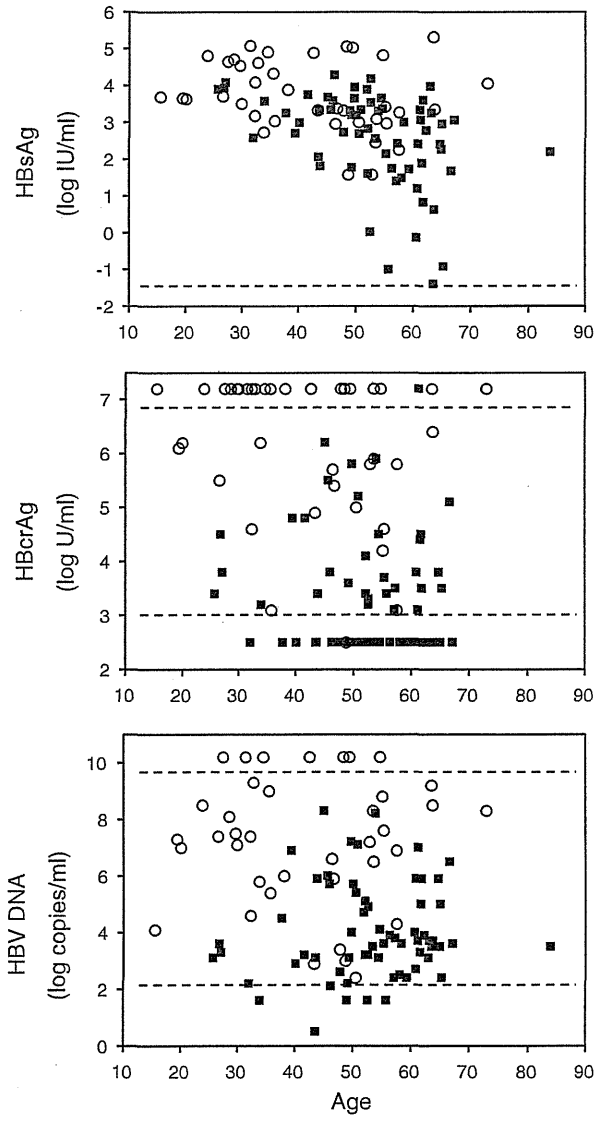

Fig. 3 HBsAg, HBcrAg, and HBV DNA levels analyzed according to HBeAg status and patient age. *Open circles* indicate patients with detectable HBeAg and *closed squares* indicate those without

ages, those in HBeAg-negative patients ($r = -0.103$, $P > 0.2$) were found in a lower range. A similar trend was seen for HBV DNA level distribution ($r = 0.015$, $P > 0.2$ and $r = 0.146$, $P > 0.2$, respectively).

Changes in HBsAg levels during the follow-up period

Positivity for HBsAg decreased gradually over the follow-up period (Fig. 4). A total of 20 patients cleared HBsAg during the follow-up period, for a disappearance rate of 2.1% per year. Clinical and virological backgrounds were compared between patients with and without clearance of HBsAg in Table 3. Patients losing HBsAg positivity were

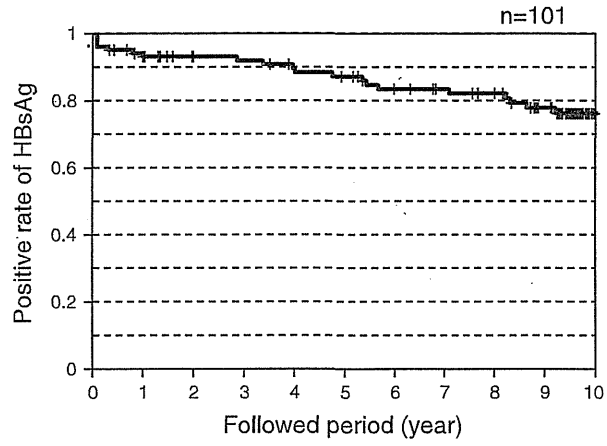

Fig. 4 Changes in HBsAg positivity during the follow-up period

significantly older than those who did not. Baseline levels of HBsAg, HBcrAg, and HBV DNA were significantly lower in these patients as well. Clearance of HBsAg was significantly associated with HBV DNA (HR 3.6, 95% CI 1.1–11.4, $P = 0.033$) and HBcrAg (HR 4.0, 95% CI 1.1–14.9, $P = 0.036$) levels at baseline by multivariate analysis. Of the 20 patients who cleared HBsAg, seven were positive for HBV DNA (range, positive 3.0 log copies/ml) and three were positive for HBcrAg (range 3.0–3.2 U/ml).

Figure 5 shows the distribution of patients according to the rate of change of HBsAg levels. Of the 98 patients analyzed, 79 (81%) showed a decrease in HBsAg. Although this level increased in 19% of patients, such changes were less than 0.2 log IU/year. The rate of change of HBsAg levels peaked at a cut-off value of −0.4 log IU/year. Accordingly, patients were tentatively classified into the rapid decrease group (rate of change <−0.4 log IU/year) and the non-rapid decrease group (rate of change ≥−0.4 log IU/year). Median age, gender distribution, prevalence of cirrhosis, ALT level, and genotype distribution did not differ between the two groups (Table 4). Levels of HBsAg, HBcrAg, and HBV DNA were significantly lower in the rapid decrease group than in the non-rapid one. Whereas all patients with persistently positive HBeAg were classified into the non-rapid group, patients with persistently negative HBeAg fell more frequently into the rapid decrease group (77%) than into the non-rapid decrease group (54%). In those patients, HBV DNA levels were significantly ($P = 0.028$) lower in the rapid decrease group (median 3.4, range <2.1–5.9 log copies/ml) than in the non-rapid decrease group (median 3.8, range <2.1–8.1 log copies/ml). Complicating HCC was lower in the rapid decrease group, but this difference was not statistically significant.

The median change in HBsAg level before NA treatment (−0.117 log IU/ml/year; range −2.4 to 1.41 log

Table 3 Comparison of clinical and virological characteristics between patients with and without clearance of HBsAg

Characteristic	Clearance of HBsAg		*P*
	Positive ($n = 20$)	Negative ($n = 81$)	
At baseline			
Age (years)[a]	56 (30 to 65)	50 (16 to 84)	0.038
Male[b]	8 (40%)	36 (44%)	>0.2
With cirrhosis[b]	4 (20%)	15 (18%)	1.000
ALT (IU/L)[a]	26 (10 to 108)	35 (13 to 447)	0.057
HBV genotype (A:B:C:UD)	0:2:18:0	3:7:69:2	>0.2
HBeAg[b]	3 (15%)	35 (43%)	0.022
HBsAg (log IU/ml)[a]	1.7 (−1.7 to 4.2)	3.3 (0.83 to 5.3)	<0.001
HBcrAg (log U/ml)[a]	3.0 (3.0 to >6.8)	4.7 (3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)[a]	3.0 (<1.7 to 7.6)	5.7 (neg. to >9.5)	<0.001
During follow-up			
Followed period (years)[a]	4.4 (0.31 to 10.0)	5.2 (0.1 to 10.0)	>0.2
Occurrence of HCC[b]	1 (5.0%)	13 (16.0%)	>0.2
Introduction of NAs[b]	0 (0%)	23 (28%)	0.006

UD undetermined

[a] Data are expressed as median (range)

[b] Data are expressed as positive number (%)

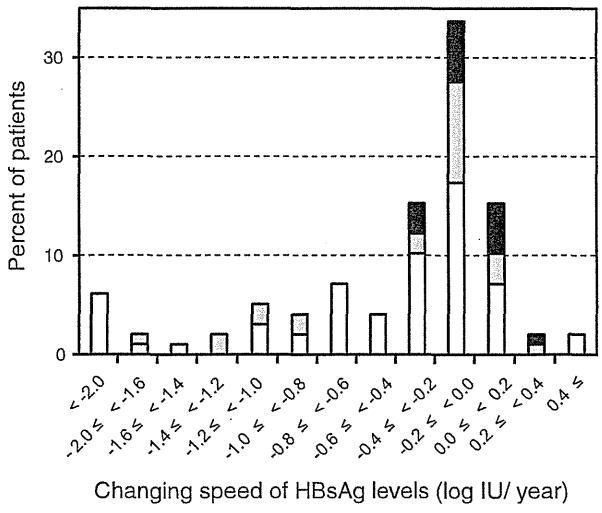

Fig. 5 Distribution of patients classified according to rate of change of HBsAg levels (log IU/year) during follow-up period. *Closed bars* indicate patients with persistent HBeAg-positive status. *Shaded bars* indicate patients who became negative for HBeAg during follow-up period. *Open bars* indicate patients with persistent HBeAg-negative status

IU/ml/year) was similar ($P > 0.2$) to that after starting NA treatment (−0.017 log IU/ml/year; range −5.18 to 0.17 log IU/ml/year) in the 20 patients who commenced therapy with NAs during the study period.

Discussion

During the natural course of HBV infection, HBsAg levels showed almost normal distribution, making a sharp peak at a median value of 3.2 log IU/ml. Lower HBsAg levels were significantly associated with older age and lower viral activity, but not with gender or genotype. A similar trend was observed in patients who cleared HBsAg in our cohort. Chan et al. [10] reported that HBsAg levels were significantly lower in HBeAg-negative patients than in HBeAg-positive ones and tended to fall in accordance with decreases in HBV DNA levels. Simonetti et al. [6] reported that clearance of HBsAg was associated with older age, but not with gender or genotype, in a prospective population-based cohort study. Chu et al. [9] also reported that HBsAg clearance was associated with older age, in which the cumulative probability of clearance increased disproportionally with a longer follow-up period. In light of these results as well as of our own, it appears that lower HBsAg levels are closely associated with older age and lower activity of HBV replication. The HBsAg clearance rate of 2.1% per year in the current study was three times higher than that of the 0.7% per year reported by Simonetti et al. [6]. However, the median age at the start of their follow-up (20 years) was considerably lower than that in our report (50 years). Chu et al. [9] followed 1965 asymptomatic HBV carriers that were positive for HBe antibodies in whom the mean age at baseline was 35.6 years, revealing a HBsAg clearance rate of 0.8% per year after 10 years of follow-up that increased to 1.8% per year over a 25-year observation period. HBsAg clearance appeared to increase as patients aged in that cohort, which may at least partly explain the higher clearance rate found in the present study.

Because HBsAg level is closely associated with age, we analyzed this relationship and compared it with those of HBcrAg and HBV DNA. HBsAg levels decreased in association with age in HBeAg-negative patients. A similar but faint association was also seen in HBeAg-positive patients. On the other hand, HBcrAg and HBV DNA levels

Table 4 Comparison of clinical and virological characteristics between patients with rapid and non-rapid decrease of HBsAg

Characteristic	Rapid decrease ($n = 31$)	Non-rapid decrease ($n = 67$)	P
At baseline			
Age (years)[a]	52 (15 to 65)	49 (19 to 83)	0.338
Male[b]	17 (55%)	38 (57%)	1.000
With cirrhosis[b]	6 (19%)	13 (19%)	1.000
ALT (IU/L)[a]	27 (10 to 108)	36 (13 to 447)	0.230
HBV genotype (A:B:C:UD)	1:4:26:0	2:4:59:2	0.617
HBeAg-positive[b]	7 (23%)	31 (46%)	0.028
HBsAg (log IU/ml)[a]	2.8 (−1.0 to 5.0)	3.3 (0.8 to 5.3)	0.001
HBcrAg (log U/ml)[a]	<3.0 (<3.0 to >6.8)	5.1 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)[a]	3.7 (<1.7 to >9.5)	5.9 (neg. to >9.5)	0.002
During follow-up			
Followed period (years)[a]	3 (1 to 9)	6 (1 to 10)	<0.001
Change in HBeAg status			0.012
Persistent positive[b]	0 (0%)	15 (22%)	
Became negative[b]	7 (23%)	16 (24%)	
Persistent negative[b]	24 (77%)	36 (54%)	
Clearance of HBsAg[b]	18 (58%)	0 (0%)	<0.001
Complication of HCC[b]	2 (7%)	12 (18%)	0.214
Introduction of NAs[b]	4 (13%)	19 (28%)	0.125

UD undetermined

[a] Data are expressed as median (range)

[b] Data are expressed as positive number (%)

were more uniformly distributed with age in both HBeAg-positive and -negative patients. Therefore, it can be inferred that HBsAg level is affected by age in the natural course of HBV, even when the factor of viral activity is excluded. The precise mechanism of this trend is at present unclear, but may be attributed to the character of HBsAg itself, and not to that of HBV antigens, because HBcrAg levels showed a similar trend as HBV DNA levels. Chan et al. [10] reported that a stronger correlation between HBV DNA and HBsAg was found in the HBeAg-positive phase than in the HBeAg-negative phase. This observation was clearly confirmed by our results in that the distribution pattern analyzed by age was similar between HBsAg and HBV DNA levels in HBeAg-positive patients but differed in HBeAg-negative ones.

The rate of change of HBsAg in the present study suggested the existence of two groups centered around a value of −0.4 log IU/year. A necessary decline in HBV replication was evident in the rapid decrease group, whose median HBV DNA level was lower than the 4.0 log copy/ml usually seen in inactive carriers of HBV. Since no patient with persistently positive HBeAg was classified into the rapid increase group, we presume that a loss of HBeAg is essential for a rapid decrease in HBsAg. In patients with persistently negative HBeAg, HBV DNA levels were significantly lower in the rapid decrease group than in the non-rapid decrease group. Therefore, not only a loss of HBeAg, but also a decline in HBV replication, appears to be fundamental factors necessary for a rapid decrease in HBsAg. Chan et al. [10] concluded that HBs antigen level remained stable in HBe antigen-positive patients and reduced slowly in HBe antigen-negative patients. Our results are similar, but further imply that a decline in HBV replication is also required. The rate of HBsAg level decrease was similar before and after starting NA treatment in the present study. However, additional studies in larger cohorts will be required to determine this particular relationship.

We analyzed HBcrAg in addition to HBsAg as an HBV-related antigen in the present study to further clarify the characteristics of HBsAg. The HBcrAg assay measures serum levels of HBcAg, HBeAg, and the 22 kDa precore protein [12] simultaneously using monoclonal antibodies that recognize the common epitopes of these denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related [14]. It is possible that levels of HBsAg and HBcrAg have different properties because transcriptions of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome [15]. Recent studies have shown that HBsAg quantification may represent a surrogate marker of cccDNA concentration in the liver and a potential tool to monitor virologic response to interferon treatment [4, 5, 16]. On the other hand, serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during nucleos(t)ide treatment [11, 17, 18], and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs [19, 20] or who had a higher possibility to develop hepatocellular carcinoma even under NA treatment [17]. Our results here suggest that there exists a

difference in natural course changes between HBsAg and HBcrAg levels. We recently reported that the combined use of these two antigens was useful for predicting the occurrence of hepatitis relapse after cessation of NAs [21]. Such results also indicated that levels of HBsAg and HBcrAg had different clinical significance despite the fact that both antigen levels are generally considered to reflect the amount of HBV cccDNA in hepatocytes.

Complicating HCC occurred during the first 6 years of follow-up in our study at an annual occurrence rate of 2.3% per year for that period. This complication was seen at similar frequencies in patients with high and low baseline HBsAg levels as well as in patients who showed rapid and non-rapid decreases in HBsAg. Patients with lower HBsAg levels and those with rapid decreases in HBsAg have been shown to have lower levels of HBV replication, which would indicate a lower risk of complicating HCC. However, such patients also tend to be older and presumably more predisposed to HCC. The similar occurrence of HCC irrespective of HBsAg status may be attributed to the existence of these two contrary factors. Yuen et al. [7] reported that the risk of HCC in patients with HBsAg seroclearance was higher in those older than 50 years of age; indeed, the single patient who developed HCC after HBsAg seroclearance in the present study was a 90 year-old woman.

In conclusion, lower HBsAg levels were significantly associated with older age and lower viral activity, but not with gender or genotype. Both a loss of HBeAg positivity and a decline in HBV replication are suggested to be fundamental factors necessary for a rapid decrease in HBsAg. Furthermore, the clinical significance of HBsAg may be different from that of HBcrAg with regard to age. Future studies are required to clarify the difference between the two antigens.

Acknowledgments This study was supported in part by a research grant on hepatitis from the Japanese Ministry of Health, Labor, and Welfare of Japan. We thank Ms. Hiroe Banno for her secretarial assistance and thank Ms. Nozomi Kamijo and Ms. Etsuko Iigahama for their technical assistance. We also thank Mr. Trevor Ralph for his English editorial assistance.

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

References

1. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat. 2004;11:97–107.
2. Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. Hepatology. 2007;45:1056–75.
3. Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology. 2007;45:507–39.
4. Brunetto MR, Moriconi F, Bonino F, Lau GK, Farci P, Yurdaydin C, et al. Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. Hepatology. 2009;49:1141–50.
5. Moucari R, Mackiewicz V, Lada O, Ripault MP, Castelnau C, Martinot-Peignoux M, et al. Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. Hepatology. 2009;49:1151–7.
6. Simonetti J, Bulkow L, McMahon BJ, Homan C, Snowball M, Negus S, et al. Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma in a cohort chronically infected with hepatitis B virus. Hepatology. 2010;51:1531–7.
7. Yuen MF, Wong DK, Fung J, Ip P, But D, Hung I, et al. HBsAg Seroclearance in chronic hepatitis B in Asian patients: replicative level and risk of hepatocellular carcinoma. Gastroenterology. 2008;135:1192–9.
8. Tseng TC, Kao JH. HBsAg seroclearance: the more and earlier, the better. Gastroenterology. 2009;136:1842–3. author reply 3–4.
9. Chu CM, Liaw YF. HBsAg seroclearance in asymptomatic carriers of high endemic areas: appreciably high rates during a long-term follow-up. Hepatology. 2007;45:1187–92.
10. Chan HL, Wong VW, Wong GL, Tse CH, Chan HY, Sung JJ. A longitudinal study on the natural history of serum hepatitis B surface antigen changes in chronic hepatitis B. Hepatology. 2010;52:1232–41.
11. Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J Med Virol. 2009;81:27–33.
12. Kimura T, Ohno N, Terada N, Rokuhara A, Matsumoto A, Yagi S, et al. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J Biol Chem. 2005;280:21713–9.
13. Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett. 1999;450:66–71.
14. Tanaka E, Matsumoto A, Yoshizawa K, Maki N. Hepatitis B core-related antigen assay is useful for monitoring the antiviral effects of nucleoside analogue therapy. Intervirology. 2008;51(Suppl 1):3–6.
15. Lee WM. Hepatitis B virus infection. N Engl J Med. 1997;337:1733–45.
16. Chan HL, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, et al. Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. Clin Gastroenterol Hepatol. 2007;5:1462–8.
17. Hosaka T, Suzuki F, Kobayashi M, Hirakawa M, Kawamura Y, Yatsuji H, et al. HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy. Liver Int. 2010;30:1461–70.
18. Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J Clin Microbiol. 2007;45:3942–7.
19. Matsumoto A, Tanaka E, Minami M, Okanoue T, Yatsuhashi H, Nagaoka S, et al. Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy. Hepatol Res. 2007;37:661–6.
20. Shinkai N, Tanaka Y, Orito E, Ito K, Ohno T, Hirashima N, et al. Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. Hepatol Res. 2006;36:272–6.
21. Matsumoto A, Tanaka E, Suzuki Y, Kobayashi M, Tanaka Y, Shinkai N, et al. Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogues in patients with chronic hepatitis B. Hepatol Res. 2012;42:139–49.

Ann Surg Oncol (2012) 19:418–425
DOI 10.1245/s10434-011-1866-1

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Impact of Pegylated Interferon Therapy on Outcomes of Patients with Hepatitis C Virus-Related Hepatocellular Carcinoma After Curative Hepatic Resection

Yoshisato Tanimoto, MD[1], Hirotaka Tashiro, MD[1], Hiroshi Aikata, MD[2], Hironobu Amano, MD[1], Akihiko Oshita, MD[1], Tsuyoshi Kobayashi, MD[1], Shintaro Kuroda, MD[1], Hirofumi Tazawa, MD[1], Shoichi Takahashi, MD[2], Toshiyuki Itamoto, MD[3], Kazuaki Chayama, MD[2], and Hideki Ohdan, MD[1]

[1]Department of Gastroenterological Surgery, Hiroshima University Hospital, Hiroshima, Japan; [2]Department of Gastroenterology, Hiroshima University Hospital, Hiroshima, Japan; [3]Department of Surgery, Prefectural Hiroshima Hospital, Hiroshima, Japan

ABSTRACT

Background. Several published reports investigating the effects of interferon (IFN) therapy on survival and tumor recurrence after curative resection of hepatocellular carcinoma (HCC) have been inconclusive. The aim of this study is to investigate the efficacy of pegylated-IFN (peg-IFN) therapy after curative hepatic resection for HCC in patients infected with hepatitis C virus (HCV).

Methods. Data from 175 patients who underwent curative hepatic resection for HCC associated with HCV were retrospectively collected and analyzed; 75 patients received peg-IFN therapy after surgery, whereas 100 patients did not receive IFN therapy. To overcome biases resulting from the different distribution of covariates in the two groups, a one-to-one match was created using propensity score analysis. After matching, patient outcomes were analyzed.

Results. After one-to-one matching, patients ($n = 38$) who received peg-IFN therapy after surgery and patients ($n = 38$) who did not receive IFN therapy had the same preoperative and operative characteristics. The 3- and 5-year overall survival rates of patients who received peg-IFN therapy after hepatic resection were significantly higher than those of patients who did not receive IFN therapy ($P = 0.00135$). The 3- and 5-year overall survival rates were 100 and 91.7% and 76.6 and 50.6% in the peg-IFN group and non-IFN group, respectively. There was no significant difference in disease-free survival between the two matched groups ($P = 0.886$).

Conclusion. Peg-IFN therapy may be effective as an adjuvant chemopreventive agent after hepatic resection in patients with HCV-related HCC.

First Received: 14 February 2011;
Published Online: 28 June 2011

H. Tashiro, MD
e-mail: htashiro@hiroshima-u.ac.jp

Hepatic resection is a well-accepted therapy for hepatocellular carcinoma (HCC), but many patients show cancer recurrence and the cumulative 5-year HCC recurrence rate exceeds 70%.[1–3] This high incidence of tumor recurrence after hepatic resection remains a major drawback. Some benefits of interferon (IFN) therapy on tumor recurrence and survival have been reported.[4–10] IFN suppresses replication of hepatitis C virus (HCV) and exerts a tumoricidal effect on a number of tumors, including HCC.[10,11] However, several randomized controlled trials (RCTs) have revealed inconclusive results regarding the effects of IFN on survival and tumor recurrence after curative resection or ablation of HCC, either because the effects were not statistically significant or because they were considered only with respect to defined subpopulations.[12–15]

Recently, combination therapy consisting of pegylated interferon (peg-IFN) plus ribavirin (RBV) has been developed, and the effect of this combination has been reported to be higher than that of conventional IFN therapy.[16,17] Peg-IFN has an extended serum half-life that provides viral suppression for 7 days, thus allowing weekly administration and enhanced clinical efficacy.[17] Most Japanese patients infected with HCV are infected with HCV genotype Ib and have high viral load. Moreover, treatment with conventional IFN is complicated by a low sustained viral response (SVR) rate of 20–30%.[18–20]

However, peg-IFN plus RBV combination therapy has good tolerability in Japanese patients with HCV and resulted in an SVR rate of approximately 40–50%.[21–23] The impact of adjuvant immunotherapy with IFN after curative resection of HCC is debatable, and few studies have investigated the effects of peg-IFN plus RBV combination therapy on survival and recurrence after curative resection of HCC.

In the present study, we aim to investigate the impact of peg-IFN plus RBV combination therapy on survival and HCC recurrence after curative resection in patients infected with HCV.

PATIENTS AND METHODS

Patients and HCV Diagnosis

From June 2003 to June 2009, 370 HCC patients underwent hepatectomy as initial treatment at the Department of Gastroenterological Surgery, Hiroshima University Hospital, Japan. Of the 370 patients, 175 patients who were HCV RNA-positive/hepatitis B surface antigen-negative underwent curative hepatectomy. Of the 175 patients, 75 patients received IFN therapy after hepatectomy, and 100 patients did not receive any IFN therapy. Of the 75 patients who received IFN, 20 patients who received IFNs such as IFN-α or IFN-β were excluded. Of the 55 patients who received peg-IFN therapy, 43 patients who started peg-IFN within 9 months after curative resection were enrolled in this analysis. Twenty-four patients who had early recurrence of HCC within 9 months after surgery were excluded from the 100 patients who did not receive any IFN therapy, because these patients could lose the opportunity to receive IFN therapy for HCC recurrence if these patients were assigned to the peg-IFN therapy. Consequently, 119 patients were eventually enrolled in this study. Of these 119 patients, 43 received peg-IFN therapy within 9 months after hepatectomy, and 76 did not receive any IFN therapy.

Curative hepatectomy was defined as removal of all recognizable tumors. HCV RNA levels were measured by quantitative reverse-transcription polymerase chain reaction (RT-PCR; Amplicor, Roche Diagnostic Systems, CA, USA). HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of the NS5 region. HCV negativity was evaluated by quantitative RT-PCR. The lower limit of the assay was 5 kIU/ml (equivalent to 5,000 copies/ml) in the quantitative method and 50 IU/ml (equivalent to 50 copies/ml) in the qualitative method. SVR was defined as undetectable HCV RNA at 24 weeks after completion of IFN therapy. The study was approved by the concerned institutional review boards. Written informed consent was obtained from all patients.

Preoperative Diagnosis and Evaluation of HCC

Hepatocellular carcinoma was diagnosed on the basis of routine imaging modalities such as Doppler ultrasonography (US), computed tomography (CT) during hepatic angiography (CTHA) and CT during arterial portography (CTAP), and magnetic resonance imaging. Tumor stage, liver damage classification, and surgical procedures were defined according to the General Rules for Clinical and Pathologic Study of Primary Liver Cancer, fifth edition, by the Liver Cancer Study Group of Japan.[24]

Hepatectomy

The surgical procedure was determined according to tumor extent and hepatic reserve function. Liver function was assessed by liver damage classification, Child–Pugh classification, and indocyanine green retention rate at 15 min (ICGR 15).[25,26] If permitted by liver function, anatomic resection was performed.[27,28] In patients with insufficient hepatic reserve, limited resection was performed. We divided the liver parenchyma by using an ultrasonic dissector.[29] Postoperative complications were graded according to the method described by Clavien et al.[30]

Follow-Up

Follow-up evaluation after the surgery consisted of monthly blood chemistry tests and measurements of levels of tumor markers, including alpha-fetoprotein and des-gamma-carboxy prothrombin. Patients were examined by US every 3 months and by CT every 6 months. When recurrence was indicated by any of these examinations, patients were examined by CTAP and CTHA.

Patient Selection for IFN Therapy

Patients with HCV genotype 1b in the IFN group received peg-IFNα-2b (Pegintron; Schering-Plough, NJ, USA) at weekly dosage of 1.5 μg/kg subcutaneously for 48 weeks. Daily RBV (Rebetrol, Schering-Plough) was administered orally for 48 weeks, and the dosage was adjusted according to weight (600 mg for patients weighing $\leq$60 kg, 800 mg for those weighing 60–80 kg). Patients with HCV genotype 2 received IFN monotherapy for 24 weeks. Blood samples were obtained every 4 weeks and analyzed for HCV RNA levels. All patients were informed about IFN therapy after hepatectomy, and only consenting patients received IFN therapy. The eligibility criteria for IFN therapy were as follows: (1) detectable serum HCV RNA level, (2) Eastern Cooperative Oncology

Group (ECOG) performance score of 0 or 1, (3) platelet count ≥70,000/μl, (4) patients with no uncompensated cirrhosis (Child class C), and (5) hemoglobin concentration ≥10 g/dl. Peg-IFN therapy was commenced within 24 weeks of surgery or after the eligibility criteria were fulfilled.

Safety Assessments and Dose Modification of Peg-IFN Therapy

Adverse events were graded as mild, moderate, severe, or potentially life-threatening according to a modified World Health Organization grading system. The dose of peg-IFN was decreased by 50% and that of RBV was lowered to half in case of severe adverse events or when laboratory results revealed any of the following: hemoglobin concentration <10 g/dl in patients with no cardiac disease, decrease in hemoglobin concentration >2 g/dl in patients with cardiac disease, white blood cell count <3,000/mm^3, or platelet count <50,000/mm^3. Full dosage could be resumed on resolution of the adverse events. Treatment was permanently discontinued in case of life-threatening events or when laboratory results revealed hemoglobin concentration <7.5 g/dl after 4 weeks of dose reduction, white blood cell count <1,500/mm^3, or platelet count <30,000 mm^3.

Treatment for Recurrence

Patients with intrahepatic HCC recurrence were managed with ablative therapies such as radiofrequency ablation (RFA), percutaneous ethanol injection therapy, transarterial chemoembolization, or surgery including living-donor liver transplantation according to the tumor characteristics (number, size, and location of the tumors) and liver function.

Statistical Analyses

Categorical variables were compared using the chi-square test, and continuous variables were compared using the Mann–Whitney *U*-test. Overall survival and disease-free survival analyses were performed using Kaplan–Meier methods; comparisons between different groups were performed using the log-rank test. *P* value of less than 0.05 was considered significant. Calculations were performed using SPSS software (version 16; SPSS Inc., IL, USA).

Propensity analysis was performed using logistic regression to create a propensity score for the IFN and non-IFN therapy groups.[31,32] Variables entered in the propensity model were age, sex, HCV genotype, liver function test, tumor factors, and operative factors. The model was then used to provide a one-to-one match between the two groups by using the nearest-neighbor matching method.[33,34] Survival and disease-free survival analyses were performed in each matched subgroup to assess the impact of peg-IFN therapy on mortality after adjusting for the confounding factors.

RESULTS

Characteristics and Postoperative Course of the Entire Population

Differences in the characteristics of patients who received peg-IFN therapy after hepatic resection and those who did not receive IFN therapy after hepatic resection are presented in Table 1. Patients who received peg-IFN therapy were younger (65 vs. 71 years; $P = 0.0003$). Regarding tumor characteristics, there was no significant difference between the two groups. Operation times tended to be longer in patients who received peg-IFN therapy than in those who did not receive IFN therapy (260 vs. 242 min; $P = 0.05$). There were no hospital-related deaths in this study. Postoperative complications did not differ between the two groups. In the entire population, the 3- and 5-year overall survival rates of patients who received peg-IFN therapy after hepatic resection were significantly higher than those of patients who did not receive IFN therapy ($P = 0.0024$) (Fig. 1a). However, there was no significant difference in disease-free survival between the two groups ($P = 0.795$) (Fig. 1b).

Results After Propensity Score Matching

Characteristics of the patients after propensity score analysis are presented in Table 1. Thirty-eight of the 43 patients who received peg-IFN therapy after hepatic resection and an equal number of the 76 patients who did not receive IFN therapy were matched after covariate adjustment. The study group of 76 patients was well matched; in particular, all covariates that significantly affected recurrence and postoperative liver failure in the entire study group were equally distributed between the two matched groups. Matched patients who received peg-IFN therapy after hepatic resection had similar total bilirubin and serum albumin levels and similar platelet counts to matched patients who did not receive IFN therapy. Similarly, the tumor characteristics, the surgical procedure, operation times, and blood loss during the operation in matched patients who received peg-IFN therapy were almost similar to those in patients who did not receive IFN therapy. There were no hospital-related deaths in the matched groups. Postoperative complications also did not differ between the two groups. The median follow-up period for patients who received peg-IFN and those who

TABLE 1 Baseline characteristics and operative data on patients who underwent hepatectomy: data are reported for whole study and for the matched study population after propensity score analysis

	Overall series		*P* value	Propensity-matched series		*P* value
	IFN (+) $n = 43$	IFN (−) $n = 76$		Peg-IFN (+) $n = 38$	IFN (−) $n = 38$	
Age (years)	65 (53–78)	71 (48–83)	0.0003	65.5 (53–75)	69 (51–80)	0.2
Sex (male/female)	27/16	47/29	0.918	23/15	25/13	0.634
Preoperative IFN	24 (55.8%)	29 (38.1%)	0.06	20 (52.6%)	14 (36.8%)	0.16
HCV genotype			0.876			0.6
1b	34	61		29	27	
2b	9	15		9	11	
Diabetes mellitus	11 (25.6%)	22 (28.9%)	0.856	11 (28.9%)	13 (34.2%)	0.621
ECOG PS			0.831			0.644
0	39	68		36	35	
1	4	8		2	3	
Platelet ($104/mm^3$)	10.3 (3.3–26.6)	10.3 (3.8–40.3)	0.381	9.75 (3.3–21.5)	11.2 (3.8–40.3)	0.454
T-Bil (mg/dl)	0.7 (0.3–1.4)	0.8 (0.3–1.7)	0.292	0.7 (0.4–1.4)	0.7 (0.3–1.7)	0.798
AST (IU/l)	42 (18–121)	48 (16–150)	0.152	43.5 (18–127)	41.5 (6–150)	0.567
ALT (IU/l)	38 (13–127)	41.5 (10–196)	0.987	40.5 (11–127)	37.5 (10–196)	0.226
Albumin (g/dl)	3.8 (2.8–5.2)	3.8 (2.5–4.9)	0.215	3.8 (2.8–5.2)	3.8 (2.5–4.5)	0.469
ICGR 15 (%)	17.9 (7.4–77.4)	18.7 (4.6–50.5)	0.734	17.65 (7.4–40.0)	17.55 (4.6–40.0)	0.561
AFP (ng/ml)	11.6 (0.5–3405)	27.6 (0.5–36572)	0.176	13.95 (0.5–3405)	22.9 (0.5–513)	0.635
Child–Pugh grade			0.665			0.556
A	41 (95.3%)	69 (90.8%)		37 (97.4%)	36 (94.7%)	
B	2 (4.7%)	7 (9.2%)		1 (2.6%)	2 (5.3%)	
Hepatic resection			0.322			0.373
Hr0	20 (46.5%)	49 (64.5%)		18 (47.4%)	23 (60.5%)	
HrS	13 (30.2%)	18 (23.7%)		12 (31.6%)	9 (23.7%)	
Hr1	3 (7.0%)	4 (5.3%)		2 (5.3%)	3 (7.9%)	
Hr2	7 (16.3%)	5 (6.6%)		6 (15.8%)	2 (5.3%)	
Hr3	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
Operation time (min)	260 (128–623)	242 (90–580)	0.0514	257 (128–623)	247.5 (90–580)	0.18
Blood loss (ml)	200 (20–1900)	225 (10–960)	0.996	210 (20–1900)	210 (10–960)	0.803
Postoperative complications			0.933			0.798
IIIa	4	6		2	2	
IIIb	1	1		1	1	
IVa	1	1		1	0	
Stage			0.315			0.293
I	14 (32.6%)	19 (25.0%)		13 (34.2%)	9 (23.7%)	
II	18 (41.9%)	44 (57.9%)		15 (39.5%)	23 (60.5%)	
III	9 (20.9%)	12 (15.8%)		9 (23.7%)	6 (15.8%)	
IV-A	2 (4.7%)	1 (1.3%)		1 (2.6%)	0 (0.0%)	
Single tumor	28 (65.1%)	57 (75.0%)	0.252	25 (65.8%)	29 (76.3%)	0.312
Tumor size			0.712			0.589
≥3 cm	15 (34.9%)	24 (31.6%)		10 (26.3%)	8 (21.1%)	
<3 cm	28 (65.1%)	52 (68.4%)		28 (73.7%)	30 (78.9%)	
Vascular invasion	4 (9.3%)	3 (3.9%)	0.233	3 (7.9%)	0 (0.0%)	0.239

Continuous variables expressed as median (range)

Hepatic resection and stage were according to General Rules for the Clinical and pathological Study of Primary Liver Cancer, by Liver cancer Study Group of Japan, 5th edition, Kanehara Co., Ltd

Hr0: limited resection, HrS: segmentectomy, Hr1: sectionectomy, Hr2: hemihepatectomy, Hr3: more than hemihepatectomy

T-Bil total bilirubin, *PS* performance status, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ICGR 15* indocyanine green retention rate at 15 min, *AFP* alpha-fetoprotein,

did not receive IFN therapy was 3.8 (1.2–6.9) and 3.5 (1.3–6.8) years, respectively. In the matched study groups, the 3- and 5-year overall survival rates of patients who received peg-IFN therapy after hepatic resection were significantly higher than those of patients who did not receive IFN therapy ($P = 0.00135$) (Fig. 1c). However, there was no significant difference in disease-free survival between the two matched groups ($P = 0.886$) (Fig. 1d).

In the matched 38 patients of the peg-IFN group, peg-IFN therapy was initiated at a median of 4.3 (0.9–9.6) months after hepatic resection. Thirty-one of 38 HCC patients began peg-IFN therapy within 6 months after hepatectomy. Seven patients required more than 6 months to commence peg-IFN therapy. Two patients required a longer time to recover platelet counts of more than 70,000/μl. Five patients required a longer time to decide to receive peg-IFN therapy. Sixteen (42.1%) of the matched 38 patients who received peg-IFN therapy after hepatectomy attained SVR. Among 16 patients who attained SVR, 10 patients received full-dose peg-IFN therapy without dose reduction, whereas 6 patients received a reduced dose of peg-IFN and/or RBV until completion of treatment. Nine patients discontinued peg-IFN therapy because of adverse events such as thrombocytopenia and neutropenia ($n = 2$), skin eruption ($n = 1$), depression ($n = 2$), and severe malaise ($n = 4$). Three patients discontinued peg-IFN therapy because of HCC recurrence. Adherence to peg-IFN therapy was 68.4% in this study. No life-threatening adverse events were observed, and none of the total 15 deaths in both sets of matched patients were related to the IFN treatment or to surgical procedures. The 3- and 5-year overall survival rates of patients ($n = 16$) who attained SVR after peg-IFN therapy were 100% and 100%, respectively; those of patients who did not attain SVR ($n = 22$) were 100 and 85.7%, respectively; and those of patients who did not receive IFN therapy were 76.6 and 50.6%, respectively. There was a statistically significant difference in overall survival among the three groups ($P = 0.005$) (Fig. 2a). However, there was no statistically significant difference in disease-free survival among the three groups ($P = 0.90$) (Fig. 2b).

Table 2 presents the patterns of cancer recurrence and the treatment details of the recurrences in both groups. Twenty-one (55.3%) of the patients who received peg-IFN therapy after hepatic resection and 17 (44.7%) of the patients who did not receive IFN therapy had HCC recurrences after hepatic resection. Regarding the pattern of recurrence, the proportion of patients who had multiple

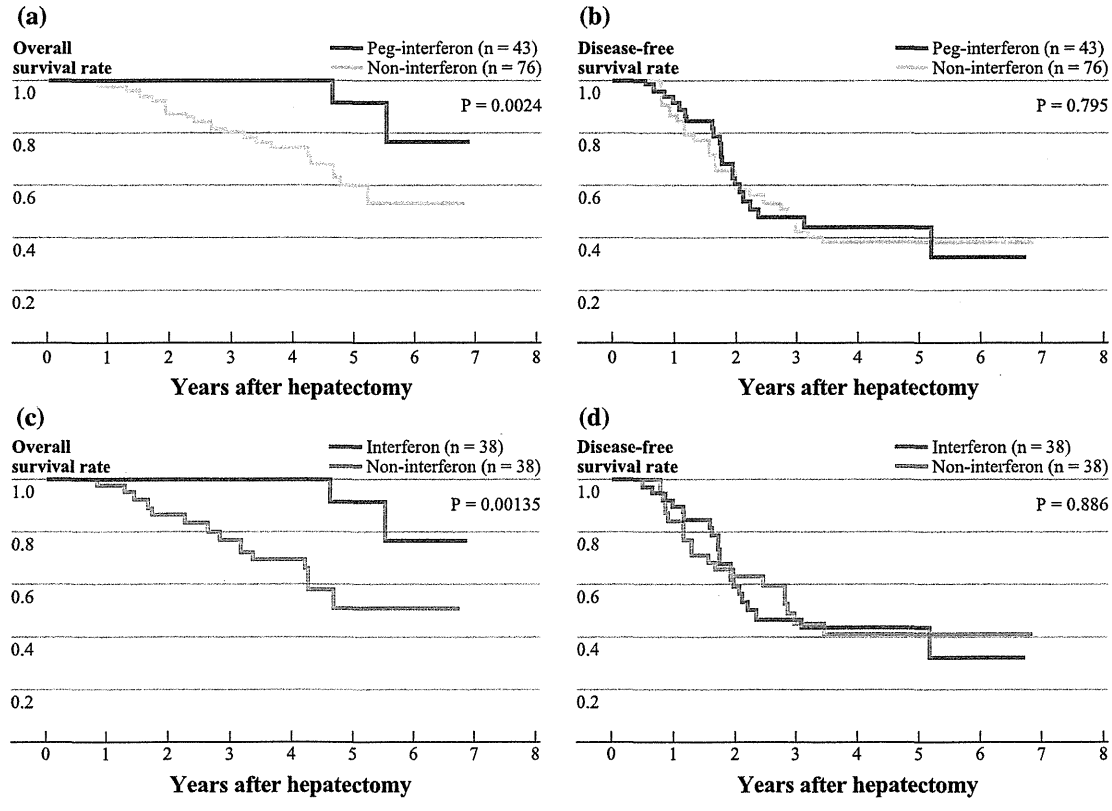

FIG. 1 Overall survival (**a**) and disease-free survival (**b**) of the entire study population of 175 patients with hepatitis C-related HCC with respect to IFN therapy after hepatic resection. Overall survival (**c**) and disease-free (**d**) survival of the matched study population of 76 patients with hepatitis C-related HCC with respect to IFN therapy after hepatic resection

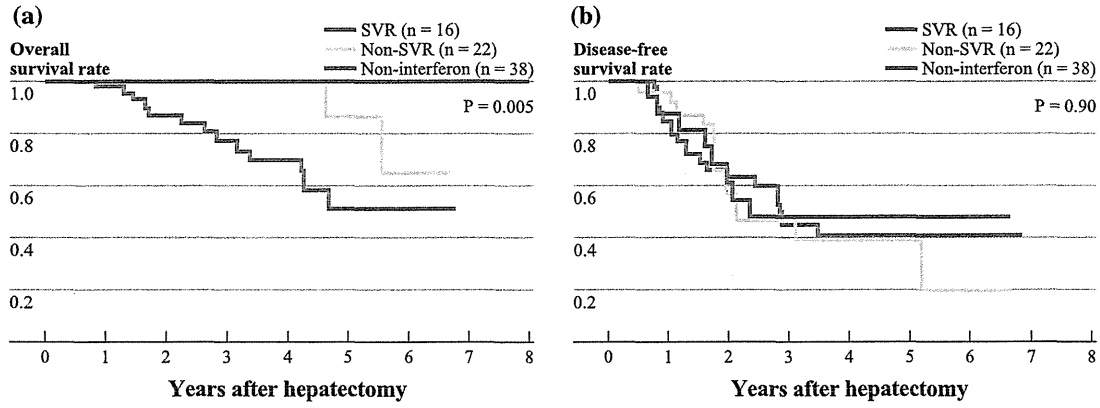

FIG. 2 Overall survival and disease-free survival of patients with hepatitis C-related HCC with respect to SVR after IFN therapy

intrahepatic recurrences (more than four nodules) was significantly lower in the peg-IFN group than in the non-IFN group ($P = 0.0047$). The proportion of patients in whom surgery or RFA was selected for treatment was significantly higher in the peg-IFN group than in the non-IFN group ($P = 0.0346$). Furthermore, regarding re-recurrence of HCC after treatment of the first-recurrent HCC, the 1-year disease-free survival rates of patients after treatment of the first-recurrent HCC was 48.5% in patients ($n = 21$) who received peg-IFN therapy and 12.5% in patients ($n = 17$) who did not receive IFN therapy. There was a statistically significant difference in disease-free survival between the two groups ($P = 0.0012$) (Fig. 3).

A comparison of results of the preoperative liver function test with those of postoperative 1-year liver function tests is presented in Table 3. In patients who received peg-IFN therapy, total bilirubin levels 1 year after surgery were significantly decreased compared with preoperative total bilirubin levels ($P = 0.018$), whereas in patients who did not receive IFN therapy, the total bilirubin level at 1 year after surgery was similar to the total bilirubin level before surgery ($P = 0.107$).

TABLE 2 Recurrence and treatments for recurrence after hepatic resection

	Peg-IFN (+) ($n = 38$)	IFN (−) ($n = 38$)	*P* value
HCC recurrence[a]: yes	21 (55.3%)	17 (44.7%)	0.359
Pattern of recurrence[b]			0.0047
Intrahepatic (single)	9 (42.9%)	8 (47.1%)	
Intrahepatic (2–3)	10 (47.6%)	1 (5.9%)	
Intrahepatic (multiple)	2 (9.5%)	8 (47.1%)	
Main modalities[b]			0.0346
Repeat hepatectomy	8 (38.1%)	2 (11.8%)	
RFA	8 (38.1%)	4 (23.5%)	
TACE	5 (23.8%)	11 (64.7%)	

peg-IFN pegylated interferon, *RFA* radiofrequency ablation, *TACE* transcatheter arterial chemoembolization

[a] Data expressed as number of patients (percentage of total patients)

[b] Data expressed as number of patients (percentage of patients who had a recurrence)

DISCUSSION

Our results revealed that peg-IFN therapy after hepatic resection improved the outcomes of HCV patients, although the interval of disease-free survival was not prolonged. Peg-IFN therapy after hepatectomy improved hepatic reserve function and suppressed multiple HCC recurrences (more than four nodules). Furthermore, re-recurrence after treatment of first-recurrent HCC after hepatic resection was significantly suppressed in the peg-IFN group compared with that in the non-IFN group. IFN has been reported to exert antitumor effects. IFN increases natural killer cell activity and exhibits antiangiogenic properties.[35,36] IFN has also been reported to be effective in eradicating HCV RNA

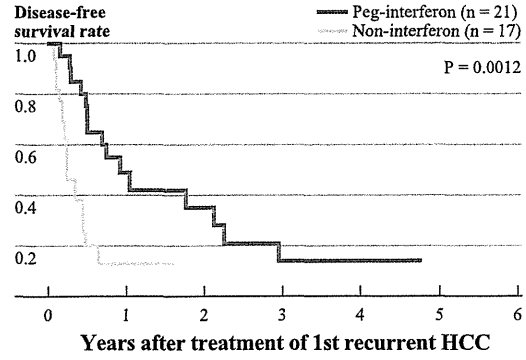

FIG. 3 Comparison of disease-free survival rate after treatment of first-recurrent HCC in patients who received peg-IFN therapy or in those who did not receive IFN therapy

TABLE 3 Comparison of preoperative liver function with 1-year liver function after hepatic resection

	Peg-IFN (+)		*P* value	IFN (−)		*P* value
	Preoperative	1 Year after surgery		Preoperative	1 Year after surgery	
T-Bil (mg/dl)	0.82 ± 0.29	0.71 ± 0.26	0.0189	0.81 ± 0.32	0.92 ± 0.35	0.107
AST (IU/l)	50.1 ± 24.1	45.8 ± 23.5	0.310	42.1 ± 18.9	56.1 ± 26.7	0.0110
ALT (IU/l)	51.3 ± 28.6	36.4 ± 22.8	0.00809	40.3 ± 24.3	49.7 ± 25.8	0.0918
Albumin (g/dl)	3.89 ± 0.80	3.99 ± 0.71	0.251	3.73 ± 0.45	3.75 ± 0.44	0.807

peg-IFN pegylated interferon, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase

from serum and hepatic tissue, thereby preventing deterioration of liver function in patients with HCV infection.[37] IFN prevents worsening of compensated cirrhosis.[18,37] Our results are compatible with those reported in those studies. In the peg-IFN group, most patients with HCC recurrence could undergo curative treatments such as repeat hepatectomy or RFA as a recurrence treatment, because the number of recurrent tumors was usually limited to three. IFN therapy appears to increase survival not only by improving residual liver function and increasing the possibility of radical treatment of recurrences but also by suppressing re-recurrence after the first recurrence of HCC.

The current study also revealed that the overall survival of patients with SVR was significantly better than that of patients without SVR. This result suggests that IFN prolongs the outcomes of patients with HCC after hepatic resection by causing remission of active hepatitis and eradication of HCV RNA in patients who attained SVR after hepatic resection.

In this study, to clarify the impact of peg-IFN therapy on outcomes of HCV-related HCC after hepatic resection, patients who received IFNs such as IFN-α or IFN-β were excluded. RCTs investigating adjuvant effects of IFN after resection or ablation of HCC were performed using IFN-α. Few studies have investigated the effects of peg-IFN plus RBV combination therapy on survival and recurrence after curative resection of HCC. Combination therapy with peg-IFN and RBV has recently been developed, and peg-IFN therapy has resulted in significantly higher SVR rates and better tolerability than treatment with IFN-α.[21,23] In our study, incidence of SVR after hepatic resection was 42.1%, which was higher than that in previous studies that reported an SVR rate of 0–10%.[12–14] The compliance of patients to peg-IFN therapy observed in the present study (68.4%) was higher than that reported elsewhere (approximately 40%).[14] This enhanced efficacy of the peg-IFN formulations might contribute to the prolonged survival of HCC patients after hepatic resection.

In this study, HCC patients who received peg-IFN therapy within 9 months after surgery were enrolled, and HCC patients who experienced recurrence of HCC within 9 months after hepatic resection were excluded from the non-IFN group, because these patients could lose the opportunity to receive IFN therapy for HCC recurrence on being assigned to the peg-IFN therapy group.

Before matching by using the propensity score, the clinical characteristics of the entire study population that can strongly influence outcomes differed significantly between the peg-IFN group and non-IFN group. The proportion of older patients was higher in the non-IFN group than in the peg-IFN group, whereas the proportion of patients who had longer operation times tended to be lower in the non-IFN group than in the peg-IFN group. To overcome bias due to the different distribution of the severity of liver function impairment between the two groups, a one-to-one match was created using propensity score analysis. After matching by propensity score, prognostic variables were appropriately handled, and there was no significant difference in prognostic factors between the two matched groups. This study had a limitation related to the small sample size after propensity score matching. To overcome this, further examination with larger sample sizes is necessary, and the potential efficacy of peg-IFN therapy must be validated in larger prospective RCTs.

CONCLUSIONS

Several previous RCTs investigating the effects of IFN on survival and tumor recurrence after hepatic resection were inconclusive. However, in the current study, peg-IFN therapy following hepatic resection improved the survival rates of hepatectomized patients with HCV-related HCC. The results of this study suggest that peg-IFN therapy is effective as an adjuvant chemopreventive agent after hepatic resection in patients with HCV-related HCC.

ACKNOWLEDGMENT The authors thank Prof. Junko Tanaka of the Department of Epidemiology, Infectious Disease Control and Prevention, Hiroshima University, for assistance in performing the propensity score analysis.

CONFLICT OF INTEREST The authors have no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements) that might pose a conflict of interest related to the submitted manuscript.

REFERENCES

1. Poon RTP, Fan ST, Lo CM, Liu CL, Wong J. Intrahepatic recurrence after curative resection of hepatocellular carcinoma: long-term results of treatment and prognostic factors. *Ann Surg.* 1999;229:216–22.
2. Minagawa M, Makuuchi M, Takayama T, Kokudo N. Selection criteria for repeat hepatectomy in patients with recurrent hepatocellular carcinoma. *Ann Surg.* 2003;238:703–10.
3. Itamoto T, Nakahara H, Amano H, et al. Repeat hepatectomy for recurrent hepatocellular carcinoma. *Surgery.* 2007;141:589–97.
4. Ikeda K, Saitoh S, Arase Y, Chayama K, et al. Effects of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology.* 1999;29:1124–30.
5. Imai Y, Kawata S, Tamura S, et al. Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. *Ann Intern Med.* 1998;129:94–9.
6. Camma C, Giunta M, Andreone P, Craxì A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J Hepatol.* 2001;34:593–602.
7. Nishiguchi S, Shiomi S, Nakatani S, et al. Prevention of hepatocellular carcinoma in patients with chronic active hepatitis C and cirrhosis. *Lancet.* 2001;357:196–7.
8. Tomimaru Y, Nagano H, Eguchi H, et al. Effects of preceding interferon therapy on outcome after surgery for hepatitis C virus-related hepatocellular carcinoma. *J Surg Oncol.* 2010;102: 308–14.
9. Jeong SC, Aikata H, Katamura Y, et al. Low-dose intermittent interferon-alpha therapy for HCV-related liver cirrhosis after curative treatment of hepatocellular carcinoma. *World J Gastroenterol.* 2007;13:5188–95.
10. Jeong SC, Aikata H, Kayamura Y, et al. Effects of a 24-week course of interferon-α therapy after curative treatment of hepatitis C virus-associated hepatocellular carcinoma. *World J Gastroenterol.* 2007;13:5343–50.
11. Harada H, Kitagawa M, Tanaka N, et al. Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. *Science.* 1993;259:971–4.
12. Liedtke C, Grogner N, Manns MP, Trautwein C. Interferon-alpha enhances TRAIL-mediated apoptosis by up-regulating caspase-8 transcription in human hepatoma cells. *J Hepatol.* 2006;44: 342–9.
13. Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Kinoshita H. Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by postoperative interferon therapy. *Br J Surg.* 2002;89:418–22.
14. Ikeda K, Arase Y, Saitoh S, et al. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of primary tumor-a prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology.* 2000;32: 228–32.
15. Mazzaferro V, Romito R, Schiavo M, et al. Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. *Hepatology.* 2006;44:1543–54.
16. Lo CM, Liu CL, Chan SC, et al. A randomized, controlled trial of postoperative adjuvant interferon therapy after resection of hepatocellular carcinoma. *Ann Surg.* 2007;245:831–42.
17. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet.* 2001;358:958–65.
18. Fried MW, Shiffman ML, Rajender Reddy K, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med.* 2002;347:975–82.
19. Yoshida H, Arakawa Y, Sata M, et al. Interferon therapy prolonged life expectancy among chronic hepatitis C patients. *Gastroenterology.* 2002;123:483–91.
20. Kiyosawa K, Uemura T, Ichijo T, et al. Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology.* 2004;127: S17–26.
21. McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med.* 1998;339:1485–92.
22. Muir AJ, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. *N Eng J Med.* 2004;350:2265–71.
23. Shirakawa H, Matsumoto A, Joshita S, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis c patients using viral and host factors. *Hepatology.* 2008;48:1753–60.
24. Kogure T, Ueno Y, Fukushima K, et al. Pegylated interferon plus ribavirin for genotype 1b chronic hepatitis C in Japan. *World J Gastroentrol.* 2008;14:7225–30.
25. Liver Cancer Study Group of Japan. General Rules for the Clinical and Pathological Study of Primary Liver Cancer. 5th ed. Tokyo: Kanehara; 2008.
26. Itamoto T, Nakahara H, Tashiro H, et al. Indications of partial hepatectomy for transplantable hepatocellular carcinoma with compensated cirrhosis. *Am J Surg.* 2005;189:167–72.
27. Oishi K, Itamoto T, Kobayashi T, et al. Hepatectomy for hepatocellular carcinoma in elderly patients aged 75 years or more. *J Gastrointest Surg.* 2009;13:695–701.
28. Makuuchi M, Hasegawa H, Yamazaki S. Ultrasonically guided subsegmentectomy. *Surg Gynecol Obstet.* 1985;161:346–50.
29. Yamamoto M, Takasaki K, Otsubo T, et al. Favorable surgical outcomes in patients with early hepatocellular carcinoma. *Ann Surg.* 2004;239:395–9.
30. Itamoto T, Katayama K, Nakahara H, Tashiro H, Asahara T. Autologous blood storage before hepatectomy for hepatocellular carcinoma with underlying liver disease. *Br J Surg.* 2003;90: 23–8.
31. Clavien PA, Barkun J, de Oliveira ML, et al. The Clavien–Dindo classification of surgical complications: five-year experience. *Ann Surg.* 2009;250:187–96.
32. Zinsmeister AR, Connor JT. Ten common statistical errors and how to avoid them. *Am J Gastroenterol.* 2008;103:262–6.
33. Rosenbaum PR, Rubin DB. The central role of the propensity score in observational studies for causal effects. *Biometrika.* 1983;70:41–55.
34. Rubin DB. Estimating causal effects from large data sets using propensity scores. *Ann Intern Med.* 1997;127:757–63.
35. D'Agostino RB Jr. Propensity score methods for bias reduction in the comparison of a treatment to non-randomized control group. *Stat Med.* 1998;17:2265–81.
36. Brinkmann V, Geiger T, Alkan S, Heusser CH. Interferon alpha increases the frequency of interferon gamma-producing human CD4+T cells. *J Exp Med.* 1993;178:1655–63.
37. Wang L, Tang ZY, Qin LX, et al. High-dose and long-term therapy with interferon-alfa inhibits tumor growth and recurrence in nude mice bearing human hepatocellular carcinoma xenografts with high metastatic potential. *Hepatology.* 2000;32:43–8.
38. Shiratori Y, Shiina S, Teratani T, et al. Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C. *Ann Intern Med.* 2003;138: 299–306.

J Gastroenterol (2012) 47:834–844
DOI 10.1007/s00535-012-0550-y

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

IL28B polymorphism is associated with fatty change in the liver of chronic hepatitis C patients

Mayu Ohnishi · Masataka Tsuge · Tomohiko Kohno · Yizhou Zhang · Hiromi Abe · Hideyuki Hyogo · Yuki Kimura · Daiki Miki · Nobuhiko Hiraga · Michio Imamura · Shoichi Takahashi · Hidenori Ochi · C. Nelson Hayes · Shinji Tanaka · Koji Arihiro · Kazuaki Chayama

Received: 17 November 2011 / Accepted: 18 January 2012 / Published online: 18 February 2012

Abstract
Background Several single nucleotide polymorphisms (SNPs) within the *interleukin 28B* (*IL28B*) locus are associated with sustained viral response in chronic hepatitis C (HCV) patients who were treated with pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. Recently, an association between γ-GTP level and IL28B genotype was identified. In this study, the relationship between IL28B genotype and liver steatosis was analyzed.
Methods One hundred fifty-three patients who underwent liver biopsy before PEG-IFN plus RBV combination therapy were enrolled. The level of liver steatosis was measured using a BIOREVO BZ-9000 microscope, and the proportion of fatty change and clear cell change were calculated using Dynamic cell count BZ-H1C software. IL28B SNP genotype (rs8099917) was determined using the Invader Assay.
Results Vesicular change was significantly associated with body mass index (BMI), HCV RNA titer, serum aspartate aminotransferase, γ-GTP, IL28B genotype and liver fibrosis level ($P < 0.05$). Clear cell change was significantly associated with serum aspartate aminotransferase, γ-GTP and IL28B genotype by univariate logistic regression analysis ($P < 0.05$). Under multiple logistic regression, IL28B genotype ($OR_{adj} = 8.158$; 95% CI 2.412–27.589), liver fibrosis ($OR_{adj} = 2.541$; 95% CI 1.040–6.207) and BMI ($OR_{adj} = 1.147$; 95% CI 1.011–1.301) were significant independent factors for vesicular change and IL28B genotype ($OR_{adj} = 3.000$; 95% CI 1.282–7.019) for clear cell change.
Conclusion In this study, a new quantitative method to objectively evaluate hepatic steatosis was described. IL28B genotypes were significantly associated with both vesicular and clear cell changes of livers in chronic hepatitis C patients.

Keywords HCV · Core substitution · IL28B · Fatty change · SNP

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon

M. Ohnishi · M. Tsuge · T. Kohno · Y. Zhang · H. Abe · H. Hyogo · Y. Kimura · D. Miki · N. Hiraga · M. Imamura · S. Takahashi · H. Ochi · C. N. Hayes · S. Tanaka · K. Chayama
Programs for Biomedical Research, Division of Frontier Medical Science, Department of Gastroenterology and Metabolism, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

M. Ohnishi · M. Tsuge · T. Kohno · Y. Zhang · H. Abe · H. Hyogo · Y. Kimura · D. Miki · N. Hiraga · M. Imamura · S. Takahashi · H. Ochi · C. N. Hayes · K. Chayama
Liver Research Project Center, Hiroshima University, Hiroshima, Japan

M. Tsuge
Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan

D. Miki · H. Ochi · K. Chayama
Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN, Hiroshima, Japan

K. Arihiro
Department of Pathology, Hiroshima University Hospital, Hiroshima, Japan

K. Chayama (✉)
Programs for Biomedical Research, Division of Frontier Medical Science, Department of Medical and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, Japan
e-mail: chayama@hiroshima-u.ac.jp

PEG-IFN	Pegylated interferon
RBV	Ribavirin
ISDR	IFN-sensitivity determining region
IL28	Interleukin 28
SNP	Single nucleotide polymorphism
BMI	Body mass index
γ-GTP	Gamma glutamyl transpeptidase
aa	Amino acid
HOMA-IR	Homeostasis model assessment of insulin resistance

Background

Hepatitis C virus (HCV) is one of the most serious global health problems, affecting more than 170 million people worldwide [1–3]. Chronic HCV infection leads to the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [4–8]. To attempt to eradicate the virus and prevent the development of advanced liver diseases and HCC, interferon is administered to chronic hepatitis C patients, with success in a subset of patients in which marked biochemical and histological improvements can be obtained [9, 10]. However, patients with a high virus titer and those who are infected with genotype 1b, which is the major genotype affecting about 70% of Japanese patients, show poor response to interferon monotherapy. Less than 20% of patients treated with interferon monotherapy show sustained virological response [11–14]. With the advent of pegylated interferon (PEG-IFN) and ribavirin (RBV) combination therapy, the eradication rate of the virus has improved. However, the eradication rate of genotype 1b with high viral load still remains only 40–50% [15–17].

Several viral and host factors have been identified that are predictive of the outcome of PEG-IFN plus RBV combination therapy. HCV genotype, HCV RNA level and HCV amino acid (aa) substitutions in the core region and the interferon-sensitivity determining region (ISDR; aa positions 237–276 of the NS5A region) have been reported as viral factors for achieving sustained viral response. On the other hand, host factors, such as patient age, gender, liver fibrosis stage, liver steatosis and homeostasis model assessment of insulin resistance (HOMA-IR), are also known to be associated with IFN response. Okanoue et al. demonstrated that the frequency of liver steatosis and HOMA-IR were significantly lower in patients who achieved sustained virological response than those who did not [18–23].

Recently, genome-wide association studies (GWAS) have examined the association between human genetic variation and the response to IFN treatment, and several common polymorphisms in the interleukin 28B (IL28B) locus were found to predict successful HCV clearance with IFN therapy [24–27]. IL28B single nucleotide polymorphisms (SNPs) (rs12979860, rs8099917, and rs12980275) are strongly associated with sustained virological response in patients who undergo PEG-IFN plus RBV combination therapy. In a recent study, the serum γ-GTP level was also found to be associated with the IL28B SNP (rs8099917) genotype in patients infected with HCV genotype 1b [28]. Serum γ-GTP levels were significantly lower in patients homozygous for the major allele (TT) than in patients with the minor allele (GG or GT) [28]. On the other hand, the serum γ-GTP level is well known to be related to liver steatosis; therefore, it was hypothesized that the levels of liver steatosis may be associated with the IL28B genotype. In the present study, a quantitative method to evaluate fatty change was established, and the association between fatty change and host or viral factors was analyzed.

Patients and methods

Patients

Three hundred fifty-three adult Japanese patients infected with HCV genotype 1b provided written informed consent to participate in the present study at Hiroshima University Hospital, Hiroshima, Japan. Among these patients, 153 who underwent liver biopsy or hepatic resection from December 2001 to August 2009 before commencing antiviral therapy were selected for the study based on the following exclusion criteria: (1) not co-infected with other viruses, such as human immunodeficiency virus or hepatitis B virus; (2) no other liver diseases such as hemochromatosis, auto-immune hepatitis or decompensated cirrhosis; (3) no co-existing diseases such as de-compensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, hemophilia, autoimmune diseases or post-transplantation. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hiroshima University Hospital ethics committee. Written informed consent was obtained from each patient. Patients whose ethanol intake was more than 20 g/day were defined as alcoholic [29, 30]. Baseline characteristics of the 153 patients are shown in Table 1.

Evaluation of liver steatosis

Liver tissues with hematoxylin–eosin stain were used in this study. Liver fibrosis and activity stages were diagnosed by a pathologist at Hiroshima University Hospital according to the criteria of Desmet et al. [31]. The level of liver steatosis was measured using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan), and the proportion of

Table 1 Clinical backgrounds of patients

Characteristics	
Age (years)[a]	60 (15–76)
Gender (M:F)	83:70
BMI (kg m^{-2})[a]	22.7 (16.2–39.4)
Alcohol (+:−)	47:103
Laboratory data	
Platelet count ($\times 10^3$ μl^{-1})[a]	99 (23–759)
Prothrombin activity (%)[a]	99 (12–166)
Total bilirubin (mg dl^{-1})[a]	0.7 (0.4–14.7)
Aspartate aminotransferase (IU l^{-1})[a]	46 (14–2617)
Alanine aminotransferase (IU l^{-1})[a]	51 (2–2707)
Lactate dehydrogenase (IU l^{-1})[a]	202 (43–899)
Gamma glutamyl transpeptidase (IU l^{-1})[a]	43 (12–295)
Albumin (g dl^{-1})[a]	4.3 (2.9–5.3)
Total cholesterol (mg dl^{-1})[a]	174 (100–312)
Triglycerides (mg dl^{-1})[a]	100 (33–2466)
Glucose (mg dl^{-1})[a]	101 (68–334)
HCV RNA (log IU ml^{-1})[a]	6.3 (4.2–7.9)
Core 70 (wild:mutant)	77:39
Core 91 (wild:mutant)	54:62
ISDR (0:≥1)	38:76
IL28B (TT:TG:GG)	116:33:4
Liver histology	
Fibrosis (F0–1:F2–3)	42:111
Inflammatory activity (A0–1:A2–3)	59:94

[a] Median (range)

fatty change was calculated using Dynamic cell count BZ-H1C software (Keyence). The average of the observed area was 3.03 mm^2 (0.74–6.77 mm^2). The distribution of brightness values in liver tissue images was evaluated using a brightness distribution histogram as shown in Fig. 1a. The brightness was divided into 256 gradation sequences. Regions with a brightness value over 60% between minimum and maximum brightness were considered to represent fatty change areas in the liver tissue. The area of interest was calculated, and <5 μm^2 of the extracted areas were excluded as noise as determined by particle elimination technology (Dynamic cell count BZ-H1C software) (Fig. 1b). The remaining highlighted noise, including glycogen nuclei and vascular spaces, was excluded as much as possible by visual observation. After two exclusion steps, the remaining noise spaces were sufficiently narrow (far <5% of total fatty changed spaces) that they could be considered negligible. The observed area of the sample was also calculated with BZ-H1C software (Fig. 1c). All threshold values of vesicular change or clear cell change ratio were defined based on the approximate median values of each change.

Determination of amino acid sequences in the HCV core region and ISDR

HCV RNA was extracted from 100 µl of stored serum samples by SepaGene RV-R (Sanko Junyaku Co., Ltd, Tokyo, Japan), dissolved in 20 µl of H_2O and converted to cDNA by RT with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The cDNA was then amplified by nested PCR to determine the nucleotide sequences in the HCV core region. The PCR protocol was as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation for 30 s at 94°C, annealing of primers for 1 min at 57°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 7 min. The primers for the first round PCR were cc11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and the primers for the second-round PCR were cc9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse), as described previously [19, 21, 32, 33]. To determine the nucleotide sequences of the HCV ISDR region, we also performed nested PCR with the same protocol as in the HCV core region. The primers used were IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5A02KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′) for the first round PCR, and 5A05KI (forward, 5′-GGG TCA CAG CTC CCA TGT GAG CC -3′), and IM10 (reverse, 5′-GAG GGT TGT AAT CCG GGC GTG C-3′) for the second round PCR. After amplification, the final PCR products were separated in 2% agarose gel and purified with the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany). Sequence analysis was performed by ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Amino acid sequences were compared with the genotype 1b HCV-J reference sequence (Gene Bank accession no. D90208) [34]. Arginine and leucine were considered wild type for amino acids 70 and 91, respectively, and the most frequent amino acid substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Determination of genotype in IL28B

In Asian populations, the genotype of rs8099917, a SNP within the *IL28B* locus, has been reported to be strongly correlated to nearby SNPs rs12979860 and rs12980275 because of strong linkage disequilibrium [35]. In this study, rs8099917 was used to represent the genotype of the *IL28B* SNP. Genotypes of rs8099917 were determined using TaqMan® Pre-Designed SNP Genotyping Assays as described previously [28].

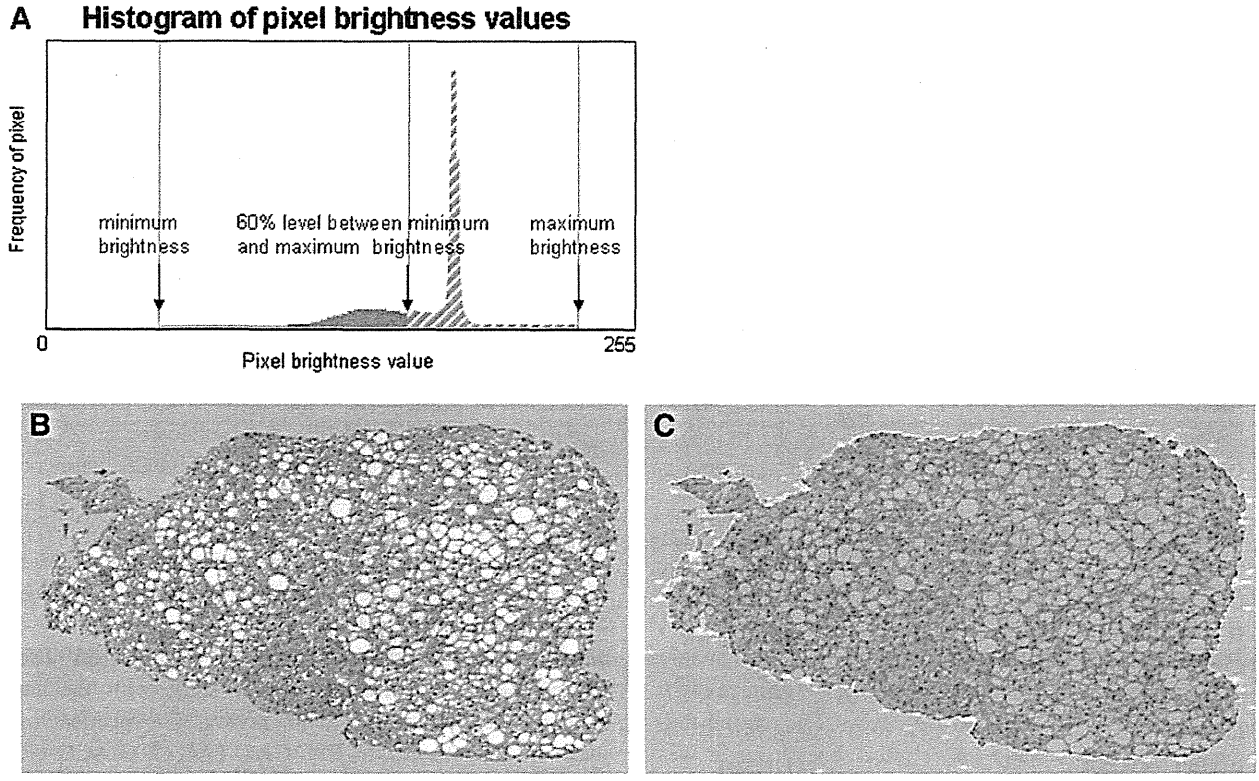

Fig. 1 Evaluation of fatty change in liver tissue. **a** The distribution of brightness in liver tissue images was represented with a brightness distribution histogram using BZ-H1C software. The relative cumulative frequency of pixels with brightness values over 60% between minimum and maximum brightness was interpreted as the proportion of fatty change in the liver tissue. **b** The area of the fatty change is depicted in *yellow*. **c** The area inside the *yellow line* is defined as the observed area (color figure online)

Statistical analysis

In univariate analysis, the clinical backgrounds of the patients in the two groups were compared, and differences were assessed by chi-square test with Yate's correction for categorical variables and by Mann–Whitney *U* test for continuous variables. All thresholds were determined based on median values. All *P* values <0.05 by two-tailed test were considered statistically significant. To analyze the association between clinical characteristics and fatty change, multivariate logistic regression analysis was performed. To identify significant independent predictive factors, variables with statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) in univariate analysis were used as the starting model for forward stepwise logistic regression model using the likelihood ratio test. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Patterns of histological change in the liver

Comparing the liver tissues between chronic hepatitis C and non-alcoholic fatty liver disease, we identified two different histological change patterns. As shown in Fig. 2, histological change in patients with non-alcoholic fatty liver disease consisted mainly of vesicular change (Fig. 2a), but histological change in the chronic hepatitis C patients consisted of both vesicular change and clear cell change (Fig. 2b), suggesting that clear cell change was the distinguishing change in chronic hepatitis C. Accordingly, we further analyzed liver specimens from patients with these diseases to determine the best criteria to distinguish clear cell change areas from vesicular change areas. Most of the vesicular change areas were found to be more than 200 μm^2 (data not shown). Therefore, we defined areas with <200 μm^2 of fatty change as areas of clear cell change (Fig. 3). Although narrow noise spaces (<200 μm^2), including small vacuoles or hepatocyte nucleus, remained after these automatic steps, the remaining noise spaces were then excluded as much as possible by visual observation. As shown in Fig. 4, the distribution of vesicular change was associated with clear cell change.

Association between vesicular change and clinical background

To analyze the relationship between fatty change and clinical background, we compiled the clinical backgrounds

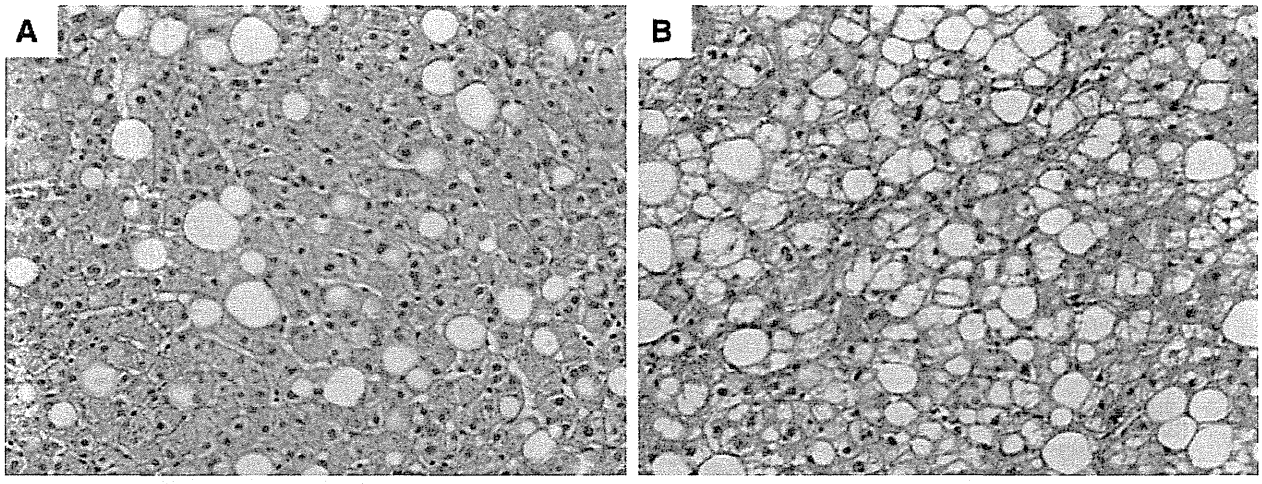

Fig. 2 Different fatty change patterns between NAFLD and chronic hepatitis C. The fatty change pattern was compared between NAFLD (**a**) and chronic hepatitis C (**b**). The liver tissues were stained with H&E

of 153 participating patients (Table 1). Because the minor allele frequency of the IL28B rs8099917 SNP was only 0.14 and the IL28B rs8099917 GG genotype was observed in only four patients, the IL28B rs8099917 genotypes were split into two groups (TT vs. TG/GG) in all analyses. The relationship between vesicular change and clinical backgrounds was examined. The proportion of vesicular change (vesicular change ratio) was defined as the ratio of the area of vesicular change and the observed area (= area of vesicular change/observed area). As shown in Table 2, in univariate analysis the greater vesicular change ratio was significantly associated with γ-GTP, *IL28B* genotype, aspartate aminotransferase, body mass index (BMI), liver fibrosis stage and HCV RNA at the point of liver biopsy ($P < 0.05$). The association between IL28B genotype and vesicular change was examined using multivariate analysis, and *IL28B* genotype, liver fibrosis stage and BMI were retained in the final model (Table 2). These results indicate that the vesicular change was not only associated with *IL28B* genotype, but also associated with BMI. To confirm the association between *IL28B* genotypes and vesicular change, multiple regression analysis was also performed using continuous values. *IL28B* genotype, HCV RNA titer and liver fibrosis stage were identified as independent factors for vesicular change ($P = 0.003$, $P = 0.022$, $P = 0.047$, respectively).

Association between clear cell change and clinical background

Next, the relationship between clear cell change and clinical background was analyzed. The proportion of clear cell change (clear cell change ratio) was defined as the ratio of the area of the clear cell change and the observed area (= area of clear cell change/observed area). We divided the study subjects into two groups according to the clear cell change ratio. As shown in Table 3, in univariate analysis the greater clear cell change ratio was significantly associated with aspartate aminotransferase, IL28B genotype and γ-GTP at the point of liver biopsy ($P < 0.05$). In multivariate analysis, the IL28B genotype was found to be a significant independent predictor, and liver inflammatory activity and gender were marginally associated (Table 3). By using continuous values in multiple regression, the *IL28B* genotype was also identified as an independent factor for clear cell change ($P < 0.001$). These results suggest that the *IL28B* genotype is significantly associated with clear cell change in livers of chronic hepatitis C patients.

The association between fatty degeneration and IL28B genotype in the non-obese group

To analyze the association between fatty change and IL28B genotype, the study subjects were divided into two groups. Thirty-six patients whose BMI was more than 25 kg m^{-2} were assigned to the obese group, and the other 114 patients were assigned to the non-obese group. In the obese group, no association between fatty change and IL28B genotype was observed (Table 4). On the other hand, each measure of fatty change (vesicular change, and clear cell change) was significantly associated with IL28B genotype in the non-obese group ($P = 0.001$, $P = 0.009$, respectively, Table 4).

Discussion

It is generally difficult to compare evaluations of liver fatty change because such studies depend on the respective assessments of individual pathologists [36, 37]. In the

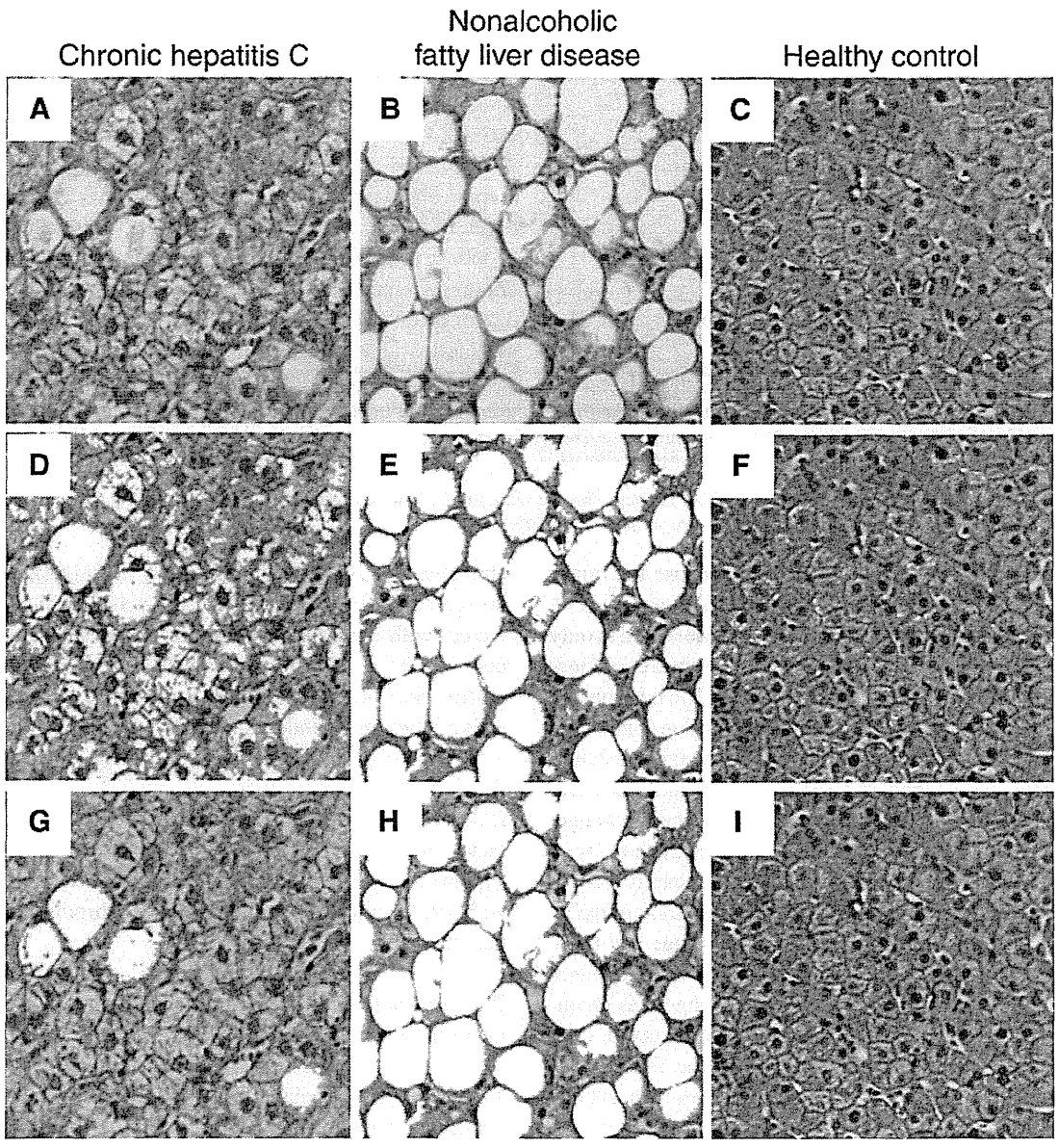

Fig. 3 Discrimination between vesicular and clear cell change. The evaluation results of three cases are shown. The *upper panels* are H&E staining of liver specimen from NAFLD (**a**), chronic hepatitis C (**b**) and a healthy control (**c**). In the *middle panels*, the fatty change area is depicted in *yellow* (**d–f**). In the *lower panels*, the vesicular change area is preferentially depicted with *yellow* using a criteria of <200 μm^2 of fatty change (**g–i**) (color figure online)

present study, we established criteria by which the degree of liver fatty change can be evaluated objectively. It is well known that the proportions of fatty change differ by location in liver tissues obtained by liver biopsy or operation. For quantitative evaluation of fatty change, an efficient algorithm was devised using the BIOREVO BZ-9000 microscope and Dynamic cell count BZ-H1C software. Using this method, fatty changes were analyzed in whole tissue specimens in chronic hepatitis C patients. With this algorithm, it was possible to evaluate fatty changes of whole tissues in the slides and obtain the same results by different operators. Furthermore, vesicular changes and clear cell changes could be evaluated separately by the distribution of brightness in liver tissues.

The main limitation of this study is that some selection biases and confounders such as alcohol consumption and the definition of fatty degeneration might affect the internal validity of the study. The study subjects were selected based on a history of liver biopsy or liver resection and infection with HCV genotype 1b. As levels of steatosis have been shown to differ among HCV genotypes, the present study was restricted to HCV genotype 1b, which

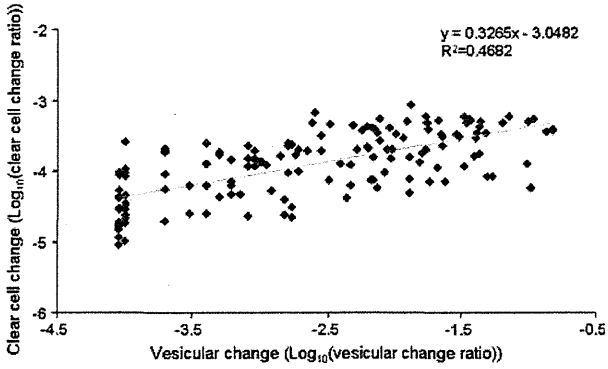

Fig. 4 Association between vesicular and clear cell change. The vesicular and clear cell change ratios were performed with logarithmic transformation, and the distributions were compared. The association between the distribution of vesicular and clear cell change are shown by a scatter diagram

accounts for more than 70% of chronic hepatitis C cases in Japan and is the best studied with respect to IL28B polymorphisms. Because ethanol intake of more than 20 g/day is associated with progressive liver damage [29, 30], patients whose daily alcohol intake exceeded this threshold were defined as alcohol positive. To evaluate fatty degeneration objectively, the distribution of brightness in liver tissue images was used. The brightness was calculated with Dynamic cell count BZ-H1C software and divided into 256 gradation sequences, and the fatty degeneration area was determined with the fixed brightness range. To evaluate the association between fatty degeneration and other factors, statistical analysis using continuous values may be more precise than using thresholds, assuming a large sample size with homoskedastic, normally distributed data. When the data were analyzed using continuous values in univariate

Table 2 Association between vesicular change and clinical background

	Vesicular change ratio		Univariate analysis	Multiple logistic regression analysis		
	<0.002 ($N = 76$)	≥0.002 ($N = 77$)	*P* value	*P* value	Adjusted odds ratio	95% CI
Age (years)[a]	60 (22–76)	61 (15–76)	0.774			
Gender (M:F)	44:32	39:38	0.368**			
BMI (kg m^{-2})[a]	22.0 (16.2–30.6)	23.5 (17.7–39.4)	0.009	0.033	1.147	1.011–1.301
Alcohol (+:−)	26:49	21:54	0.379**			
Platelet count ($\times 10^3$ μl^{-1})[a]	144 (23–759)	143 (49–327)	0.436			
Prothrombin activity (%)[a]	99 (56–124)	100 (12–166)	0.727			
Total bilirubin (mg dl^{-1})[a]	0.7 (0.4–14.7)	0.7 (0.4–2.1)	0.922			
Aspartate aminotransferase (IU l^{-1})[a]	42 (20–2617)	53 (14–250)	0.008	0.317		
Alanine aminotransferase (IU l^{-1})[a]	48 (11–2707)	61 (2–327)	0.093	0.318		
Lactate dehydrogenase (IU l^{-1})[a]	197 (129–899)	204 (43–473)	0.286			
Gamma glutamyl transpeptidase (IU l^{-1})[a]	34 (12–187)	55 (14–295)	<0.001	0.110		
Albumin (g dl^{-1})[a]	4.4 (2.9–5.3)	4.2 (3.1–5.1)	0.069			
Total cholesterol (mg dl^{-1})[a]	174 (100–312)	174 (122–263)	0.975			
Triglycerides (mg dl^{-1})[a]	97 (33–339)	113 (35–2466)	0.141			
Glucose (mg dl^{-1})[a]	98 (68–334)	103 (70–237)	0.533			
HCV RNA (log IU ml^{-1})[a]	6.4 (4.2–7.9)	6.2 (4.2–7.5)	0.031	0.068		
Core 70 (wild:mutant)	37:12	40:27	0.075**	0.230		
Core 91 (wild:mutant)	21:28	33:34	0.495**			
ISDR (0:≥1)	15:33	23:43	0.687**			
IL28B (TT:TG or GG)	65:11	51:26	0.005**	0.001	8.158[b]	2.412–27.589
Fibrosis (F0–1:F2–3)	36:40	23:54	0.026**	0.041	2.541[c]	1.040–6.207
Inflammatory activity (A0–1:A2–3)	24:51	17:60	0.168**			

Univariate analysis was performed with Mann–Whitney *U* test and **chi-square test

Multiple logistic regression analysis was performed using variables that were significant ($P < 0.05$) or marginally significant ($P < 0.10$) in univariate analysis

[a] Median (range)

[b] IL28B genotypes were coded as 0 or 1 depending on whether the subject carried the minor allele

[c] Fibrosis was coded as 0 for patients with mild fibrosis (F0–1) and 1 for patients with severe fibrosis (F2–3)

Table 3 Association between clear cell change and clinical background

	Clear cell change ratio		Univariate analysis	Multiple logistic regression analysis		
	<0.03 ($N = 77$)	≥0.03 ($N = 76$)	P value	P value	Adjusted odds ratio	95% CI
Age (years)[a]	60 (22–76)	61 (15–76)	0.408			
Gender (M:F)	47:29	36:41	0.061**	0.057		
BMI (kg m^{-2})[a]	23.0 (16.2–32.3)	22.7 (17.6–39.4)	0.477			
Alcohol (+:−)	27:47	20:56	0.179**			
Platelet count ($\times 10^3$ μl^{-1})[a]	152 (23–759)	131 (49–327)	0.175			
Prothrombin activity (%)[a]	99 (56–166)	100 (12–136)	0.829			
Total bilirubin (mg dl^{-1})[a]	0.7 (0.4–14.7)	0.7 (0.4–2.1)	0.815			
Aspartate aminotransferase (IU l^{-1})[a]	42 (14–2617)	56 (15–250)	<0.001	0.824		
Alanine aminotransferase (IU l^{-1})[a]	48 (11–2707)	65 (2–327)	0.018	0.809		
Lactate dehydrogenase (IU l^{-1})[a]	197 (123–899)	209 (43–473)	0.193			
Gamma glutamyl transpeptidase (IU l^{-1})[a]	37 (12–187)	47 (13–295)	0.049	0.928		
Albumin (g dl^{-1})[a]	4.3 (2.9–5.1)	4.2 (3.0–5.3)	0.376	0.200		
Total cholesterol (mg dl^{-1})[a]	174 (122–270)	173 (100–312)	0.161			
Triglycerides (mg dl^{-1})[a]	102 (33–2466)	97 (35–517)	0.861			
Glucose (mg dl^{-1})[a]	97 (68–334)	104 (70–284)	0.092	0.456		
HCV RNA (log IU ml^{-1})[a]	6.3 (4.2–7.9)	6.2 (4.2–7.5)	0.060	0.101		
Core 70 (wild:mutant)	35:14	42:25	0.325**			
Core 91 (wild:mutant)	20:29	34:33	0.290**			
ISDR (0:≥1)	14:34	24:42	0.421**			
IL28B (TT:TG or GG)	64:12	52:25	0.016**	0.011	3.000[b]	1.282–7.019
Fibrosis (F0–1:F2–3)	33:43	26:51	0.220**			
Inflammatory activity (A0–1:A2–3)	25:50	16:61	0.081**	0.066		

Univariate analysis was performed with Mann–Whitney U test and **chi-square test

Multiple logistic regression analysis was performed using variables that were significant ($P < 0.05$) or marginally significant ($P < 0.10$) in univariate analysis

[a] Median (range)

[b] IL28B genotypes were coded as 0 or 1 depending on whether the subject carried the minor allele

Table 4 Association between fatty degeneration and IL28B genotype

	IL28B		Univariate analysis
	TT	TG or GG	P value
Obesity group (BMI ≥25 kg m^{-2}, $N = 36$)			
Vesicular change ratio (<0.002:≥0.002)	12:19	2:3	1.000*
Clear cell change ratio (<0.033:≥0.033)	20:11	2:3	0.357*
Non-obesity group (BMI <25 kg m^{-2}, $N = 114$)			
Vesicular change ratio (<0.002:≥0.002)	52:30	9:23	0.001
Clear cell change ratio (<0.033:≥0.033)	48:34	10:22	0.009

Univariate analysis was performed with chi-square test and *Fisher's exact test

analysis using the Mann–Whitney U test, the IL28B genotype was found to be significantly associated with clear cell change and vesicular change ($P = 0.001$, $P < 0.001$, respectively). When continuous values were used in multiple regression, the association between IL28B genotype and fatty degeneration was also observed. *IL28B* genotype, HCV RNA titer and liver fibrosis stage were identified as independent factors for vesicular change ($P = 0.003$, $P = 0.022$, $P = 0.047$, respectively), and the *IL28B* genotype was identified as an independent factor for clear cell change ($P < 0.001$). Genotypes of rs738409 within the Patatin-like phospholipase domain-containing 3 (PNPLA3) locus were also recently reported to be associated with hepatic steatosis in chronic hepatitis C patients