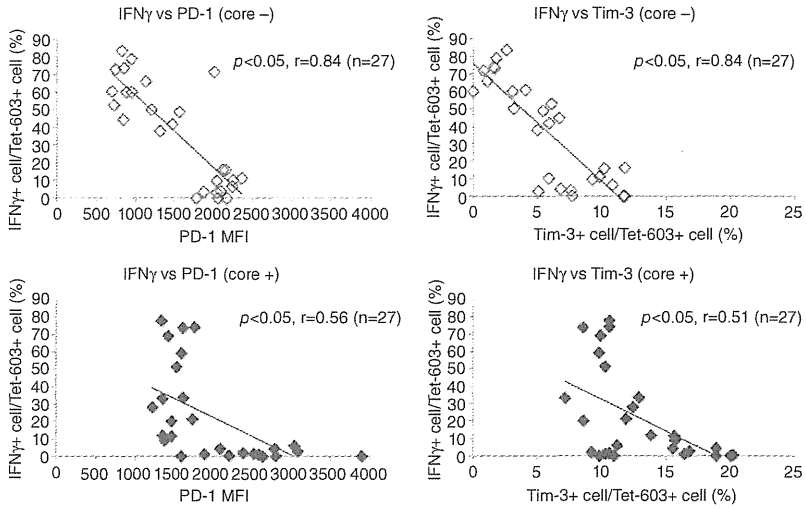

Figure 4 Inverse correlation between the percentages of interferon (IFN)-γ-producing cells and expression of regulatory molecules in antigen-specific intrahepatic CD8 T cells.

When core (+) and core (−) mice were compared, the expression of PD-1 and Tim-3 by Ad-HCV-NS3-specific intrahepatic CD8 T cells was significantly higher in core (+) than core (−) at various time points following Ad-HCV-NS3 infection. Furthermore, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in Ag-specific intrahepatic CD8 T cells (Fig. 4).

To determine whether suppression ligand expression by intrahepatic APC is altered in core (+) mice, the intensity of PD-L1 expressed by $CD11^+$ cells was analyzed at 7 and 14 days post-infection. Intrahepatic APC showed the infectious dose-dependent augmentation of PD-L1 expression. We observed elevated expression of PD-L1 by APC in core (+) mice infected with 10^{10} PFU at both time points (Fig. 5a,b). In PD-L1 expression, we did not find a significant difference between Ad-HCV-NS3 infection and Adψ5 control vector infection (Fig. 5c,d).

Taken together, these data suggest that the existence of HCV core gene suppress T-cell-mediated immune response by causing higher expression of suppression molecules.

Ag persistence after Ad-HCV-NS3 infection

To determine the Ag persistence after Ad-HCV-NS3 infection, we analyzed the expression of FLAG-tagged HCV-NS3 protein in the liver by IP-western blot after administration of 2×10^7, 1×10^9 or 1×10^{10} PFU of the virus. The Ag expression in the liver could be found in both core (+) and core (−) mice on 21 days after infection with 1×10^{10} PFU. When 1×10^9 PFU of Ad-HCV-NS3 was administrated, HCV NS3-protein was almost cleared from the liver of core (−) mice at day 21 post-infection, whereas the Ag expression persisted in the liver of core (+) mice until day 21 post-infection (Fig. 6).

It is important to note that the loss of Ag expression in the liver of core (−) mice after infection with 1×10^9 PFU coincided with the high HCV-NS3-specific CD8 T-cell response at 14 days post-infection (Fig. 2c), whereas Ag persistence in the liver of core (+) mice after infection with 1×10^9 PFU or the liver of core (−) and core (+) mice after infection with 1×10^{10} PFU was associated with strongly diminished Ag-specific CD8 T-cell response (Fig. 2c). It is likely that the expression of core protein and the high amount of Ag in the liver contributed to the functional exhaustion of HCV-NS3-specific CD8 T cells.

DISCUSSION

IN THIS STUDY, we found an impaired response of HCV-NS3-specific intrahepatic CD8 T cell in a high dose setting (1×10^{10} PFU) of Ad-HCV-NS3 infection. Furthermore, higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and PD-L1 by intrahepatic APC were observed in HCV core Tg mice and the expression increased dependent on infectious dose. In addition, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules

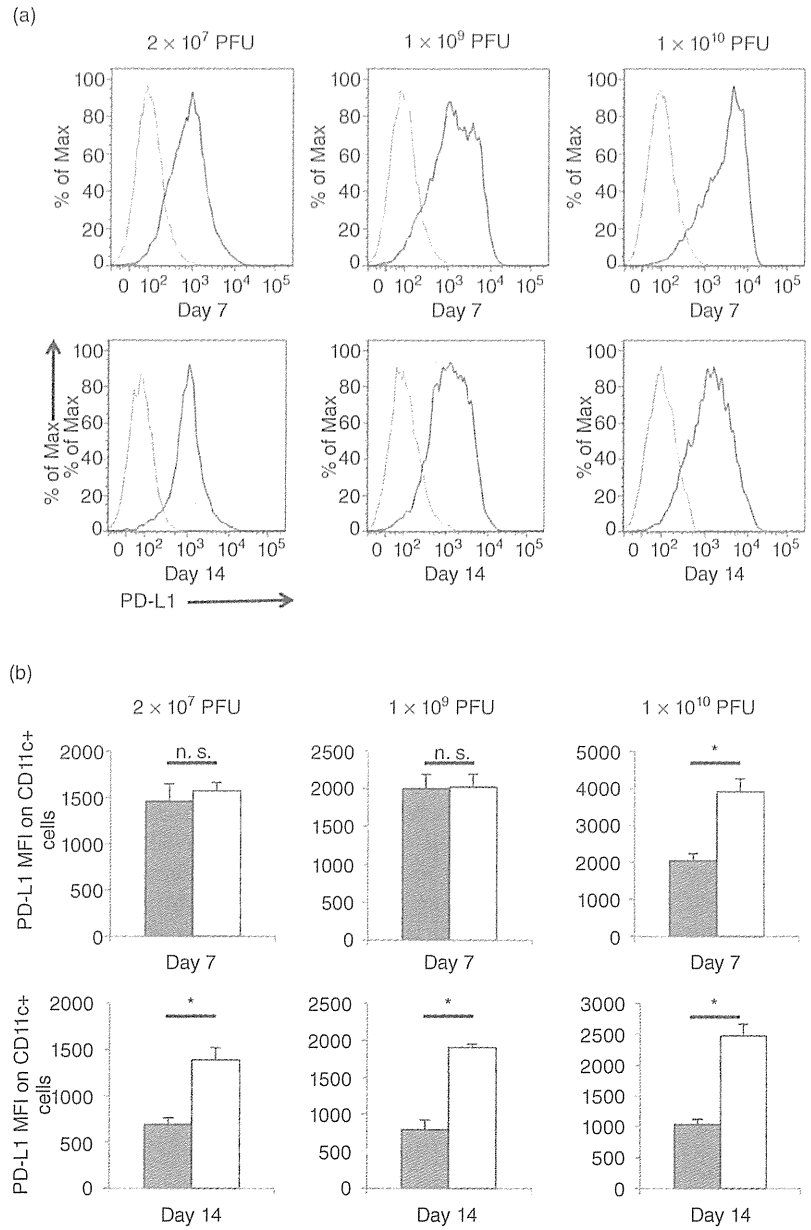

Figure 5 PD-L1 expression in the liver of core (+) and core (−) mice. Core (+) and core (−) mice were injected with 2×10^7, 1×10^9 and 1×10^{10} plaque-forming units (PFU) of adenovirus (Ad)-hepatitis C virus (HCV)-NS3 or Adψ5 control vector. (a) PD-L1 expression by intrahepatic antigen-presenting cells (APC) from core (+) and core (−) mice infected with Ad-HCV-NS3. The % of Max is the number of cells in each sample divided by the number of cells in the sample that contains the largest number of cells. (b) The median fluorescence index (MFI) expression of PD-L1 by intrahepatic $CD11c^+$ leukocyte from core (+) and core (−) mice infected with Ad-HCV-NS3 ($^*P < 0.05$; n.s., not statistically significant). (c) PD-L1 expression by intrahepatic APC from core (+) and core (−) mice infected with Ad-HCV-NS3 or Adψ5 control vector. (d) The MFI expression of PD-L1 by intrahepatic $CD11c^+$ leukocyte from core (+) and core (−) mice infected with Ad-HCV-NS3 or Adψ5 control vector (n.s., not statistically significant). (a) ····, isotype; , core (−); ——, core (+); (b) ▨, core (−); □, core (+); (c) ····, isotype; , Adψ5; ——, Ad-NS3; (d) ▨, Adψ5; □, Ad-NS3.

in Ag-specific intrahepatic CD8 T cells. These results indicated that high infectious dose and the presence of HCV core gene were strongly involved in ineffective CD8 T-cell responses.

Recently, a novel mechanism of T-cell dysfunction was demonstrated in a murine model of chronic LCMV infection.[24] It was found that the expression of PD-1 was upregulated on dysfunctional LCMV-specific CD8 T cells in mice.[24] *In vivo* blockade of PD-1/PD-L1 interaction restored the functions of LCMV-specific CD8 T cells and reduced the viral titer.[24] More recently, other inhibitory receptors such as Tim-3 have also been studied as the factors that can cause T-cell impairments in chronic viral infections.[25] These influential discoveries led to

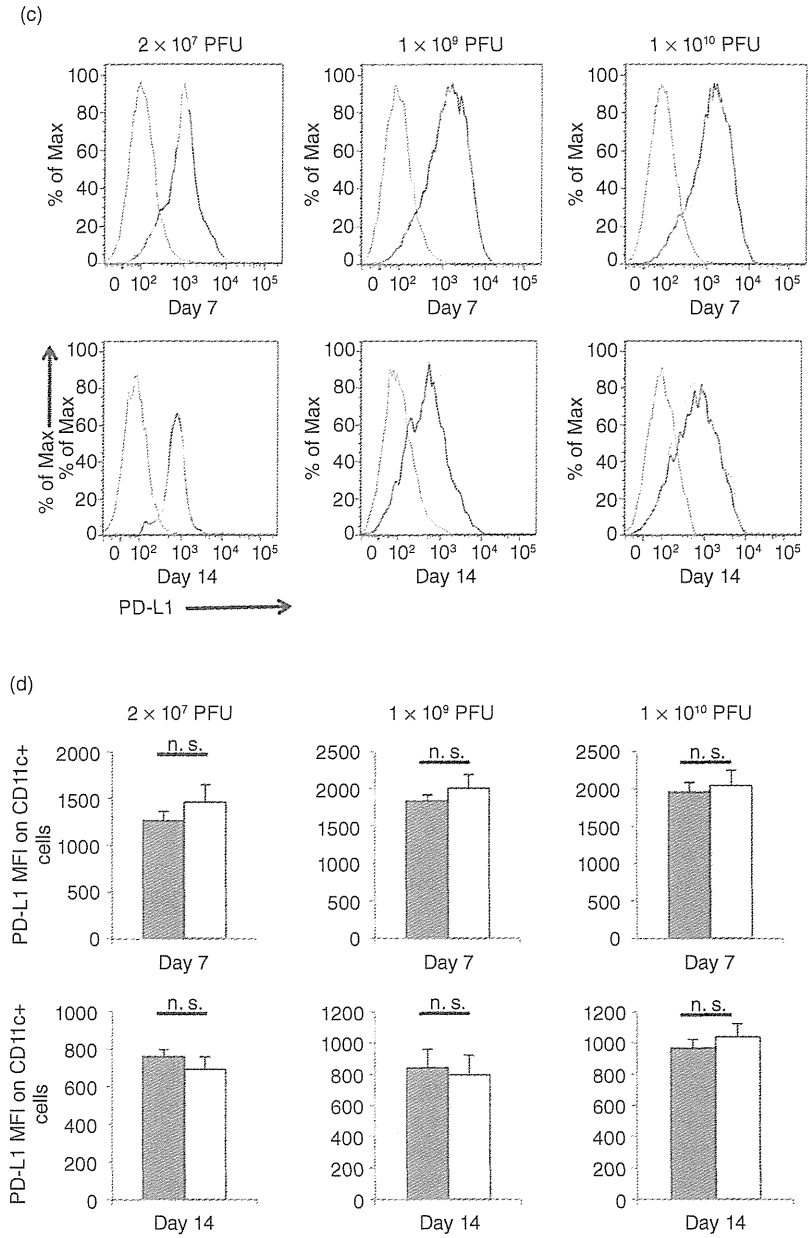

Figure 5 *Continued*

extensive investigations of inhibitory receptors in the regulation of T cells in human chronic viral infections.[25,26]

Chronic HCV infection in humans is characterized by CD8 T-cell exhaustion and dysfunction.[27] As in chronic LCMV infection, the expression of PD-1 is similarly upregulated on the virus-specific CD8 T cells in chronic HCV infection, and HCV-specific PD-1^{high} T cells are functionally impaired.[28–30] Also, Tim-3 is overexpressed on HCV-specific dysfunctional CD8 T cells.[25] In addition, a blockade of PD-1/PD-L1 or Tim-3/galectin9 (Gal9) interaction restores T-cell functions such as proliferation, cytolytic activity and cytokine (IFN-γ and tumor necrosis factor-α) production.[25,28–30] As was

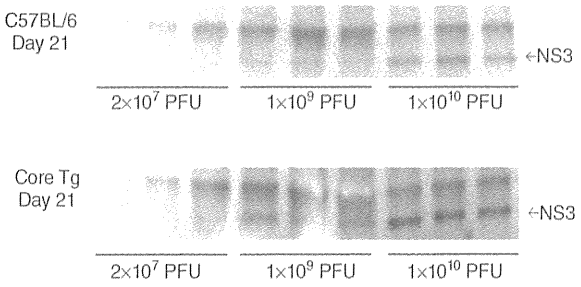

Figure 6 Persisting hepatitis C virus (HCV)-NS3 antigen detection was performed on the liver sections isolated 21 days post-infection. Liver sections were analyzed by IP-western blot assay using anti-FLAG antibody.

mentioned above, it has been reported that increased expression of inhibitory receptors is associated with the impaired HCV-specific CD8 T cells observed in chronic HCV patients. However, the underlying mechanisms for HCV-mediated impaired CD8 T-cell responses have yet to be determined. Based on our finding that lower level of activation and higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and higher levels of expression of PD-L1 by intrahepatic APC were observed in core (+) mice in comparison with core (−) mice, it is possible that HCV core-induced T-cell dysfunction is one of the viral factors that contributes to impaired CD8 T-cell responses as seen in chronic HCV patients. Our speculation is in accordance with the study by Lukens *et al.*[31]

Suppression of CTL responses via highly expressed Ag was found in chronic HCV infection. Inverse relationships between HCV viral titer and HCV-specific T cells have been reported.[7,32,33] In this study, we found higher levels of expressions of PD-L1 by intrahepatic APC and an impaired intrahepatic CD8 T-cell response in high infectious dose setting. Moreover, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in Ag-specific intrahepatic CD8 T cells. It is likely that the PD-1/PD-L1 or Tim-3/Gal9 pathway play a major inhibitory role in our model. High-dose Ad-HCV NS3 infection may inhibit the NS3-specific CD8 T-cell responses not at the induction phase but at the effector phase because Ag-specific-MHC tetramer$^+$ T cells were observed, and most Ag-specific MHC tetramer$^+$ T cells was anergic to PMA/ionophore stimulation and these T cells expressed PD-1 and Tim-3. The role of PD-1/PD-L1 as mechanism for liver tolerance has been well established. PD-1 expression by T cells has been shown to inhibit intrahepatic antiviral immune responses at the effector phase.[34–36]

Hepatitis C virus infection affects approximately 170 million people in the world and is a major global health problem because infected individuals can develop liver cirrhosis and hepatocellular carcinoma. Pegylated interferon and ribavirin therapy, although beneficial in approximately half of treated patients, are expensive and associated with significant side-effects.[37] In this clinical context, there is an urgent need for the development of a therapeutic and/or prophylactic HCV vaccine.[38] Because HCV infects only humans and chimpanzees, it is difficult to evaluate effective therapeutic vaccine candidates. Recently, as a small animal model for HCV infection study, chimeric humanized mouse harboring a human hepatocyte and hematolymphoid system was established by xenotransplantation technique.[39,40] The xenograft model provides a unique opportunity for HCV vaccine development. However, the generation of this chimeric humanized mouse requires advanced technical skills and the scarcity of adequate human primary material remains a significant logistical challenge.[41,42] Our model showed in the present study is easy to create, and it has Ag-specific T-cell exhaustion and Ag persistent in the liver seen in chronic HCV patients. These features suggest that this system is useful for therapeutic HCV vaccine development.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical characteristics, treatment, and prognosis of non-B, non-C hepatocellular carcinoma: a large retrospective multicenter cohort study

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Abstract
Background The number of hepatocellular carcinoma (HCC) patients with non-viral etiologies is increasing in Japan. We conducted a nation-wide survey to examine the characteristics of those patients.
Methods After we assessed the trend of patients who were first diagnosed with HCC at 53 tertiary care centers in Japan from 1991 to 2010, we collected detailed data of 5326 patients with non-viral etiology. The etiologies were categorized as autoimmune hepatitis, primary biliary cirrhosis, alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), unclassified, and other. Baseline characteristics at initial diagnosis, the modality of the initial treatment, and survival status were collected via a website. Survival of the patients was assessed by the Kaplan–Meier method and Cox proportional hazard regression.
Results The proportion of patients with non-viral etiologies increased from 10.0 % in 1991 to 24.1 % in 2010. Of the patients, 92 % were categorized as ALD, NAFLD, or unclassified. Body mass index (BMI) was $\geq$ 25 kg/m^2 in 39 %. Diabetes was most prevalent in NAFLD (63 %), followed by unclassified etiology (46 %) and ALD (45 %). Approximately 80 % of patients underwent radical therapy, including resection, ablation, or transarterial chemoembolization. Survival rates at 3, 5, 10, 15, and 20 years were 58.2, 42.6, 21.5, 15.2, and 15.2 %, respectively. Multivariate analysis revealed that patients with BMI > 22 and $\leq$ 25 kg/m^2 showed the best prognosis versus other BMI categories, after adjusting by age, gender, tumor-related factors, and Child-Pugh score.
Conclusions Most cases of non-B, non-C HCC are related to lifestyle factors, including obesity and diabetes. Slightly overweight patients showed the best prognosis.

Keywords Hepatocellular carcinoma · Non-alcoholic fatty liver disease · Non-alcoholic steatohepatitis · Alcoholic liver disease · Retrospective study

For the INUYAMA NOBLESSE Study group. Members of the INUYAMA NOBLESSE Study group are listed in "Appendix".

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Introduction

Hepatocellular carcinoma (HCC) is a typical example of an infection-associated malignancy [1]. The geographical

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distribution of the highly endemic area of HCC overlaps that of chronic hepatitis B and C [2]. Rigorous efforts to control horizontal transmission of hepatitis B virus (HBV) by vaccination since the mid-1980s succeeded in reducing hepatitis B-related HCC in children [3]. Screening for hepatitis C virus (HCV) and the ending of paid blood donations markedly reduced the incidence of transfusion-associated hepatitis [4]. In those with active chronic hepatitis B, long-term suppression using nucleotide analogs may reduce the incidence of HBV-related HCC [5, 6], and the eradication of HCV by interferon-based therapy can reduce HCV-related HCC [7, 8]. It can reasonably be concluded that hepatitis virus-related HCC will continue to decrease in the future [9, 10].

While HCC is a typical example of a virus-related cancer, it is also well known to be strongly related to life style. Chronic alcoholism is a classical risk factor [11]; more recently, obesity has been recognized to strongly affect HCC development in males, versus various other malignancies [12]. There is also growing evidence suggesting that type 2 diabetes increases the incidence of HCC [13, 14]. Due to the globally increasing proportion of the obese population over the past 30 years [15], obesity-related HCC will likely continue to increase.

Unlike virus-related HCC, in which the high-risk populations and surveillance programs are well established, little is known about the characteristics of virus-unrelated HCC. To reduce the forthcoming global burden of obesity-related HCC, to clarify its clinical features is quite important. The Non-B, Non-C Liver Cancer, Etiology, Prognosis and Treatment (NOBLESSE) study was conducted as a special project of the Inuyama Symposium, an assembly of 56 gastroenterology and hepatology units in university hospitals and tertiary care hospitals in Japan, to investigate the characteristics of non-B, non-C HCC patients.

Patients and methods

Patients

This retrospective study complied with the ethical guidelines for epidemiological research designed by the Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour, and Welfare. The study protocol was approved by the University of Tokyo Medical Research Center Ethics Committee (approval number 3710) and the Institutional Review Board or Ethics Committee of each participating institution. Informed consent was waved because of the retrospective design. This study was registered with the University Hospital Medical Information Network (UMIN) Clinical Trial Registry (UMIN-CTR000007570).

First we collected the number of patients with HCC who were first diagnosed with HCC in the participating hospitals from 1991 to 2010 and categorized them as HBV-related, HCV-related, both HBV and HCV-related, and non-B, non-C to assess trends in the proportion of background etiologies. Next we collected detailed data of non-B, non-C HCC patients defined as negative for both hepatitis B surface antigen (HBsAg) and anti-HCV antibody. Patients who lost HBsAg before the diagnosis of HCC or who were positive for HBV DNA were excluded.

Diagnosis of HCC

The diagnosis of HCC was made by dynamic computed tomography (CT) or dynamic magnetic resonance imaging (MRI) with consideration of hyperattenuation in the arterial phase, with washout in the late phase as a definite sign of this disease [16] or pathology. In years when dynamic CT was not available, the diagnosis was also made by angiography.

Data collection

The patients were registered via a website specially designed by the investigators. The following characteristics at diagnosis were collected: age, gender, body height, body weight, etiology of background liver disease, daily alcohol consumption; comorbidities including liver cirrhosis, fatty liver by ultrasonography, hypertension, dyslipidemia, and diabetes; tumor factors including tumor size of the maximal nodule, number of tumor nodules, the presence of vascular invasion, and extrahepatic metastasis; symptoms including ascites and hepatic encephalopathy, laboratory data, including serum albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), platelet count, prothrombin activity, alpha-fetoprotein (AFP), des-gamma-carboxyprothrombin (DCP), and lens culinaris agglutinin-reactive fraction of AFP (AFP-L3); and treatment modality for the first time, including hepatic resection, liver transplantation, ablation, transarterial chemoembolization (TACE), transarterial chemotherapy, systemic chemotherapy, radiation therapy, and supportive therapy. Body mass index (BMI), Child–Turcotte–Pugh (CTP) score, and Barcelona-Clinic–Liver-Cancer (BCLC) stages were calculated automatically using the data obtained above.

The etiology of background liver diseases was categorized as follows: autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), AIH–PBC overlap syndrome, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), Budd-Chiari syndrome, hemochromatosis, Wilson disease, and others. The diagnosis of the

background liver disease, hypertension, dyslipidemia, and diabetes was made by the attending physician, based on the Japanese clinical guidelines for each disease. Daily alcohol consumption was calculated from forms of alcohol and frequency. Alcoholic liver disease was defined as chronic liver injury with daily alcohol consumption $\geq$ 80 g/day without another definite etiology. NAFLD was defined as a history of fatty liver or who were diagnosed with fatty liver, radiologically or pathologically, with alcohol consumption $\leq$ 20 g/day. Those with cryptogenic chronic liver disease who did not meet the criteria described above for alcoholic liver disease or NAFLD were categorized as unclassified.

Patient survival status was also registered. Status was defined as alive, dead, or lost to follow-up. Observations were censored on 31 December 2011. In diseased patients, the cause of death was categorized according to the criteria of the Liver Cancer Study Group of Japan [17], as follows: liver cancer progression, liver failure, gastrointestinal bleeding, gastro-esophageal varices rupture, rupture of liver cancer, operative death, other, and unknown.

Statistical analysis

Data are expressed as medians with 25th to 75th percentiles, unless otherwise indicated. Numbers and percentages were used for qualitative variables. Student's *t* test was used for comparisons of two continuous variables. Differences among groups were assessed with one-way analysis of variance (ANOVA) for continuous data, and with the Chi squared test for categorical data. The Cochran–Armitage trend test was used to evaluate increasing or decreasing trends in etiology. Survival time was defined as the interval between the day of the first diagnosis and death or the last visit to the hospital until 31 December 2011. Cumulative survival curves were constructed with the Kaplan–Meier method and compared with the log-rank test. To assess the hazard ratios of various factors on overall survival, the Cox proportional hazard model was used.

Statistical analyses were performed using the 'R' software (ver. 2.13.0; http://www.R-project.org). All tests were two-sided, and p values < 0.05 were considered to indicate statistical significance.

Results

Patient profiles

Of 33,782 patients who were first diagnosed with HCC at the 53 participating hospitals from 1991 to 2010, 5326 (15.8 %) were categorized as non-B, non-C. A marked increase in the proportion of patients categorized as non-B, non-C was observed ($p < 0.001$ by Cochran–Armitage test; Fig. 1). The proportion of non-B, non-C patients was 24.1 % in 2010, whereas it was only 10.0 % in 1991. The distribution of background liver diseases among non-B, non-C patients was as follows: AIH in 161 (3.0 %), PBC in 164 (3.1 %), AIH–PBC overlap syndrome in 18 (0.3 %), alcoholic liver disease in 1423 (26.7 %), NAFLD in 596 (11.2 %), Budd-Chiari Syndrome in 20 (0.4 %), hemochromatosis in 9 (0.2 %), Wilson's disease in 5 (0.1 %), unclassified in 2875 (54.0 %), and other in 53 (1.0 %). 'Other' included schistosomiasis japonica, suspicion of autoimmune liver diseases, and normal liver. As few patients were categorized as AIH–PBC overlap syndrome, Budd-Chiari syndrome, hemochromatosis and Wilson's disease, they were combined with 'others' in Table 1. Among non-B, non-C patients, 31 and 10 % were diagnosed as HCC at the department of gastroenterology or hepatology and other department in the participating hospital, respectively. The remaining 59 % were diagnosed at other hospitals and referred to the participating hospitals. Forty-one percent of patients were followed by imaging modalities before the diagnosis of HCC.

The median [interquartile range (IQR)] age in the entire cohort was 70.0 (63.0–75.0) years and approximately three-quarters were males. Patients with alcoholic liver disease were significantly younger than other etiologies ($p < 0.001$). The male to female ratio was different among the etiologies: females predominated in autoimmune liver diseases. The vast majority were non drinkers or light drinkers, except for those with alcoholic liver disease or unclassified etiology. Among those judged as unclassified, 41 % were moderate drinkers.

The distribution of BMI varied across the etiologies and gender. The median BMI was the highest in those with

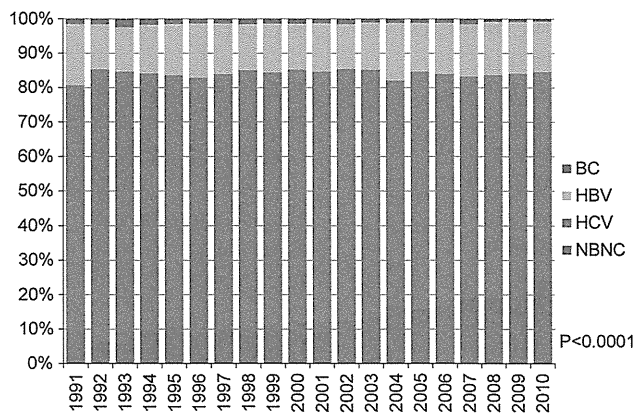

Fig. 1 Trend in background liver disease in hepatocellular carcinoma in Japan. A marked increase in the proportion of patients categorized as non-B, non-C in the participating hospitals was observed ($p < 0.001$ by Cochran–Armitage test)

Table 1 Baseline characteristics of the HCC patients analyzed in this study ($n = 5,326$)

	ALL	AIH	PBC	Alcoholic liver disease	NAFLD	Unclassified	Others
Number of patients	5,326	161	166	1,423	596	2,875	105
Age (year)							
Median	70.0	70.0	71.5	66.0	72.0	71.0	70.0
IQR	63.0–75.0	66.0–76.0	66.0–77.0	60.0–72.0	66.0–77.0	64.0–76.0	58.0–76.0
Male gender [*n* (%)]	4,022 (75.5)	43 (26.7)	52 (31.3)	1,327 (93.3)	348 (58.4)	2,188 (76.1)	64 (61.0)
Alcohol consumption (g/day)[a]							
≤ 20 [*n* (%)]	2623 (50.9)	144 (90.0)	146 (90.7)		596 (100.0)	1661 (59.0)	80 (86.0)
21–79 [*n* (%)]	1179 (22.9)	9 (5.6)	9 (5.6)			1154 (41.0)	7 (7.5)
≥ 80 [*n* (%)]	1351 (26.2)	7 (3.7)	6 (3.7)	1423 (100.0)			6 (6.5)
Diabetes [*n* (%)][b]	2345 (46.1)	48 (30.6)	27 (17.0)	621 (45.2)	359 (62.7)	1264 (46.4)	26 (27.1)
Hypertension [*n* (%)][c]	2063 (42.7)	51 (35.4)	42 (26.8)	493 (38.0)	313 (55.5)	1135 (44.1)	29 (31.9)
Dyslipidemia [*n* (%)][d]	720 (14.6)	26 (17.1)	12 (7.6)	171 (12.7)	125 (22.9)	374 (14.2)	12 (12.6)
Fatty liver [*n* (%)][e]	936 (24.0)	18 (15.5)	7 (5.5)	219 (20.7)	280 (64.4)	403 (19.3)	9 (13.4)
Liver Cirrhosis [*n* (%)][f]	3439 (67.0)	127 (80.9)	145 (87.9)	1115 (80.2)	368 (63.4)	1619 (59.0)	65 (67.0)
Anti-HBcAb positive [*n* (%)][g]	1501 (40.3)	27 (23.5)	35 (31.3)	410 (40.8)	159 (34.6)	837 (43.0)	33 (40.7)
ALT (U/L)							
Median	32	29	29	33	33	32	29
IQR	22–50	20–44	20–41.3	22–50	22–51	22–51	20–54
Platelet count ($\times 10^9/\mu L$)[h]							
Median	135	105	103	123	138	148	124
IQR	90–193	72–166	74–139	84–173	94–189	97–205	81–183
Child-Pugh class[i]							
A [*n* (%)]	3500 (69.0)	89 (57.4)	83 (52.9)	843 (62.1)	439 (76.5)	1976 (72.4)	70 (72.2)
B [*n* (%)]	1231 (24.3)	54 (34.8)	57 (36.3)	383 (28.2)	120 (20.9)	595 (21.8)	22 (22.7)
C [*n* (%)]	338 (6.7)	12 (7.7)	17 (10.8)	131 (9.7)	15 (2.6)	158 (5.8)	5 (5.2)
Tumor characteristics							
Maximal tumor size (cm)[j]							
Median	3.2	3.0	2.8	3.0	3.0	3.5	3.0
IQR	2.0–6.0	2.0–4.3	1.7–3.5	2.0–5.0	2.0–5.0	2.2–7.0	2.0–5.1
Diffuse type [*n* (%)]	209 (4.0)	6 (3.7)	1 (0.6)	62 (4.4)	17 (2.9)	119 (4.2)	4 (3.8)
Number of nodules[k]							
Single [*n* (%)]	2700 (51.1)	87 (54.0)	110 (66.3)	664 (46.8)	340 (57.0)	1443 (50.8)	56 (53.8)
2–3 [*n* (%)]	1368 (25.9)	46 (28.6)	40 (24.1)	402 (28.3)	156 (26.2)	697 (24.5)	27 (26.0)
> 3 [*n* (%)]	1220 (23.1)	28 (17.4)	16 (9.6)	353 (24.9)	100 (16.8)	702 (24.7)	21 (20.2)
Vascular invasion [*n* (%)][l]	187 (3.5)	3 (1.9)	1 (0.6)	52 (3.7)	13 (2.2)	116 (4.1)	2 (1.9)
Extrahepatic metastasis [*n* (%)][m]	401 (7.6)	8 (5.0)	2 (1.2)	114 (8.0)	26 (4.4)	244 (8.6)	7 (6.7)
AFP (ng/mL)[n]							
≤20 [*n* (%)]	2908 (59.4)	80 (54.1)	71 (51.4)	827 (62.4)	361 (63.1)	1515 (58.0)	54 (55.7)
21–200 [*n* (%)]	820 (16.8)	33 (22.3)	29 (21.0)	229 (17.3)	92 (16.1)	423 (16.2)	14 (14.4)
>200 [*n* (%)]	1164 (23.8)	35 (23.6)	38 (27.5)	270 (20.4)	119 (20.8)	673 (25.8)	29 (29.9)
DCP (mAU/mL)[o]							
≤100 [*n* (%)]	2121 (45.8)	75 (53.6)	81 (59.1)	593 (46.8)	299 (53.9)	1032 (42.1)	41 (47.7)
101–400 [*n* (%)]	787 (17.0)	23 (16.4)	25 (18.2)	227 (17.9)	95 (17.1)	400 (16.3)	17 (19.8)
>400 [*n* (%)]	1727 (37.3)	42 (30.0)	31 (22.6)	448 (35.3)	161 (29.0)	1017 (41.5)	28 (32.6)
AFP-L3 (%)[p]							
≤10 [*n* (%)]	1765 (67.7)	53 (64.6)	39 (55.7)	498 (69.6)	263 (73.5)	881 (66.1)	31 (66.0)
10.1–15 [*n* (%)]	74 (2.8)	3 (3.7)	4 (5.7)	17 (2.4)	7 (2.0)	43 (3.2)	0 (0)
>15 [*n* (%)]	767 (29.4)	26 (31.7)	27 (38.6)	201 (28.1)	88 (24.6)	409 (30.7)	16 (34.0)

As few patients were categorized as having the AIH–PBC overlap syndrome, Budd-Chiari syndrome, hemochromatosis or Wilson's disease, they were combined with 'others'. Data were missing in [a]173, [b]241, [c]498, [d]388, [e]1434, [f]193, [g]1606, [h]61, [i]257, [j]42, [k]38, [l]28, [m]26, [n]434, [o]691, and [p]3677 patients

AFP alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive fraction of AFP, *ALT* alanine aminotransferase, *Anti-HBcAb* anti-hepatitis B core antibody, *DCP* des-gamma-carboxy prothrombin, *IQR* interquartile range

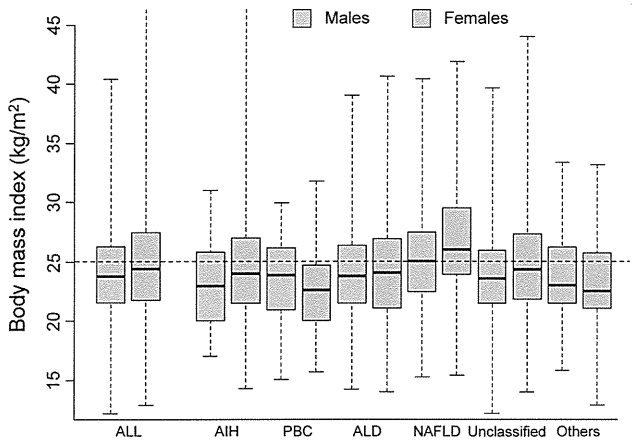

Fig. 2 Body mass index according to background liver disease. Median (25th–75th percentiles) BMI values in all categories were 23.8 (21.6–26.3) kg/m^2 in males and 24.4 (21.8–27.5) kg/m^2 in females. *Box plot 'whiskers'* show the minimum and maximum values; the *horizontal line* in each box plot shows the median, and the *colored* segment shows the interquartile range. *AIH* autoimmune hepatitis, *PBC* primary biliary cirrhosis, *ALD* alcoholic liver disease, *NAFLD* non-alcoholic fatty liver disease

NAFLD. Females had significantly higher BMI than males in NAFLD and those unclassified ($p = 0.01$ and <0.001, respectively; Fig. 2).

Nearly half of the patients were complicated with diabetes (Table 1, Supplementary Fig. 1). The proportion of those with diabetes was highest in NAFLD patients. A similar trend was observed in the proportions of hypertension and dyslipidemia. The presence of fatty liver, judged by ultrasonography at the diagnosis of HCC, varied across the etiologies. The proportion was approximately 20 % in alcoholic liver disease and unclassified etiology, while it was lower in autoimmune liver diseases, especially PBC. It was also suggested that fatty liver could not be detected by ultrasonography in approximately 30 % at the diagnosis of HCC in NAFLD.

Approximately two-thirds of the patients were complicated with cirrhosis. The proportion of those with cirrhosis was lower in those with NAFLD and unclassified etiology compared with other etiologies ($p < 0.001$). Reflecting the proportion of cirrhosis, platelet counts were highest in those with unclassified etiology, followed by those with NAFLD.

Regarding the diagnosis process, 30.3 % of the patients had their tumor pointed out for the first time in the participating department, 10.6 % in another department of the same hospital, and 59.1 % at other hospitals. Patients were diagnosed at more advanced stages in those with unclassified etiology; the tumor size was the largest and the proportion of patients with vascular invasion and extrahepatic metastasis was also the largest. The sensitivity of DCP was superior to that of AFP (54.2 vs. 40.6 % with cutoff values of 100 mAU/mL and 20 ng/mL, respectively).

Treatment and survival

Among 5058 patients in whom BCLC staging could be determined, 2533 (50.1 %), 1913 (37.8 %), 283 (5.6 %), and 329 (6.5 %) were categorized as stages A, B, C, and D, respectively (Table 2). The distribution of the initial treatment was as follows: resection in 1073 (20.3 %), ablation in 1060 (20.0 %), TACE + ablation in 470 (8.9 %), TACE in 1590 (30.1 %), transarterial chemotherapy with one-shot and continuous infusion in 99 (1.9 %), systemic therapy in 20 (0.3 %), radiation therapy in 20 (0.4 %), liver transplantation in 17, others in 30 (0.6 %), and supportive care in 429 (8.1 %).

During the mean follow-up period of 2.6 years, 2225 patients died and 670 patients were lost to follow-up. The causes of death were cancer progression in 1411 (58.0 %), liver failure in 359 (14.8 %), gastrointestinal bleeding, including varices rupture, in 87 (3.6 %), tumor rupture in 71 (2.9 %), operative death in 13 (0.5 %), and other in 284 (11.7 %). The cause of death was unspecified in 206 (8.5 %). Median survival time [95 % confidence interval (CI)] after the initial diagnosis of HCC was 4.03 (3.82–4.20) years. Overall survival rates at 1, 3, 5, 7, 10, 15, and 20 years were 80.1, 58.2, 42.6, 32.2, 21.5, 15.2,

Table 2 Distribution of treatments according to BCLC stage

	A	B	C	D
Number of patients	2533	1913	283	329
Hepatic resection [*n* (%)]	616 (24.3)	398 (20.8)	30 (10.6)	3 (0.9)
Ablation [*n* (%)]	887 (35.0)	81 (4.2)	4 (1.4)	52 (15.8)
TACE + ablation [*n* (%)]	335 (13.2)	116 (6.1)	3 (1.1)	4 (1.2)
TACE [*n* (%)]	517 (17.1)	840 (43.9)	78 (27.6)	83 (25.2)
Transarterial chemotherapy [*n* (%)]	83 (3.2)	278 (14.5)	87 (30.7)	27 (8.2)
Systemic therapy [*n* (%)]	5 (0.2)	50 (2.6)	25 (8.8)	7 (2.1)
Radiation therapy [*n* (%)]	5 (0.2)	4 (0.2)	3 (1.1)	5 (1.5)
Liver transplantation [*n* (%)]	11 (0.4)	6 (0.3)	0(0.0)	0 (0.0)
Others [*n* (%)]	12 (0.5)	5 (0.3)	2 (0.7)	4 (1.2)
Supportive therapy [*n* (%)]	64 (2.5)	135 (7.1)	51 (18.0)	144 (43.8)

BCLC stage could not be determined in 268 patients

TACE transarterial chemoembolization

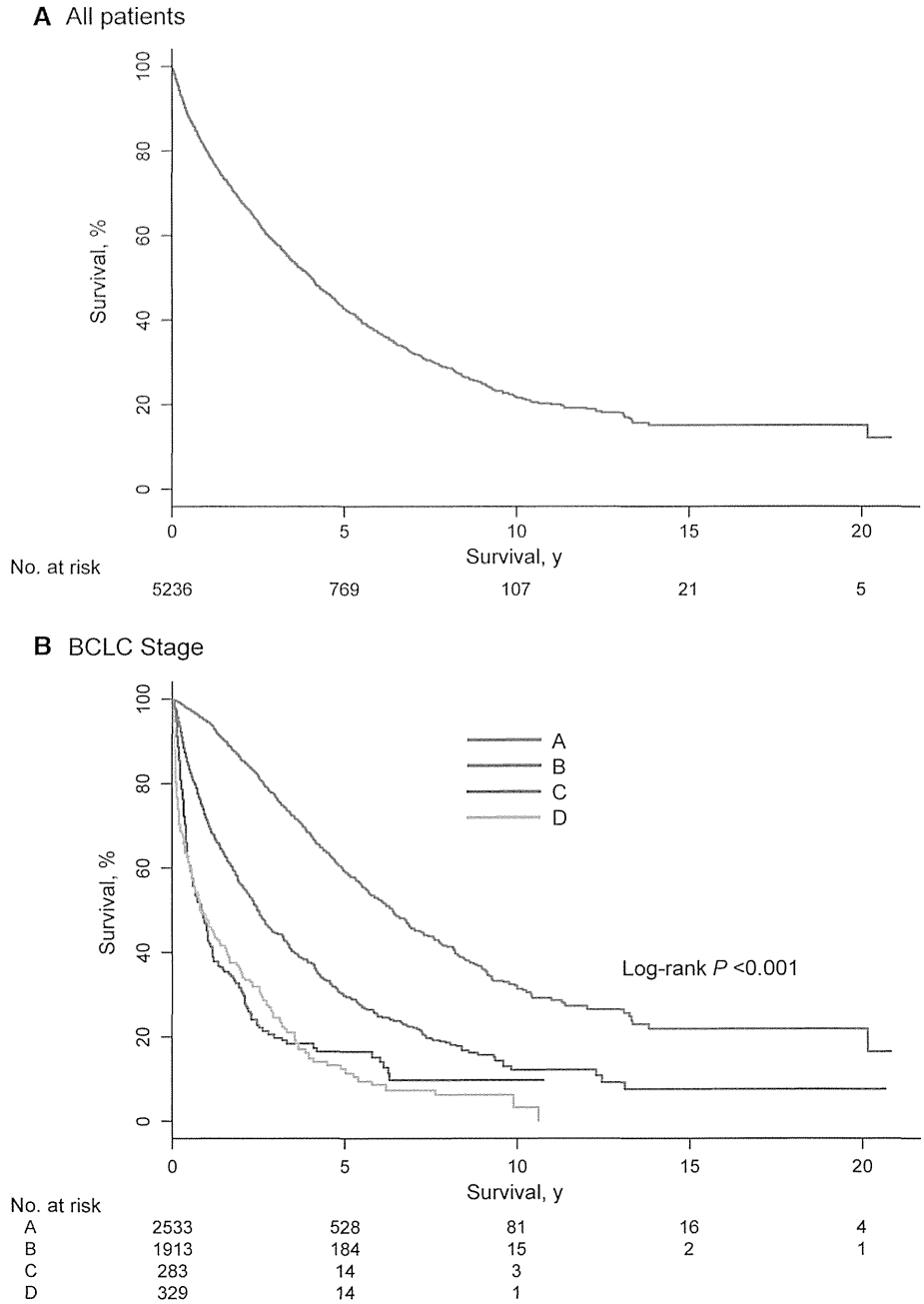

Fig. 3 Overall survival. **A** Overall survival of the entire patient cohort. Overall survival rates at 1, 3, 5, 7, 10, 15, and 20 years were 80.1, 58.2, 42.6, 32.2, 21.5, 15.2, and 15.2 %, respectively. **B** Overall survival according to BCLC stage. Survival rates at 1, 3, 5, 7, 10, 15, and 20 years were 94.5, 76.4, 58.7, 44.7, 30.7, 21.9, and 21.9 % in stage A, 71.1, 44.1, 29.1, 22.2, 13.0, 9.0, and 9.0 % in stage B, 44.6, 18.8, 15.5, 9.3, and 9.3 % in Stage C, and 48.0, 24.4, 12.3, 7.3, 3.1 %, respectively, in Stage D

and 15.2 %, respectively (Fig. 3a). When stratified by BCLC stage, the median (95 % CI) survival times were 6.39 (5.96–6.85), 2.48 (2.34–2.68), 0.83 (0.61–1.03), and 0.80 (0.64–1.23) years in BCLC stages A, B, C, and D, respectively. There was a significant difference in survival among the stages (Fig. 3b, $p < 0.001$).

Univariate Cox regression analysis revealed that the following factors were significantly related to poor survival: old age ($p < 0.001$), male gender ($p = 0.003$), alcohol consumption $\geq$80 g/day ($p < 0.001$), BMI ($p = 0.001$), Child-Pugh score ($p < 0.001$), maximal tumor size ($p < 0.001$), number of nodules ($p < 0.001$), the presence of vascular invasion ($p < 0.001$), the presence of extrahepatic metastasis ($p < 0.001$), AFP ($p < 0.001$), DCP ($p < 0.001$), and AFP-L3 ($p < 0.001$). The presence of diabetes was indicated as a better prognosis factor, though with marginal significance (hazard ratio, 0.93; 95 % CI, 0.86–1.01; $p = 0.08$). BMI showed a V-shaped hazard distribution: those with BMIs of 22.1–25 kg/m^2 had the best outcomes, whereas those with higher and lower BMI showed worse prognoses. We plotted relative hazard against BMI using cubic splines. The V-shape hazard distribution was also observed in the plot (Supplementary Fig. 2).

We performed a multivariate analysis using the variables above, except that AFP-L3 was excluded because of missing values. The results showed that age, BMI, alcohol consumption, Child-Pugh score, tumor size, number of tumor nodules, extrahepatic metastasis, AFP, and DCP were significant factors related to poor prognosis (Fig. 4). The presence of diabetes again showed no statistical significance.

Discussion

In the present study, a rapidly increasing proportion of HCC patients with non-viral etiologies was found. A similar trend was reported in a national survey by the Liver Cancer Study Group of Japan [18]. As the number of newly diagnosed HCC cases in Japan was almost at a plateau throughout the study period [19], not only the proportion,

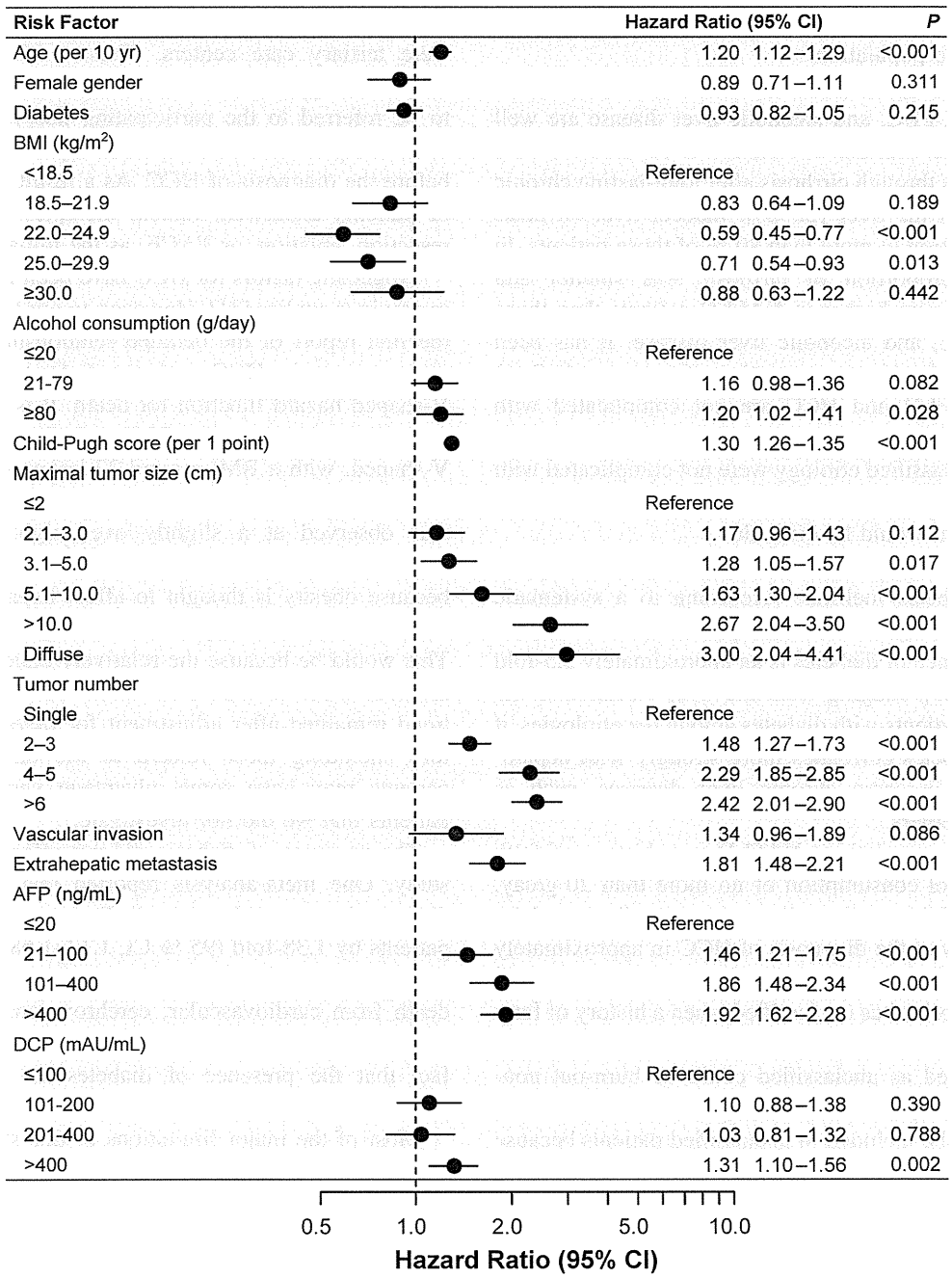

Fig. 4 Multivariate Cox proportional hazard regression analysis of survival. *AFP* alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive fraction of AFP, *DCP* des-gamma-carboxy prothrombin *AFP* alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive fraction of AFP, *ALT* alanine aminotransferase, *Anti-HBcAb* anti-hepatitis B core antibody, *DCP* des-gamma-carboxy prothrombin, *IQR* interquartile range

but also the number, of patients with non-viral etiologies was increasing. As a risk factor of HCC, alcohol consumption has not increased over the last two decades in Japan according to statistics from the Ministry Labour and Welfare in Japan [20]. In contrast, the size of the obese population is increasing rapidly due to changes in the diet in Japan. The proportion of patients with diabetes has also increased in the past three decades [21]. It seems reasonable that the rapidly increasing number of HCC patients with non-viral etiologies was largely due to the rapidly increasing obese population.

Among non-viral chronic liver diseases, the natural history of AIH, PBC, and alcoholic liver disease are well known compared with that of NAFLD. In these three, HCC ordinarily arises through cirrhosis after long-lasting chronic inflammation in the liver [22–24]. Indeed, liver cirrhosis was a complication in more than 80 % of those patients. In contrast, the proportion of cirrhosis was smaller and platelet counts were higher in NAFLD patients than those with AIH, PBC, and alcoholic liver disease. It has been reported that a significant proportion of patients (41.7 %) with both NAFLD and HCC are not complicated with cirrhosis [25]. That a significant proportion of patients with NAFLD or unclassified etiology were not complicated with cirrhosis suggests that to characterize a high-risk population within them would be difficult.

In this study, almost half of the patients were complicated with diabetes mellitus. According to a systematic review investigating the relationship between diabetes and HCC, the presence of diabetes is an approximately 2.5-fold risk of HCC [26]. Judging from the wide variation in the proportion of patients with diabetes among the etiologies, it seems that diabetes correlates more strongly with hepatocarcinogenesis in some chronic liver diseases, such as NAFLD, than others.

In this study, we defined NAFLD as a history of fatty liver and alcohol consumption of no more than 20 g/day. As shown in Table 1, fatty liver was not diagnosed by ultrasonography at the diagnosis of HCC in approximately 30 % of patients with NAFLD-related HCC. Those patients would be categorized as unclassified when a history of fatty liver was not confirmed. That is, a significant proportion of those categorized as unclassified could be burn-out non-alcoholic steatohepatitis (NASH). Similarly, alcohol-related HCC could be included in unclassified patients because approximately 40 % of the patients in the category were moderate drinkers. In the first place, it might be unreasonable to categorize those patients clearly, because moderate alcohol intake, obesity, and fatty liver are mutually correlated and may have a synergistic effect on hepatocarcinogenesis.

Occult infection with HBV represented by the presence of antibody to hepatitis B core antigen (anti-HBc) has been considered as a risk factor of non-B, non-C HCC defined as negative for both HBsAg and anti-HCV antibody [27, 28]. Indeed the prevalence of anti-HBc antibody was higher in this study compared to a previous report in blood donors [29]. It is also to be noted that those with anti-HBc antibody may include chronic HBV carriers with HBsAg loss before the diagnosis of HCC, who had significant risk for HCC [30].

Patients were diagnosed at less-advanced stages than we expected. This is partly because all participating hospitals were tertiary care centers. Those with terminal stages diagnosed in primary or secondary hospitals were unlikely to be referred to the participating hospitals. In addition, 41 % of patients were followed by imaging modalities before the diagnosis of HCC. As a result, a large majority of patients underwent radical therapies, such as hepatic resection, ablation, or TACE, as the initial treatment.

Prognostic factors for HCC have been investigated fully in previous studies [31]. However, to our knowledge, this is the first report of the detailed relationship between BMI and survival in HCC patients. Indeed, BMI showed a V-shaped hazard function for death. It is well known that the relationship between BMI and all-cause mortality is V-shaped, with a BMI around 22 kg/m^2 showing the best prognosis. However in this study, the lowest relative hazard was observed at a slightly overweight BMI. We had expected that the best BMI would be around 22 kg/m^2, because obesity is thought to affect hepatocarcinogenesis in this cohort and may affect recurrence after treatment. This would be because the relatively underweight patients included those with more advanced disease. However, the trend remained after adjustment for other significant factors, including those related to the tumor. Overweight patients may have some advantage versus underweight patients that we did not investigate.

The presence of diabetes did not affect survival in this study. One meta-analysis reported that the presence of diabetes increased the risk of all-cause mortality in HCC patients by 1.38-fold (95 % CI, 1.13–1.68) [32]. It is quite reasonable that those with diabetes had additional risk for death from cardiovascular, cerebrovascular, infectious or renal diseases. Some kind of biases might exist behind the fact that the presence of diabetes did not worsen the patients' survival, which needs further investigation.

Most of the major limitations of this study relate to its retrospective design.

(1) Because the major data source was a database maintained by each participating hospital, some data were missing. Patients who were not registered in the database could not be entered into this study. However, the proportion of patients with missing data on important items, such as alcohol consumption, was less than 5 %; this would not affect the overall results. (2) As the amount of daily

alcohol intake was self-reported, some patients might have underreported their alcohol intakes. Some should possibly have been categorized as having alcoholic liver disease. (3) Similarly, because the diagnosis of NAFLD was based on a past history or ultrasound examination at the diagnosis of HCC, undiagnosed burn-out NASH patients were included in those unclassified, especially when not followed in clinics or hospitals. Based on the high proportion of those with lifestyle diseases and moderate drinkers, at least a majority of those unclassified would be related to chronic alcoholism, obesity, or both.

In conclusion, the proportion of HCC patients without chronic viral hepatitis in Japan is increasing rapidly. Most had lifestyle disease-related backgrounds, especially related to obesity. Narrowing down a high-risk population would be difficult because one-third of the patients were non-cirrhotic, and obesity, fatty liver, and diabetes are prevalent in Japan.

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

Appendix

The following investigators enrolled patients in the Inuyama NOBLESSE Study: Joji Toyota, Yoshiyasu Karino (Hokkaido P.W.F.A.C Sapporo-Kosei General Hospital, Sapporo); Kazuyuki Suzuki, Hidekatsu Kuroda (Iwate Medical University, Iwate); Yoshiyuki Ueno, Hisayoshi Watanabe (Yamagata University Faculty of Medicine, Yamagata); Yutaka Aoyagi, Hirokazu Kawai (Niigata University Graduate School of Medical and Dental Science, Niigata); Eiji Tanaka, Takefumi Kimura (Shinshu University School of Medicine, Matsumoto); Kendo Kiyosawa, Hiromitsu Mori (Nagano Red Cross Hospital, Nagano); Nobuyuki Enomoto (University of Yamanashi Faculty of Medicine, Chuo); Masao Omata, Hitoshi Mochizuki (Yamanashi Central Hospital, Kofu); Satoshi Mochida, Mie Inao (Saitama Medical University, Iruma-gun); Kunihiko Hino, Hiromi Hoshino (Delta Clinic, Tokorozawa); Masashi Mizokami, Kazumoto Murata (Kohnodai Hospital, National Center for Global Health and Medicine, Ichikawa); Osamu Yokosuka, Fumihiko Kanai (Chiba University Graduate School of Medicine, Chiba); Ryosuke Tateishi, Kenichiro Enooku, Koji Uchino, Masaya Sato, Shintaro Kayaki, Tatsuya Minami, Shintaro Mikami, Naoto Fujiwara (University of Tokyo Graduate School of Medicine, Tokyo); Sumio Watanabe, Kazuyoshi Kon (Juntendo University, Tokyo); Michio Imawari, Junichi Eguchi (Showa University, Tokyo); Hajime Takikawa, Masaki Mikami (Teikyo University, Tokyo); Shunji Mishiro, Masahiro Arai (Toshiba General Hospital, Tokyo); Hiromitsu Kumada, Yusuke Kawamura (Department of Hepatology, Toranomon Hospital, Kawasaki); Namiki Izumi, Takanori Hosokawa (Musashino Red-Cross Hospital, Musashino); Mitsuhiko Moriyama, Jumpei Hayashi (Nihon University School of Medicine, Tokyo); Michihiro Suzuki, Kotaro Matsunaga (Kawasaki City Tama Hospital, Kawasaki); Katsuaki Tanaka, Manabu Morimoto (Yokohama City University Medical Center, Yokohama); Takafumi Ichida, Katsuharu Hirano (Juntendo University Shizuoka Hospital, Izunokuni); Yasuhito Tanaka, Kei Fujiwara (Nagoya City University Graduate School of Medical Sciences, Nagoya); Takashi Kumada (Ogaki Municipal Hospital, Ogaki); Hisataka Moriwaki, Koji Takai (Gifu University Graduate School of Medicine, Gifu); Shuichi Kaneko, Masaaki Kitahara (Kanazawa University Graduate School of Medical Sciences, Kanazawa); Hiroshi Fukui, Masao Fujimoto (Nara Medical University, Kashihara); Yukio Osaki, Akihiro Nasu (Osaka Red Cross Hospital, Osaka); Takeshi Okanoue, Toshihide Shima (Saiseikai Suita Hospital, Suita); Toshihito Seki, Rinako Kawanura (Kansai Medical University Takii Hospital, Moriguchi); Masatoshi Kudo, Yasunori Minami (Kinki University School of Medicine, Osaka-Sayama); Tetsuo Takehara, Takayuki Yakushijin (Osaka University Graduate School of Medicine, Suita); Michio Kato, Seiji Morioka (Minami Wakayama Medical Center, Tanabe); Shuhei Nishiguchi, Hironori Tanaka (Hyogo College of Medicine, Nishinomiya); Keisuke Hino, Yasuyuki Tomiyama (Kawasaki Medical School, Kurashiki); Kazuhide Yamamoto, Kazuhiro Nouso (Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama); Kazuaki Chayama, Hiroshi Aikata (Hisoshima University Graduate School of Biomedical Sciences, Hiroshima); Isao Sakaida, Makoto Segawa (Yamaguchi University Graduate School of Medicine, Ube); Kiwamu Okita, Akira Kato (Shimonoseki Kohsei Hospital, Shimonoseki); Yoshikazu Murawaki, Naoto Maeda (Tottori University Faculty of Medicine, Yonago); Morikazu Onji, Yoichi Hiasa (Ehime University Graduate School of Medicine, Tōon); Michio Sata, Takumi Kawaguchi (Kurume University School of Medicine, Kurume); Masaru Harada, Michihiko Shibata (University of Occupational and Environmental Health, Kitakyushu); Hideyuki Nomura (Shin-Kokura Hospital, Kokura); Shotaro

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Impact of IL28B Genetic Variation on HCV-Induced Liver Fibrosis, Inflammation, and Steatosis: A Meta-Analysis

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Abstract

Background & Aims: IL28B polymorphisms were shown to be strongly associated with the response to interferon therapy in chronic hepatitis C (CHC) and spontaneous viral clearance. However, little is known about how these polymorphisms affect the natural course of the disease. Thus, we conducted the present meta-analysis to assess the impact of IL28B polymorphisms on disease progression.

Methods: A literature search was conducted using MEDLINE, EMBASE, and the Cochrane Library. Integrated odds ratios (OR) were calculated with a fixed-effects or random-effects model based on heterogeneity analyses.

Results: We identified 28 studies that included 10,024 patients. The pooled results indicated that the rs12979860 genotype CC was significantly associated (vs. genotype CT/TT; OR, 1.122; 95%CI, 1.003–1.254; P = 0.044), and that the rs8099917 genotype TT tended to be (vs. genotype TG/GG; OR, 1.126; 95%CI, 0.988–1.284; P = 0.076) associated, with an increased possibility of severe fibrosis. Both rs12979860 CC (vs. CT/TT; OR, 1.288; 95%CI, 1.050–1.581; P = 0.015) and rs8099917 TT (vs. TG/GG; OR, 1.324; 95%CI, 1.110–1.579; P = 0.002) were significantly associated with a higher possibility of severe inflammation activity. Rs8099917 TT was also significantly associated with a lower possibility of severe steatosis (vs. TG/GG; OR, 0.580; 95%CI, 0.351–0.959; P = 0.034), whereas rs12979860 CC was not associated with hepatic steatosis (vs. CT/TT; OR, 1.062; 95%CI, 0.415–2.717; P = 0.901).

Conclusions: IL28B polymorphisms appeared to modify the natural course of disease in patients with CHC. Disease progression seems to be promoted in patients with the rs12979860 CC and rs8099917 TT genotypes.

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Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In epidemiological studies of chronic HCV infection, age, duration of infection, alcohol consumption, coinfection with human immune deficiency virus, low CD4 count, male gender, and HCV genotype 3 have been shown to be associated with histological activity [2–7]. Although these factors explain part of the extreme variability seen in the progression of fibrosis among HCV-infected patients, they do not completely account for the differences. Genetic host factors have long been suspected to play a role in chronic hepatitis C (CHC) [8–10]. Two genome-wide association studies recently reported the susceptible loci for the progression of liver cirrhosis [11,12].

Currently, patients with CHC are treated with a combination of peg-interferon (peg-IFN) and ribavirin [13,14]. Telaprevir and boceprevir, two protease inhibitors, were recently approved for patients with genotype 1 in combination with peg-IFN and ribavirin. This combination has been shown to lead to substantial improvement in the sustained virologic response rate [15,16]. Genetic variations near the interleukin 28B (IL28B) gene, encoding type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [17–20] and with spontaneous clearance of HCV [21]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via retinoic acid-inducible gene-I and toll-like receptor 3 and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [22,23].

Polymorphisms of genes involved in innate immunity are likely to influence the strength and nature of this defense system [24]. Moreover, IL28B polymorphisms were shown to be associated with lipid metabolism [25]. Thus, this genetic factor is thought to influence the natural course of HCV infection including liver fibrosis, inflammation activity, or steatosis. However, associations between IL28B polymorphisms and the state of background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC remain controversial. Single studies may have limited statistical power to detect the modest effects of IL28B polymorphisms on disease progression.

Thus, we conducted the present meta-analysis to integrate the results of eligible studies and provide statistically reliable evidence of the role of IL28B polymorphisms in patients with CHC.

Materials and Methods

2.1 Search strategy

An electronic search was conducted in MEDLINE, EMBASE, and the Cochrane Library for articles published prior to 30 April, 2012. Search terms included *IL28B*, *IL28*, *IL-28B*, *interleukin-28B*, *interleukin 28B*, *rs12979860*, and *rs8099917*. The search was limited to the English language.

2.2 Inclusion criteria

A study was included in the current analysis if it satisfied the following criteria: (1) It evaluated the associations between IL28B polymorphisms (rs12979860 or rs8099917) and liver fibrosis, inflammation activity, or steatosis. We also included studies that evaluated fibrosis or inflammation activity using the aminotransferase platelet ratio index or ALT. (2) It provided sufficient published data for estimating odds ratios (OR) with 95% confidence intervals (CIs). In case of multiple studies based on the same population, we selected the study with the largest number of participants. A study was excluded if (1) it dealt only with co-infection of HCV and human immunodeficiency virus, (2) it dealt only with patients with a specific condition such as a comorbid disease (e.g., thalassemia) or status after liver transplantation, or (3) it only used a recessive hereditary model (rs12979860 CC + CT vs. TT, or rs8099917 TT +TG vs. GG).

2.3 Data extraction

Two authors (M.S. and M.K.) independently screened titles and abstracts for potential eligibility and full texts for final eligibility. Disagreements were resolved by consultation with a third author (R.T.). The following information was extracted or calculated from each study: first author, year of publication, country of origin, ethnicity, sex, HCV genotype, and background liver information (fibrosis, inflammation activity, or steatosis) for each genotype. The analysis was based on the dominant model (CC vs. CT and TT in rs12979860; TT vs. TG and GG in rs8099917).

2.4 Definition

In some studies, mild or severe fibrosis or inflammation activity was not defined. To compare results among studies on these outcomes, we defined Ishak level F4 to F6; METAVIR, Ludwig Batts, and Inuyama level F3 to F4; and Knodell histology activity index as severe fibrosis. We also defined METAVIR A2 to A3 as severe inflammation activity.

2.5 Statistical analysis

The association of liver fibrosis, inflammation activity, or steatosis with the IL28B genotype in patients with CHC was assessed by summary ORs and corresponding 95% CIs. Heterogeneity among studies was examined with I^2 statistics interpreted as the proportion of total variation contributed by between-study variation [26]. If there was no or low statistical heterogeneity among studies ($I^2<50\%$ and $P>0.05$), the ORs and 95% CIs were calculated by the fixed-effects model. Otherwise, the random-effects model was adopted. When significant heterogeneity was observed, we performed a meta-regression analysis to investigate relationships between the effect of IL28B polymorphisms on liver fibrosis, inflammation activity, or steatosis; and continuous variables (proportion of patients with genotype 1 or 4 virus infection, proportion of males; and proportion of Caucasian, African-American, and Asian patients) to explore the possible reason for heterogeneity between studies [27,28]. To check for publication bias, we used the linear regression approach described by Egger et al. [29]. All calculations were performed using Comprehensive Meta-Analysis software (Biostat, Englewood, NJ).

Results

3.1 Characteristics of articles

Figure 1 shows the literature search and study selection procedures. A total of 471 potentially relevant publications up to 30 April, 2012, were initially identified through MEDLINE, EMBASE, and the Cochrane Library, 443 of which were excluded because they did not meet our inclusion criteria. Therefore, 28 studies involving a total number of 10,024 patients were included in the meta-analysis. Study characteristics are shown in Table 1. There were 5616 males and 3974 females, and the sex was not reported in the remaining 434 patients (1 study). Nineteen studies (7542 patients) evaluated liver fibrosis according to rs12979860 polymorphism and 16 studies (5052 patients) according to rs8099917 polymorphism; four studies (2301 patients) evaluated inflammation activity according to rs12979860 polymorphism and eight studies (2904 patients) according to rs8099917 polymorphism; and four studies (962 patients) evaluated steatosis according to rs12979860 polymorphism and five studies (1308 patients) according to rs8099917 polymorphism.

3.2 Fibrosis

For rs12979860, the between-study heterogeneity was not significant ($I^2=25\%$, $P=0.147$); thus, the fixed-effects model was applied. The pooled results indicated that IL28B rs12979860 genotype CC was associated with an increased possibility of severe fibrosis (OR, 1.122; 95%CI, 1.003–1.254; $P=0.044$) (Fig. 2-a). For rs8099917, there was no or low heterogeneity ($I^2=31\%$, $P=0.111$), and IL28B rs8099917 genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference did not reach statistical significance (OR, 1.126; 95%CI, 0.988–1.284; $P=0.076$) (Fig. 2-b). Egger's test showed no evidence for publication biases for either rs12979860 ($P=0.839$) or rs8099917 ($P=0.342$). When restricted to studies in which only treatment-naïve patients were included, 12 studies (5865 patients) according to rs12979860 polymorphism and eight studies (3333 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2=0\%$, $P=0.615$) and rs8099917 ($I^2=16\%$, $P=0.304$). For rs12979860, fixed-effect model analyses showed a higher probability of severe fibrosis in genotype CC (OR, 1.184; 95%CI, 1.040–1.348; $P=0.010$) (Fig. 2-c), and for rs8099917, genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference was not statistically significant (OR, 1.154; 95%CI, 0.985–1.351; $P=0.076$) (Fig. 2-d). Egger's test showed no evidence of publication bias ($P=0.394$ for rs12979860 and $P=0.295$ for rs8099917).

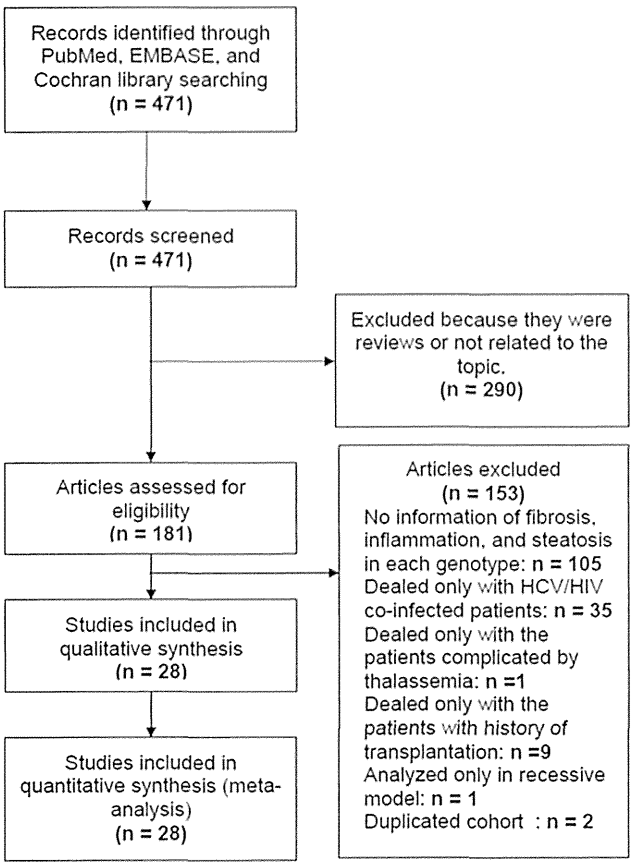

Figure 1. Literature search and study selection process. Twenty-eight individual studies that met all of the inclusion and exclusion criteria.
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3.3 Inflammation activity

The between-study heterogeneity was not significant ($I^2 = 35\%$, $P = 0.204$) for rs12979860. In the fixed-effects model, the pooled results indicated that IL28B rs12979860 genotype CC was associated with a higher possibility of severe inflammation activity (OR, 1.288; 95%CI, 1.050–1.581; $P = 0.015$) (Fig. 3-a). For rs8099917, there was no or low heterogeneity ($I^2 = 0\%$, $P = 0.598$), and IL28B rs8099917 genotype TT was also associated with a higher possibility of severe inflammation activity (OR, 1.324; 95%CI, 1.110–1.579; $P = 0.002$) (Fig. 3-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.448$) and rs8099917 ($P = 0.531$). When restricted to studies in which only treatment-naïve patients were included, three studies (2192 patients) according to rs12979860 polymorphism and two studies (1769 patients) according to rs8099917 polymorphism were extracted. Significant heterogeneities were found for rs12979860 ($I^2 = 53\%$, $P = 0.120$); thus, the random-effect model was applied. The pooled results indicated that IL28B rs12979860 genotype was not associated with inflammatory activity (OR, 1.340; 95%CI, 0.938–1.916; $P = 0.108$) (Fig. 3-c). For rs8099917, the between-study heterogeneity was not significant ($I^2 = 0\%$, $P = 0.585$). In the fixed-effects model, genotype TT tended to be associated with a higher possibility of severe inflammation activity (OR, 1.217; 95%CI, 0.978–1.515; $P = 0.079$) (Fig. 3-d). Egger's test showed no evidence of publication bias in rs12979860 ($P = 0.646$). For rs8099917, Egger's test was not applicable because only 2 studies were included. We also performed a meta-regression analysis for rs12979860 because significant heterogeneities were observed. Table 2 shows the results of these meta-regression analyses. Significant correlation was observed between rs12979860 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, 2.992 ± 1.497; $P = 0.046$).

3.4 Steatosis

Significant heterogeneities were found for rs12979860 ($I^2 = 86\%$, $P<0.001$) and rs8099917 ($I^2 = 52\%$, $P = 0.082$); thus, we applied the random-effects model for this outcome. The pooled results indicated that IL28B rs12979860 genotype CC was not associated with hepatic steatosis (OR, 1.062; 95%CI, 0.415–2.717, $P = 0.901$) (Fig. 4-a), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.580; 95%CI, 0.351–0.959; $P = 0.034$) (Fig. 4-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.238$) or rs8099917 ($P = 0.182$). We also performed a meta-regression analysis because significant heterogeneities were observed. Table 3 shows the results of these meta-regression analyses. In terms of the effect of rs12979860 on steatosis, significant correlations were observed between the proportion of patients with genotype 1 or 4 virus (slope, -4.947 ± 1.086; $P<0.001$), the proportion of Caucasian patients (slope, 7.361 ± 1.569; $P<0.001$), and the proportion of African-American patients (slope, -8.996 ± 1.918; $P<0.001$). We also observed a significant correlation between the effect of rs8099917 polymorphism on steatosis and the proportion of male patients (slope, 6.225 ± 2.530; $P = 0.014$) (Fig. 5). Finally, we observed significant correlations between rs8099917 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, -2.704 ± 1.277; $P = 0.034$), the proportion of Caucasian patients (slope, 1.168 ± 0.422; $P = 0.006$), and the proportion of Asian patients (slope, -1.049 ± 0.398; $P = 0.008$). When restricted to studies in which only treatment-naïve patients were included, two studies (495 patients) according to rs12979860 polymorphism and four studies (812 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2 = 0\%$, $P = 0.823$) and rs8099917 ($I^2 = 41\%$, $P = 0.166$). For rs12979860, fixed-effect model analyses showed that rs12979860 genotype CC was significantly associated with a higher possibility of severe steatosis (OR, 1.708; 95%CI, 1.047–2.787; $P = 0.032$) (Fig. 4-c), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.675; 95%CI, 0.474–0.960; $P = 0.026$) (Fig. 4-d). Egger's test showed no evidence of publication bias in rs8099917 ($P = 0.554$). For rs12979860, Egger's test was not applicable because only 2 studies were included.

Discussion

In the present study, we evaluated the association between IL28B polymorphisms and the background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC. The rs12979860 CC genotype was significantly associated with a higher probability of severe fibrosis (Fig. 2-c), and the rs8099917 TT genotype tended to be associated with a higher possibility of severe fibrosis (Fig. 2-d). The accumulation of liver inflammation promotes liver fibrosis, and these polymorphisms are associated with the effect of IFN-based treatment; therefore, past treatment might alter the results. Thus, we also analyzed studies involving only patients without a history of IFN-based treatment; however, the results were not changed.

The rs12979860 CC and rs8099917 TT genotypes were also associated with a higher possibility of severe inflammation activity. Genetic variations near the IL28B gene were originally reported as