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Short Communication

Changes in levels of hepatitis B virus markers in patients positive for low-titer hepatitis B surface antigen

Chiaki Okuse,[1] Hiroshi Yotsuyanagi,[2] Norie Yamada,[4] Hiroki Ikeda,[1] Minoru Kobayashi,[1] Yasunobu Fukuda,[1] Hideaki Takahashi,[1] Kotaro Matsunaga,[1] Nobuyuki Matsumoto,[1] Masaru Okamoto,[5] Toshiya Ishii,[5] Akira Sato,[5] Kazuhiko Koike,[3] Michihiro Suzuki[1] and Fumio Itoh[1]

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Aim: Recently, patients positive for the low-titer hepatitis B surface antigen (HBsAg) have been found occasionally owing to the increase in the accuracy of detection methods. The aim of this study is to clarify the clinical status of acute hepatitis B virus (HBV) infection in patients positive for low-titer HBsAg.

Method: Eight patients, who were positive for HBsAg at low titers and diagnosed as having acute HBV infection, were enrolled in this study. Assays of HBsAg, hepatitis B core antibody (anti-HBc), hepatitis B e-antigen (HBeAg), hepatitis B e-antibody (anti-HBe), hepatitis B surface antibody (anti-HBs) and HBV DNA, and biochemical tests were basically conducted every 4 weeks for at least 24 weeks.

Result: The average cut-off index of HBsAg was 8.7 ± 9.6 (range, 1.0–25.7). All the patients were negative for anti-HBc, HBeAg, anti-HBe and HBV DNA on their initial visit. The genotype of HBV could be determined in four patients: two were infected with genotype B/HBV, one was infected with genotype A/HBV, and the remaining patient was infected with genotype C/HBV. Although HBsAg clearance was observed within 4 months in all the patients, none of the other HBV markers seroconverted during the observation period.

Conclusion: HBV infection terminating with seronegativity for HBV markers may occur in transient HBV infection.

Key words: hepatitis B virus, low-titer hepatitis B surface antigen, seronegativity

INTRODUCTION

CHRONIC INFECTION BY hepatitis B virus (HBV) is a major global health problem, affecting more than 400 million people worldwide.[1] Approximately 15–40% of infected patients develop cirrhosis, liver failure or hepatocellular carcinoma.[2] Although the number of new patients with persistent HBV infection by vertical transmission has decreased owing to the development and widespread use of hepatitis B (HB) vaccines,[3,4] HBV remains the major cause of acute hepatitis that is sexually transmitted in most cases at present.[5]

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In a recent epidemiological study in Japan, the proportion of patients with acute hepatitis B (AHB) was approximately 40% between 2006 and 2010, which was approximately 25% between 1980 and 1995, thus showing an increasing trend in Japan.[6]

Seventy to ninety percent of patients with acute HBV infection show an asymptomatic course that ends as a subclinical infection.[7,8] However, the epidemiology of subclinical HBV infection in Japan has not been fully understood. The clearance of the HB surface antigen (HBs antigen: HBsAg) and the appearance of the HB surface antibody (anti-HBs: HBsAb) are generally accepted as evidence of clinical and serological recovery from AHB. However, HBV replication has been shown to persist and HBV exists at low levels in the liver for decades.[9–11] Recently, HBV reactivation and development of fatal fulminant liver failure in patients with resolved infection during or after chemotherapy and transplantation have been reported.[12–14]

Thus, it is considered important to estimate the current status of patients with HBV infection, including those with subclinical infection. We focused on the patients positive for low-titer HBsAg, and studied their clinical characteristics.

METHODS

Patients

A TOTAL OF 33555 patients were examined for HBsAg in St Marianna University School of Medicine between January 2004 and June 2006, among whom 493 patients (1.5%) were found to be positive for HBsAg. Four hundred and forty patients regularly visited our hospital owing to chronic HBV infection. The remaining 53 patients were found to be positive for HBsAg on their initial visit to our hospital. Among these 53 patients, 25 (47.2%) were presumably diagnosed as having acute HBV infection on the basis of the following: (i) positivity for HBsAg and/or HBV DNA; (ii) positivity for the immunoglobulin M (IgM) hepatitis B core antibody (anti-HBc); and/or (iii) negativity for HBsAg determined in a previous examination. The others were diagnosed as having chronic HBV infection. Eight of the 25 patients were referred to our department and were enrolled in this prospective cohort study. These eight patients visited our hospital not for HBV infection but for examination or treatment of non-hepatic diseases. The eight patients were diagnosed as having acute HBV infection on the basis of the following: (i) negativity for HBsAg determined in a previous examination; and (ii) positivity for HBsAg after re-examination of their plasma samples mixed with ethylenediamine tetraacetic acid (EDTA) collected at the same time as their serum samples. None of the patients were vaccinated for HB. Written informed consent was obtained from all the patients in this study.

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The assays of HBsAg, anti-HBc, HB e-antigen (HBeAg), HB e-antibody (anti-HBe), anti-HBs and HBV DNA, and biochemical tests were basically conducted every 4 weeks for at least 24 weeks. The upper normal level of serum ALT was defined as 30 IU/L.

HBV markers

The levels of HBsAg and anti-HBs were determined using commercially available chemiluminescence enzyme immunoassay kits (LUMIPULSE II HBsAg, LUMIPULSE II HBsAb; Fujirebio, Tokyo, Japan). The cut-off index (COI) of the assay of HBsAg was 1.0. The levels of anti-HBc, HBeAg and anti-HBe were determined using other commercial chemiluminescence immunoassay kits (LUMIPULSE II HBcAb, LUMIPULSE II HBeAg, LUMIPULSE II HBeAb; Fujirebio). Serum HBV DNA level was determined using a commercial transcription-mediated amplification kit or a polymerase chain reaction kit (DNA probe FR-HBV; Fujirebio). The lowest detection limit of the assay was 3.7 LGE/mL. The HBV genotype was determined using a commercial enzyme-linked immunosorbent assay kit (SMITEST HBV genotype detection kit; Genome Science Laboratories, Fukushima, Japan).

RESULTS

Baseline characteristics of patients

QUANTITATIVE VARIABLES WERE expressed as mean ± standard deviation. The baseline characteristics of the enrolled patients on their initial visit are shown in Table 1. The patients comprised five men and three women with a mean age of 37.8 ± 12.5 years. The average COI of HBsAg was 8.7 ± 9.6 (range, 1.0–25.7). None of the patients were positive for anti-HBc, HBeAg, anti-HBe or HBV DNA on their initial visit. Elevation of serum ALT level was observed in three patients. The serum ALT levels in patients 3 and 5 on their initial visit were 51 IU/L and 47 IU/L, respectively, whose liver dysfunction was presumably caused by their transient use of a non-steroidal anti-inflammatory drug and by alcohol overconsumption, respectively. In patient 8, the elevated serum ALT level was presumably caused by infectious mononucleosis, because this patient was positive for the IgM Epstein–Barr virus antibody to the viral capsid antigen at the time of examination. The genotype of HBV could be determined in four patients: two patients were infected with genotype B/HBV, one was infected with genotype A/HBV and the remaining patient was infected with genotype C/HBV (Table 1).

Changes in levels of HBV markers

Among these eight patients, HBV marker levels were monitored in principle for 24 weeks in four patients and for 48 weeks in four patients after their initial visit. HBsAg was cleared from serum within 4 months in all these patients (Table 1). Unfortunately, periodic determination of HBsAg level 1, 2 or 3 months after the referral to our department was not conducted in patient 7. The period of HBsAg clearance in patient 7 was

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Table 1 Characteristics of patients studied on their initial visit and period of verified HBsAg clearance

Patient	Sex	Age (years)	ALT (IU/L)	HBsAg (COI)	HBeAg (COI)	Anti-HBe (% inhibition)	Anti-HBc (S/CO)	HBV DNA (LGE/mL)	HBV genotype	Confirmation of HBsAg negative in the past	Period of verified HBsAg clearance (day)
1	M	31	18	19.7	0.4	2.6	0.16	<3.7	B	March 2004	12
2	M	63	14	12.7	0.5	0.0	0.27	<3.7	B	September 2003	26
3	M	45	51	5.4	ND	ND	ND	<3.7	ND	January 2004	21
4	F	39	28	2.4	0.5	0.0	0.26	<3.7	A	January 2003	29
5	M	37	47	1.5	0.53	18.1	0.38	<3.7	ND	December 2004	14
6	F	34	24	1.0	0.5	0.0	0.20	<3.7	ND	November 1999	54
7	F	33	26	1.1	0.6	0.0	ND	<3.7	ND	July 2004	113
8	M	20	526	25.7	0.6	0.0	0.43	<3.7	C	June 2004	38

ALT, alanine aminotransferase; COI, cut-off index; F, Female; anti-HBc, hepatitis B core antibody; anti-HBe, hepatitis B e-antibody; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; M, Male; ND, not determined; *S/CO, sample/cut-off.*

verified in the second test for HBsAg conducted 113 days (4 months) after the patient's initial visit. Anti-HBs and anti-HBc remained undetectable during the observation period (Table 2).

DISCUSSION

IN THE PRESENT cohort, HBsAg and HBV DNA were transiently detected during the observation period of 24–48 weeks, and neither anti-HBs nor anti-HBc was detected even at the final observation time point. We consider that false-positive results and cross-contamination were excluded as much as possible because of the following four reasons. First, re-examination of the different plasma samples mixed with EDTA, which removes fibrin clots that might yield false-positive results, yielded the same results as that of serum samples in all eight patients. Second, neutralization of HBsAg with a neutralization solution yielded negativity for HBsAg in 10 other samples with low-titer HBsAg (ratios of absorption, 85–100%). Although the neutralization of HBsAg could not be verified in the samples presented here owing to the shortage of these samples, the probability of false-positive results for low-titer HBsAg may be considered minimal. Third, the HBV genotype with variability could be determined in four of eight patients. Fourth, the chance for cross-contamination was very small because the assays were conducted automatically after applying the serum samples. Thus, the eight patients may be regarded as actually infected by HBV. However, careful attention is required in the diagnosis based on low-titer HBsAg without other serum markers related to HBV infection.

Our findings imply that a substantial portion of cases of acute HBV infection might be subclinical without the appearance of serum anti-HBc or anti-HBs. The existence of HBV DNA besides HBsAg shows that HBV virions, presumably produced in infected hepatocytes, circulate in peripheral blood. Anti-HBs may be expected to appear after HBsAg clearance. However, taking into consideration that nearly 10–15% of patients with acute symptomatic hepatitis remain negative for anti-HBs even after recovery,[15] the patients in our present study may lack anti-HBs after recovery.

As for anti-HBc, the diagnosis of AHB is based on the presence of serum IgM anti-HBc, which presumably reacts with HBeAg or core-gene-related proteins secreted in the blood. Considering that none of the patients in our present study were positive for HBeAg, the amount of anti-HBc secreted in the blood might be too small to be detected.

Table 2 Summary of the changes of HBV markers

Patient	HBsAg/anti-HBs (COI / mIU/mL)	HBeAg/anti-HBe (COI / % inhibition)	Anti-HBc (S/CO)	HBV DNA (LGE/mL)	ALT (IU/L)	HBsAg/anti-HBs (COI / mIU/mL)	HBeAg/anti-HBe (COI / % inhibition)	Anti-HBc (S/CO)	HBV DNA (LGE/mL)	ALT (IU/L)
	1 month					3 months				
1	–/–	–/–	–	<3.7	16	–/–	–/–	–	<3.7	29
2	–/–	–/–	–	<3.7	14	–/–	–/–	–	<3.7	14
3	–/–	–/–	–	<3.7	55	–/ND	ND/ND	–	<3.7	51
4	–/–	–/–	–	<3.7	16	–/–	–/–	–	<3.7	14
5	–/–	–/–	–	<3.7	40	–/–	–/–	–	<3.7	24
6	+/–	–/–	–	<3.7	14	–/–	–/–	–	<3.7	11
7	ND/–	–/–	–	ND	26	ND/ND	ND/ND	–	ND	20
8	+/–	–/–	–	<3.7	23	–/–	–/–	–	<3.7	24
	6 months					12 months				
1	–/–	–/–	–	<3.7	20	–/–	–/–	–	<3.7	22
2	–/–	–/–	–	<3.7	12	–/–	–/–	–	<3.7	10
3	–/ND	ND/ND	ND	ND	82	–/–	–/–	–	<3.7	14
4	–/–	–/–	–	<3.7	12	–/–	–/–	–	<3.7	9
5	–/–	–/–	–	<3.7	27	ND/ND	ND/ND	ND	ND	ND
6	–/–	–/–	–	<3.7	12	ND/ND	ND/ND	ND	ND	ND
7	–/–	–/–	–	ND	24	ND/ND	ND/ND	ND	ND	ND
8	–/–	–/–	–	<3.7	14	ND/ND	ND/ND	ND	ND	ND

ALT, alanine aminotransferase; anti-HBc, hepatitis B core antibody; anti-HBe, hepatitis B e-antibody; COI, cut-off index; anti-HBs, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; ND, not determined; S/CO, sample/cut-off.

The severity of symptoms and liver injury is related to the amount of HBV DNA, and it is reported that the minimum 50% dose in chimpanzees was estimated to be approximately 10 copies for genotype A/HBV and C/HBV.[16] Therefore, it is suggested that although transient viremia or transmission of HBV to partial hepatocytes is possible in the case of HBV infection by an extremely small amount of HBV, the clearance of HBV without concurrent liver injury may occur.

Hepatitis B virus replication may persist at low levels in the liver for decades[9–11] after recovery from acute hepatitis and may lead to fatal fulminant liver failure after chemotherapy or organ transplantation.[12–14] It is unclear whether patients whose HBV infection terminates without the appearance of serum anti-HBc or anti-HBs clear HBV from their infected liver or might experience HBV reactivation. Careful follow-up examinations of the patients with transient HBV viremia without anti-HBs or anti-HBc are necessary.

According to the data from our hospital, 25 of 33 115 (33 555 minus 440 regular visitors) persons (0.08%) suffered from transient HBV infection. At least eight of them cleared HBV from their blood and were without serum anti-HBs or anti-HBc. The proportion of asymptomatic patients, diagnosed on the basis of positivity for anti-HBc without symptomatic hepatitis, is in the range 70–90%.[7,8] Our findings imply that the actual number of patients who experienced acute HBV infection may be larger than previously thought, which should be studied in other cohorts.

The incidence of AHB that required hospitalization was estimated to be approximately 2100–2400 cases/year in Japan.[17] The actual number of patients with acute HBV infection including those with asymptomatic hepatitis was calculated to be nearly 10 000. Our pilot study suggests that the actual number of patients with transient HBV infection might be far larger and requires to be confirmed.

In conclusion, our findings suggest that HBV infection terminating with seronegativity for HBV markers may occur in transient HBV infection.

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Letter to the Editor

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α2-HS glycoprotein is an essential component of cryoglobulin associated with chronic hepatitis C

Keywords: α2-HS glycoprotein; cryoglobulin; hepatitis C; jacalin.

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It is well-known that cryoglobulinemia is associated with hepatitis C [1–4]. We previously invented a cooling gel diffusion method as a highly sensitive method for detecting a small amount of cryoglobulin that appears in chronic hepatitis C [5]. In the novel cooling gel diffusion method, the cryoglobulin ring was formed around the well in the cooling gel. In order to clarify the physicochemical characteristics of cryoglobulin proteins, both the cryoglobulin-negative and -positive serum were treated as follows: heating at 56°C and the addition of βME or lectins, jacalin (EY Laboratories, San Mate, CA, USA), and protein A (Bio Vision, Milpitas, CA, USA) and applying to the well of an agarose gel plate. The gel plate was stored at 4°C for 48 h (Figure 1A). From the results, the density of the cryoglobulin ring from the cryoglobulin-positive serum that underwent heat treatment and the addition of βME or protein A was not changed. However, the cryoglobulin ring disappeared with the addition of jacalin lectin, which is a plant-based lectin for capturing O-glycoproteins, similar to IgA1 with the galactose residue. Cryoglobulin consisted of jacalin reactive protein. For the purification of jacalin reactive protein, a mini column packed with agarose-bound jacalin (InvivoGen, San Diego, CA, USA) was prepared. The serum sample was poured onto the chilled column and left for 6 h. This column was sufficiently washed with phosphate-glycine buffer, and non-reactive proteins were removed from the agarose-bound jacalin column. Next, elution buffer including 0.1 M melibiose was added to the column to obtain the jacalin reactive proteins in the serum, culminating in 30 fractions with 2 mL. The absorbance at 280 nm of each fraction was measured, and the fraction with the highest absorbance was used for SDS-polyacrylamide gel electrophoresis. For the cryoglobulin-negative sample, one band appeared near 65 kDa, and for the positive sample, two bands with molecular weights of about 57 and 65 kDa appeared (Figure 1B). These bands were analyzed using liquid chromatography-matrix assisted laser desorption ionization-time of flight/mass spectrometer (LC-MS/MS) and the analytical software MASCOT, and it was clarified using Western blotting that the 65-kDa band was an α heavy chain of IgA and the 57-kDa band was α2-HS glycoprotein (AHSG) (Figure 1C). It is known that AHSG is a carrier protein of calcium phosphate, forming particles with a 30–150-nm diameter [6], and the AHSG concentration increases 10-fold during HCV replication in hepatocellar Huh-7 in vitro [7]. It was assumed that cryoprecipitate associated with chronic hepatitis C consisted of AHSG complexes with calcium phosphate and large molecular serum proteins like IgM combined with hemolytic complements [8]. The consumption of AHSG by the forming cryoprecipitate may relate to the progression from chronic hepatitis C to cirrhosis by the accumulation of calcium phosphate in the liver.

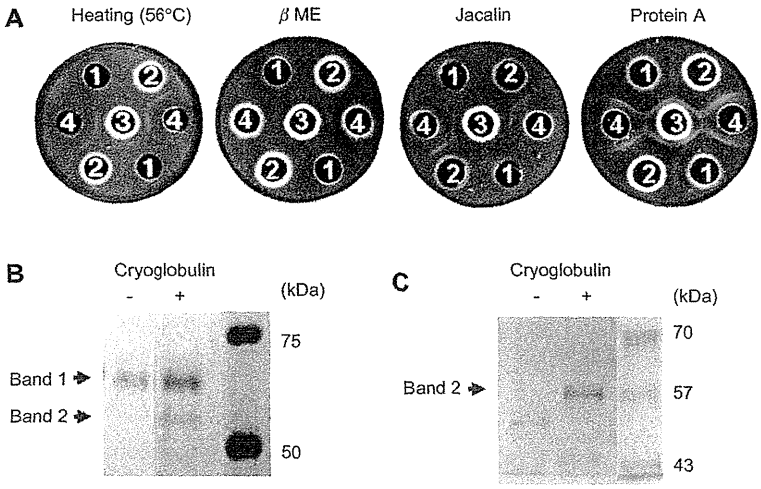

Figure 1 Analysis of essential components of cryoglobulin with chronic hepatitis C.
(A) Before cryoglobulin detection using the cooling gel diffusion method, serum samples from a cryoglobulin-negative healthy volunteer (57-year-old man) and a positive patient (74-year-old woman with chronic hepatitis C) were treated at 56°C, with the addition of βME or lectins, like jacalin and protein A. Treated and non-treated samples were poured into wells of the gel plate as follows: 1) treated healthy volunteer's serum sample; 2) treated chronic hepatitis C patient's serum sample; 3) non-treated chronic hepatitis C patient's serum sample; 4) non-treated healthy volunteer's serum sample. These plates were stored at 4°C for 48 h, and then analyzed. (B) Affinity column chromatography of serum samples from a cryoglobulin-negative patient (70-year-old man with chronic hepatitis C who developed liver cirrhosis) and a positive patient (74-year-old woman with chronic hepatitis C) was performed using the jacalin/agarose column, and jacalin-affinity serum proteins were fractioned with SDS-polyacrylamide gel electrophoresis. (C) Western blotting analyses of jacalin-affinity serum proteins were performed using the anti-α2-HS glycoprotein mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Non-invasive prediction of hepatocellular carcinoma development using serum fibrosis marker in chronic hepatitis C patients

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Abstract
Background The FIB-4 index is a simple formula to predict liver fibrosis. This study aimed to evaluate the utility of the FIB-4 index and associated time-course changes as a predictor of hepatocellular carcinoma (HCC) development.
Methods A total of 171 chronic hepatitis C patients who underwent paired liver biopsies and 875 patients who underwent a single liver biopsy (validation group) were investigated during mean follow-up periods of 6.4 and 5.9 years, respectively. All patients had received interferon therapy and had not achieved a sustained virological response. Factors associated with HCC development were analyzed in these patients.
Results HCC developed in 30 patients in the paired biopsy group and 89 patients in the validation group. Univariate analysis demonstrated that the FIB-4 index >3.25 and change in the FIB-4 index per year (ΔFIB-4/year) ≥0.3 were predictive factors for HCC development in both groups. Multivariate analysis in the combined population revealed that these two factors were independent. The hazard ratio (HR) for the FIB-4 index >3.25 was 2.7 ($p < 0.001$) and ΔFIB-4/year ≥0.3 was 1.8 ($p = 0.003$). Patients with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 were defined as high risk, and those with a FIB-4 index ≤3.25 and a ΔFIB-4/year <0.3 were defined as low risk. The HR of HCC development in patients at high risk was 7.3 (95 % confidence interval 4.3–12.5, $p < 0.001$).
Conclusions It was possible to define a group at high risk of developing HCC by intermittently measuring the FIB-4 index and considering time-course changes in this index.

Keywords FIB-4 index · Hepatocellular carcinoma · Chronic hepatitis C · Liver fibrosis · Non-invasive

Introduction

Persistent hepatitis C virus infection induces chronic hepatitis and eventually develops into liver cirrhosis and hepatocellular carcinoma (HCC) [1]. An advanced stage of liver fibrosis in chronic hepatitis C is associated with HCC development and complications such as esophageal variceal bleeding and liver failure [2, 3]. Therefore, accurate evaluation of the stage of liver fibrosis is necessary to predict its progression to liver cirrhosis and HCC development for optimal clinical disease management.

Although the gold standard for evaluating liver fibrosis is liver biopsy [4, 5], it has been reported that this method may be inaccurate because of sampling errors and inter-observer variations [6, 7]. Moreover, because the invasiveness of liver biopsy precludes repeated examinations [8], evaluation of liver fibrosis time-course changes is difficult.

Recently, various non-invasive methods for evaluating liver fibrosis have rapidly improved as alternatives to liver biopsy. Liver fibrosis was reportedly predicted by transient elastography [9, 10], acoustic radiation force impulse imaging [11], and real-time tissue elastography [12]. In

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addition, methods using blood test data, including the aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio [13], AST/platelet ratio index [14], the Forns test [15], and the Fibro test [16] have been reported to be useful. These tests have exhibited high accuracy in predicting severe liver fibrosis.

The FIB-4 index is a simple formula used for predicting liver fibrosis based on the standard biochemical values (AST, ALT and platelet count) and age, and is reported to be significantly useful for predicting advanced liver fibrosis [17–19]. Because the FIB-4 index can be repeatedly calculated using age and general biochemistry results, it offers the advantage of easy follow-up of time-course changes with repeated measurements. We have reported that time-course changes in the FIB-4 index correlate with changes in liver fibrosis, and advancement of liver fibrosis can be predicted by changes in the FIB-4 index [20].

Progression of liver fibrosis in chronic hepatitis C is closely associated with the high risk of developing HCC [2, 3]. In addition, we have reported that the risk of developing HCC increases with aging [21]. Because the FIB-4 index correlates with liver fibrosis and considers age, it is possible that it can also be used to predict the risk of developing HCC. In this study we investigated the significance of the FIB-4 index and time-course changes in the FIB-4 index as predictors of HCC development.

Methods

Paired biopsy group

Study subjects comprised 314 chronic hepatitis C patients who underwent liver biopsies twice between 1991 and 2010 at Musashino Red Cross Hospital. The average interval between two biopsies was 4.9 ± 2.9 years. The subject characteristics were detailed previously [20]. All patients were treated by interferon after the first liver biopsy and had non-sustained virological response. They underwent the second biopsy and were treated again by interferon. After excluding 110 patients who achieved a sustained virological response with the second interferon therapy, 171 patients were followed-up ≥1 year and were included in this analysis. Exclusion criteria comprised the follows: (1) co-infection with hepatitis B virus or human immunodeficiency virus, (2) alcohol abuse, (3) the presence of nonalcoholic steatohepatitis, (4) the presence of HCC at entry, (5) interval between paired biopsies <1.5 years, and (6) length of biopsy sample <15 mm. The relationship between HCC development and the FIB-4 index at liver biopsy or change in the FIB-4 index between the two liver biopsies was investigated. To determine the optimal cut-off values of change in the FIB-4 index for prediction of HCC development, patients with HCC development within 10 years were considered. Time zero was set at the date of the second biopsy.

Single liver biopsy group (validation group)

A total of 1,377 patients received interferon therapy after liver biopsy at Musashino Red Cross Hospital between 1991 and 2010 and were followed-up for ≥1 year after treatment. Of those in follow-up, 875 patients who exhibited non-sustained virological response were included in the validation group. Exclusion criteria were the same as those for the paired biopsy group. Because these patients did not undergo a second liver biopsy, change in the FIB-4 index was calculated between the liver biopsy and 1, 2 and 3 years after the end of interferon therapy. The relationship between HCC development and the FIB-4 index at liver biopsy or change in the FIB-4 index was investigated. Time zero was set at the date of liver biopsy.

Ethical approval

Written informed consent was obtained from each patient in the paired biopsy group and in the validation group, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Histological evaluation

Liver biopsy specimens were obtained using 13G needles laparoscopically, or by percutaneous ultrasound-guided liver biopsy using 15G needles. Specimens were fixed, paraffin-embedded, and stained with hematoxylin–eosin and Masson's trichrome. A minimum 15 mm biopsy sample was required for diagnosis. All liver biopsy samples were independently evaluated by two senior pathologists who were blinded to the clinical data. Fibrosis staging was categorized according to the METAVIR score: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis [22]. When staging was inconsistent between the two pathologists, an appropriate stage was determined by discussion between the two. Fibrosis progression was defined as a 1 point or more increase in the METAVIR score, and fibrosis non-progression was defined as no change or a 1 point or more decrease in the METAVIR score.

HCC surveillance and diagnosis

Ultrasonography and a blood test including tumor markers were performed every 3–6 months for HCC surveillance. When tumor marker levels showed an abnormal rise and/or

abdominal ultrasonography suggesting a lesion suspicious for HCC, contrast-enhanced computed tomography, magnetic resonance imaging or angiography were performed. HCC was diagnosed for tumors showing vascular enhancement at an early phase with washout at a later phase. Tumor biopsy was used to diagnose tumors with non-typical imaging findings.

Clinical and biological data

The age and gender of the patients were recorded. Serum samples were collected within 1 month prior to the liver biopsy. The following variables were obtained by analyzing the serum samples: AST, ALT, and platelet count. The FIB-4 index was calculated according to the following formula: FIB-4 index = age [years] × AST [IU/L]/(platelets [10^9/L] × ALT [IU/L]$^{1/2}$). Cutoff value of the FIB-4 index was set at 3.25 according to the previously established value for the prediction of advanced fibrosis [17]. Change in the FIB-4 index per year (ΔFIB-4 index/year) in the paired biopsy group was calculated by the following formula: ΔFIB-4 index/year = (the FIB-4 index at the second liver biopsy − the FIB-4 index at the first liver biopsy)/the interval between paired biopsies (years). ΔFIB-4 index/year in the validation group was calculated similarly between the liver biopsy and 1, 2, and 3 years after the end of interferon therapy. Change in AST, ALT, platelets per year (ΔAST/year, ΔALT/year, ΔPlatelets/year) were calculated similarly.

Statistical analysis

Categorical data were compared using the Chi-square and Fisher's exact test. Distributions of continuous variables were analyzed using the Student's t test or the Mann–Whitney U test. A p value of <0.05 was considered statistically significant. The cumulative incidence curve was determined by the Kaplan–Meier method and differences among groups were assessed using a log-rank test. Receiver operating characteristic (ROC) curves were constructed, and the area under the ROC curve (AUROC) was calculated. Optimal cut-off values were selected using Youden's index. Factors associated with HCC risk were determined by the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 15.0 (SPSS Inc., Chicago, IL, USA)

Results

Patient characteristics

Table 1 shows the characteristics of patients in the paired biopsy group and validation group. There were no significant differences in the FIB-4 index between the two groups. Mean follow-up periods were 6.4 years in the paired biopsy group and 5.9 years in the validation group, respectively. HCC developed in 30 patients (14 %) in the

Table 1 Patient characteristics

	Paired biopsy group		Validation group	p value*	p value**
	First biopsy	Second biopsy			
Patients (n)	171		875		
Age (SD) (years)	56.1 (8.5)	60.8 (8.1)	58.0 (10.3)	0.02	<0.001
Gender [n (%)]					
Female	95 (56)		493 (56)		
Male	76 (44)		382 (44)	0.85	
Fibrosis stage [n (%)]					
F0–1	67 (39)	57 (33)	388 (44)		
F2	57 (34)	60 (35)	269 (31)		
F3	43 (25)	43 (25)	186 (21)		
F4	4 (2)	11 (7)	32 (4)	0.41	0.04
AST (SD) (IU/L)	68.3 (38.2)	60.3 (38.7)	62.9 (35.4)	0.08	0.51
ALT (SD) (IU/L)	90.8 (63.2)	68.3 (54.0)	77.9 (52.7)	0.008	0.06
Platelets (SD) (10^9/L)	159 (48)	153 (51)	157 (50)	0.71	0.28
FIB-4 index	2.90 (1.6)	3.38 (1.9)	3.20 (2.1)	0.08	0.28
Interferon response (relapse/no response/ND)		71/100	335/366/174		0.15
HCC development [n (%)]		30 (14)	89 (10)		0.01
Follow-up period (SD) (years)		6.4 (2.7)	5.9 (2.8)		0.04

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ND* not determined

* Comparison between paired biopsy group at first biopsy and validation group

** Comparison between paired biopsy group at second biopsy and validation group

paired biopsy group and 89 patients (10 %) in the validation group during the follow-up.

Prediction of HCC development by a single-point assessment in the paired biopsy group

The incidence of HCC development was compared between patients with F0–2 and F3–4 at the second liver biopsy. The 3-year, 5-year, and 7-year cumulative incidence of HCC was 13.3, 26.6, and 39.4 %, respectively, in patients with F3–4, which was significantly higher than those with F0–2 (1.7, 4.9, and 7.3 %, respectively; $p < 0.001$, Fig. 1a). Similarly, using the FIB-4 index at the second biopsy, the 3-year, 5-year, and 7-year cumulative incidence of HCC after interferon therapy was 1.0, 5.5 and 6.9 %, respectively, in patients with a FIB-4 index ≤ 3.25, whereas it was 11.9, 20.9, and 32.0 %, respectively, in those with a FIB-4 index >3.25 ($p < 0.001$, Fig. 1b).

Prediction of HCC development by time-course changes in FIB-4 index in the paired biopsy group

HCC development was compared with time-course changes in the fibrosis stage from repeated liver biopsies. For this analysis, 4 patients who were diagnosed as having cirrhosis at the first liver biopsy were excluded. The cumulative incidence of HCC was not significantly different between patients with fibrosis progression and those without (Fig. 1c). In contrast, when time-course changes in the FIB-4 index (ΔFIB-4/year) were considered, HCC developed more frequently in patients with large time-course changes in the FIB-4 index. Of 30 patients with HCC development, 28 patients developed HCC within 10 years. The AUROC of the ΔFIB-4 index/year for prediction of HCC development within 10 years was 0.61. Using a cut-off value for a ΔFIB-4/year of 0.3, the sensitivity and specificity for the prediction of HCC

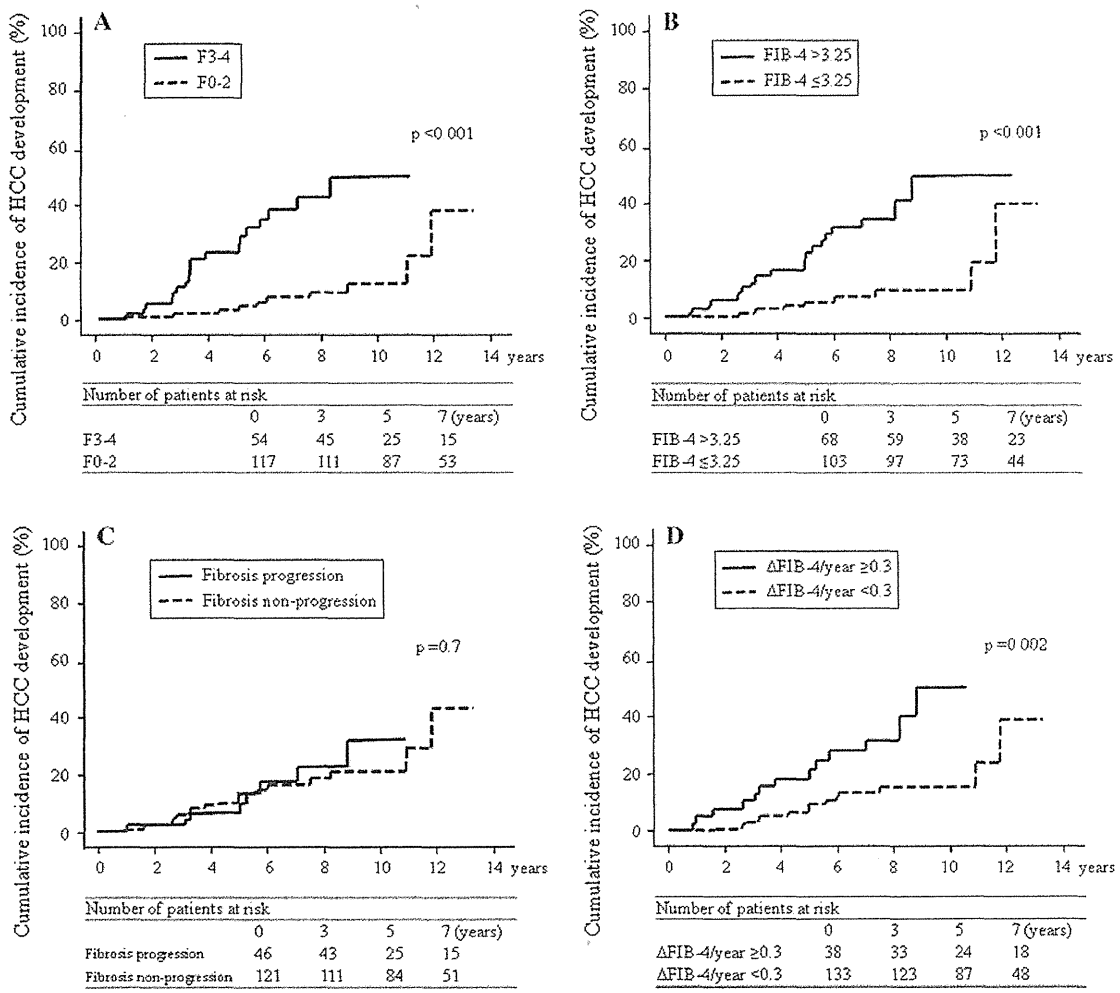


Fig. 1 Cumulative incidence of HCC development in the paired biopsy group. Patients were categorized into two groups according to **a** fibrosis stage, **b** FIB-4 index, **c** time-course change in fibrosis stage, and **d** time-course change in the FIB-4 index (ΔFIB-4/year)

development was 46 and 82 %, and the 3-year, 5-year and 7-year cumulative incidence of HCC was 13.2, 21.9, and 28.4 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas it was 3.1, 9.6, and 13.1 % in those with a ΔFIB-4/year <0.3 ($p = 0.002$, Fig. 1d).

Validation by independent patients

The cumulative HCC incidence rate was similarly examined in the validation group using the FIB-4 index at the time of biopsy. In the group with a FIB-4 index >3.25, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 3.9, 11.2, and 22.0 %, respectively, whereas, in the group with a FIB-4 index ≤3.25, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 0.6, 4.0, and 6.4 %, respectively. The rate was significantly higher ($p < 0.001$) in the group with a FIB-4 index >3.25.

Time-course changes in the FIB-4 index and HCC incidence were examined with the cut-off value of a ΔFIB-4/year at 0.3. Cumulative incidence of HCC development was examined using the ΔFIB-4/year calculated by using data at 1 year after the interferon therapy. The 3-year, 5-year, and 7-year cumulative incidences of HCC were 2.8, 15.0, and 20.1 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas they were 1.4, 4.3, and 9.8 % in patients with a ΔFIB-4/year <0.3. The cumulative incidence rate was significantly higher ($p = 0.008$) in the group with a ΔFIB-4/year ≥0.3. Similarly, using the ΔFIB-4/year calculated by using data at 2 years after the interferon therapy, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 3.3, 11.1, and 16.9 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas they were 1.4, 5.3, and 10.7 % in patients with a ΔFIB-4/year <0.3 ($p = 0.04$). Using the ΔFIB-4/year calculated by using data at 3 years after the interferon therapy, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 4.4, 9.7, and 17.1 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas they were 1.4, 5.0 and 10.4 % in patients with a ΔFIB-4/year <0.3 ($p = 0.005$). Because similar results were obtained by the ΔFIB-4/year at 1, 2, and 3 years after the interferon therapy, the ΔFIB-4/year calculated by using data at 1 year after the interferon therapy was used for subsequent analysis.

Factors associated with HCC development

Univariate analysis demonstrated factors that increase the hazard ratio (HR) for the development of HCC (Table 2). In the paired biopsy group for advanced liver fibrosis detected by liver biopsy, a high FIB-4 index level, and a ΔFIB-4/year ≥0.3 were risk factors for HCC development. Compared with patients with a FIB-4 index ≤3.25, the HR of those with a FIB-4 index >3.25 was 4.8 [95 % confidence interval (CI) 2.0–10.7, $p < 0.001$]. In terms of change in fibrosis stage, there was no significant difference between the progression and non-progression groups. In contrast, in terms of change in the FIB-4 index, compared with patients with a ΔFIB-4/year <0.3, the HR of those with a ΔFIB-4/year ≥0.3 was 3.1 (95 % CI 1.3–5.7, $p = 0.002$). Similar results were obtained in the validation group; a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 were risk factors for HCC development (Table 2). These two groups of patients were combined and univariate and multivariate analysis were performed (Table 3). Because AST, ALT, platelets and age are contained in the FIB-4 index, these factors were excluded in the multivariate analysis. Multivariate analysis revealed that gender, fibrosis stage, the FIB-4 index and ΔFIB-4/year were independent factors associated with HCC development. The HR of HCC development with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 was 2.7 (95 % CI 1.7–4.2, $p < 0.001$) and 1.8 (95 % CI 1.2–2.6, $p = 0.003$), respectively. ΔAST/year, ΔALT/year, and ΔPlatelets/year were not associated with HCC development.

Evaluation of HCC risk by a combining the FIB-4 index and ΔFIB-4/year in the whole group

Multivariate analysis demonstrated that a high FIB-4 index level by single-point assessment and a time-course increase in the FIB-4 index were independent risk factors for HCC development. Their combined risk was examined in four groups, with the cut-off values of a FIB-4 index at 3.25 and a ΔFIB-4/year at 0.3. Patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year <0.3 were defined as the low risk group. Patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year ≥0.3 were defined as the intermediate risk-1 group. Similarly, patients with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 were defined as the high risk group. Patients with a FIB-4 index >3.25 and a ΔFIB-4/year <0.3 were defined as the intermediate risk-2 group. The 3-year, 5-year, and 7-year cumulative incidence of HCC in patients within the high risk group was 7.6, 21.0, and 30.0 %. Similarly, the 3-year, 5-year, and 7-year cumulative incidence of HCC was 4.1, 9.6, and 21.1 % in patients within the intermediate risk-2 group. It was 0.8, 10.1, and 13.8 % in the patients within the intermediate risk-1 group and 0.6, 2.9, and 4.8 % in patients within the low risk group ($p < 0.001$, Fig. 2). The HR of HCC development in patients at high risk was 7.3 (95 % CI 4.3–12.5, $p < 0.001$, Table 4). Sensitivity of prediction for HCC development by the liver biopsy and the FIB-4 index was 58 and 61 %, respectively. The combined risk classification by the FIB-4 index and the ΔFIB-4/year had higher sensitivity (72 %, Table 5).

Table 2 Factors associated with HCC development in the paired biopsy group and the validation group

Risk factor value	Paired biopsy group		Validation group	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Risk factor at baseline[a]				
Age (by every 10 years)	1.3 (0.8–2.1)	0.3	1.9 (1.5–2.5)	<0.001
Gender				
Female	1		1	
Male	1.5 (0.7–3.2)	0.2	1.5 (0.9–2.2)	0.06
Fibrosis stage				
F0/F1/F2	1		1	
F3/F4	5.4 (2.5–11.3)	<0.001	4.7 (3.1–7.1)	<0.001
AST (by every 1× ULN)	1.3 (1.1–1.5)	0.01	1.4 (1.1–1.7)	<0.001
ALT (by every 1× ULN)	1.1 (1.0–1.3)	0.04	1.1 (0.9–1.2)	0.1
Platelets (10^9/L)				
≥150	1		1	
<150	3.0 (1.3–6.5)	0.006	2.6 (1.6–4.5)	<0.001
FIB-4 index				
≤3.25	1		1	
>3.25	4.8 (2.2–10.7)	<0.001	3.8 (2.4–5.8)	<0.001
Change of risk factor				
ΔAST/year (IU/L)				
<0	1		1	
≥0	0.9 (0.4–1.9)	0.8	1.4 (0.8–2.4)	0.2
ΔALT/year (IU/L)				
<0	1		1	
≥0	0.8 (0.4–1.8)	0.6	0.8 (0.4–1.6)	0.6
ΔPlatelets/year (10^9/L)				
>−0.5	1		1	
≤−0.5	2.4 (1.1–5.0)	0.01	0.7 (0.4–1.3)	0.3
ΔFIB-4/year				
<0.3	1		1	
≥0.3	3.1 (1.5–6.6)	0.002	1.8 (1.2–2.9)	0.008
Fibrosis stage change				
Non-progression	1			
Progression	1.2 (0.5–2.7)	0.7		

AST aspartate aminotransferase, *ALT* alanine aminotransferase

[a] Data at the second biopsy was used for the paired biopsy group

Discussion

Recently, non-invasive methods substituting liver biopsy for the diagnoses of liver fibrosis have been developed. It has been elucidated that non-invasive liver fibrosis markers are related to HCC development and mortality [23–25]. In addition, it was reported that after interferon therapy for chronic hepatitis C, some non-invasive liver fibrosis markers correlated with HCC development [26]. However, it remains unclear whether time-course changes in these markers correlate with HCC development and mortality.

Previously, we reported that time-course changes in the FIB-4 index correlated with liver fibrosis progression [20]. Because the FIB-4 index correlates with liver fibrosis, a risk factor for HCC development, and it considers age, another risk factor, it was presumed that the index could be closely correlated with HCC development. In this study, the significance of the FIB-4 index and time-course changes in the FIB-4 index were investigated in relation to HCC development.

The most important finding in this study was that it was possible to predict HCC development by time-course changes in the FIB-4 index. The cumulative HCC incidence rate was lower in patients with a ΔFIB-4/year <0.3 compared with those with a ΔFIB-4/year ≥0.3. It has been reported that a high level of AST and ALT levels correlate with progression of liver fibrosis, and improved levels prevent HCC development [27–29]. It is also known that liver fibrosis progression and the risk of HCC development is increased with a decrease in platelet count [30].

Table 3 Factors associated with HCC development in the combined population

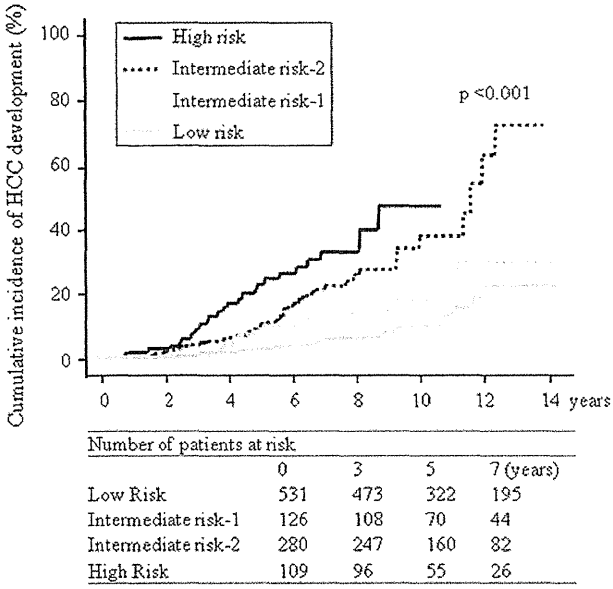
Risk factor value	Univariate		Multivariate	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Risk factor at baseline				
Age (by every 10 years)	1.8 (1.4–2.3)	<0.001		
Gender				
Female	1		1	
Male	1.5 (1.0–2.1)	0.03	2.0 (1.4–2.9)	<0.001
Fibrosis stage				
F0/F1/F2	1		1	
F3/F4	4.9 (3.5–7.2)	<0.001	3.0 (2.0–4.6)	<0.001
AST (by every 1× ULN)	1.4 (1.2–1.6)	<0.001		
ALT (by every 1× ULN)	1.1 (1.0–1.2)	0.04		
Platelets (10^9/L)				
≥150	1			
<150	2.7 (1.7–4.1)	<0.001		
FIB-4 index				
≤3.25	1		1	
>3.25	4.0 (2.8–5.9)	<0.001	2.7 (1.7–4.2)	<0.001
Change of risk factor				
ΔAST/year (IU/L)				
<0	1			
≥0	1.3 (0.8–2.1)	0.2		
ΔALT/year (IU/L)				
<0	1			
≥0	0.9 (0.6–1.6)	0.8		
ΔPlatelets/year (10^9/L)				
>−0.5	1			
≤−0.5	1.0 (0.6–1.6)	0.8		
ΔFIB-4/year				
<0.3	1		1	
≥0.3	2.1 (1.4–3.1)	<0.001	1.8 (1.2–2.6)	0.003

AST aspartate aminotransferase, *ALT* alanine aminotransferase

However, time-course changes of AST, ALT, and platelet count were not significantly associated with HCC development in this study. On the other hand, the FIB-4 index, which considers these factors together; had its time-course changes useful for real-time monitoring of disease progression. As the disease advances, the FIB-4 index deteriorates and the risk of HCC development increases.

One advantage of the FIB-4 index is the feasibility of repeated measurements for evaluating disease status. Needless to say, liver biopsy is the gold standard for diagnosis of liver fibrosis and is still important to predict the progression of liver disease. However, there are problems associated with liver biopsies including sampling errors and inter-observer variations [6, 7]. In addition, it is difficult to repeat biopsies, making it challenging to evaluate time-course changes because of the invasiveness of the procedure. In contrast, the FIB-4 index can be calculated using age and general biochemistry results and making it markedly easy to follow up time-course changes. In this study, changes in fibrosis stage between two liver biopsies failed to stratify HCC development. These results suggest that the FIB-4 index, rather than the liver biopsy, was more useful for real-time monitoring of disease advancement.

Correlations of non-invasive liver fibrosis markers including the FIB-4 index with HCC incidence risk have been reported previously [23–26, 31]. A similar result was shown in this study using a single-point assessment of the FIB-4 index. Since the FIB-4 index correlates with liver fibrosis, a high FIB-4 index indicates a high risk for HCC development similar to other liver fibrosis markers. Furthermore, an important fact in this study was that combining the FIB-4 index and time-course changes in the FIB-4 index could stratify patients with high risk of HCC development. A high FIB-4 index level by single-point assessment and a time-course increase in the FIB-4 index were independent risk factors for HCC development. Patients with a low baseline FIB-4 index and a time-course



Number of patients at risk

	0	3	5	7 (years)
Low Risk	531	473	322	195
Intermediate risk-1	126	108	70	44
Intermediate risk-2	280	247	160	82
High Risk	109	96	55	26

Fig. 2 Cumulative incidence of HCC development in the combined population. Patients were categorized into four groups using the FIB-4 index and time-course change in the FIB-4 index (ΔFIB-4/year). Low risk: FIB-4 index ≤3.25 and ΔFIB-4/year <0.3, intermediate risk-1: FIB-4 index ≤3.25 and ΔFIB-4/year ≥0.3, intermediate risk-2: FIB-4 index >3.25 and ΔFIB-4/year <0.3, high risk: FIB-4 index >3.25 and ΔFIB-4/year ≥0.3

Table 4 Evaluation of HCC risk by combining the FIB-4 and the ΔFIB-4/year

	Number of patients	Hazard ratio (95 % CI)	*p* value
Low risk	531	1	
Intermediate risk-1	126	2.1 (1.1–4.0)	0.03
Intermediate risk-2	280	4.1 (2.6–6.5)	<0.001
High risk	109	7.3 (4.3–12.5)	<0.001

Low risk: patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year <0.3

Intermediate risk-1: patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year ≥0.3

Intermediate risk-2: patients with a FIB-4 index >3.25 and a ΔFIB-4/year <0.3

High risk: patients with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3

improvement in the FIB-4 index had a low risk of HCC development, whereas those with a high baseline FIB-4 index and worsening of the FIB-4 index had a markedly high risk for HCC development. In addition to the utility of predicting liver fibrosis and HCC development by single-point assessment, the combination with real-time monitoring enables stratification of a group with a high risk of HCC development, which is a great advantage of the FIB-4 index over a liver biopsy.

With regard to diagnosis capabilities for liver fibrosis, it has been reported that other non-invasive liver fibrosis markers have higher diagnostic capabilities than the FIB-4 index [32, 33]. However, the FIB-4 index has several advantages. Although, it has been reported that transient elastography has high diagnostic capabilities when it comes to liver fibrosis, measurements are sometimes impossible in patients with severe obesity [34]. Reproducibility of transient elastography was reportedly reduced in patients with steatosis, increased body mass index, and lower degrees of liver fibrosis [35]. Moreover, these modalities for measurement of elasticity of the liver using ultrasonography are not widely available, especially in countries where resources are limited. In contrast, the FIB-4 index can be determined by a general blood test, and it can be measured in almost all patients. The parameters required for calculation are only age, AST, ALT, and platelet count, which are measured during the routine examination of patients with liver disease. Therefore, additional blood collection is unnecessary, and the index can be calculated at no extra cost.

Table 5 Sensitivity of prediction for HCC development

	Patients with HCC development	Patients without HCC development
F0–2	50	724
F3–4	69	203
	Sensitivity: 58 %	Specificity: 78 %
FIB-4 index ≤3.25	42	615
FIB-4 index >3.25	77	312
	Sensitivity: 61 %	Specificity: 66 %
Low risk	29	502
Other risk	90	425
	Sensitivity: 72 %	Specificity: 54 %

Other risk: high risk, intermediate risk-2, and intermediate risk-1

In conclusion, it was possible to define a group with a high risk of HCC development by calculating the FIB-4 index and considering time-course changes in the FIB-4 index. Because measurement of the FIB-4 index is simple and easy to repeat, it is useful for non-invasive, real-time monitoring of HCC development.

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Conflict of interest The authors declare that they have no conflict of interest.

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BRIEF COMMUNICATION

Genetic polymorphism in *IFNL4* and response to pegylated interferon-α and ribavirin in Japanese chronic hepatitis C patients

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Key words
hepatitis C virus; interferon-λ 4; IL28B; pegylated interferon

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Abstract

A genetic polymorphism of the newly discovered interferon-λ 4 (*IFNL4*) gene was associated with hepatitis C virus (HCV) clearance in individuals of African ancestry. To assess whether a dinucleotide variant of *IFNL4* (ss469415590) also affected treatment outcome of antiviral therapy in Japan, we genotyped 213 patients with chronic genotype 1 HCV infection and 176 healthy subjects. The ΔG allele was associated with treatment failure [odds ratio (OR) 4.73, $P = 0.019$], as was the IFL3 rs8099917 single nucleotide polymorphism (SNP) (OR 5.06, $P = 0.068$). The correlation between ss469415590 and rs8099917 was high ($r^2 = 0.92$, $D' = 0.98$). Multivariate analysis revealed that the rs8099917 SNP was independently associated with treatment failure (OR 5.28, $P = 0.009$). Therefore, ss469415590 may be another predictive marker of antiviral therapy outcome in the Japanese population.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection often develops into chronic hepatitis, leading to liver cirrhosis and/or hepatocellular carcinoma (HCC) (1, 2). As approximately 70–80% of Japanese HCC patients are infected with HCV (3), the successful eradication of HCV, defined as a sustained virological response (SVR), is considered important to decrease the incidence of HCC. Approximately 50% of Japanese patients with genotype 1 HCV infection do not achieve an SVR by conventional pegylated interferon-α (PEG-IFN) and ribavirin (RBV) therapy. Although the addition of a direct-acting antiviral agent to this regimen has increased response rate (4), reliable markers are needed to better predict treatment outcome.

The strongest genetic factors associated with patient response to PEG-IFN and RBV therapy and spontaneous clearance are single nucleotide polymorphisms (SNPs) around *IFNL3* (*IL28B*) [reviewed in (5)]. In particular, the rs8099917 SNP has been shown as a good predictive marker for the response to PEG-IFN and RBV therapy in Japanese patients (6–8). Prokunina-Olsson et al. (9) recently discovered a new transiently induced region that harbors a dinucleotide variant ss469415590 (TT or ΔG) between *IFNL3* and *IFNL2*. ss469415590 (ΔG) is a frameshift variant that