Table 2 Univariate and multivariate analysis of variables associated with waiting list mortality

Variables	Univariate			Multivariate		
	HR	95 % CI	*P* value	HR	95 % CI	*P* value
Age (per year of age)	1.04	1.03–1.05	<0.001	1.04	1.03–1.05	<0.001
Male gender	0.93	0.77–1.13	0.48			
Blood type						
A	1.00	Reference				
B	1.07	0.83–1.43	0.61			
O	1.13	0.90–1.43	0.29			
AB	1.26	0.90–1.77	0.17			
Etiology						
HCV	1.00	Reference				
BA	0.40	0.22–0.72	0.002			
PBC	1.62	1.21–2.16	0.001	1.79	1.34–2.39	<0.001
PSC	0.79	0.54–1.17	0.24			
HBV	0.77	0.56–1.05	0.10			
Alcohol	0.95	0.59–1.53	0.83			
AIH	0.77	0.34–1.74	0.52			
NASH	1.11	0.76–1.63	0.59			
HCC	1.46	1.05–2.05	0.003			
Metabolic disease	0.40	0.22–0.75	0.004			
Polycystic disease	0.26	0.10–0.70	0.008	0.27	0.10–0.73	0.01
Vascular disease	0.009	0.01–0.67	0.002			
Others	0.70	0.34–1.43	0.33			

AIH autoimmune hepatitis, *BA* biliary atresia, *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma, *HCV* hepatitis C virus, *HR* hazard ratio, *NASH* non-alcoholic steatohepatitis, *PBC* primary biliary cirrhosis, *PSC* primary sclerosing cholangitis

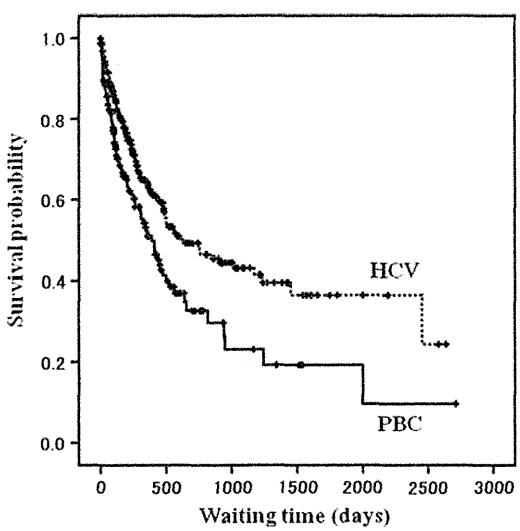


Fig. 2 Kaplan–Meier curves comparing the cumulative waiting list survival probability of patients with chronic hepatitis C (HCV, $n = 254$) and primary biliary cirrhosis (PBC, $n = 156$)

Table 3 Comparison of patient characteristics between HCV and PBC

Variable	HCV ($n = 189$)	PBC ($n = 81$)	*P* value
Age (years)	55 (29–69)	52 (27–69)	0.02[a]
Gender (male/female)	143/46	15/66	<0.001[b]
Platelet count ($\times 10^4/\mu L$)	6.0 (1.7–49.0)	10.2 (2.2–42.3)	<0.001[a]
Albumin (g/dL)	2.8 (1.8–4.4)	2.8 (1.4–4.2)	0.96[a]
Total bilirubin (mg/dL)	2.7 (0.4–39.8)	7.2 (0.7–41.2)	<0.001[a]
Creatinine (mg/dL)	0.78 (0.4–7.4)	0.67 (0.37–2.83)	<0.001[a]
Prothrombin time (%)	54.7 (11.0–103.0)	62.2 (16.0–120.0)	0.001[a]
INR	1.51 (0.98–6.24)	1.32 (0.91–4.31)	0.001[a]
MELD score	15 (7–52)	17.5 (8–39)	0.002[a]
CTP score	10 (6–15)	10 (5–15)	0.27[a]
Medical point (1, 3/6, 9)	54/135	22/59	0.81[b]

Data are shown as median (range). Data were available for patients who were listed after June 22, 2006

CTP Child–Turcotte–Pugh, *HCV* hepatitis C virus, *INR* international normalized ratio, *MELD* model of end-stage liver disease, *PBC* primary biliary cirrhosis

[a] Mann–Whitney *U* test

[b] Chi-square test

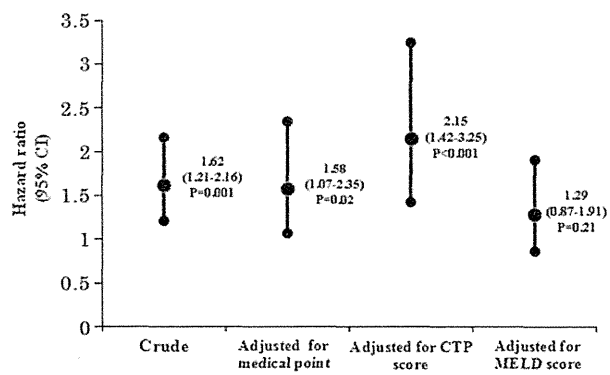


Fig. 3 Adjusted risk of waiting list mortality for patients with primary biliary cirrhosis compared with patients with chronic hepatitis C

To examine which disease severity index was able to assess the risk of PBC patients accurately, we estimated their relative hazards with adjustment for each index. We did not estimate age-adjusted relative hazard because age was not included in the allocation measures. Figure 3 indicates the crude and disease severity index-adjusted HR for waiting list mortality of PBC patients with reference to HCV patients. In univariate analysis, PBC patients were at 62 % (HR 1.62; 95 % CI 1.21–2.16, $P = 0.001$) increased risk of waiting list mortality

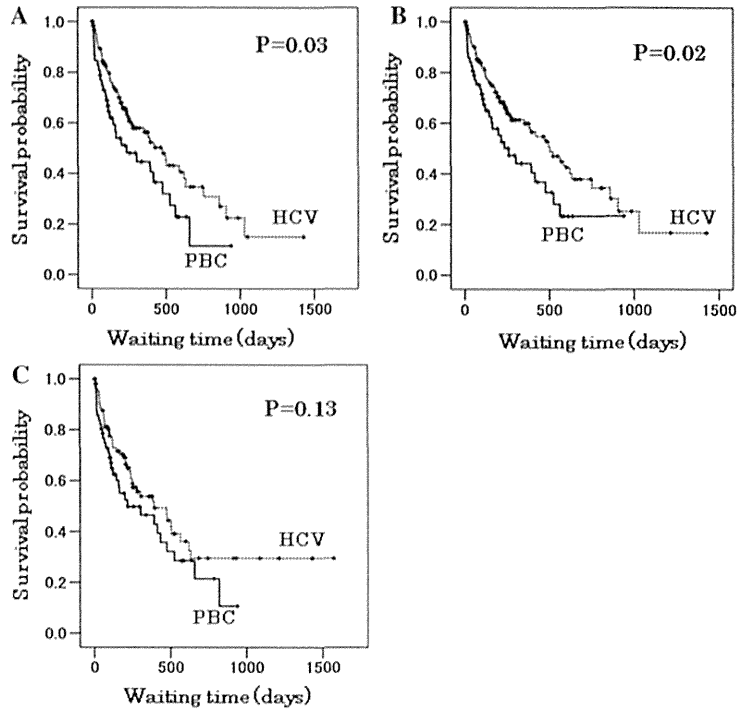


Fig. 4 Kaplan–Meier curves comparing the cumulative waiting list survival probability of patients with chronic hepatitis C (HCV) and primary biliary cirrhosis (PBC). Patients stratified medical point = 6 (**a**), and Child–Turcotte–Pugh score ≥10 (**b**), and Model of End-Stage Liver Disease (MELD) score ≥15 (**c**)

compared with HCV patients. In bivariate analysis, the medical point-adjusted HR of waiting list mortality of PBC patients was significantly higher than that of HCV patients (HR 1.58; 95 % CI 1.07–2.35, $P = 0.02$). The CTP score-adjusted HR also showed a significantly increased risk of waiting -list mortality in PBC patients (HR 2.15; 95 % CI 1.42–3.25, $P < 0.001$). However, the MELD score-adjusted HR did not show a statistically significant risk of waiting list mortality in PBC patients (HR 1.29; 95 % CI 0.87–1.91, $P = 0.21$).

Waiting list survival of patients with HCV and PBC was compared with stratification by each of the disease severity indices (Fig. 4). Patients with medical point 6, for which most PBC and HCV patients were registered, showed a significantly shorter waiting list survival for PBC patients than of HCV patients (median 261 vs. 503 days, $P = 0.02$). In patients with CTP score ≥10, the score classified as C, the shorter waiting list survival of PBC patients was also significant (median 235 vs. 475 days, $P = 0.03$). On the other hand, when they were selected by MELD ≥15, the score indicating patients who can be expected to achieve improved survival with liver transplantation [12], there was no significant difference in the waiting list survival rate between them ($P = 0.13$).

Discussion

The result of this study clearly indicated that the most common reason for removal from the waiting list in Japan was "waiting list death", which was a combination of death and becoming too sick for transplantation. The waiting list death included 58.1 % of all the patients removed from the list. In the United States, a recent report indicated that waiting list death was the reason for removal from the list in 25.9 % of adult patients [1]. Although this report included patients with acute liver failure and re-transplantation, high waiting list mortality in Japan was evident. Thus, the high mortality rate on the liver transplant waiting list is a major challenge in Japan. Moreover, severe donor organ shortage in Japan should contribute to the high waiting list mortality [13]; an improved organ allocation policy will be necessary to cause a decrease in waiting list death.

In this study, we found that PBC patients had a significantly higher risk of waiting list mortality compared with patients with other etiologies in the JOT registry. Since PBC is currently the third most common diagnosis in the JOT registry for liver transplantation, poor waiting list survival of PBC patients would contribute to the high waiting list mortality in Japan. PBC is a cholestatic liver disease that causes bile duct deterioration and progresses slowly to a terminal phase characterized by hyperbilirubinemia, signs of decompensated cirrhosis, ascites, and variceal bleeding. Only one type of medical therapy, involving the use of ursodeoxycholic acid (UDCA), is now widely recognized to improve the prognosis of PBC patients. Many studies have shown that UDCA therapy not only improves biochemical indices, but also delays histologic progression and improves survival without transplantation [14–16]. However, evidence has also accumulated that the

favorable effect of UDCA therapy is limited to patients with early-stage disease. In histologically advanced patients or biochemical non-responders, the transplant-free survival rate of UDCA-treated patients was not different from spontaneous survival [16, 17]. This means that PBC patients have no effective medical therapeutic option to prolong their survival when they have progressed to end-stage liver disease, and liver transplantation remains the only hope of a cure [18, 19]. PBC patients in our cohort also showed a consistently poor survival of a median period of 392 days.

The reason why PBC patients have a higher risk for waiting list mortality compared with patients with other etiologies of chronic liver disease is not clearly understood. Interestingly, PBC patients were younger, and their INR and serum creatinine levels were lower than for HCV patients at registration. This indicated that neither age nor liver and renal function at registration alone caused poor waiting list survival of PBC patients; the registration of PBC patients was not later than that for HCV patients. The rate of disease progression and lethal complications might be involved in their short waiting list survival rate. Moreover, the actual waiting list survival rate in PBC patients was not greater than the updated Mayo score-predicted spontaneous survival rate. This observation indicated that the PBC patients on the waiting list were refractory to the medical therapy and their waiting list survival suddenly deteriorated. Further analyses, particularly on the cause of death, are required to clarify the pathophysiology of PBC patients who have progressed to end-stage liver disease.

In general, deceased donor livers are allocated for transplantation on the basis of "sickest first", i.e., those who are more likely to die without a liver transplantation are assigned the highest priority. Therefore, the disease severity index used in the liver allocation system should consider the urgency of PBC patients for liver transplantation. However, our results have clarified the inability of the currently used Japanese allocation system to identify the risk of PBC patients. The medical point-adjusted HR of PBC patients revealed that they were at 58 % increased risk of waiting list mortality compared with HCV patients. In addition, the CTP score-adjusted HR showed that PBC patients were at 115 % increased risk for waiting list mortality. Thus, it is not only the current allocation system but also the CTP score-based allocation that cannot capture the risk for waiting list mortality in PBC patients. On the other hand, we found that the MELD score-adjusted HR of PBC patients lost statistical significance, and stratification by MELD score revealed comparable survival curves between patients with PBC and HCV. These results indicated that PBC patients had a similar risk of waiting list mortality compared with patients with other etiologies when they were stratified by MELD score. At the time of registration, the patients with HCV and PBC had different characteristics; however, only the MELD score accurately evaluated their disease severity, and therefore, MELD-based allocation would adequately assign priority to the patients according to their risk of waiting list mortality. Thus, our results demonstrated that the MELD score was superior to both the current Japanese allocation and CTP score-based allocation for ranking patients in the JOT registry by their risk of waiting list mortality.

In addition, patients should be re-evaluated according to their chronological change of hepatic failure to improve allocation. However, most patients with chronic liver disease were waiting at medical point 6 as an upper limit, because the highest priority at medical point 9 was generally awarded to the patients with acute liver failure or early graft failure in the current Japanese allocation system. Therefore, the current allocation system did not completely reflect the chronological change in the degree of liver failure. Thus, the MELD score, which was expressed numerically as a continuous variable with a wide dynamic range in the evaluation of hepatic decompensation, would have an advantage over the medical point system for assessing the chronological change in patients' risk of death.

In conclusion, this study demonstrated that patients with PBC, the third most common indication for liver transplantation in Japan, have a high risk for waiting list mortality in the current Japanese allocation system. The allocation system should be changed to accurately prioritize the patients with a higher mortality risk; MELD-based allocation would be suitable for this purpose and could reduce the waiting list mortality of PBC patients.

Acknowledgments This study was supported by a Health Labor Sciences Research Grant, Research on Measures for Intractable Diseases, from the Ministry of Health, Labor and Welfare of Japan.

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

References

1. OPTN/SRTR 2010 Annual Data Report. Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation; 2011. Available at: http://www.srtr.org/annual_reports/2010/. Accessed 3 Sept 2012.
2. Dickson ER, Grambsch PM, Fleming TR, Fisher LD, Langworthy A. Prognosis in primary biliary cirrhosis: model for decision making. Hepatology. 1989;10:1–7.
3. Murtaugh PA, Dickson ER, Van Dam GM, Malinchoc M, Grambsch PM, Langworthy AL, et al. Primary biliary cirrhosis: prediction of short term survival based on repeated patients visits. Hepatology. 1994;20:126–34.
4. Dickson ER, Murtaugh PA, Wiesner RH, Grambsch PM, Fleming TR, Ludwig J, et al. Primary sclerosing cholangitis: refinement

and validation of survival models. Gastroenterology. 1992;103: 1893–901.
5. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg. 1973;60:646–9.
6. Malinchoc M, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. Hepatology. 2000; 31:864–71.
7. Kamath PS, Wiesner R, Malinchoc M, Kremers W, Therneau TM, Kosbery CL, et al. A model to predict survival in patients with end-stage liver disease. Hepatology. 2001;33:464–70.
8. Forman LM, Lucey MR. Predicting the prognosis of chronic liver disease: an evolution from Child to MELD. Mayo End-stage Liver Disease. Hepatology. 2001;33:473–5.
9. Said A, Williams J, Holden J, Remington P, Gangnon R, Musat A, et al. Model for end-stage liver disease score predicts mortality across a broad spectrum of liver disease. J Hepatol. 2004;40: 897–903.
10. Wiesner R, Edwards E, Freeman R, Harper A, Kim R, Kamath P, et al. Model for end-stage liver disease (MELD) and allocation of donor livers. Gastroenterology. 2003;124:91–6.
11. Organ distribution: allocation of livers. Available at: http://optn.transplant.hrsa.gov/PoliciesandBylaws2/policies/pdfs/policy_8.pdf. Accessed 3 Sept 2012.
12. Murray KF, Carithers RI Jr. AASLD practice guidelines: evaluation of the patient for liver transplantation. Hepatology. 2005; 41:1–26.
13. Japan Organ Transplant Network. Available at: http://www.jotnw.or.jp/datafile/index.html in Japanese. Accessed 3 Sept 2012.
14. Poupon RE, Poupon R, Balkau B. Ursodiol for the long-term treatment of primary biliary cirrhosis. The UDCA-PBC study group. N Engl J Med. 1994;330:1342–7.
15. Corpechot C, Carrat F, Bonnand AM, Poupon RE, Poupon R. The effect of ursodeoxycholic acid therapy on liver fibrosis progression in primary biliary cirrhosis. Hepatology. 2000;32:1196–9.
16. Corpechot C, Carrat F, Bahr A, Chretien Y, Poupon RE, Poupon R. The effect of ursodeoxycholic acid therapy on the natural course of primary biliary cirrhosis. Gastroenterology. 2005;128: 297–303.
17. Corpechot C, Abenavoli L, Rabahi N, Chretien Y, Andreani T, Johanet C, et al. Biochemical response to ursodeoxycholic acid and long-term prognosis in primary biliary cirrhosis. Hepatology. 2008;48:871–7.
18. Markus BH, Dickinson E, Grambsh P, Fleming TR, Mazzaferro V, Klintmalm GB, et al. Efficacy of liver transplantation in patients with primary biliary cirrhosis. N Engl J Med. 1989;320: 1709–13.
19. Kim WR, Wiesner RH, Therneau TM, Poterucha JJ, Porayko MK, Evans RW, et al. Optimal timing of liver transplantation for primary biliary cirrhosis. Hepatology. 1998;28:33–8.

JGH Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.12401

HEPATOLOGY

Prediction of liver stiffness hepatocellular carcinoma in chronic hepatitis C patients on interferon-based anti-viral therapy

Yutaka Narita,* Takuya Genda,* Hironori Tsuzura,* Shunsuke Sato,* Yoshio Kanemitsu,* Sachiko Ishikawa,* Tetsu Kikuchi,* Katsuharu Hirano,* Katsuyori Iijima,* Ryo Wada† and Takafumi Ichida*

Departments of *Gastroenterology and Hepatology and †Pathology, Juntendo University Shizuoka Hospital, Shizuoka, Japan

Key words
chronic hepatitis C, hepatocellular carcinoma, liver stiffness, risk factor.

Accepted for publication 31 August 2013.

Correspondence
Dr Takuya Genda, Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, 1129 Nagaoka Izunokuni-shi, Shizuoka 410-2295, Japan.
Email: genda@rice.ocn.ne.jp

Disclosure: The authors declare no conflict of interest.

Abstract

Background and Aim: The purpose of this study was to evaluate the usefulness of liver stiffness measurement (LSM) for assessing the risk of hepatocellular carcinoma (HCC) in chronic hepatitis C (CHC) patients receiving interferon (IFN) therapy.

Methods: One hundred fifty-one CHC patients who underwent LSM and received IFN therapy were included in the estimation cohort, and 56 were included in the validation study. The cumulative HCC incidences were evaluated using Kaplan–Meier plot analysis and the log-rank test. Multivariate Cox proportional hazard analyses were used to estimate the hazard ratios (HRs) of variables for HCC.

Results: In the estimation cohort, 9 of 151 patients developed HCC during the median follow-up time of 722 days. Multivariate analysis identified three independent risk factors for HCC: LSM ($\geq$ 14.0 kPa, HR 5.58, $P = 0.020$), platelet count ($< 14.1 \times 10^4/\mu L$, HR 5.59, $P = 0.034$), and non-sustained virological response (HR 8.28, $P = 0.049$). The cumulative incidence of HCC development at 3 years was 59.6%, 8.2%, and 0.0% in patients with all three risk factors, one to two risk factors, and none of these risk factors, respectively. The incidence of HCC was significantly different between these groups ($P < 0.001$). In the validation cohort, HCC incidence was also significantly different with respect to these risk factors ($P = 0.037$).

Conclusion: LSM, platelet count, and IFN-therapeutic effect could be used to successfully stratify the risk of HCC in patients receiving IFN therapy and demonstrate the usefulness of LSM before IFN therapy for the management of CHC patients.

Introduction

Persistent hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease leading to the development of HCC, the fifth most common cancer, and the third most common cause of cancer-related death worldwide.[1] HCV is responsible for 27–75% of the HCC cases in Europe and the United States and > 80% of the HCC cases in Japan.[2,3] In fact, HCV-positive patients have a 20-fold higher risk of developing HCC than HCV-negative patients,[4] indicating a significant carcinogenic role for persistent HCV infection. Because of this connection, many chronic hepatitis C (CHC) patients are treated with interferon (IFN)-based antiviral therapy because it not only eradicates HCV but also reduces the rate of HCC development. IFN therapy is most effective at decreasing the risk of developing HCC in patients that achieve a sustained virological response (SVR);[5–7] however, the risk of HCC development persists after IFN therapy even in patients who do achieve SVR.[8] HCC might develop immediately after IFN therapy in some cases, or during long-term IFN therapy in others.[9,10] Because assessing the risk of developing HCC is clinically important in the management of CHC patients, it is necessary to establish predictors for HCC development in patients who receive IFN therapy.

Some factors reported to predict the risk of HCC development after IFN therapy are older age, male gender, and severe fibrosis,[11,12] with advanced fibrosis and cirrhosis significantly correlating with the risk of HCC development.[13] To date, liver biopsy has been the gold standard for assessing the severity of liver fibrosis and cirrhosis,[14] although sampling errors and intraobserver and interobserver variability can lead to understaging.[15,16] In addition, it is difficult to perform liver biopsy for all patients because of its invasiveness and rare but potentially life-threatening complications.[14] As a result, liver stiffness measurement (LSM), a type of transient elastography, has become a reliable alternative for assessing hepatic fibrosis and cirrhosis mainly in patients with CHC.[17,18] LSM is non-invasive, reproducible, can be expressed numerically as continuous values, and has a wide dynamic range in the evaluation of hepatic fibrosis. These advantages over liver biopsy

suggest the clinical usefulness of LSM for predicting HCC development. Here, we evaluated factors that affect the occurrence of HCC in CHC patients receiving IFN therapy, with a special focus on the predictive value of LSM.

Methods

Patients. Between October 2007 and April 2011, a total of 207 consecutive CHC patients who underwent a successful LSM and then received IFN-based antiviral therapy at the Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, Shizuoka, Japan, were retrospectively enrolled in this study. CHC diagnosis was based on serum HCV-RNA positivity. Exclusion criteria were as follows: (i) hepatitis B surface antigen positivity; (ii) other causes of liver disease of mixed etiologies, including autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease; (iii) evidence of hepatocellular carcinoma (HCC) on ultrasonography or computed tomography; (iv) previous history of liver transplantation; and (v) treatment for HCC. This study was approved by the Ethics Committee of Juntendo University Shizuoka Hospital in accordance with the Helsinki Declaration, and all patients provided written informed consent.

Of these 207 patients, 151 underwent ultrasonography-guided percutaneous liver biopsy within a week before treatment initiation. Liver biopsy specimens were embedded in paraffin and stained with hematoxylin-eosin, Azan-Mallory, and reticulin silver impregnation. The specimens were evaluated by an experienced pathologist who was blinded to the patients' clinical data. Histological evaluation was based on the METAVIR criteria.[19] Hepatic fibrosis was defined as follows: F0, no fibrosis; F1, periportal fibrous expansion; F2, portal fibrous widening with bridging fibrosis; F3, bridging fibrosis with lobular distortion; and F4, liver cirrhosis. On the basis of the degree of lymphocyte infiltration and hepatocyte necrosis, inflammation was scored from A0 to A3, with higher scores indicating more severe inflammation. The 151 patients who underwent liver biopsy were enrolled into the estimation group for the identification of risk factors for HCC development, and the remaining 56 patients who did not undergo liver biopsy were enrolled into a group for the validation of these identified risk factors.

All laboratory tests were performed for each patient just before initiation of IFN therapy. Blood cell counts, serum alanine transaminase, gamma-glutamyl transpeptidase, hemoglobin A1c, total bilirubin, albumin, prothrombin time, and alpha-fetoprotein (AFP) were measured using commercially available assays. The HCV genotype was determined using polymerase chain reaction with the HCV Genotype Primer Kit (Institute of Immunology Co., Ltd., Tokyo, Japan) and classified as genotype 1, genotype 2, or other, according to Simmonds' classification system. Serum HCV viral load was determined using quantitative reverse transcription polymerase chain reaction using the COBAS TaqMan HCV Test (Roche Diagnostics, Branchburg, NJ, USA).

Treatment protocol. The treatment protocol for CHC patients consisted of 1.5 μg/kg of pegylated IFN-α-2b or 180 μg of pegylated IFN-α-2a once a week, combined with ribavirin at an oral dose of 600–1000 mg/day. Duration of the treatment was 48–72 weeks for those with HCV genotype 1 and a serum HCV viral load > 5 log IU/mL. For all other patients, treatment lasted for 24 weeks. SVR was defined as undetectable serum HCV-RNA at 24 weeks after the end of treatment.

Measurement of liver stiffness. Measurement of liver stiffness by transient elastography was performed using FibroScan (Echosens, Paris, France) within a week before treatment initiation. Technical details of the examination and procedure have been reported previously.[17] Ten validated measurements were made on each patient, and results were expressed in kilopascals (kPa). Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable, and the median value was considered representative of the liver elastic modulus.

Patient follow-up and HCC diagnosis. Serum AFP was measured every month, and ultrasonography or computed tomography were performed at least every 3–6 months for HCC surveillance during and after treatment, with a minimum follow-up duration of 6 months after the initiation of IFN therapy. HCC was diagnosed by histological examination and/or triphasic computerized tomography, in which hyperattenuation in the arterial phase with washout in the late phase is pathognomonic for HCC.[20] The status of patients enrolled in this study was confirmed as of March 2012.

Statistical analyses. All analyses were conducted using IBM SPSS version 19 (IBM SPSS, Chicago, IL, USA), and P values less than 0.05 were considered statistically significant. Continuous variables and categorical variables were summarized as median (range) and percentage, respectively. Mann–Whitney U and chi-square tests were used when appropriate. The strength of the association between LSM and the histological fibrosis stage was estimated using the Spearman's rank correlation coefficient. Cumulative incidences of HCC development were estimated by Kaplan–Meier analysis and compared using the log-rank test. Cox logistic regression analysis was used for multivariate analysis to identify factors that were independently associated with HCC development. The cut-off value of each factor for predicting the development of HCC was determined using receiver operator characteristics analysis.

Results

Patient characteristics. A total of 229 patients received LSM followed by IFN-based antiviral therapy at Juntendo Shizuoka Hospital during the study period. Twenty-two patients (9.6%) were excluded because of LSM failure and/or an invalid LSM. Of the remaining 207 patients, 151 underwent liver biopsy prior to IFN therapy and together formed the risk factor-estimation cohort. The clinical, anthropometric, and laboratory data of the estimation cohort are summarized in Table 1. The 151 patients (83 male and 68 female) had a median age of 62 years (range 22–82 years) and a median LSM of 8.8 kPa (range 2.8–45.7 kPa). There was a significant positive association between LSM and histological fibrosis stage ($r = 0.59$, $P < 0.001$). The prevalence of genotype

Table 1 Baseline characteristics of the estimation cohort

Variables	All	HCC development (+)	HCC development (–)	*P*-value
Number of patients	151	9	142	
Age (years)	62 (22–82)	67 (60–82)	61 (22–80)	0.010†
Male (%)	55	55.6	54.9	1.000‡
BMI (kg/m^2)	23.5 (18.1–36.8)	23.8 (23.3–25.7)	23.4 (18.1–36.8)	0.217†
Habitual drinker (%)	10.6	11.1	10.6	1.000‡
Fibrosis stage (F0–2/F3–4)	115/36	5/4	110/32	0.048‡
Inflammatory grade (A0–1/A2–3)	33/118	0/9	33/109	0.101‡
LSM (kPa)	8.8 (2.8–45.7)	14.8 (9.8–45.7)	8.7 (2.8–34.8)	0.002†
Observation period (days)	722 (189–1378)	688 (189–1217)	733 (190–1378)	0.467†
Genotype 1 (%)	56.3	100	53.5	0.065‡
HCV-RNA (log IU/mL)	6.4 (0.0–7.7)	6.5 (2.9–7.2)	6.3 (0.0–7.7)	0.168†
Albumin (g/dL)	4.1 (3.4–4.8)	4.1 (3.5–4.6)	4.1 (3.4–4.8)	0.390†
ALT (IU/L)	59 (10–410)	75 (27–181)	57 (10–410)	0.467†
Total bilirubin (mg/dL)	0.7 (0.3–1.8)	0.8 (0.5–1.3)	0.7 (0.3–1.8)	0.070†
γGTP (IU/L)	44 (4–517)	75 (31–129)	41 (4–517)	0.120†
Hemoglobin A1c (%)	5.1 (3.7–8.2)	5.1 (3.7–6.1)	5.1 (4.2–8.2)	0.561†
Ferritin (ng/mL)	134 (8–2096)	215 (8–1026)	134 (9–2096)	0.675†
White blood cell count (× 10^3/μL)	4.9 (2.0–10.3)	4.3 (3.0–7.3)	4.9 (2.0–10.3)	0.496†
Hemoglobin (g/dL)	13.8 (8.9–17.5)	13.3 (9.9–17.5)	13.8 (8.9–17.1)	0.376†
Platelet count (× 10^4/μL)	16.3 (5.2–37.0)	9.6 (5.2–19.4)	16.5 (5.8–37.0)	0.004†
Prothrombin time (%)	100 (70–157)	93 (79–120)	102 (70–157)	0.185†
AFP (ng/mL)	6 (1–306)	14 (4–109)	6 (1–306)	0.004†
SVR rate (%)	55	11.1	57.7	0.011‡

Scale data are shown as median (range). *P* values are for comparisons between patients with and without HCC development.
†Mann–Whitney *U* test.
‡Chi-square test.
γGTP, γ-glutamyl transpeptidase; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BMI, body mass index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LSM, liver stiffness measurement; SVR, sustained virological response.

1 HCV infection was 56.3%. Following IFN-based antiviral therapy, SVR was obtained in 83 of the 151 patients (55%). During the median follow-up period of 722 days (range 189–1378 days), nine patients (6.0%) developed HCC. The cumulative incidence of HCC estimated using the Kaplan–Meier method was 1.3%, 4.5%, and 9.0% at 1, 2, and 3 years, respectively (Fig. 1). Compared with patients who had not developed HCC, HCC patients were of advanced age and had a high LSM, a high fibrosis stage, a low platelet count, and a low SVR rate (Table 1).

Risk analyses. Univariate analysis revealed that age ($P = 0.029$), LSM ($P = 0.005$), platelet count ($P = 0.002$), AFP ($P = 0.003$), and non-SVR ($P = 0.011$) were associated with HCC development (Table 2). Multivariate Cox logistic regression analysis identified three independent risk factors: LSM ≥ 14.0 kPa (hazard ratio [HR] 5.58, 95% confidence interval [CI] 1.32–23.64, $P = 0.02$), non-SVR (HR 8.28, 95% CI 1.01–68.05, $P = 0.049$), and platelet count < 14.1 × 10^4/μL (HR 5.59, 95% CI 1.14–27.53, $P = 0.034$), Table 3. The 1-, 2-, and 3-year cumulative incidence rates of HCC development in patients with LSM < 14.0 kPa were 0.8%, 2.3%, and 4.6%, respectively, whereas those with LSM ≥ 14.0 kPa were 3.2%, 12.0%, and 22.2%, respectively ($P = 0.005$) (Fig. 2a). The cumulative incidence rates of HCC development in patients with SVR were 0.0%, 2.0%, and 2.0%, respectively, whereas those without SVR were 3.0%, 7.4%, and 17.1%, respectively ($P = 0.011$) (Fig. 2b). The cumulative incidence rates of HCC development in patients with a platelet count ≥ 14.1 × 10^4/μL were 0.0%, 0.0%, and 4.2%, respectively, whereas those with a platelet count < 14.1 × 10^4/μL were 4.0%, 13.4%, and 19.1%, respectively ($P = 0.002$) (Fig. 2c).

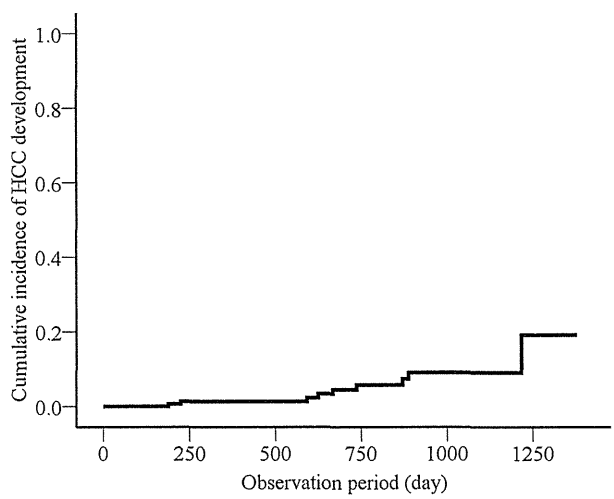


Figure 1 Incidence of hepatocellular carcinoma (HCC) in 151 patients with chronic hepatitis C receiving interferon-based anti-viral therapy estimated using the Kaplan–Meier method.

Table 2 Univariate analysis of factors associated with hepatocellular carcinoma development

Variables	*n*	Cumulative incidence of HCC (%)		*P*-value
		1 year	3 years	
Age (years)				
< 60	63	0.0	0.0	0.029
≥ 60	88	2.3	13.6	
Sex				
Female	68	1.5	12.1	0.910
Male	83	1.2	6.7	
BMI† (kg/m²)				
< 23.8	50	0.0	5.3	0.250
≥ 23.8	42	2.4	6.0	
Habitual drinker				
No	135	0.8	9.6	0.905
Yes	16	6.2	6.2	
Fibrosis stage				
F0–2	115	0.9	6.7	0.228
F3–4	36	2.9	15.0	
LSM (kPa)				
< 14	119	0.8	4.6	0.005
≥ 14	32	3.2	22.2	
ALT (IU/L)				
< 55	71	0.0	4.9	0.123
≥ 55	80	2.5	12.9	
γGTP† (IU/L)				
< 55	83	0.0	5.2	0.057
≥ 55	67	3.0	13.5	
Hemoglobin A1c† (%)				
< 5.5	109	0.9	6.8	0.219
≥ 5.5	25	0.0	18.8	
Ferritin† (ng/mL)				
< 210	74	1.4	10.0	0.175
≥ 210	43	2.3	16.3	
Platelet count (× 10^4/μL)				
≥ 14.1	101	0.0	4.2	0.002
< 14.1	50	4.0	19.1	
AFP† (ng/mL)				
< 10	95	0.0	5.6	0.003
≥ 10	38	4.9	22.3	
SVR				
Yes	83	0.0	2.0	0.011
No	68	3.0	17.1	

†Data not available for all patients.
AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BMI, body mass index; γGTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; LSM, liver stiffness measurement; SVR, sustained virological response.

Number of risk factors and HCC development. The number of risk factors varied between patients: 12 patients (7.9%) had all three risk factors, 32 patients (21.2%) had two, 50 patients (33.1%) had one, and 57 patients (37.7%) had none of these risk factors (Fig. 3). Patients without these risk factors did not develop HCC during the study period. In patients with 1 or 2 risk factors, the cumulative incidence rates at 1, 2, and 3 years were 1.2%, 3.1%, and 8.2%, respectively, whereas patients with all three risk

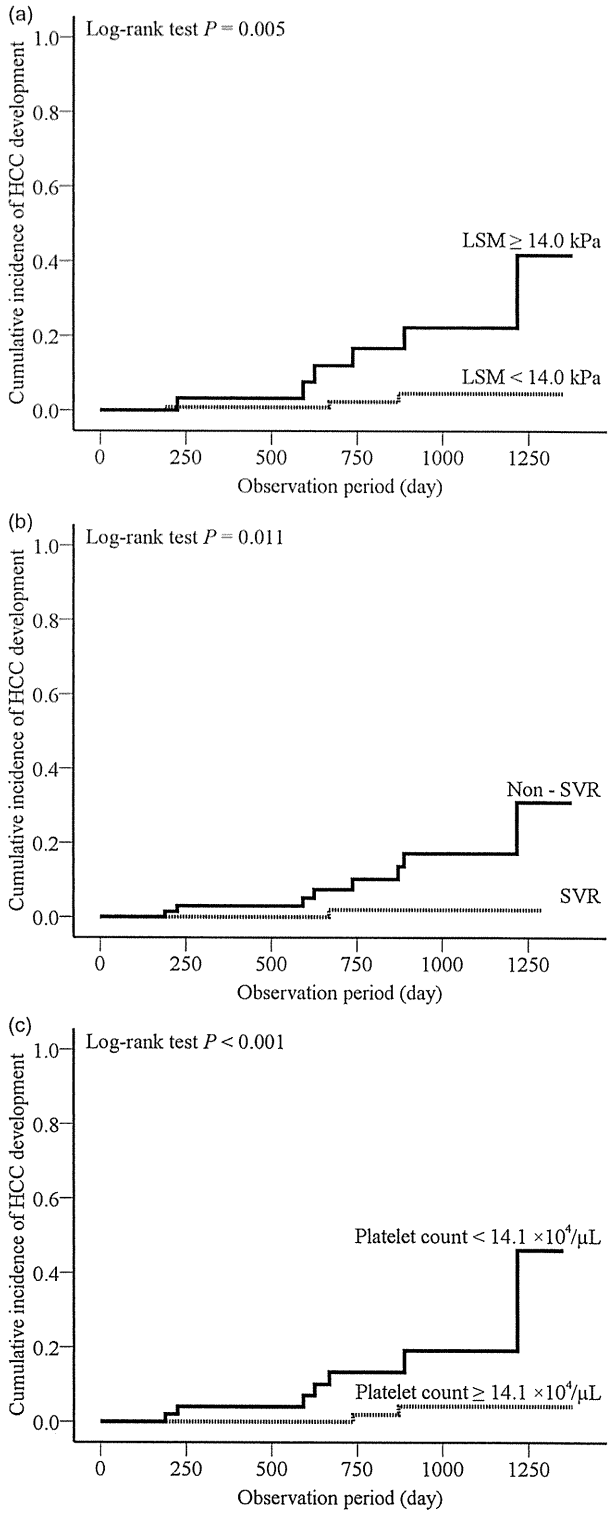


Figure 2 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were stratified according to liver stiffness measurement (LSM) (a), sustained virological response (SVR) (b), and platelet count (c).

Table 3 Multivariate analysis of factors associated with hepatocellular carcinoma development

Variable		Hazard ratio (95% CI)	*P*-value
LSM (kPa)	< 14.0	1.00	0.020
	≥ 14.0	5.58 (1.32–23.64)	
SVR	SVR	1.00	0.049
	Non-SVR	8.28 (1.01–68.05)	
Platelet count (× 10^4/μL)	> 14.1	1.00	0.034
	≤ 14.1	5.59 (1.14–27.53)	

CI, confidence interval; LSM, liver stiffness measurement; SVR, sustained virological response.

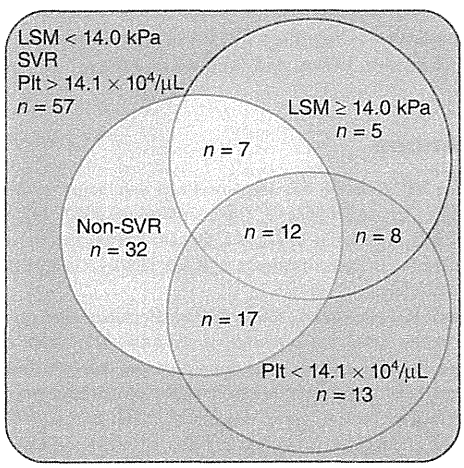


Figure 3 Patient distribution at each risk factor. LSM, liver stiffness measurement; Plt, platelet count; SVR, sustained virological response.

factors had significantly higher cumulative incidence rates (9.1%, 39.4%, and 59.6% at 1, 2, and 3 years, respectively; log-rank test, $P < 0.001$) (Fig. 4).

The relationship between the number of risk factors and HCC development in the validation cohort. Fifty-six patients who received IFN therapy without liver biopsy were enrolled into the validation group for analysis of these three risk factors. The 56 patients (33 male and 23 female) had a median age of 65 years (range 35–79 years) and a median LSM of 8.0 kPa (range 2.6–32.0 kPa). There were no significant differences in clinical, anthropometric, and laboratory findings between the validation and estimation cohorts (data not shown). In the validation cohort, seven patients (12.5%) had all three risk factors, 25 patients (44.6%) had one or two risk factors, and 24 patients (42.9%) had none of these risk factors. Patients without these risk factors did not develop HCC during the study period. In patients with one or two risk factors, and patients with all three risk factors, the cumulative incidence rates at 3 years were 12.7% and 28.6%, respectively. There was also a significant difference in the cumulative incidences of HCC development according to the number of risk factors ($P = 0.037$, Fig. 5).

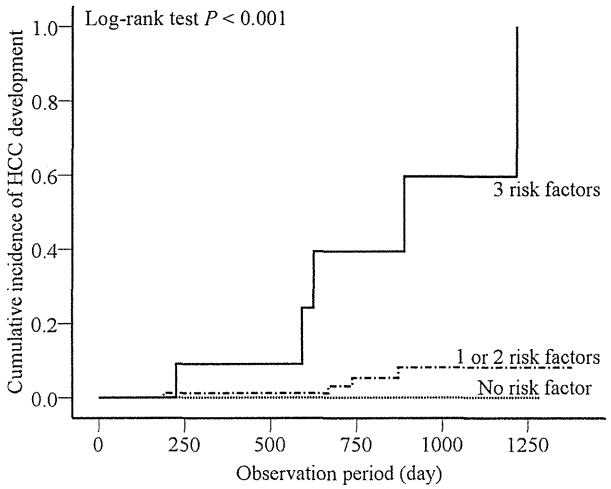


Figure 4 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were stratified according to the number of risk factors.

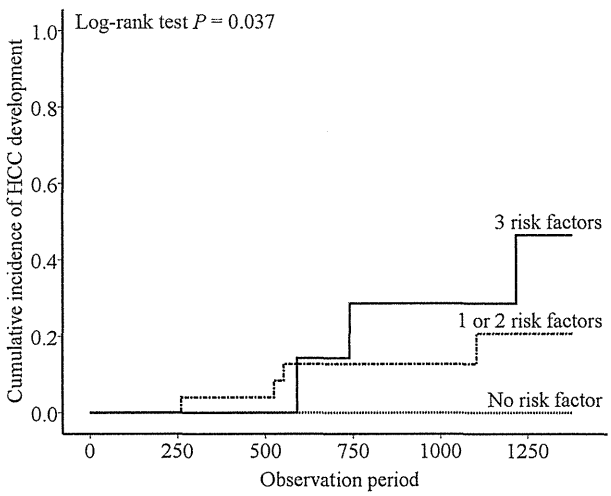


Figure 5 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development in the validation cohort. Patients were stratified according to the number of risk factors they had.

Discussion

Patients with liver cirrhosis or pre-existing severe hepatic fibrosis have a higher risk of developing HCC,[2] even after IFN-based therapy with SVR.[9,10] Clinical diagnosis of liver cirrhosis can be easily made in cases showing stigmata of end-stage liver disease, such as ascites, jaundice, variceal bleeding, and hepatic encephalopathy; however, diagnosis becomes difficult if the liver shows compensation, and normal or near-normal laboratory findings. Liver biopsy has been considered the only diagnostic method for the assessment of early compensated cirrhosis, although

several studies have pointed out sampling variability as a potential limitation of biopsy to diagnose cirrhosis.[21,22] Given the importance of assessing the HCC risk factors in managing CHC patients, we evaluated factors that affect the occurrence of HCC in CHC patients receiving IFN therapy, with a special focus on the predictive value of LSM as an alternative to liver biopsy.

Our data identified three risk factors for developing HCC after IFN therapy. Consistent with previous reports,[5–7] we found that failure to achieve SVR was a significant predictor of HCC development among patients receiving IFN therapy. Although it is possible that IFN therapy itself reduces the risk of HCC,[6,7] non-SVR patients had an approximately eightfold higher risk of developing HCC than SVR patients. In addition, we identified both high LSM and low platelet count as significant predictors of HCC development independently of non-SVR. The LSM threshold ≥ 14.0 kPa identified here as a risk factor for HCC is in agreement with previously reported cut-off values for liver cirrhosis,[15,16] further supporting the idea that pre-existing liver cirrhosis increases the risk of HCC development. Similar to LSM, the platelet count reflects the severity of CHC[21] and is used to estimate the degree of fibrosis.[23–25] Previous reports have also shown low platelet counts to represent a risk of HCC.[23,24] Our cohort showed that LSM was sometimes high even in patients without a low platelet count, whereas other patients had a low platelet count without LSM elevation. Such patients are nevertheless at risk of HCC, suggesting that LSM and platelet count indicate advanced fibrosis or compensated cirrhosis in a complementary manner.

In agreement with a previous report, our findings indicate that LSM could be used to stratify the risk of HCC development in CHC patients.[26] Moreover, combination of LSM with platelet count and the IFN-therapeutic effect could be used to stratify the risk of HCC in patients receiving IFN therapy. Patients without all three risk factors had a very low risk of HCC development, and patients with 1 or 2 risk factors had a moderate risk. Conversely, patients with all three risks had an extremely high risk. In clinical practice, frequency of HCC surveillance should be decided based on HCC risk. Indeed, each of these three factors has previously been shown to be associated with the risk of developing HCC. However, here, we have proposed a new, non-invasive risk assessment based on the combination of LSM and two other factors. In the present study, we did not identify advanced histological fibrosis stage F3–4 as a risk factor for HCC likely because of liver biopsy sampling variability because patients were not excluded based on the length of liver biopsy samples, an important factor affecting variability in histological assessment of liver fibrosis.[15] Taken together, these findings suggest that LSM would be more useful than liver biopsy for diagnosis of patients with liver cirrhosis who are at high risk of HCC, especially those with compensated cirrhosis.

Our data indicate patients with all of the three risk factors require the most intensive HCC surveillance; however, this study does have a few limitations. One drawback is that LSM failure and unreliable results occur in some patients. In our cohort, 9.0% of patients who received LSM did not yield reliable results. Because subcutaneous fat attenuates the transmission of share waves and the ultrasonic signals into the liver used to determine LSM, obesity is the principal reason for LSM failure.[27] In addition, it is likely that obesity itself is associated with an increased risk of HCC.[28] As a result, our findings might not reflect the risk of HCC in obese patients. Another recent report demonstrated that a new FibroScan XL probe, designated for use in obese patients, could reduce LSM failure and facilitate reliable results.[29] A study using this new probe will more accurately evaluate the predictive value of LSM for the risk of HCC development.

In conclusion, our findings indicate that LSM, platelet count, and IFN-therapeutic effect could be used to successfully stratify the risk for HCC development in patients receiving IFN-based antiviral therapy and demonstrate the usefulness of LSM before IFN therapy for the management of CHC patients.

Acknowledgment

This study was supported by a Health Labor Sciences Research Grant, Research on Measures for Intractable Diseases, from the Ministry of Health, Labor, and Welfare of Japan.

References

1 El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557–76.
2 Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127** (Suppl.): S35–50.
3 Kiyosawa K, Umemura T, Ichijo T *et al.* Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127** (Suppl.): S17–26.
4 Sun CA, Wu DM, Lin CC *et al.* Incidence and cofactors of hepatitis C virus-related hepatocellular carcinoma: a prospective study of 12,008 men in Taiwan. *Am. J. Epidemiol.* 2003; **157**: 674–82.
5 Cammà C, Giunta M, Andreone P, Craxi A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J. Hepatol.* 2001; **34**: 593–602.
6 Papatheodoridis GV, Papadimitropoulos VC, Hadziyannis SJ. Effect of interferon therapy on the development of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis: a meta-analysis. *Aliment. Pharmacol. Ther.* 2001; **15**: 689–98.
7 Shiratori Y, Ito Y, Yokosuka O *et al.* Antiviral therapy for cirrhotic hepatitis C: association with reduced hepatocellular carcinoma development and improved survival. *Ann. Intern. Med.* 2005; **142**: 105–14.
8 Morgan TR, Ghany MG, Kim HY *et al.* Outcome of sustained virological responders with histologically advanced chronic hepatitis C. *Hepatology* 2010; **52**: 833–44.
9 Kanda T, Imazeki F, Mikami S *et al.* Occurrence of hepatocellular carcinoma was not a rare event during and immediately after antiviral treatment in Japanese HCV-positive patients. *Oncology* 2011; **80**: 366–72.
10 Lok AS, Everhart JE, Wright EC *et al.* Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. *Gastroenterology* 2011; **140**: 840–9.
11 Makiyama A, Itoh Y, Kasahara A *et al.* Characteristics of patients with chronic hepatitis C who develop hepatocellular carcinoma after a sustained response to interferon therapy. *Cancer* 2004; **101**: 1616–22.
12 Ikeda M, Fujiyama S, Tanaka M *et al.* Risk factors for development of hepatocellular carcinoma in patients with chronic hepatitis C after sustained response to interferon. *J. Gastroenterol.* 2005; **40**: 148–56.
13 Yoshida H, Shiratori Y, Moriyama M *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis

C in Japan. IHIT study group. Inhibition of hepatocarcinogenesis by interferon therapy. *Ann. Intern. Med.* 1999; **131**: 174–81.
14 Bravo AA, Sheth SG, Chopra S. Liver biopsy. *N. Engl. J. Med.* 2001; **344**: 495–500.
15 Bedossa P, Dargère D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 2003; **38**: 1449–57.
16 Rousselet MC, Michalak S, Dupré F *et al.* Sources of variability in histological scoring of chronic viral hepatitis. *Hepatology* 2005; **41**: 257–64.
17 Ziol M, Handra-Luca A, Kettaneh A *et al.* Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48–54.
18 Castéra L, Vergniol J, Foucher J *et al.* Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343–50.
19 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513–20.
20 Bruix J, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208–36.
21 Abdi W, Millan JC, Mezey E. Sampling variability on percutaneous liver biopsy. *Arch. Intern. Med.* 1979; **139**: 667–9.
22 Maharaj B, Maharaj RJ, Leary WP *et al.* Sampling variability and its influence on the diagnostic yield of percutaneous needle biopsy of the liver. *Lancet* 1986; **1**: 523–5.
23 Matsumura H, Moriyama M, Goto I, Tanaka N, Okubo H, Arakawa Y. Natural course of progression of liver fibrosis in Japanese patients with chronic liver disease type C—a study of 527 patients at one establishment. *J. Viral Hepat.* 2000; **7**: 268–75.
24 Pohl A, Behling C, Oliver D, Kilani M, Monson P, Hassanein T. Serum aminotransferase levels and platelet counts as predictors of degree of fibrosis in chronic hepatitis C virus infection. *Am. J. Gastroenterol.* 2001; **96**: 3142–6.
25 Degos F, Christidis C, Ganne-Carrie N *et al.* Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47**: 131–6.
26 Masuzaki R, Tateishi R, Yoshida H *et al.* Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. *Hepatology* 2009; **49**: 1954–61.
27 Castéra L, Foucher J, Bernard PH *et al.* Pitfalls of liver stiffness measurement: a 5-year prospective study of 13,369 examinations. *Hepatology* 2010; **51**: 828–35.
28 Polesel J, Zucchetto A, Montella M *et al.* The impact of obesity and diabetes mellitus on the risk of hepatocellular carcinoma. *Ann. Oncol.* 2009; **20**: 353–7.
29 Myers RP, Pomier-Layrargues G, Kirsch R *et al.* Feasibility and diagnostic performance of the FibroScan XL probe for liver stiffness measurement in overweight and obese patients. *Hepatology* 2012; **55**: 199–208.

Int. J. Mol. Sci. **2014**, *15*, 6556-6568; doi:10.3390/ijms15046556

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

Expression of Aldo-Keto Reductase Family 1 Member B10 in the Early Stages of Human Hepatocarcinogenesis

Hironori Tsuzura [1], Takuya Genda [1,*], Shunsuke Sato [1], Ayato Murata [1], Yoshio Kanemitsu [1], Yutaka Narita [1], Sachiko Ishikawa [1], Tetsu Kikuchi [1], Masashi Mori [1], Katsuharu Hirano [1], Katsuyori Iijima [1], Ryo Wada [2] and Takafumi Ichida [1]

[1] Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, 1129 Nagaoka, Izunokuni-shi, Shizuoka 410-2295, Japan; E-Mails: htudura@juntendo.ac.jp (H.T.); syusato@juntendo.ac.jp (S.S.); perseverance3@hotmail.co.jp (A.M.); yoshio-k.s52@live.jp (Y.K.); ynarita@juntendo.ac.jp (Y.N.); s-ogawa@juntendo.ac.jp (S.I.); klinsmann1011@yahoo.co.jp (T.K.); ten2617@gmail.com (M.M.); khirano-tym@umin.ac.jp (K.H.); katsu0716@shore.ne.jp (K.I.); takafumi@air.ocn.ne.jp (T.I.)

[2] Department of Pathology, Juntendo University Shizuoka Hospital, 1129 Nagaoka, Izunokuni-shi, Shizuoka 410-2295, Japan; E-Mail: mdksmrwjunten522@ybb.ne.jp

* Author to whom correspondence should be addressed; E-Mail: genda@rice.ocn.ne.jp; Tel.: +81-559-48-3111; Fax: +81-559-48-5088.

Received: 25 February 2014; in revised form: 28 March 2014 / Accepted: 3 April 2014 / Published: 17 April 2014

Abstract: Aldo-keto reductase family 1, member B10 (AKR1B10), a cancer-related oxidoreductase, is expressed in well-differentiated hepatocellular carcinomas (HCCs). However, AKR1B10 levels are minimal in normal liver tissues (NLs), similar to the 70-kilodalton heat shock protein (HSP70) and glypican-3. Moreover, the role of AKR1B10 in chronic hepatitis or cirrhosis, which are considered preneoplastic conditions for HCC, has not been fully elucidated. The aim of this study was to evaluate the expression of AKR1B10, HSP70, and glypican-3 in 61 HCC tissue samples compared to corresponding non-tumorous liver tissues (NTs), comprising 42 chronic hepatitis and 19 cirrhosis cases to clarify the significance of molecular changes at the preneoplastic stages of HCC. Immunohistochemical analysis demonstrated that the median expression levels of AKR1B10 were higher in HCCs than in NTs ($p < 0.001$) and higher in NTs than NLs ($p < 0.001$) with 54.8%, 2.1%, and 0.3% expression in HCCs, NTs, and NLs, respectively. HSP70 and glypican-3 were expressed in HCCs, but minimally in NTs and NLs with no significant difference between expression in NTs and NLs. Furthermore, a multivariate

analysis identified an association between hepatic steatosis and AKR1B10 expression in NTs (p = 0.020). Of the three protein expressed in well-differentiated HCCs, only AKR1B10 was upregulated in preneoplastic conditions, and a steatosis-related factor might influence its expression.

Keywords: AKR1B10; HSP70; glypican-3; hepatocellular carcinoma; chronic hepatitis; cirrhosis

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death worldwide [1]. HCC is characterized by a multistep morphological developmental process, progressing from early, well-differentiated HCC to moderately or poorly differentiated, advanced HCC [2]. Until now, several molecular alterations have been found to be associated with morphological developments in HCC. The 70-kilodalton heat shock protein (HSP70) and glypican-3 (GPC3) are upregulated even in early, well-differentiated HCC [3,4], while accumulations of mutated beta-catenin and p53 are mainly observed in moderately to poorly differentiated HCC [5,6]. On the other hand, the majority of HCCs arise in chronically diseased livers, including livers with chronic hepatitis and cirrhosis resulting from hepatitis B or C infection or exposure to other carcinogenic factors. These chronic liver diseases are widely considered to be preneoplastic conditions for HCC, potentially leading to molecular alterations that predispose hepatocytes to malignant transformation. However, the molecular alterations underlying preneoplastic conditions that predispose to HCC remain poorly understood.

Aldo-keto reductase family 1 member B10 (AKR1B10) is a member of the AKR superfamily, which are NAD(P)H-dependent oxidoreductases that catalyze the reduction of carbonyl compounds and various physiological and xenobiotic substrates [7,8]. AKR1B10 was originally isolated as a gene with increased expression in human HCC [9,10], including well-differentiated HCC, but is minimal in normal liver tissue [11,12]. This is similar to findings regarding the expression of HSP70 and GPC3. Recently, we analyzed the expression profiles of approximately 41,000 genes in patients with chronic hepatitis C and found that AKR1B10 was upregulated in the livers of chronic hepatitis C patients at high risk of HCC [13]. These observations suggest that an alteration of AKR1B10 expression occurs even in preneoplastic conditions that predispose to HCC. To further study the significance of AKR1B10 alterations in the early stages of hepatocarcinogenesis, we evaluated AKR1B10 expression in 61 HCCs and corresponding non-tumorous liver tissues (NTs), which comprised 42 chronic hepatitis and 19 cirrhosis cases, and compared AKR1B10 expression with the expression of the other two molecules known to be upregulated in well-differentiated HCC—HSP70 and GPC3—both of which are widely used as immunohistochemical molecular markers of early HCC [14]. In addition, because a strong association has been reported between smoking and AKR1B10 expression in malignant and non-malignant lung airway epithelium [15–17] and several studies have identified smoking as a risk factor for HCC development [18,19], we further evaluated the correlation of AKR1B10 expression with NT-associated factors, including patients' smoking history.

2. Results

2.1. Clinicopathological Patient Features

Table 1 summarizes demographic, biochemical, and pathological data for all 61 patients enrolled in this study. Serological markers of hepatitis virus were distributed as follows: positive for HBsAg, n = 13 (21%); positive for anti-HCV (hepatitis C virus), n = 33 (54%); and negative for HBsAg and anti-HCV, n = 15 (25%). The median HCC size was 48 mm (range: 12–170 mm), and 19 HCCs were classified as well differentiated, 38 were moderately differentiated, and 4 were poorly differentiated. The distribution of the degree of hepatic fibrosis in NTs was as follows: no fibrosis or periportal fibrous expansion (F0-1), n = 18; portal fibrous widening with bridging fibrosis (F2), n = 14; bridging fibrosis with lobular distortion (F3), n = 10; and liver cirrhosis (F4), n = 19. Steatosis in NTs ranged between 0.0% and 23.3% (median 1.3%).

Table 1. Clinicopathological characteristics of patients included in this study.

Characteristics	Units	N = 61
Age	(years) *	67 (38–83)
Sex	(male/female)	47/14
Habitual drinker	(yes/no)	27/34
Smoking	(0/<40/≥40 pack-years)	38/11/12
Hepatitis virus	(B/C/NBNC)	13/33/15
Albumin	(g/mL) *	3.8 (3.0–4.9)
ALT	(IU/L) *	44 (8–224)
Platelet count	(10^4/μL) *	14.9 (6.0–45.6)
Total bilirubin	(mg/dL) *	0.7 (0.3–1.7)
Prothrombin time	(%) *	91 (68–136)
AFP	(ng/mL) *	13 (2–60,514)
DCP	(mAU/mL) *	184 (0–129,000)
Tumor size	(mm) *	48 (12–170)
Grade of HCC	(W/M/P)	19/38/4
Fibrosis stage of NT	(F0-1/F2/F3/F4)	18/14/10/19
Steatosis in NT	(%) *	1.3 (0.0–23.3)

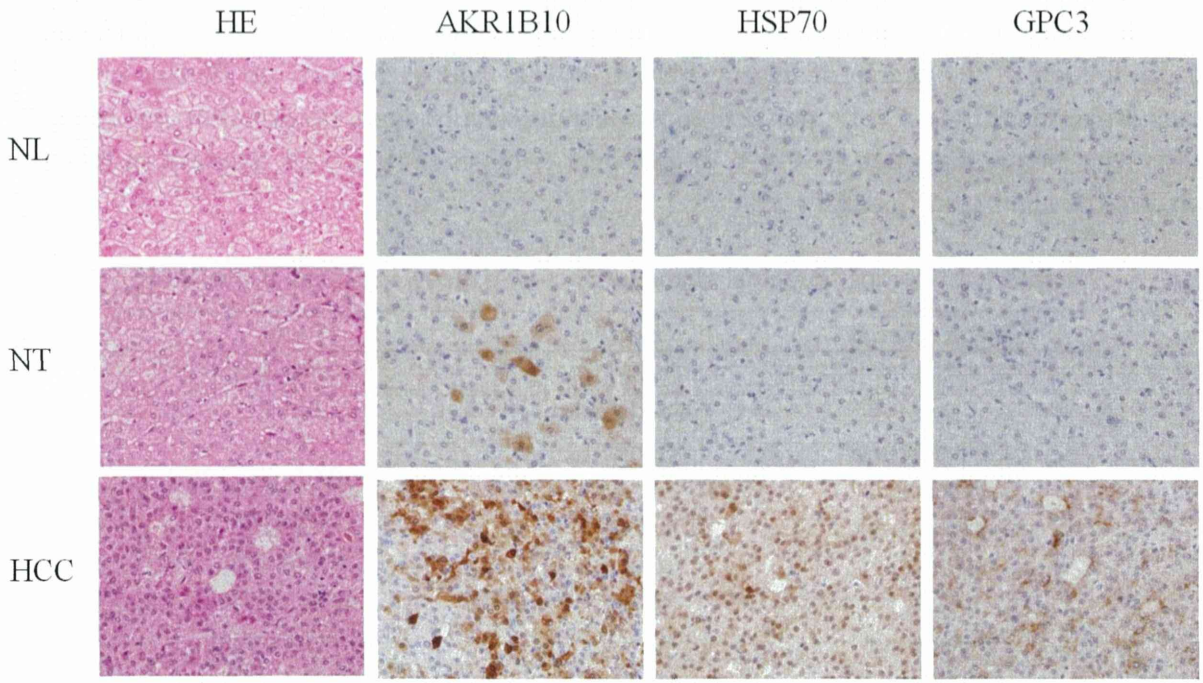
* Date shown as median value (range). Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; B, positive for HBsAg; C, positive for anti-HCV; DCP, des-gamma-carboxy prothrombin; HCC, hepatocellular carcinoma; M, moderately differentiated; NBNC, negative for HBsAg and anti-HCV; NT, non-tumorous liver tissue; P, poorly differentiated; W, well differentiated.

2.2. Immunohistochemical Analyses

Figure 1 shows representative immnohistochemical staining of AKR1B10, HSP70, and GPC3 in liver tissues. No AKR1B10 immunoreactivity was observed in any of the 8 NLs, but there was detectable nucleocytoplasmic AKR1B10 immunoreactivity in single, scattered hepatocytes or some clustered hepatocytes in 44 of 61 NTs. More widespread nucleocytoplasmic immnoreactivity of AKR1B10 was seen in 55 of the 61 HCCs. In addition, HSP70 immunoreactivity was observed in

50 of the 61 HCCs and GPC3 immnoreactivity in 42 of the 61 HCCs. However, HSP70 and GPC3 immunoreactivity was minimal in both NTs and NLs.

Figure 1. Representative immunostaining of aldo-keto reductase family 1 member B10 (AKR1B10), 70-kilodalton heat shock protein (HSP70), and glypican-3 (GPC3). HCC, hepatocellular carcinoma; HE, hematoxylin-eosin; NL, control normal liver tissue; NT, non-tumorous liver tissue. Original magnification ×100.



Quantification of the immunoreactivity by image analyses showed areas staining positive for AKR1B10 ranging from 0.3% to 93.6% (median 54.8%) in HCCs, 0.2% to 47.0% (median 2.1%) in NTs, and 0.1% to 0.9% (median 0.3%) in NLs. The AKR1B10 expression level was significantly higher in HCCs than in NTs ($p < 0.001$) and higher in NTs than in NLs ($p < 0.001$, Figure 2A). The HSP70 expression level ranged between 0.0% and 82.5% (median 14.9%) in HCCs, 0.0% and 5.4% (median 0.2%) in NTs, and 0.0% and 0.4% (median 0.1%) in NLs. The HSP70 expression level was significantly higher in HCCs than in NTs ($p < 0.001$), but did not differ significantly between NTs and NLs ($p = 0.80$, Figure 2B). The GPC3 expression level ranged between 0.0% and 74.4% (median 7.2%) in HCCs, 0.0% and 0.8% (median 0.0%) in NTs, and 0.0% and 0.0% (median 0.0%) in NLs. The GPC3 expression level was significantly higher in HCCs than in NTs ($p < 0.001$), but did not differ significantly between NTs and NLs ($p = 0.80$, Figure 2C).

2.3. Factors Associated with Aldo-Keto Reductase Family 1 Member B10 (AKR1B10) Expression in Non-Tumorous Liver Tissues (NTs)

Of the 3 molecules upregulated in well-differentiated HCC, only AKR1B10 expression increased in NTs. Therefore, we next evaluated the association between AKR1B10 expression and various clinicopathological parameters in NTs (Table 2). In the univariate analysis, patients' clinical and biochemical factors, viral markers, fibrosis stage of NTs, and grade of HCC were not associated with

AKR1B10 expression in NTs, and hepatic steatosis was the only parameter significantly associated with AKR1B10 expression. The multivariate analysis confirmed this significant association between AKR1B10 expression and hepatic steatosis ($p = 0.020$). Figure 3A shows a comparison of AKR1B10 expression levels in NTs among HBsAg positive, anti-HCV positive, and HBsAg and anti-HCV negative patients. Median AKR1B10 expression levels in these 3 patient groups were 1.9%, 2.1%, and 4.1%, respectively. AKR1B10 expression tended to be higher in HBsAg and anti-HCV negative patients, but the difference was not statistically significant. Figure 3B shows the regression analysis of AKR1B10 expression *versus* hepatic steatosis in NTs, with a significant positive correlation between the 2 ($r = 0.40$, $p = 0.001$). Our immnohistochemical examination also found a tendency towards more prominent AKR1B10 immunoreactivity in hepatocytes showing fatty change than in hepatocytes with no fatty change (Figure 4).

Figure 2. Comparison of AKR1B10, HSP70, and GPC3 expression levels among control normal liver tissues (NLs), non-tumorous liver tissues (NTs), and hepatocellular carcinomas (HCCs). The box encompasses the 25th through 75th percentiles, and the horizontal line through the middle of the box indicates the fiftieth percentile (median). The 10th and 90th percentiles are shown as whisker caps. (**a**) AKR1B10 expression; (**b**) HSP70 expression; and (**c**) GPC3 expression.

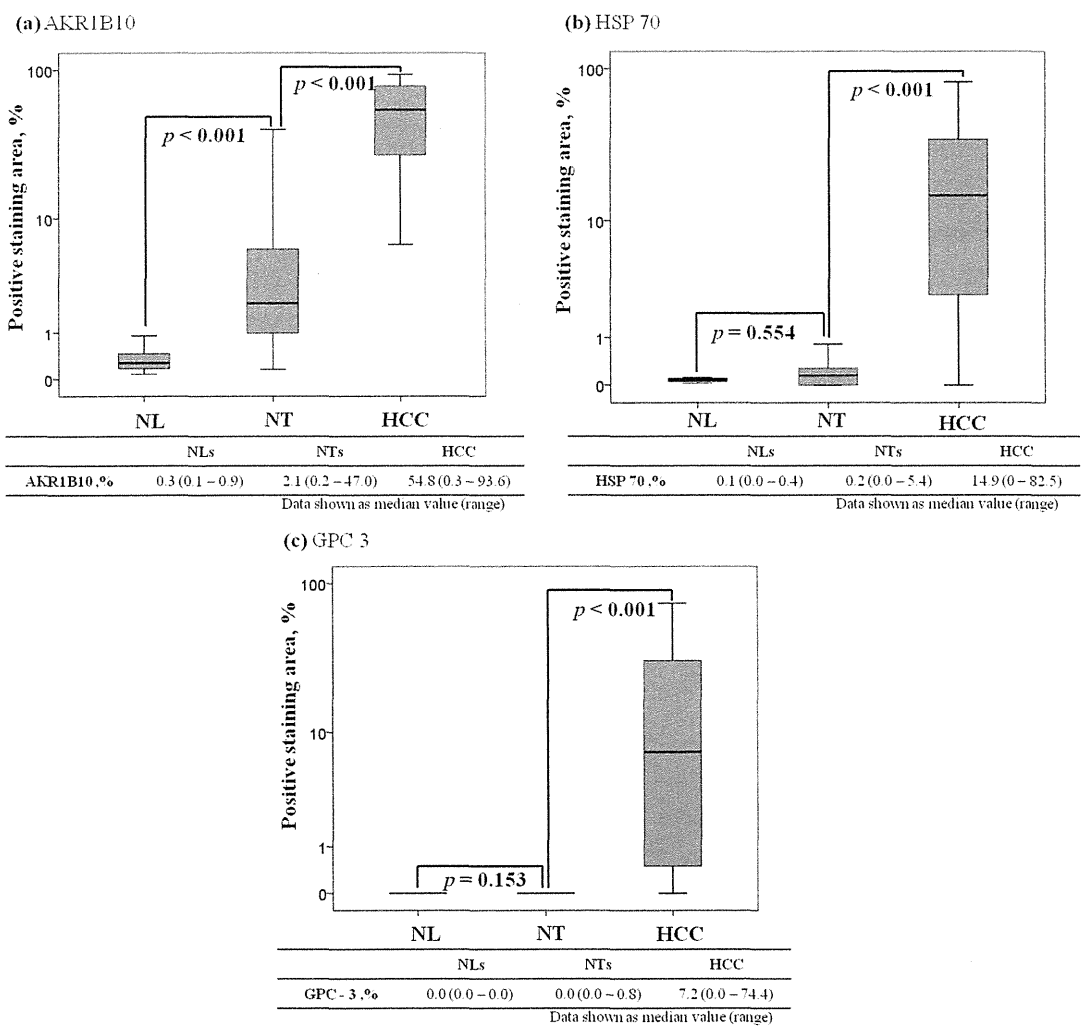


Table 2. Univariate and multivariate analysis of factors associated with AKR1B10 expression in non-tumorous liver tissues.

Variables	Univariate		Multivariate	
	Coefficient	*p* Value	Coefficient	*p* Value
Age	−0.109	0.403		
Male gender	0.033	0.802		
Habitual drinker	0.069	0.598		
Smoking	0.003	0.980		
Hepatitis virus	−0.111	0.395		
Albumin	−0.160	0.218		
ALT	0.133	0.305		
Platelet count	−0.173	0.182		
Total bilirubin	−0.068	0.604		
Prothrombin time	−0.105	0.427		
AFP	−0.107	0.415		
DCP	−0.100	0.466		
Grade of HCC	−0.032	0.805		
Fibrosis stage of NT	0.130	0.318		
Steatosis in NT	0.306	0.004	0.317	0.021

Stepwise linear regression analysis was used in the univariate and multivariate analysis. Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; DCP, des-gamma-carboxy prothrombin; HCC, hepatocellular carcinoma; NT, non-tumorous liver tissue.

Figure 3. (**a**) ARK1B10 expression level in non-tumorous liver tissue. HBV, positive for HBsAg; HCV, positive for anti-HCV; NBNC, negative for HBsAg and anti-HCV. The box encompasses the twenty-fifth through seventy-fifth percentiles, and the horizontal line through the middle of the box indicates the fiftieth percentile (median). The tenth and ninetieth percentiles are shown as whisker caps; and (**b**) Regression analysis of the relationship between AKR1B10 expression and hepatic steatosis in non-tumorous liver tissues.

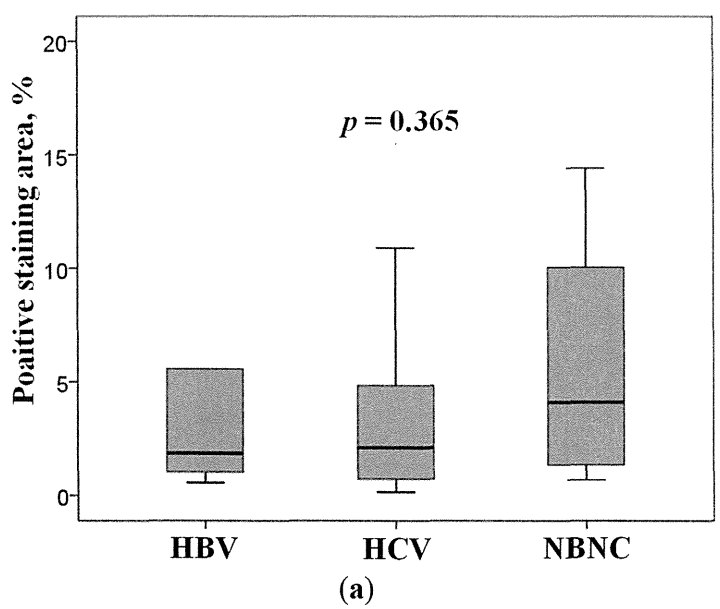
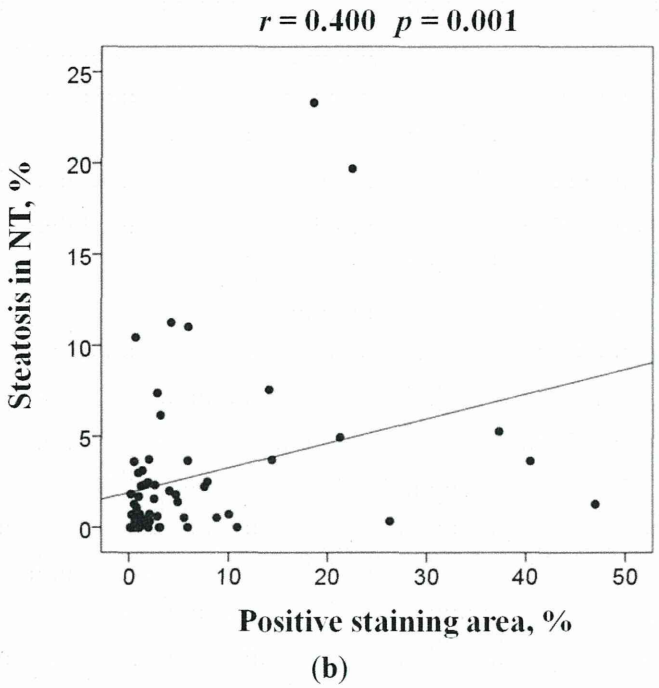
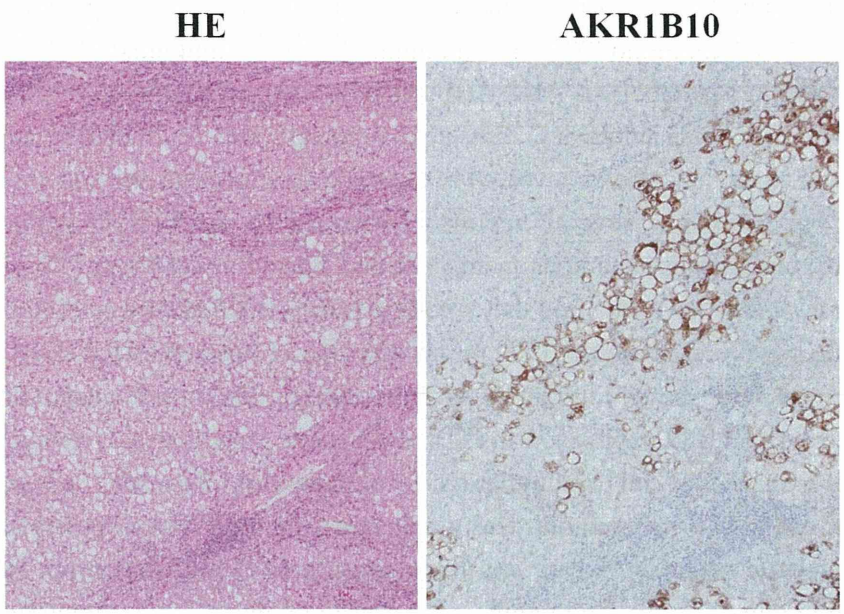


Figure 3. *Cont.*



(b)

Figure 4. Representative immunostaining of AKR1B10 in hepatocytes containing fatty change. AKR1B10, aldo-keto reductase family 1 member B10; HE, hematoxylin-eosin. Original magnification ×100.



3. Discussion

In the present study, we demonstrated that AKR1B10 expression levels were significantly higher in livers with chronic hepatitis or cirrhosis, which are preneoplastic conditions underlying HCC, than in normal livers, and that AKR1B10 expression was still higher in HCCs. Because the AKR superfamily consists of more than 100 members, and several AKR members, such as AKR1B15, show high amino

acid sequence identity with AKR1B10, cross-reactivity of the anti-AKR1B10 antibody used in this study may become an issue in immunohistochemical analysis. However, both our previous study and another study performed quantitative reverse transcription polymerase chain reaction for AKR1B10, and both reported results consistent with AKR1B10 immunohistochemistry [13,20]. HSP70 and GPC3 also showed prominent immunoreactivities in HCC, but minimal immunoreactivities in NTs and NLs, consistent with previous reports [3,4]. These results indicate that HSP70 and GPC3, but not AKR1B10, are useful markers for distinguishing HCC from chronic hepatitis or cirrhosis. However, the stepwise upregulation of AKR1B10 from chronic hepatitis or cirrhosis to HCC might indicate its potential role in the early stage of hepatocarcinogenesis.

Many studies have demonstrated AKR1B10 upregulation in several types of cancer, including several recent reports on HCC [11,20–22]. AKR1B10 was shown to have a high catalytic efficiency for the reduction of all *trans*-, 9-*cis*-, and 13-*cis*-retinals to their corresponding retinols *in vitro* and *in vivo* [23,24], and the conversion of retinals to retinols via AKR1B10 can deprive retinoic acid receptors of their ligands, and can presumably inhibit the retinoic acid signaling pathway [25,26]. Retinoic acid is thought to be essential for the maintenance of normal epithelial differentiation. Retinoic acid depletion causes cell proliferation and loss of differentiation, thereby inducing neoplastic phenotypes in normal epithelium [27–29]. On the other hand, retinoic acid exposure inhibits proliferation of normal and transformed cells *in vitro* [30,31], and dietary retinoic acid reduced the development of premalignant and malignant lesions in a chemically induced mouse carcinogenesis model [32]. AKR1B10 was shown to be downregulated using small, interfering, RNA-inhibited cancer cell proliferation both *in vitro* and *in vivo* [22,33]. Furthermore, oral administration of acyclic retinoids was reported to prevent human HCC [34]. These observations suggest the involvement of AKR1B10 in cancer cell dedifferentiation and proliferation via inhibition of retinoic acid signals. In addition to its hypothetical role in retinoid metabolism, AKR1B10 has been reported to perform other potential functions such as detoxification of toxic aldehydes, fatty acid synthesis, and resistance to carbonyl-containing drugs [15]. These functions may also be involved in the molecular mechanisms underlying carcinogenesis. Consistent with our findings, several reports demonstrated AKR1B10 upregulation in some preneoplastic conditions such as squamous metaplasia and Barrett's esophagus [15,16,35].

Although several studies have reported that smoking affects AKR1B10 expression in malignant and non-malignant lung airway epithelium [15–17], no association between AKR1B10 expression in the liver and smoking was demonstrated in this study. Interestingly, the present study showed a significant association between AKR1B10 expression in NTs and hepatic steatosis: a cytological change marked by clear vacuoles because of fat accumulation in hepatocytes. Recently, we conducted a study demonstrating that AKR1B10 upregulation was a risk factor for HCC development in chronic hepatitis C patients [13]. Hepatic steatosis *per se* has been considered a risk factor for HCC development. Presence of hepatic steatosis is associated with increased frequency of HCC in patients with HCV-related cirrhosis [36]. Alcoholic and non-alcoholic steatohepatitis is recognized as an important liver disease preceding cirrhosis and HCC [37,38]. Taken together, all of these findings suggest that AKR1B10 might play a role in the molecular basis of steatosis-related hepatocarcinogenesis. Indeed, Starmann *et al.* reported that AKR1B10 expression was upregulated during the progression of simple steatosis to steatohepatitis with increased risk of HCC [39]. The present study showed that AKR1B10 expression in NTs was not significantly different according to hepatitis B or C viral infection status.

Interestingly, of the 15 NT liver samples in this study that were negative for hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus antibody (anti-HCV), 9 showed histological features of steatohepatitis. In addition, AKR1B10 expression tended to be higher in HBsAg- and anti-HCV-negative NT samples, compared to HBsAg- or anti-HCV-positive NT samples, although the difference was not statistically significant. These results partially confirm the results reported by Starmann *et al.* Because hepatic steatosis is generally associated with metabolic disorders such as obesity and type 2 diabetes, some metabolic disorders might affect AKR1B10 expression in preneoplastic conditions [40].

4. Patients and Methods

4.1. Tissue Samples

We obtained paired samples of primary HCCs and their corresponding NTs from 61 patients who underwent hepatic resection at Juntendo University Shizuoka Hospital, Izunokni, Japan between 2004 and 2012. None of the patients were previously treated. The following laboratory parameters were measured using commercially available assays immediately prior to hepatic resection: HBsAg and anti-HCV levels (LUMIPULSE Presto®, FUJIREBIO Inc., Tokyo, Japan); blood cell count (COULTER LH780, Beckman coulter, Inc., Brea, CA, USA); prothrombin time (Thromborel S®, SYSMEX Co., Kobe, Japan); and serum albumin, alanine aminotransferase (ALT), total bilirubin (AQUAAUTO, KAINOS Laboratories, Inc., Tokyo, Japan), alpha-fetoprotein (ARCHITECT®, ABBOT JAPAN Co., Tokyo, Japan), and des-gamma-carboxy prothrombin (BML, Inc., Tokyo, Japan) levels. HCC histological grades were determined according to the World Health Organization criteria [41]. In instances where different tumor grades were found within the same nodule, the predominant histological grade was used. Histological evaluation of NTs was based on the METAVIR criteria, as reported previously [42]. Steatosis in NTs was quantitatively assessed by computer-assisted morphometric image analysis as previously described [43]. Control normal liver tissues (NLs) showing no unusual histological features were also obtained from surgically resected materials obtained from 8 patients with liver metastasis of colorectal cancer.

This study was approved by the Ethical Committee of Juntendo University Shizuoka Hospital in accordance with the Helsinki Declaration, and written informed consent was obtained from all patients.

4.2. Immunohistochemistry

Immunohistochemical analyses for AKR1B10, HSP70, and GPC3 were performed on formalin-fixed, paraffin-embedded tissue sections using an immunoperoxidase method. In brief, deparaffinized and rehydrated sections were processed by heat-induced antigen retrieval in 0.1 M citrate buffer at pH 6.0. After blocking endogenous peroxidase activity with 0.3% H_2O_2 in methanol solution, the sections were treated with 2% normal swine serum and incubated with primary antibody overnight at room temperature, followed by incubation with biotinylated secondary antibody (Ventana iVIEW DAB Universal Kit; Ventana Medical Systems Inc., Tucson, AZ, USA). Staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride and hematoxylin counterstain. Negative controls were prepared by replacing the primary antibody with mouse immunoglobin (Sigma–Aldrich Biochemicals, St. Louis, MO, USA). Immunostaining was quantitatively assessed as the mean percentage of the