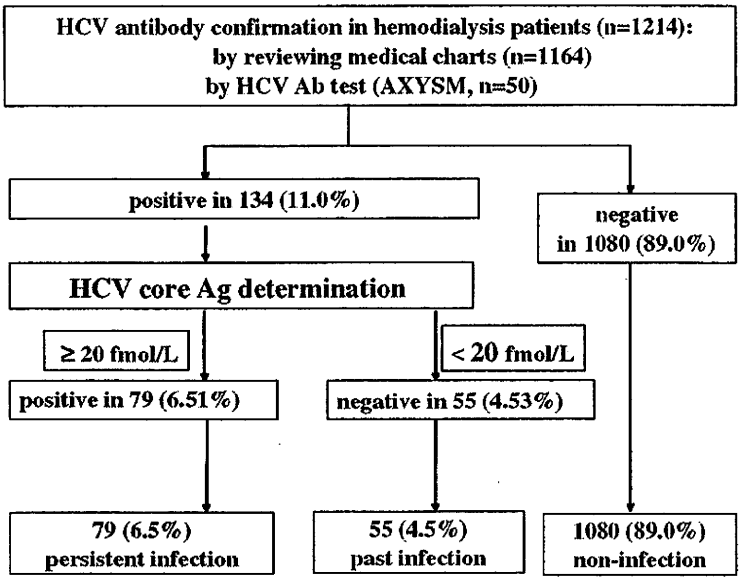

Figure 3. Flow chart of HCV antibody and HCV core antigen screening in hemodialysis patients in the KAREN Study. Information on HCV serology tests was not collected from 50 subjects in the KAREN Study. For those 50 subjects, we defrosted frozen serum samples and performed HCV antibody tests using Architect HCV (Abbott, Japan). A total of 134 subjects (11.0%) were positive for HCV antibody. HCV core antigen tests were then performed for those subjects. A total of 79 were positive for HCV core antigen and were classified with persistent HCV infection (6.0%).

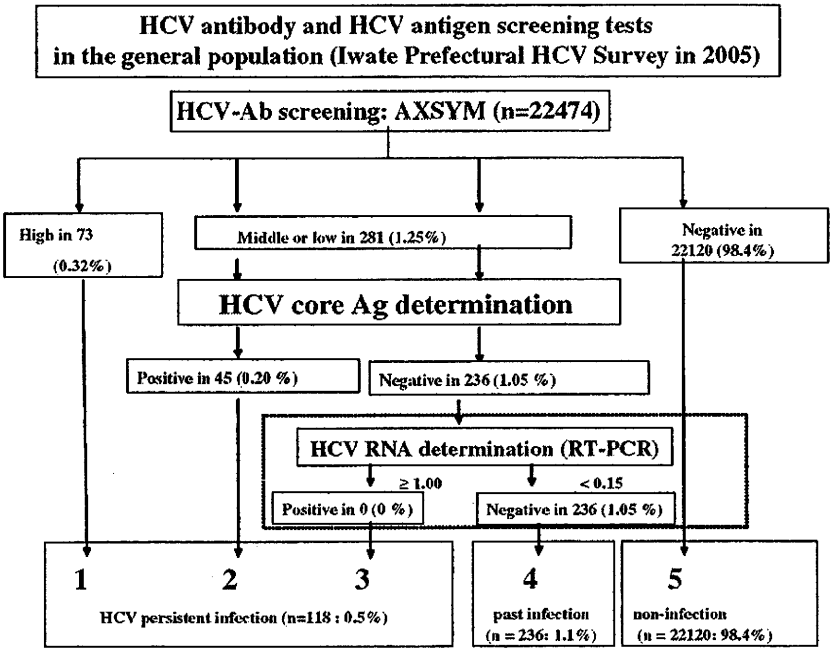

Figure 4. Flow chart of HCV antibody and HCV core antigen screening in population-based controls (Iwate Prefectural HCV survey in 2005).
There were 22474 participants who underwent annual health check-ups and HCV screening. A total of 354 subjects were positive for HCV antibody (1.57%). HCV core antigen tests were performed in subjects with low- or middle-range positivity for HCV antibody. A total of 45 were positive for HCV core antigen. HCV-RNA determination using the RT-PCR method was performed in 236 subjects, but none were positive. Ultimately, 118 subjects were classified with persistent HCV infection (0.53%).

Hemodialysis patients were also divided into 6 groups according to dialysis "vintage" (length of time on dialysis): <6 months, 6 to 23 months, 2 to 4 years, 5 to 9 years, 10 to 14 years, or 15 years or longer. Prevalences of anti-HCV antibody and HCV core antigen in each group were estimated. Differences in prevalences by sex or dialysis vintage (vintage ≥10 years vs <10 years) were tested using the chi-square test. To examine whether each risk factor was

Table 1. Sex- and age-specific prevalences of anti-HCV antibody in hemodialysis patients and a general population

Age group	General population		HD patients	
	Total No.	HCV Ab-positive (%)	Total No.	HCV Ab-positive (%)
Men				
20–39	36	0 (0.0%)	52	4 (7.7%)
40–49	890	16 (1.8%)	96	13 (13.5%)
50–59	1564	14 (0.9%)	191	38 (19.9%)
60–69	3001	43 (1.4%)	233	27 (11.6%)
≥70	2159	50 (2.3%)	207	15 (7.2%)
total	7650	123 (1.6%)	779	97 (12.5%)
Women				
20–39	62	0 (0.0%)	22	0 (0.0%)
40–49	2662	22 (0.8%)	55	5 (9.1%)
50–59	3980	40 (1.0%)	121	5 (4.1%)
60–69	4927	87 (1.8%)	116	1 (0.9%)
≥70	3193	82 (2.6%)	121	11 (9.1%)
total	14824	231 (1.6%)	435	37 (8.5%)

Abbreviations: HCV, hepatitis C virus; HD, hemodialysis; No., number; Ab, antibody.

Table 2. Sex- and age-specific prevalences of HCV core antigen in hemodialysis patients and normal controls

Age group	General population		HD patients	
	Total No.	HCV core Ag-positive (%)	Total No.	HCV core Ag-positive (%)
Men				
20–39	36	0 (0.0%)	52	3 (5.8%)
40–49	890	8 (0.9%)	96	8 (8.3%)
50–59	1564	5 (0.3%)	191	32 (16.8%)
60–69	3001	16 (0.5%)	233	12 (5.2%)
≥70	2159	21 (1.0%)	207	6 (2.9%)
total	7650	50 (0.7%)	779	61 (7.8%)
Women				
20–39	62	0 (0.0%)	22	0 (0.0%)
40–49	2662	5 (0.2%)	55	2 (3.6%)
50–59	3980	5 (0.1%)	121	5 (4.1%)
60–69	4927	28 (0.6%)	116	4 (3.4%)
≥70	3193	30 (0.9%)	121	7 (5.8%)
total	14824	68 (0.5%)	435	18 (4.1%)

Abbreviations: HCV, hepatitis C virus; HD, hemodialysis; No., number; Ag, antigen.

independently associated with chronic HCV infection or past HCV infection, logistic regression analysis was performed using presence of chronic HCV infection or history of HCV infection as the dependent variable and age, sex, and dialysis vintage as explanatory variables. A P value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS software package (SPSS, Japan Inc., Version 14.0).

RESULTS

Table 1 shows sex- and age-specific prevalences of anti-HCV antibody in hemodialysis patients and population-based controls. Among population-based controls, the prevalence of anti-HCV antibody increased with advancing age; however, no such association was observed among hemodialysis patients. A sex difference in the prevalence of anti-HCV antibody was not found in the population-based controls; however, among the hemodialysis patients, the prevalence of anti-HCV antibody was higher in men than in women (12.5% vs 8.5%, $P < 0.05$).

The prevalence of anti-HCV antibody was considerably higher in hemodialysis patients than in controls. The SPR (95% CI) for anti-HCV antibody was 8.39 (6.72–10.1) in male hemodialysis patients and 5.42 (3.67–7.17) in female hemodialysis patients.

Table 2 shows sex- and age-specific prevalences of HCV core antigen in hemodialysis patients and population-based controls. A positive association between the prevalence of HCV core antigen and age was found in controls but not in hemodialysis patients. The prevalence of HCV core antigen was also higher in male hemodialysis patients than in female hemodialysis patients (7.8% vs 4.1%, $P < 0.05$). The SPR (95% CI) for HCV core antigen was 12.9 (9.66–16.1) in male hemodialysis patients and 8.77 (4.72–12.8) in female hemodialysis patients.

Table 3. Prevalences of anti-HCV antibody and HCV core antigen among hemodialysis patients, stratified by hemodialysis vintage

HD vintage	No.	HCV Ab-positive (%)	HCV core Ag-positive (%)
Men			
<6 months	44	4 (9.1%)	3 (6.8%)
6–23 months	158	14 (8.9%)	8 (5.1%)
2–4 yrs	218	18 (8.3%)	10 (4.6%)
5–9 yrs	176	15 (8.5%)	7 (4.0%)
10–14 yrs	75	10 (13.3%)	8 (10.7%)
≥15 yrs	108	36 (33.3%)	25 (23.1%)
total	779	97 (12.5%)	61 (4.6%)
Women			
<6 months	18	1 (5.6%)	1 (5.6%)
6–23 months	74	4 (5.4%)	3 (4.1%)
2–4 yrs	129	8 (6.2%)	4 (3.1%)
5–9 yrs	109	8 (7.3%)	4 (3.7%)
10–14 yrs	49	3 (6.1%)	3 (6.1%)
≥15 yrs	56	13 (23.2%)	3 (5.4%)
total	435	37 (8.5%)	18 (4.1%)

Abbreviations: HCV, hepatitis C virus; HD, hemodialysis; No., number; Ab, antibody; Ag, antigen.

Table 3 shows prevalences of anti-HCV antibody and HCV core antigen by dialysis vintage. Male and female patients with longer hemodialysis vintages (10–14 years or ≥15 years) had high prevalences of anti-HCV antibody than did male and female patients with a dialysis vintage less than 10 years ($P < 0.05$). Male and female patients with a dialysis vintage of 15 years or more had extremely high prevalences of anti-HCV antibody. However, among the dialysis vintage subgroups,

Table 4. Odds ratios for each risk factor for past or chronic HCV infection

Risk factor	Chronic HCV infection			Past HCV infection		
	OR	95%CI	*P*	OR	95%CI	*P*
Age (per 1 year increase)	0.99	(0.97–1.01)	0.484	1.02	(0.99–1.05)	0.107
Male sex	1.99	(1.14–3.44)	0.014	1.06	(0.60–1.89)	0.843
Dialysis vintage (per 1 year increase)	1.09	(1.06–1.12)	<0.001	1.09	(1.06–1.13)	0.006

Odds ratios and their 95% confidence intervals were estimated by logistic regression analysis.
Abbreviations: OR, odds ratio; CI, confidence interval.

Table 5. Prevalences of anti-HCV antibody and HCV core antigen (or RNA) among hemodialysis patients from various countries

Country	Author or name of study	Sample size	HCV Ab-positive (%)	Positive for HCV Ag or RNA (%)	Years tested
Japan	Washio[15]	540	24.3	—	1990
	Nakayama[24]	1470	18.8	—	1993
	DOPPS[8]	not obtained	19.9	—	1997–2001
	Kumagai[6]	1882	—	12.9[a]	1999–2003
	KAREN	1214	11.0	6.5[b]	2003–2004
United States	DOPPS[8]	not obtained	14.4	—	1997–2001
	Da Vita[14]	13664	11.6	—	2001–2004
Belgium	Jadoul[13]	629	6.8	—	2000
France	DOPPS[8]	not obtained	14.7	—	1997–2001
Germany	Hinrichsen[9]	2796	7.0	—	1996–1997
United Kingdom	DOPPS[8]	not obtained	2.7	—	1997–2001
Italy	DOPPS[8]	not obtained	22.2	—	1997–2001
Iran	Shamshirsaz[10]	593	—	8.6[a]	2004[c]
Tunisia	Hmaied[11]	395	20	14[a]	2001–2003
Thailand	Luengrojanakul[12]	221	—	19.9[a]	1994

Abbreviations are the same as those used in Tables 1, 2, and 3. Italics indicate the present study.
Superscript numbers correspond to the reference used in the present study.
[a], determined by HCV-RNA test by the PCR method; [b], determined by HCV core antigen test.
[c], Not clearly described when blood sampling was performed (published in 2004).

male patients with a dialysis vintage of 15 years or more had the highest prevalence of HCV core antigen.

Both male and female patients in the 4 groups with the shortest dialysis vintage (ie, <10 years) had similar prevalences of HCV antibody, regardless of dialysis vintage (approximately 9% in male hemodialysis patients and 5% in female hemodialysis patients in each of the 4 groups).

Table 4 shows the odds ratios attributable to each factor for having chronic HCV infection or past HCV infection. Male sex and dialysis vintage were independently associated with a higher prevalence of chronic HCV infection. The prevalence of chronic HCV infection among male hemodialysis patients was double that of female patients. However, only hemodialysis vintage was independently associated with an increased prevalence of past HCV infection.

DISCUSSION

In this study, we analyzed the prevalences of HCV antibody and HCV core antigen in adult hemodialysis patients. We estimated SPRs for both anti-HCV antibody and HCV core antigen among hemodialysis patients, and compared these estimates to those of the general population living in the same area.

Patients who are positive for HCV core antigen all have chronic HCV infection, whereas patients with anti-HCV antibody include those who have recovered from HCV infection, as well as those with chronic HCV infection. In a general population, patients who have recovered from HCV infection never develop liver cirrhosis or hepatocellular carcinoma (HCC) due to HCV, whereas patients with chronic HCV infection will develop liver cirrhosis or HCC 20 to 30 years after initial infection.[29] Therefore, in a general population, information regarding chronic HCV infection is more important than information on anti-HCV antibody.

In their study of Tunisian hemodialysis patients, Bouzgarrou et al reported that an HCV core antigen assay based on the HCV-RNA test had high sensitivity and high specificity; however, they were unable to provide an accurate estimate of the prevalence of chronic HCV infection and past HCV infection because of the large number of missing cases.[30]

Table 5 shows prevalences of anti-HCV antibody and chronic HCV infection (positivity for HCV core antigen or HCV RNA) in several studies with large sample sizes.[6,8–15,24] Hmaied reported the prevalences of both anti-HCV antibody and HCV-RNA.[11] The proportion of patients with HCV-RNA

among patients with anti-HCV antibody was 70% in their study, and this proportion is similar to that of patients with HCV core antigen among patients with anti-HCV antibody in our study; it is also similar to the proportion of patients with chronic infection among all patients with HCV infection in the general population.[31]

We determined the prevalences of anti-HCV antibody and HCV core antigen in hemodialysis patients who were divided into 6 groups according to hemodialysis vintage. Patients with a hemodialysis vintage of 10 years or more had significantly higher prevalences of anti-HCV antibody and HCV core antigen than did patients with shorter hemodialysis vintages. Furthermore, patients with a hemodialysis vintage of 15 years or more had significantly higher prevalences of anti-HCV antibody than did other groups.

Since 1981, the Japanese Red Cross Blood Transfusion Service has excluded blood samples from donors with high serum ALT levels (≥36 KU/mL) in order to prevent transfusion of blood with non-A non-B hepatitis virus. Erythropoietin has been used clinically for treatment of anemia since 1986. In 1989, the Japanese Red Cross Blood Transfusion Service began using a first generation assay to screen blood donors for anti-HCV antibody.[32] The timing of the introduction of these programs explains the relatively low prevalence of HCV infection among patients with a dialysis vintage less than 10 years and the extremely high prevalence of HCV infection among patients with a dialysis vintage of 15 years or more.

Choo and Kuo first developed a specific assay for HCV in 1989,[33,34] and a second-generation ELISA, which was more sensitive than the first-generation ELISA, was developed in 1992 and became widely used as a clinical diagnostic tool and for epidemiological and other investigative purposes. As a result, the risk of nosocomial HCV infection has dramatically decreased among hemodialysis patients who started hemodialysis treatment after 1992. Our results showing a high prevalence of HCV infection among patients with a hemodialysis vintage of 10 years or more are consistent with the fact that risks for HCV infection have been reduced by the development and widespread use of HCV assays.

However, as compared to the general population, patients with a hemodialysis vintage of less than 10 years had a significantly higher prevalence of HCV infection, even though they would be expected to be at low risk of HCV infection due to blood transfusion and dialysis. This cross-sectional analysis also showed that prevalences were similar among the groups of patients with a dialysis vintage less than 10 years (ie, <6 months, 6–23 months, 2–4 years, 5–9 years), which suggests that most hemodialysis patients with HCV infection became infected before initiation of hemodialysis treatment, and that only a few patients with HCV infection developed the infection after initiation of hemodialysis treatment.

The incidence rate of HCV infection among hemodialysis patients is reported to be lower than 0.5 percent per year,[6,35] indicating that the very high prevalence of HCV infection among hemodialysis patients is not entirely due to the elevated risk of nosocomial infection associated with dialysis therapy. There are several possible pathways for HCV transmission before initiation of hemodialysis. Patients with renal failure may have a high prevalence of HCV infection, regardless of the severity of renal failure, or, alternatively, patients with HCV infection may have a high prevalence of renal failure. It has been shown that HCV is associated with an increased prevalence of renal insufficiency.[36] Renal diseases associated with HCV infection may also contribute to the high prevalence of HCV infection among patients with kidney disease.[37]

Another possible explanation is that patients with mild-to-moderate renal failure (ie, patients with chronic kidney disease) tend to develop ESRD after HCV infection, which may contribute to the high prevalence of HCV among patients with ESRD. Two studies have shown that HCV infection contributed to an increased risk of developing ESRD.[38,39] If HCV infection does indeed contribute greatly to the development of ESRD, better prevention and treatment strategies for HCV infection should not only decrease liver disease-related mortality, they should also decrease the development of ESRD and its related mortality in patients with CKD and in the general population.

Although there was no sex-based difference in the prevalence of HCV infection in the general population, the prevalences of anti-HCV antibody and HCV core antigen were higher in male hemodialysis patients than in female hemodialysis patients. This suggests that male hemodialysis patients are at greater risk for HCV infection, perhaps due to the presence of predisposing factors for HCV infection.

Male hemodialysis patients with a long hemodialysis vintage (≥10 years) had a high rate of chronic HCV infection (70%: the percentage of patients who were positive for HCV core antigen among those were positive for anti-HCV antibody); however, female patients with a similarly long hemodialysis vintage had a lower rate of chronic HCV infection (37.5%). Male sex was independently associated with a high prevalence of HCV core antigen in logistic regression analysis. These data suggest that male hemodialysis patients have a greater risk of HCV infection, and a greater risk of persistent HCV infection, than do female hemodialysis patients.

Thomas et al reported that the spontaneous clearance rate of HCV among female patients was 1.58 times that of male subjects; however, the finding was of only marginal statistical significance.[40] Women are less likely to be regular alcohol drinkers.[27,31] In addition, they have higher levels of serum HDL cholesterol[27,41] and perhaps other unknown protective factors. This may attenuate their risks of initial and chronic HCV infection, and may explain the observed sex-based differences.

Another possible explanation is that women who had recovered from HCV were selectively registered in the study

because of a very high mortality rate for women with chronic HCV infection. However, to our knowledge, no studies have shown that female patients with chronic HCV infection have a higher mortality rate than that of patients who have recovered from HCV infection.

One major feature of this study is the long dialysis vintage of the participants. Mean dialysis vintage of the study participants exceeded 7 years; mean dialysis vintage was only approximately 3 years in reports from the United States and Europe.[42] The generous medical insurance reimbursement system for Japanese dialysis patients and the high quality of hemodialysis treatment, which includes legal controls that strictly restrict re-use of a dialyzer, may have contributed to the longevity of hemodialysis patients. More than 20% of patients in the present study had long dialysis vintage (≥10 years), and long dialysis vintage was associated with a high prevalence of HCV infection in our study.

Since hemodialysis patients have a short life expectancy, there are few cases in which liver cirrhosis or HCC develops long after initiation of hemodialysis. Nakayama and Fabrizi found that hemodialysis patients who were anti-HCV antibody-positive had higher rates of liver disease-related deaths.[24,26] However, the authors did not reveal whether an elevated mortality rate among hemodialysis patients with anti-HCV antibody was totally attributable to the increase in liver disease-related deaths. It is necessary to determine which cause of death contributes to the increase in mortality among hemodialysis patients with HCV infection.

This study was based on data from a population-based study and the sample size was sufficient to satisfy our objectives. Indeed, the large sample size of population-based controls living in the same area is one of the strengths of the study. However, several limitations to our study should be noted. The cross-sectional design of the present study cannot prove causal relationships. In addition, the lack of HCV-RNA data on the hemodialysis subjects who were positive for HCV antibody and negative for HCV core antigen is a major limitation in our study. It is possible that hemodialysis patients who are negative for HCV core antigen nevertheless have very low levels of HCV-RNA; however, the possibility of missing such cases in the present study is very low because, among the population-based controls, none were simultaneously positive for both HCV-RNA and HCV antibody and negative for HCV core antigen (Figure 4). Therefore, we believe that the results of the study were not distorted by lack of data regarding HCV-RNA. A history of blood transfusion is a strong predisposing factor for HCV infection. Thus, lack of information about past history of blood transfusion is also a major limitation. In addition, people who did not participate in the annual health check-ups may have been in poor health and might have had liver disease. This would have resulted in an underestimation of HCV infection in the general population and overestimation of the SPR for HCV among hemodialysis patients.

In conclusion, the prevalences of chronic HCV infection in male and female hemodialysis patients are 13 times and 9 times those of men and women in the general population. Further studies should therefore be carried out to determine the extent of chronic HCV infection in hemodialysis patients in other populations and to determine whether chronic HCV infection contributes to increased mortality in hemodialysis patients.

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The KAREN Study Group

Chairman: Tomoaki Fujioka.

Principal Investigators: Karen Kato, Masaki Ohsawa, Ryuichiro Konda, Kazuyoshi Itai, Kozo Tanno, Yosuke Fujishima, Akira Okayama, Toshiyuki Onoda, Kiyomi Sakata, Motoyuki Nakamura.

Research Associates: Yahaba Clinic: Mikihiko Fujishima, Yuko Nakamura, Toshiko Kumagai; San-ai Hospital: Toshihiro Yamauchi, Koji Seino, Masataka Nasu, Tayo Kambayashi; Yuai Hospital: Shigeru Nagasawa, Akira Suzuki; Yamada Clinic: Ikuo Yamada; Isurugi Clinic: Takashi Isurugi; Morioka Red Cross Hospital: Susumu Terasato, Michihiko Hasegawa; Ohinata Clinic: Mitsuru Ohinata; Numakunai Clinic: Jun-ichi Matsuzaka; Ninohe Clinic: Hikaru Aoki; Iwate Prefectural Ichinohe Hospital: Kou Takada, Tadao Toda; Iwate Prefectural Kuji Hospital: Tadashi Abe, Takuji Kaneko, So Ohmori; Taneichi Hospital: Kiyoshi Urushikubo, Tamako Kasatsuki; Obara Clinic: Noriaki Obara, Nobuko Miyakawa, Masako Takahashi; Hoyo Hospital: Takao Ishihara, Satsu Utsunomiya, Toshihiro Sato; Iwate Prefectural Kitakami Hospital: Katsuya Goto, Tsuneo Kajikawa; Kitakami Saiseikai Hospital: Kazuyoshi Saito, Kaoru Suzuki; Hidakami Chuo Clinic: Shigetoshi Kanazawa; Kitakami Jin Clinic: Hiroyuki Koike; Iwate Prefectural Miyako Hospital: Sen-ichi Kanno, Hiromitsu Fujisawa; Goto Urologic Clinic: Yasufumi Goto, Yasuki Goto; Goto Clinic: Takashi Goto; Iwaizumi Saiseikai Hospital: Yoshihiro Shibano.

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□ ORIGINAL ARTICLE □

Combination Therapy of Peginterferon and Ribavirin for Chronic Hepatitis C Patients with Genotype 1b and Low-virus Load

Yasuji Arase[1], Fumitaka Suzuki[1], Norio Akuta[1], Hitomi Sezaki[1], Yoshiyuki Suzuki[1], Yusuke Kawamura[1], Masahiro Kobayashi[1], Tetsuya Hosaka[1], Hiromi Yatsuji[1], Miharu Hirakawa[1], Satoshi Saito[1], Kenji Ikeda[1], Mariko Kobayashi[2] and Hiromitsu Kumada[1]

Abstract

Objective The aim of this study was to evaluate the efficacy of combination therapy of peginterferon and ribavirin in patients infected with hepatitis C virus (HCV) genotype 1b and low virus load.

Methods Inclusion criteria were HCV-genotype 1b, serum HCV RNA level of <100 KIU/mL at the initiation time of treatment. A total of 60 were enrolled in this retrospective cohort study. The treatment period of combination therapy was 39.8±16.1 weeks.

Results Of the 60 study patients, 47 had sustained virological response (SVR) by the intention to treat analysis. SVR occurred when serum HCV RNA was negative 8 weeks after the initiation of the treatment (p=0.004) and continuance of negative HCV RNA during treatment was ≥30 week (p=0.016). In rapid virological response, all of seven patients with continuance of negative HCV RNA 20 to 29 weeks during treatment had SVR. In early virological response nine of 10 patients with continuance of negative HCV RNA of 30 to 39 week during treatment had SVR.

Conclusion The duration of combination therapy for chronic hepatitis C should be determined based on the time of attainment of negative HCV RNA in patients with genotype 1b and low-virus load.

Key words: chronic hepatitis C, peginterferon, ribavirin, HCV genotype 1b, low virus load, duration of treatment

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Introduction

Current evidence indicates that combination therapy of peginterferon and ribavirin for hepatitis C virus (HCV) is associated with a higher rate of sustained virological response (SVR) compared with interferon (IFN) alone (1-7). Hence, combination therapy of peginterferon and ribavirin has been recommended as a first choice for chronic hepatitis C patients with high virus-load. Now, the selection of duration of treatment and optimum doses of combination therapy is an area of active investigation (8-16).

However, the dropout rates in patients treated with combination therapy was higher than those treated with IFN monotherapy (17, 18). On the other hand, some authors have reported that in half of the patients with a low virus load HCV RNA is eradicated by IFN monotherapy. Thus, for patients with a low virus load IFN monotherapy has been recommended as a first choice in Japan. However, there is also controversy over which patients should be treated with what agent and what regimen as a first choice for good prolonged prognosis in chronic hepatitis C patients with a low virus load. There is an ongoing need to refine treatment strategies in patients with a low virus load.

Thus, in the present study, we performed a retrospective study to examine the efficacy of combination therapy in pa-

[1]Department of Hepatology, Toranomon Hospital, Tokyo and [2]Hepatic Research Unit, Toranomon Hospital, Tokyo
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Correspondence to Dr. Yasuji Arase, es9y-ars@asahi-net.or.jp

tients with genotype 1b and low virus load. Additionally, the relationship between attainment time of negativity of serum HCV RNA after the initiation of combination therapy and the continuance of negative HCV RNA in patients with genotype 1b and low HCV-RNA load of <100 KIU/mL were also evaluated.

Materials and Methods

Patients

Eligibility criteria for entry into the study included the following: 1) HCV genotype 1b; 2) serum level of HCV RNA of <100 KIU/mL before treatment; 3) no corticosteroid, immunosuppressive agents, or antiviral agents used within 6 months; 4) no hepatitis B surface antigens (HBsAg), antinuclear antibodies (ANA), or antimitochondrial antibodies (AMA) detectable in serum, determined by radioimmunoassay; 5) leukocytes >2,000/mm^3, platelet count >80,000/mm^3, and bilirubin <2.0 mg/mL; 6) follow up for >6 months before treatment. We excluded from the study all of the patients with the following: 1) a history of alcohol abuse; 2) advanced liver cirrhosis of encephalopathy, bleeding esophageal varices, or ascites. The physician in charge explained the purpose and method of the combination therapy as well as the potential adverse reactions to each patient and informed consent was obtained from each patient.

From December 2004 to May 2007, 60 HCV patients were enrolled in this retrospective cohort study at the study hospital.

Patients were classified into three groups according to their response to combination therapy: rapid virological response (RVR), defined as undetectable HCV RNA at week 4 after the initiation of combination therapy; early virological response (EVR), defined as undetectable HCV RNA at week, 5 to 12 of combination therapy; and late virological response (LVR), defined as undetectable HCV RNA at week 13 to 24 of combination therapy. A SVR was defined as clearance of HCV RNA by commercial amplicor HCV qualitative assay (Amplicor HCV; Ver.2.0, Roche Diagnostic Systems, Basel, Switzerland) at 6 months after the cessation of combination therapy (19).

Next, predictors of SVR in patients with undetectable HCV RNA in serum during treatment were assessed by the multiple logistic regression analysis. Finally, SVR rate based on the attainment time of negativity of HCV RNA and continuance of negative HCV RNA during combination therapy were examined.

Combination therapy of pegylated-IFN and ribavirin

For the treatment regimen, the peginterferon (Peg-intron, Schering-Plough Pharmaceutical Co., Osaka, Japan) and ribavirin (Rebetol, Schering-Plough) were given at the dose described based on body weight. At the initiation of combination therapy, patients received peginterferon at a median dose of 1.4 μg/kg (range, 1.3-1.7 μg/kg) subcutaneously each week and oral ribavirin at a median dose of 12.0 mg/kg (range, 9.9-14.9 mg/kg) daily. The peginterferon dose was adjusted according to body weight (60 μg for ≤40 kg, 80 μg for >40 kg and ≤60 kg, 100 μg for >60 kg and ≤80 kg, 120 μg for >80 kg and ≤100 kg, and 150 μg for >100 kg). The ribavirin dose was adjusted according to body weight (600 mg for ≤60 kg, 800 mg for >60 kg and ≤80 kg, and 1,000 mg for >80 kg). The regimen or treatment period was decided by the physician. A total of 39 patients were treated with a 48-week regimen and 16 patients were given combination therapy for a 24-week regimen. Treatment for the remaining five patients was discontinued because of treatment-related side effects within 26 weeks after the initiation of combination therapy.

Blood samples were obtained just before and 6 month after combination therapy. The samples were stored at -80℃ until analyzed. Using these blood samples, HCV-RNA level before IFN therapy was analyzed by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0, Roche Molecular Systems) (20). HCV-genotype was examined by polymerized chain reaction assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported previously (21). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) concentrations, and HCV RNA were measured at least once per month during therapy. Negativity of serum HCV RNA was defined as clearance of serum HCV RNA by commercial amplicor HCV qualitative assay (19). Clinical evaluation and biochemical and hematological tests were performed at 4 weekly intervals.

Liver histology before IFN therapy

Liver biopsy specimens were obtained percutaneously under the observation by laparoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with Hematoxylin and Eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The biopsy specimens were diagnosed according to the system of Desmet et al (22).

Statistical analysis

Nonparametric procedures were employed for the analysis of background features of the patients with SVR and without SVR, including the Mann-Whitney U test. Independent factors that might have influenced SVR were studied using multiple logistic regression analysis, and the following variables were evaluated as prognostic factors: sex, age, body mass index, liver staging, a history of interferon therapy, a history of HCV load of ≥100 KIU/mL, HCV RNA level, biochemical factors (AST, ALT), platelet count, HCV RNA 4, 8, 12 week after the initiation of IFN therapy, continuous negative period of HCV RNA during IFN therapy and period of IFN therapy. The SPSS software package (SPSS Inc., Chicago, IL) was used to perform statistical analysis. A p value of <0.05 was considered to indicate a significant difference.

Table 1. Clinical Backgrounds before Combination Therapy of Peginterferon and Ribavirin in Chronic Hepatitis C Patients

	Total	Response			
		RVR	EVR	LVR	p
Patients, n†	60	18	31	6	
Sex, male (%)†	42 (70%)	15 (83%)	23 (74%)	2 (33%)	0.063
Age (yrs)‡	51.9±10.1	50.8±9.3	52.1±10.8	53.9±10.9	0.713
BMI‡	21.9±3.1	23.2±3.6	21.2±2.9	21.9±2.3	0.177
A history of IFN†, (%)	28 (47%)	7 (39%)	13 (42%)	4 (67%)	0.085
History of maximum HCV RNA level of >100KIU/mL (+/-)†	43/17	13/5	21/10	4/2	0.498
HCV RNA(KIU/mL)§	52 (<5-99)	43(8-93)	58(<5-99)	72(21-90)	0.498
AST (IU/L)‡	58±32	61±47	56±24	51±18	0.480
ALT (IU/L)‡	73±52	80 ± 62	69 ± 37	82±59	0.456
FPG(mg/dL)‡	93.1±13.6	93.2±13.0	92.5±12.2	97.5±24.6	0.182
Triglyceride (mg/dL)‡	92.5±35.2	94.5±27.8	90.6±42.9	93.9±30.2	0.887
Platelet(10^4/mm^3)‡	18.7±6.3	20.9±4.7	19.6±5.9	13.7±5.6	0.106
Fibrosis staging† (Non-LC/LC)	54/6	18/0	26/5	5/1	0.067

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; EVR, early virological response; FPG, fasting plasma glucose; HCV, hepatitis C virus; IFN, interferon; LC, liver cirrhosis; LVR, late virological response; RVR, rapid virological response

Normal reference ranges 6-50 IU/L for ALT, 11-38 IU/L for AST,

†Data expressed as number of patients (percentage)

‡Data expressed as mean ± standard deviation

§Data expressed as median (range)

Result

Clinical characteristics of the patients

A total of 60 patients were enrolled on present study. Table 1 shows the characteristics of the patients who received combination therapy. Clinical profiles were as follows: mean age =52 years, male/female =42/18, and median (range) HCV-RNA=52 (<5-99) KIU/mL. Two of the patients treated with 48-regimen and three out of five discontinued combination therapy due to side effects had positive HCV RNA during combination therapy. Patients with negativity of serum HCV RNA during combination therapy were classified into three groups according to the difference of response: RVR (n=18), EVR (n=31), and LVR (n=6). There were no significant differences in several factors in three groups as shown in Table 1.

Safety and tolerance of IFN

Of the 60 patients included in this study, five discontinued combination therapy because of IFN-related adverse events: one patient each with thrombocytopenia, general fatigue, psychiatric disorder, poor appetite, and cholecystitis. The onset of IFN-related side effects ranged from one to 11 weeks after initiation of IFN therapy. These side effects in five patients disappeared one month after cessation of IFN therapy.

Next, ten of the remaining 55 patients had dose reduction of interferon and/or ribavirin because of side effects: 5 cases of thrombocytopenia, 3 cases of general fatigue, and 2 cases of poor appetite. The onset of dose reduction due to IFN-related side effects ranged from 1 to 26 weeks after initiation of IFN therapy.

Efficacy of treatment

Out of 60 patients enrolled in the present study, 47 patients (78.3%) had SVR by the intention-to-treat analysis. Table 2 shows the differences in the clinical background between patients with SVR and those without SVR. The SVR was significantly associated with the attainment time of negativity of serum HCV RNA and continuance period of negative HCV RNA. Multivariate analysis indicated that non-relapse occurred when serum HCV RNA at week 8 was negative (p=0.004) and continuance of negative HCV RNA during treatment was $\geq$30 weeks (p=0.016) (Table 3).

Table 2. The Difference of Clinical Backgrounds between Patients with SVR and Those without SVR

	SVR (n=47)	Non-SVR (n=13)	p value
Age (years old) †	52.2 ± 10.1	53.4 ± 8.9	0.346
Sex (male/female) †	35/12	8/5	0.488
BMI	21.8 ± 3.2	22.2 ± 3.0	0.732
Liver staging (non-LC /LC)	42/5	12/1	1.00
a history of interferon (+/-)	22/25	6/7	1.00
a history of HCV load of ≥100KIU/mL (+/-)	31/16	7/6	0.520
HCV-load (KIU/mL) *	58 (<5-99)	46 (6-93)	0.375
AST (IU/L) *	49 ± 34	54 ± 22	0.102
ALT (IU/L) *	70 ± 55	83 ± 39	0.082
Platelet (10^4/mm^3) *	19.0 ± 6.5	17.6 ± 3.8	0.230
HCV RNA (-) 4W	17/46 (37%)	0/10 (0%)	0.023
HCV RNA (-) 8W	35/46 (76%)	1/10 (10%)	0.002
HCV RNA (-) 12W	44/46 (96%)	3/10 (30%)	<0.001
Continuous negative period (week)	34.9 ± 11.6	10.4 ± 12.1	<0.001
Period of IFN therapy (week)	41.6 ± 12.6	28.8 ± 19.6	<0.001

Data are number of patients, median (range) or mean± standard deviation. p value calculated by the Mann-Whitney U test

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IFN, interferon; SVR, sustained virologic response

Table 3. Multivariate Analyses Identifying Predictors of SVR

Factor	Category	Odds ratio	95% Confidence interval	p value
HCV RNA week 8*	+/-	1/69.1	4.0-1201.4	0.004
Continuance period of negative HCV RNA during treatment (week)	<30 / ≥30	1/34.5	1.9-500.0	0.016

HCV, hepatitis C virus

*HCV RNA at week 8 after the initiation of treatment

SVR based on the attainment time of negativity of serum HCV RNA and continuance of negative HCV RNA

All fifty-five patients with negativity of HCV RNA after the initiation of combination therapy had continuance of negative HCV RNA during combination therapy. SVR rate based on the attainment time of negativity of serum HCV RNA and continuance of negative HCV RNA during combination therapy are shown in Table 4. In the RVR group, all of seven patients with continuance of negative HCV RNA of 20 to 29 week during treatment had SVR. In the EVR group, patients with continuance of 30 to 39 week during treatment had SVR of ≥90%. In the LVR group, patients with continuance of 30 to 39 week during treatment had SVR of 50%.

Discussion

We have described the efficacy of combination therapy of peginterferon and ribavirin in patients infected with HCV genotype 1b and low virus load. The present study was limited to patients with genotype 1 and HCV-load of <100 KIU/mL. Another limitation is that the present study was not a randomized controlled study; thus, the treatment period was varied. Moreover, half of the patients had a history of IFN monotherapy and two-thirds of the patients had a history of maximum HCV RNA level of >100 KIU/mL. Clinical backgrounds of the enrolled patients were varied.

However, several findings from the present study have direct implications for combination therapy for chronic hepatitis C in the future. First, SVR was primarily associated with attainment time of negativity of serum HCV RNA and continuance of negative HCV RNA. The period of combination

Table 4. SVR Based on the Attainment Time of Negative HCV RNA and Continuance Period of Negative HCV RNA during Combination Therapy

Response*	Continuance period of negative HCV RNA (week)					Total
	<10	10-19	20-29	30-39	40-49	
RVR	100% (1/1)	ND	100% (7/7)	ND	100% (10/10)	100% (18/18)
EVR	ND	63% (5/8)	ND	90% (9/10)	100% (13/13)	87% (27/31)
LVR	0% (0/2)	ND	ND	50% (2/4)	ND	33% (2/6)
Total	33% (1/3)	63% (5/8)	100% (7/7)	79% (11/14)	100% (23/23)	85% (47/55)

EVR, early virological response; HCV, hepatitis C virus; LVR, late virological response; ND, not done; RVR, rapid virological response

*Response of HCV RNA means attainment time of negativity of serum HCV RNA after the initiation of combination therapy

therapy is statistically significant by univariate analysis. However, multivariate analysis showed that early undetectable HCV RNA and prolonged negativity of serum HCV RNA during treatment were associated with the SVR. In the RVR group, all seven patients with continuance of negative HCV RNA for 20 to 29 week during treatment had SVR. This result suggests that a short course regimen of 24 or < 24 week in combination therapy may be suitable for patients who have genotype 1, low virus load, and RVR. Earlier studies have reported higher SVR rates in patients with undetectable HCV RNA at week 4 compared to those with detectable HCV RNA (7-9, 23). Jensen et al (8) has reported that patients with RVR should be treated for a short course regimen. On the contrary, it may be necessary to treat patients without RVR with a long course regimen. The present results coincided closely with these earlier results.

Secondly, in the EVR group, patients with continuance of negative HCV RNA of ≥30 weeks during treatment had SVR of ≥90%. However, one-third of the patients with continuance of negative HCV RNA of 10 to19 weeks relapsed after the termination of therapy. This result suggests that patient with EVR should be given combination therapy for a year. Third, in LVR group, half of the patients with continuance of negative HCV RNA of 30 to 39 weeks during treatment had SVR. This indicates that patients with delayed undetectable HCV RNA should be treated to continue the negativity of serum HCV RNA for a prolonged period of ≥ one year to obtain a high rate of SVR.

A previous study (24) indicates that the suitable treatment period of combination therapy for chronic hepatitis C should be determined based on the time of attainment of negative HCV RNA in patients with genotype 1b and a high virus load of ≥100 KIU/mL. Similarly, the present study suggests that in patients with genotype 1b and low-virus load, the period of combination therapy should be determined based on the attainment time of negativity of serum HCV RNA.

It is desirable to expose patients with chronic hepatitis C to the shortest duration of treatment possible to reduce the likelihood of adverse events and minimize costs. Long-term treatment can be associated with serious side effects and is costly. HCV treatment of combination therapy is expensive; a 24-week treatment course costs approximately 20,000 dollars. Thus, the results of this study underscore the importance of changing the duration of treatment based on the difference of attainment time of negative HCV RNA. To attain SVR rate of ≥90% in patients with undetectable HCV RNA and continuance of negative HCV RNA during treatment, it is desirable to give a short course regimen of ≤20-29 weeks in the RVR group, 30-39 week in the EVR group. Moreover, in LVR, prolonged combination therapy regimen of >48 weeks may be recommended.

In conclusion, the period of combination therapy for chronic hepatitis C should be determined based on attainment time of negativity of serum HCV RNA and continuance of negative HCV RNA in patients with genotype 1b and low-virus load.

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Occult hepatitis B virus infection increases hepatocellular carcinogenesis by eight times in patients with non-B, non-C liver cirrhosis: a cohort study

K. Ikeda, M. Kobayashi, T. Someya, S. Saitoh, T. Hosaka, N. Akuta, F. Suzuki, Y. Suzuki, Y. Arase and H. Kumada *Department of Hepatology, Toranomon Hospital, Tokyo, and the Okinaka Memorial Institute for Medical Research, Tokyo, Japan*

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SUMMARY. An impact of serum hepatitis B virus (HBV) DNA on hepatocarcinogenesis has not been investigated in a cohort of patients with non-B, non-C cirrhosis. Eighty-two consecutive Japanese patients with cirrhosis, who showed negative hepatitis B surface antigen and negative anti-hepatitis C virus, were observed for a median of 5.8 years. Hepatitis B virus core (HBc) region and HBx region were assayed with nested polymerase chain reaction. Both of HBc and HBx DNA were positive in 9 patients (11.0%) and both were negative in 73. Carcinogenesis rates in the whole patients were 13.5% at the end of the 5th year and 24.6% at the 10th year. The carcinogenesis rates in the patients with positive DNA group and negative DNA group were 27.0% and 11.8% at the end of the 5th year, and 100% and 17.6% at the 10th year, respectively ($P = 0.0078$). Multivariate analysis showed that men ($P = 0.04$), presence of HBc and HBx DNA (hazard ratio: 8.25, $P = 0.003$), less total alcohol intake ($P = 0.010$), older age ($P = 0.010$), and association of diabetes ($P = 0.005$) were independently associated with hepatocellular carcinogenesis. Existence of serum HBV DNA predicted a high hepatocellular carcinogenesis rate in a cohort of patients with non-B, non-C cirrhosis.

Keywords: hepatitis B virus, hepatocellular carcinogenesis, liver cirrhosis, occult hepatitis B virus infection, proportional hazard model.

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 [3]. Hepatitis B virus (HBV) infection is the primary cause of cirrhosis and HCC and one of the major causes of death globally [4]. Needless to say, a cohort of patients with HBV-related chronic hepatitis and cirrhosis has a significantly high risk for HCC development [5–7]. In our retrospective cohort studies concerning HBV-related disease, cumulative hepatocellular carcinogenesis rates in chronic hepatitis ($n = 610$) and cirrhosis ($n = 180$) were 2.1% and 7.2% at the end of the 5th year, and 4.9% and 27.2% at the 10th year, respectively [5,7]. Abundant epidemiological and molecular biological evidence shows that HBV is an important factor in the development of HCC [8–10], but the precise role of HBV in the oncogenesis is still unknown.

HBV infection is usually diagnosed when the circulating hepatitis B surface antigen (HBsAg) is detected. However, the availability of highly sensitive molecular biology techniques has allowed the identification of HBV infection in HBsAg-negative individuals with or without circulating antibodies to HBsAg and/or hepatitis B core antigen (anti-HBc) [11–16]. Much evidence suggests that this so-called occult HBV infection is highly prevalent in a number of patient subgroups including those with HCV infection [16,17], cryptogenic advanced liver fibrosis [18] and HCC [17,19–27]. Although Marusawa *et al.* [28] and Uetake *et al.* [29] described the relationship between anti-HBc and HCC appearance rate in each study, impact of occult HBV infection on carcinogenesis cannot be evaluated because of lack of HBV DNA assay. As all the previous studies were performed as a pilot study or a case-controlled one, actual risk ratio of occult HBV infection for hepatocellular carcinogenesis has not been reported in a cohort study until now.

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine transaminase; AST, aspartic transaminase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PCR, polymerase chain reaction.

Correspondence: Kenji Ikeda, M.D., Department of Hepatology, Toranomon Hospital, Toranomon 2-2-2, Minato-ku, Tokyo 105-8470, Japan. E-mail: ikedakenji@tora.email.ne.jp

We, therefore, analysed a retrospective cohort of consecutive patients with cirrhosis for a long period, in order to elucidate the influence of occult HBV infection on the carcinogenesis rate from non-B, non-C cirrhosis.

PATIENTS AND METHODS

Patients

Among 103 consecutive patients diagnosed as having non-B, non-C cirrhosis by peritoneoscopic liver biopsy at Toranomon Hospital, Tokyo, Japan in the period from 1976 to 1998, initial frozen sera at the time of the diagnosis of cirrhosis were available for the assay of HBV DNA in 82 patients (79.6%). The cohort of 82 patients was retrospectively observed for a long period. All the patients showed negative HBsAg, negative anti-hepatitis C virus (HCV) and negative HCV RNA. Patients with a possible association of HCC at the time of the diagnosis of cirrhosis were strictly excluded from this study. No patient received interferon or other antiviral therapy after the diagnosis of cirrhosis.

Background and laboratory data of the patients

There were 67 men and 15 women aged 34–80 with a median age of 58 years. A total of 47 patients (57.3%) had a history of alcohol intake of more than 500 kg until the diagnosis of liver cirrhosis. Fifteen patients (18.3%) had decompensated cirrhosis with ascites, a history of encephalopathy, or both. The median value of indocyanine green retention rate at 15 min (ICG R15) was 33% (range, 7–75%), and total bilirubin concentration was 1.3 mg/dL (range 0.4–20.9 mg/dL).

Measurement of hepatitis virus markers

Hepatitis virus markers were assayed using frozen sera at −80 °C. All sera were tested for HBsAg (radioimmunoassay, Dainabot, Tokyo, Japan), anti-HCV (second-generation anti-HCV, enzyme-linked immunosorbent assay, Dainabot), and HCV RNA with reverse transcription-nested polymerase chain reaction (PCR).

HBV DNA was analysed for the region of HBc and HBx by sensitive nested PCR according to Yotsuyanagi *et al.* [30]. Fifty microlitres of STE solution [100 mmol/L Tris–HCl (pH 8.0), 100 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid (pH 8.0), and 0.2% sodium dodecyl sulphate] with 20 μg of proteinase K (Boehringer, Mannheim, Germany) were added to serum samples. Mixed samples were then incubated for 2 h at 55 °C. DNA was extracted twice with phenol/chloroform, once with chloroform, and precipitated with ethanol. The DNA pellet was dissolved in 25 μL of TE buffer [10 mmol/L Tris–HCl (pH 8.0) and 1 mmol/L ethylenediaminetetraacetic acid (pH 8.0)].

Prepared DNA was subjected to amplification using nested PCR technique. HBV DNA was amplified using two independent pairs of primers, with each primer complementary to sequences in the X or core region of the HBV genome [30]. Amplification was performed using a thermal cycler for a total of 40 cycles, with each cycle consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, in 100 μL of reaction mixture containing 200 mmol/L of each dNTP, 1X PCR buffer [50 mmol/L KCl, 10 mmol/L Tris–HCl (pH 8.3), 1.5 mmol/L $MgCl_2$ and 0.001% (w/v) gelatine], and 2 units of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Norwalk, CT, USA). The PCR products were separated in a 2% agarose gel and transferred to a nylon membrane (Schleicher and Schuell, Dassel, Germany). The membrane was then probed with digoxigenin-labelled oligonucleotides, which hybridize specifically with the core or X gene. Results were considered valid only if the same results were obtained in at least two separate experiments.

We considered the cases with positivity in at least two different viral genomic regions as HBV DNA positive. Appropriate negative controls were included in each PCR. The limit of sensitivity of our nested PCR methods ranged from 10 to 1 genome equivalents/mL.

Follow-up of patients

Follow-up of the patients was made on a monthly or bimonthly basis after diagnosis of cirrhosis by monitoring alpha-fetoprotein (AFP) and other biochemical data. Imaging diagnosis was made at least once a year for each patient with CT or US. After 1988, in order to detect HCC earlier, imagings were done three or more times per year in a majority of patients.

No patient underwent interferon therapy after the diagnosis of cirrhosis, but some of the patients received an oral or intravenous administration of medicinal herbs during the follow-up period.

All patients were finally evaluated in November 2004. The cases lost to follow-up were 13 (15.9%). The median observation period of the total patients was 5.8 years with a range of 0.1–34.8 years.

Statistical analysis

Differences of background features and laboratory data between the patients with and without HBV DNA were analysed by chi-square test, Fisher's exact test and Mann–Whitney's *U*-test. The time between diagnosis of cirrhosis and appearance of HCC was analysed using the Kaplan–Meier technique [31] and differences in curves were tested using log-rank test [32]. Those patients who had been lost to follow-up were regarded as censored data at the time of missing in the statistics. Independent risk factors associated with the appearance rate of HCC were studied using the stepwise Cox regression analysis [33]. Potential risk factors

assessed for hepatocellular carcinogenesis included the following 18 variables: age, sex, association of diabetes mellitus, total alcohol intake, history of cigarette smoking, family history of liver disease, history of blood transfusion, state of cirrhosis (presence of ascites and/or a history of encephalopathy), HBc DNA, HBx DNA, aspartic transaminase (AST), alanine transaminase (ALT), albumin, bilirubin, globulin, AFP, platelet, and ICG R15. A probability less than 0.05 was considered as significant. Data analysis was performed using computer program SPSS version 11 [34].

RESULTS

HCC appearance rate in all the patients

During the observation period, HCC appeared in 16 patients (19.5%). Median interval between the diagnosis of cirrhosis and HCC was 5.6 years (range 0.7–15.6 years) in the patients with HCC development. The cumulative HCC appearance rate in the 82 patients was 13.5% at the end of the fifth year after the diagnosis of cirrhosis, 24.6% at the end of tenth year, 33.3% at the 15th year, and 41.6% at the end of 20th year.

HCC appearance rates according to serum HBV DNA

Among the 82 patients, 9 patients (11.0%) showed positive serum HBV DNA and 73 (89.0%) negative HBV DNA. The former 9 patients had both HBc DNA and HBx DNA, and the latter 73 had neither of them. Table 1 summarizes the profiles and laboratory data of each group. There was no demographic difference between the two groups. There was also no statistically significant difference between them except for ALT value, which was lower in the patient group with positive HBV DNA ($P = 0.028$).

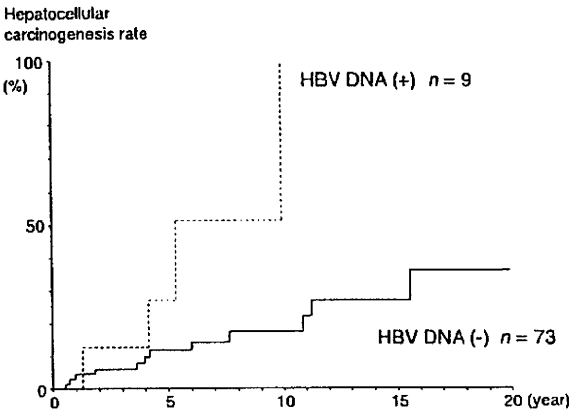

Fig. 1 Hepatocellular carcinogenesis curves of the patients with and without serum hepatitis B virus DNA. Carcinogenesis rates were 12.5% and 6.0% at the end of the third year, 27.0% and 11.8% at the fifth year, and 100% and 17.6% at the tenth year, respectively.

Figure 1 shows the curves of crude HCC appearance rate in the two patients group with and without serum HBV DNA. The third-year HCC appearance rates in the patients with and without DNA were 12.5% and 6.0%, the 5th-yr rates 27.0%, 11.8%, the tenth-yr rates 100% and 17.6%, respectively. The HCC appearance rate of the patient group

Table 1 Demography and laboratory data of patients with and without serum hepatitis B virus DNA

	HBV DNA*		
	Positive ($n = 9$)	Negative ($n = 73$)	P
Demographic and background features			
Sex – men/women	8/1	59/14	0.55
Age (median, range)	51 (45–68)	58 (34–80)	0.44
History of transfusion	1 (11.1%)	14 (19.4%)	0.55
Alcohol intake of 500 kg or more	5 (55.6%)	42 (58.3%)	0.87
Diabetes mellitus	3 (33.3%)	15 (20.8%)	0.40
Observation period (years)	5.7 (1.0–21.0)	6.1 (0.1–34.8)	0.92
Laboratory data (median, range)			
ICG R15 (%)	34 (12–51)	32.5 (7–75)	0.78
AST (IU/L)	32 (17–86)	40.5 (14–184)	0.26
ALT (IU/L)	16 (9–43)	28.5 (4–160)	0.028
Albumin (g/dL)	3.8 (2.6–4.5)	3.6 (1.7–5.2)	0.20
Bilirubin (mg/dL)	0.9 (0.5–2.8)	1.3 (0.4–20.9)	0.14
Platelet ($\times 1000/mm^3$)	142 (67–232)	104 (27–647)	0.18
AFP (ng/mL)	5 (3–9)	6 (1–98)	0.38

ICG R15, indocyanine green retention rate at 15 min; AST, aspartic transaminase; ALT, alanine transaminase; AFP, alpha-fetoprotein. *HBV DNA was assessed for HBc and HBx DNA using polymerase chain reaction

of positive HBV DNA was slightly higher than that of negative DNA (P = 0.0078, log-rank test).

Significance of serum HBV DNA in hepatocellular carcinogenesis

Cox proportional hazard model was performed for analysis of risk factors for liver carcinogenesis, using the 18 variables as mentioned above.

In the last step of stepwise regression analysis, the following five variables entered the model and could not be removed: sex (P = 0.005), serum HBV DNA (P = 0.003), past history of alcohol intake (P = 0.003), age (P = 0.035), and association of diabetes mellitus (P = 0.022) (Table 2). Accordingly, these five factors were significantly associated with hepatocellular carcinogenesis in the patients with non-B, non-C cirrhosis. Among them, gender was the strongest predictor of future HCC occurrence rate, indicating that male patients had 15.4 times as high carcinogenesis hazard as women patients. Similarly, positive HBV DNA (hazard ratio, 8.25) and little alcohol consumption of less than 500 kg (hazard ratio, 7.19) were the second and third strongest predictors for carcinogenesis, respectively. When the background factors of the cases were adjusted with the other significant factors, positive test for HBV DNA was significantly associated with the hepatocellular carcinogenesis rate.

Curves of carcinogenesis rates were generated from the multivariate analysis in an imaginary positive DNA group and an imaginary negative DNA group, with average sex ratio, average alcohol intake, average age and average association rate of diabetes (Fig. 2). The difference of the carcinogenesis curves indicated 'pure' impact of positive serum HBV DNA upon the carcinogenesis, which was adjusted with significant covariates assuming a standardized study group.

Table 2 Independent factors associated with liver carcinogenesis in the patients with non-B, non-C cirrhosis

Factors	Category	Hazard ratio (95% confidence interval)	P
Sex	Women	1	
	Men	15.4 (2.24–111.1)	0.005
Serum HBV DNA*	Negative	1	
	Positive	8.25 (2.01–33.93)	0.003
Total alcohol intake	≥500 kg	1	
	<500 kg	7.19 (1.98–26.32)	0.003
Age	<60 years	1	
	≥60 years	3.98 (1.10–14.42)	0.035
Diabetes mellitus	No	1	
	Yes	3.89 (1.22–12.47)	0.022

*Positive HBV DNA: positive for both HBc DNA and HBx DNA.

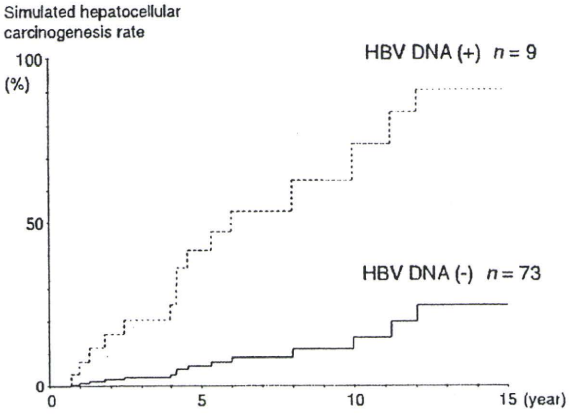

Fig. 2 'Adjusted' hepatocellular carcinogenesis rates in the positive HBV DNA group and the negative DNA group. Cox proportional hazard analysis showed that the carcinogenesis rate in the positive DNA group was significantly higher than that of the negative DNA group, when the other significant covariates were substituted with the same average parameters in the two groups.

Mortality and causes of death

During the observation period, 36 (43.9%) of 82 patients died: 5 (55.6%) of 9 patients in the positive DNA group and 31 (42.5%) of 73 patients in the negative DNA group. Cumulative survival rates in patients with and without HBV DNA were 78.8% and 74.1% at the end of the fifth year, 54.4% and 44.4% at the tenth year, 38.4% and 29.6% at the 15th year, and 33.6% and 29.6% at the 20th year, respectively. Although the survival rate in the positive HBV DNA group was lower than in the negative group, statistical significance was not shown.

Causes of death included liver failure due to liver cirrhosis in 21 (4 in positive DNA group and 17 in negative DNA group), progression of HCC in 7 patients (all in negative DNA group), and other causes in 8 (one in positive DNA group and 7 in negative DNA group).

DISCUSSION

Epidemiological and molecular virological studies in the 1970s and early 1980s established a strong aetiological association between chronic HBV infection and the hepatocellular carcinogenesis [35]. We also estimated annual carcinogenesis rates as 0.5% in chronic hepatitis and 3% in cirrhosis, from cohorts of biopsy-proven HBV disease [5,7].

Integration of HBV DNA has been reported in the majority of HBsAg positive HCCs since 1980s, and the fact suggested HBV might be oncogenic. Up to now, there is no evidence

that HBV DNA is directly oncogenic and the mechanism by which chronic HBV infection leads to carcinogenesis remains unclear. Integration of HBV DNA may stimulate cellular pro-oncogenes or suppress growth-regulating genes [36]. Integration of HBV DNA, however, has been found in varied regions of the host chromosomes and no preferential and specific site has been identified until now. The other authors suggested that integration of HBV DNA could also induce carcinogenesis via transactivation of other oncogenes [37]. Both HBx protein and the truncated pre-S/S protein are potent transactivators and are commonly found in HCC tissue but their precise role in hepatocarcinogenesis remains unknown.

Occult HBV infection is generally defined as the detection of HBV DNA in the serum or liver tissue of patients who test negative for hepatitis B surface antigen [38–41]. Occult HBV infection was first reported in the early 1980s when hybridization techniques for the detection of HBV DNA became available. These studies showed that HBV DNA could be detected in HBsAg negative patients with HCC [42]. Recent studies using more sensitive techniques confirmed the close correlation between chronic occult HBV infection and carcinogenesis. Many authors demonstrated the relationship between occult HBV infection and hepatocellular carcinogenesis, mainly by a pilot study or a case–control study [17,19–27]. Shiota *et al.* [24] reported in their case studies without control group that serum of 18 out of 26 HCC patients without HBsAg and anti-HCV were positive for either S, C, or X region on PCR and southern blotting. Pollicino *et al.* [26] described that viral DNA was detected in 68 of 107 cases of HCC tissue (63.5%) and in 63 of 192 cases of chronic hepatitis tissue (32.8%), and concluded that occult HBV is a risk factor for development of HCC. The other authors also emphasized the high incidence of HBV DNA in either serum or HCC tissue compared with that of cases without HCC development. All the literatures, except one [43] from Taipei where HBV infection was endemic and prevalent, concluded that occult HBV infection was closely associated HCC development. However, precise risk or hazard ratio for carcinogenesis has not been reported.

Current study on this topic provided strong evidence of an association between occult HBV infection and HCC. In the patient cohort of non-B, non-C cirrhosis, occult HBV infection increased the future carcinogenesis rate with a hazard ratio of 8.25 (95% confidence interval, 2.01–33.93). It has been proposed that diagnosis of occult HBV infection be made only when HBV DNA can be detected using at least two sets of primers from different areas of the HBV genome in duplicate assay [38,39]. Appropriate negative controls must be included in each assay and specificity of the amplification reaction confirmed by sequencing of the amplicons. Using this strict criterion, occult HBV infection was found in 9 (11.0%) of 82 Japanese patients with non-B, non-C cirrhosis. Background features of the nine patients with serum HBV DNA showed a slightly younger age, a lower ALT, a slightly lower bilirubin, and a slightly higher platelet count (Table 1). Although all these demographic and laboratory findings were considered to favour low carcinogenetic risk, the patients with cryptic HBV DNA infection developed HCC more frequently. After adjustment of these background covariates in the multivariate analysis, positivity of serum HBV DNA proved to be an independent risk factor for hepatocarcinogenesis (Table 2).

As this retrospective cohort consisted of only cirrhosis as an advanced liver disease, and as it included both alcoholic and non-alcoholic cirrhosis, the hazard ratio of 8.25 could not be applied for varied stages and varied aetiologies of liver disease. In order to elucidate the impact of occult HBV infection on carcinogenesis, future studies should be performed also in the other cohort of chronic liver disease, such as HCV-related disease. Although anti-HBc and anti-HBs antibody were measured in a small numbers of the patients, an exact relationship between serum HBV DNA and serum positivity of anti-HBc antibody was not analysed in this study. When we tested anti-HBc antibody in a small part of subjects, 3 of 6 patients (50.0%) with positive HBV DNA had serum anti-HBc antibody and 7 of 19 patients (36.8%) without HBV DNA had anti-HBc (Fisher's exact test, $P = 0.69$). For the convenience of clinical circumstance and practical usefulness, significance of positive anti-HBc on carcinogenesis risk should be elucidated through a large-scale cohort study with an identical assay for anti-HBc antibody.

Although a lot of epidemiological and clinicopathological evidence of the relationship has been published, precise role of occult HBV in this setting has been still unclear. Patients with occult hepatitis B overlap with those who previously have been classified as having recovered [44]. In fact, the distinction between recovery and occult hepatitis B is likely to be somewhat arbitrary, as recovery does not necessarily imply eradication of infection in all cases [30], but includes the possibility of complete suppression in some cases by a broad and vigorous immune response [44]. One of the most important clinical questions is whether occult hepatitis B merely represents a marker of past infection, or whether HBV genome persistence contributes to liver disease. It is very likely that occult HBV is a cofactor in the development of HCC. Several studies found that patients co-infected with HBV and HCV have increased risks of HCC compared with those with mono-infection. Our cohort studies [45] also showed that a risk factor of a history of heavy drinking interacted with HBV or HCV subtypes in a characteristic manner from the viewpoint of carcinogenesis in cirrhosis. The other important problem is whether occult HBV infection alone causes HCC. To address this question, studies on occult HBV infection in patients with HCC might provide details on other causes of chronic liver disease including nonalcoholic fatty liver disease, which may masquerade as cryptogenic cirrhosis, hemochromatosis, alfa-1-antitrypsin deficiency, and autoimmune liver disease [46]. Recently,

Castillo *et al.* [47] reported a clinical state of occult HCV infection, which shows negative serum anti-HCV, negative serum HCV RNA, and positive HCV RNA in liver biopsy specimen. Although we did not tested the possibility of occult HCV infection in this study, future studies should be also aimed at the influence of latent HCV infection on hepatocarcinogenesis.

In conclusion, occult HBV infection significantly increased the incidence of hepatocellular carcinogenesis in patients with non-B, non-C cirrhosis. Although non-B, non-C cirrhosis seemed to include varied aetiology of liver disease, cryptic HBV infection should be taken account in the prediction of future HCC development.

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