay and classified by severity (Child's classification) in 94 cirrhotic patients. The oxidized albumin percentage by Child class increased with progression of the disease. Vertical bars show the standard deviation. Oxidized albumin percentage: control versus liver cirrhosis (Child A, B, and C), $\#P < 0.05$; Child A versus Child B and C, $\#\#P < 0.05$. Albumin level: control versus liver cirrhosis (Child A, Bs and C), $*P < 0.05$; Child A versus Child B and C, $**P < 0.05$.

albumin percentage increases with a decrease in total albumin concentration (Child A versus B, $P < 0.001$; Child A versus C, $P < 0.001$).

Patients were assigned to one of two groups: one group in which the difference in the measured serum albumin level between the BCP method and immunoassay was 0.3 g/dL or higher and another group in which it was lower than 0.3 g/dL. The oxidized albumin percentage was higher in the group with 0.3 g/dL or higher (data not shown). This suggested that albumin determination by the BCP method is affected by the oxidized albumin percentage. In cirrhotic patients, the reduced albumin percentage was more markedly decreased than the oxidized albumin percentage (data not shown). This is one reason the oxidized albumin percentage was increased.

Figure 6 shows the relation between the serum albumin level measured by immunoassay and the glycoalbumin percentage in 77 patients with liver cirrhosis but without diabetes, and Figure 7 shows the glycoalbumin percentage by Child class. Like the oxidized albumin percentage, the glycoalbumin percentage increased with a decrease in the serum albumin level measured by immunoassay ($P < 0.001$) and increased with the severity of the disease (Child A versus B, $P < 0.001$; Child A versus C, $P < 0.001$). In cirrhotic patients with diabetes, the mean glycoalbumin percentage

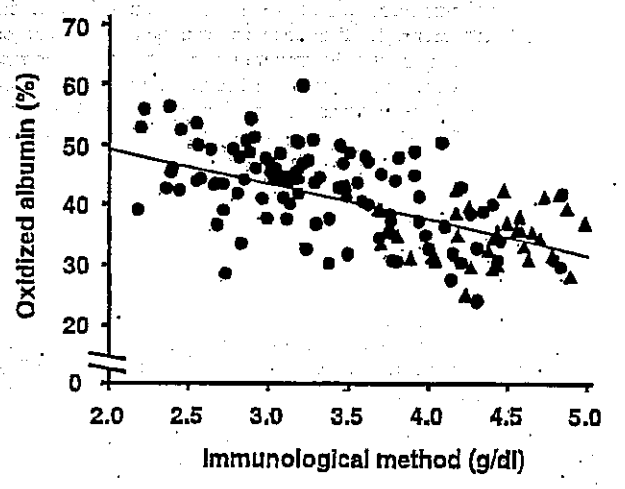

Fig. 4. Correlation between serum albumin levels measured by immunoassay and the oxidized albumin percentage method. In cirrhotic patients (circles; $n = 103$) and healthy controls (triangles; $n = 36$), the oxidized albumin percentage increased with a decrease in serum albumin level. Solid line, regression equation: $y = -0.06x + 0.61$ ($R^2 = 0.380$).

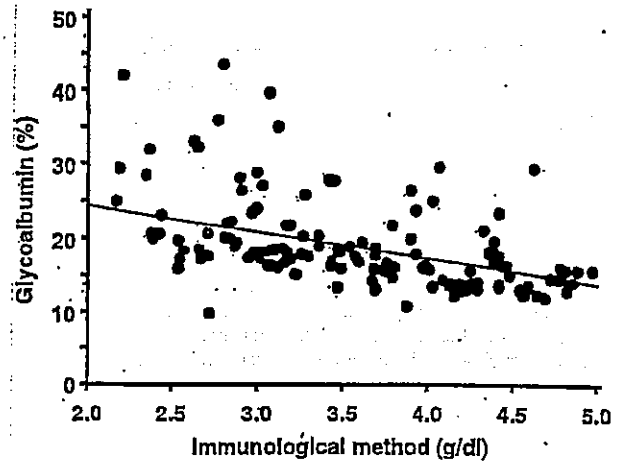

Fig. 6. Correlation between serum albumin level measured by immunoassay and the glycoalbumin percentage. In 78 cirrhotic patients without diabetes (solid circles), the glycoalbumin percentage increased as the serum albumin level decreased. Solid line, regression equation: $y = -3.56x + 31.52$ ($R^2 = 0.201$).

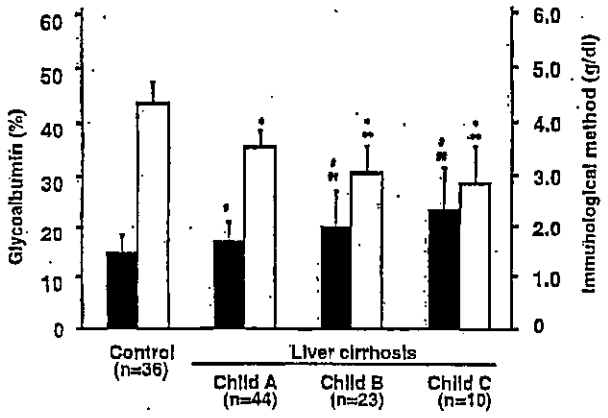

Fig. 7. Glycoalbumin percentage (solid bars) and serum albumin level (open bars) measured by immunoassay and classified by severity (Child's classification) in 78 cirrhotic patients without diabetes. In these patients, the glycoalbumin percentage by Child class increased with progression of the disease. Vertical bars show the standard deviation. Glycoalbumin percentage: control versus liver cirrhosis (Child A, B, and C), $\#P < 0.05$; Child A versus Child B and C, $\#\#P < 0.05$. Albumin level (see Figure 5 for data from control subjects): control versus liver cirrhosis (Child A, B, and C), $*P < 0.05$; Child A versus Child B and C, $**P < 0.05$.

was 27.1%, resulting in a markedly increased percentage independent of the Child class (data not shown).

In cirrhotic patients without diabetes, the oxidized albumin percentage demonstrated a correlation with the glycoalbumin percentage, as shown in Figure 8.

DISCUSSION

The BCP method was first reported by Pinnell et al. in 1978[20]; at that time, it attracted much interest as a good method highly specific for albumin. Subsequently, however, it was pointed out that the BCP method has various disadvantages,[21,22] such as negative error due to δ-bilirubin,[23] reaction with serum in patients undergoing hemodialysis[24] and a difference in reaction between reduced albumin and oxidized albumin.[25] Our study also showed that some serum albumin levels measured by the BCP method differ markedly from the levels detected by immunoassay and that this difference increased as the oxidized albumin ratio increased. Therefore, the BCP method is not ideal as a method for determination of serum albumin in cirrhotic patients. To solve these problems, Muramoto et al.[26] endeavored to eliminate the difference in reaction between reduced albumin and oxidized albumin by pretreating serum samples. As a result, a modified BCP method using an autoanalyzer (Akua-auto Kainos Albumin Test Kit, Kainos Laboratories, Inc., Tokyo, Japan; lipid-free human serum is used as the reference standard, with CRM470 as the primary standard) is now available.[27] Thus, this modified BCP method (CRM 470) will be more suitable in the future for determination of serum albumin in clinical situations because the method is simple, inexpensive, and reliable.

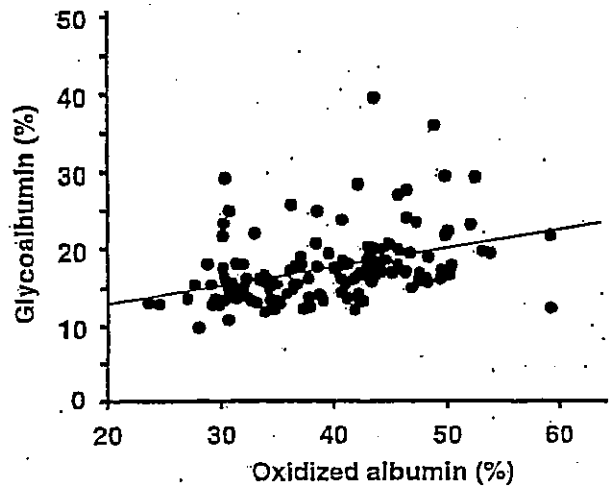

Fig. 8. Correlation between the glycoalbumin percentage and the oxidized albumin percentage in 78 cirrhotic patients without diabetes (solid circles). The glycoalbumin percentage correlated with the oxidized albumin percentage in cirrhotic patients without diabetes. Solid line, regression equation: $y = 23.65x + 8.32$ ($R^2 = 0.13$).

The serum albumin level decreases by 0.1 to 0.2 g/dL annually in most cirrhotic serum. Treatment with BCAA preparations can increase the albumin level by 0.1 to 0.2 g/dL.[28] It is expected that determination of serum albumin will be performed more frequently to assess the prognosis of cirrhotic patients, to decide the timing of liver transplantation or the start of treatment for hypoalbuminemia, and to assess the therapeutic efficacy. Therefore, improvement of methods for albumin determination is strongly desired to minimize variation in the measured values among institutions and to ensure more accurate determination of the albumin level as an important index for deciding when to start treatment, assessing the efficacy of treatment, and predicting the prognosis.

Patients with liver cirrhosis were stratified by serum albumin level in increments of 0.1 g/dL over the range of 2.9 to 4.1 g/dL. There was a significant difference in the survival rate between any two adjacent classes.[29] Because no deaths were observed at serum albumin levels of 3.8 g/dL or higher, this is likely an important threshold. In this study, cirrhotic patients in whom the serum albumin level measured by the BCG method was 3.8 g/dL or higher had complications at a lower frequency than did patients with a serum albumin level below 3.8 g/dL. It is unclear whether a serum albumin level of 3.8 g/dL by the BCG method corresponds to a value of 3.5 g/dL measured around 1964 when Child's classification was developed. However, it is currently appropriate to consider cirrhotic patients with a serum albumin level of 3.8 g/dL as hypoalbuminemic. When the method for serum albumin determination is standardized in the future using the international protein standard CRM 470 supplied by the International Federation of Clinical Chemistry, the definition of hypoalbuminemia should be reconsidered.

The prognosis of cirrhotic patients depends on the serum albumin level.[30] The clinical implications of the serum albumin level have been investigated by many researchers. However, there have been only a few studies on the properties of albumin microheterogeneity on a molecular basis. In cirrhotic patients, the percentage of oxidized albumin increases with a decrease in total albumin concentration, independently of concurrence of diabetes. Further, we found that the increase in the oxidized albumin percentage may cause an increase in the serum albumin level when measured by the BCP method. The absolute levels of oxidized albumin and reduced albumin may decrease with the progression of liver disease. However, the oxidized albumin percentage is thought to increase because the degree of the decrease in the oxidized albumin level is less than that of reduced albumin. In reduced albumin, the cysteine residue at the 34th position from the N-terminal is preserved. In oxidized albumin, this cysteine residue is oxidized or forms a reactive S-S bond with a sulfurous amino acid (−SOH, $-SO_2H$, and $-SO_3H$ are also formed). Because this S-S bond is formed by a reversible reaction within a short time,[31] both molecular species are in dynamic equilibrium in the blood. Accordingly,