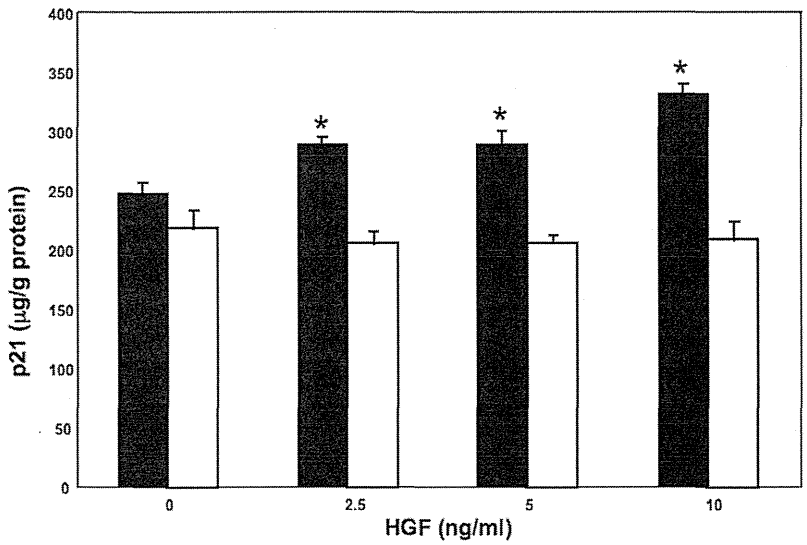

Figure 4. Cellular p21 levels in hepatocytes treated with HGF. Rat hepatocytes were cultured in WE containing 10% FCS and various concentration of HGF for 18 hours. Closed bars denote hepatocytes cultured at high density. Open bars denote hepatocytes cultured at low density. Data are mean + SEM of four dishes. *$p<0.05$ compared with the values in the absence of HGF.
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pifithrin-α, a chemical inhibitor of p53, when compared with the addition of vehicles ($p<0.01$) (Figure 6).

In contrast, the levels of p21 in hepatocytes at high density treated without HGF for 18 hours, the values of p21 protein were 211.5±24.9 μg/g protein (mean±standard error), and did not show any significant changes by the addition of pifithrin-α when compared with the addition of vehicles (202.2±57.4 μg/g protein). In addition, the levels of p21 in hepatocytes cultured at low density treated with 10 ng/mL HGF for 18 hours were not affected by pifithrin-α treatment (121.7±18.1 μg/g protein vs137.6±21.8 μg/g protein).

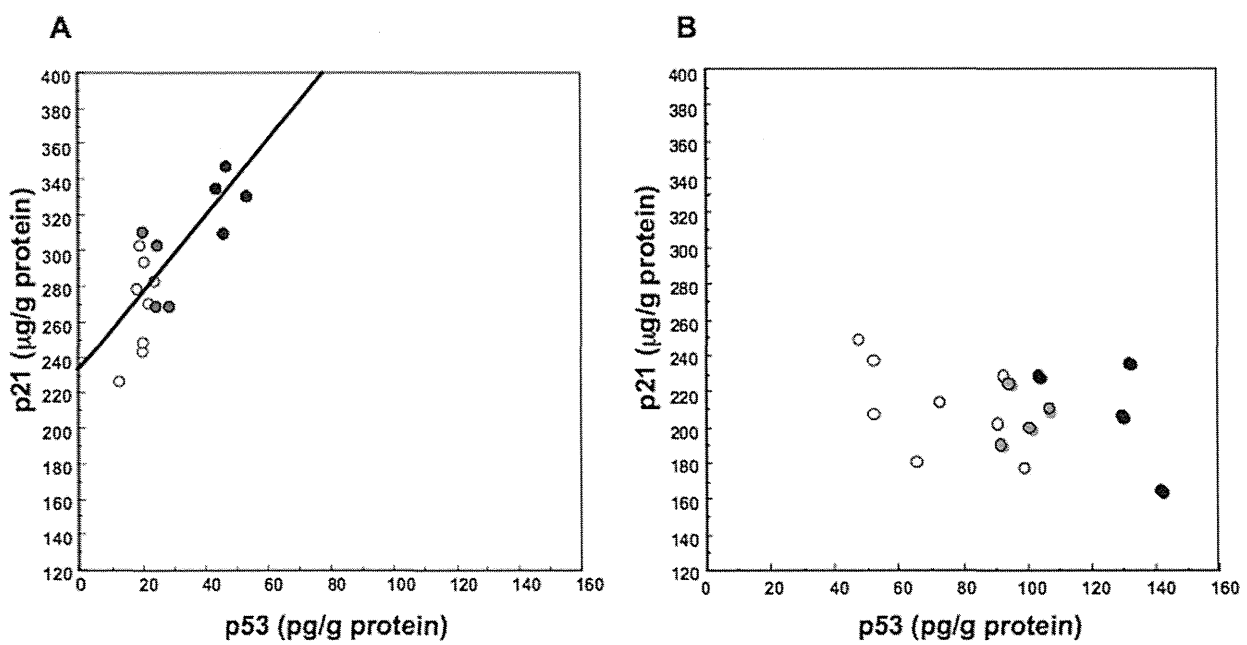

Figure 5. Cellular p53 and p21 levels in hepatocytes treated with HGF. Rat hepatocytes were cultured in WE containing 10% FCS and various concentrations of HGF for 18 hours. Open circles denote hepatocytes cultured in the absence of HGF. Dotted open circles denote hepatocytes cultured with 2.5 ng/mL HGF. Dotted closed circles denote hepatocytes cultured with 5 ng/mL HGF. Closed circles denote hepatocytes cultured with 10 ng/mL HGF. (A) Hepatocytes cultured at high density. (B) Hepatocytes cultured at low density.
doi:10.1371/journal.pone.0078346.g005

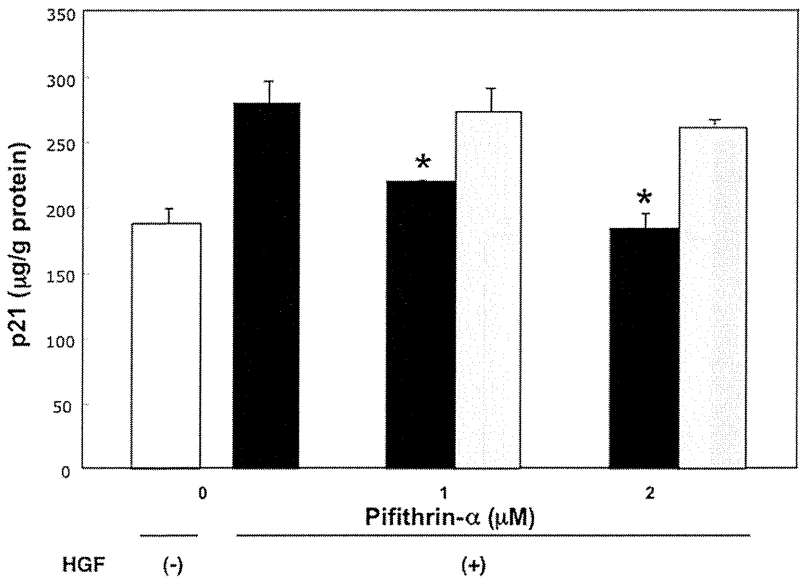

Figure 6. The effect of pifithrin-α on p21 levels of hepatocytes in the presence of HGF. Rat hepatocytes were cultured at high density in WE containing 10% FCS, 10 ng/mL HGF, along with various concentrations of pifithrin-α, the chemical inhibitor of p53, dissolved in DMSO, or DMSO of the same concentration for 18 hours. An open bar denotes hepatocytes cultured in the absence of HGF. Closed bars denote hepatocytes cultured with pifithrin-α in the presence of 10 ng/mL HGF. Dotted bars denote hepatocytes cultured with DMSO in the presence of 10 ng/mL HGF. Data are mean ± SEM of four dishes. *$p<0.05$ compared with the values treated only with HGF or values treated with HGF and DMSO.
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The levels of BrdU incorporation treated with 10 ng/mL of HGF for 24 hours were increased significantly by the addition of 2 μM of pifithrin-α, when compared with the addition of vehicles ($p<0.01$) (Figure 7). The total cellular protein levels were not affected by the addition of either pifithrin-α or vehicles (data not shown).

p21 levels were reduced and DNA synthesis was significantly increased by pifithrin-α a chemical inhibitor of p53, in the presence of HGF in high density cultured hepatocytes.

The effect of p21 antisense oligonucleotides on BrdU incorporation in rat hepatocytes cultured at high density in the presence of HGF

We investigated the effect of suppression of p21 expression on DNA synthesis in non-proliferationg hepatocytes in the presence of HGF. BrdU incorporation in hepatocytes cultured at high density in the presence of 10 ng/mL of HGF was significantly increased after a 24-hour exposure to p21 antisense oligonucleotide, when compared with that of hepatocytes treated with the nonsense oligonucleotide ($p<0.01$) (Figure 8). The total cellular protein levels were not affected by the addition of either oligonucleotide (data not shown). Suppression of p21 expression increased the DNA synthesis in the presence of HGF in high density cultured hepatocytes.

p21 levels in the liver after two-thirds PH or sham operation in rats

To study p21 expression profile in regenerating and quiescent rat liver, we determined hepatic p21 levels in rats after PH and sham operation. Hepatic p21 levels were increased to the maximal levels at 4 hours, and decreased to the basal levels at 8 hours after PH with minor increase at 48 hours. In contrast, hepatic p21 levels of sham-operated rats were increased up to 2.5-fold higher than preoperative levels at 12 to 48 hours and decreased to the

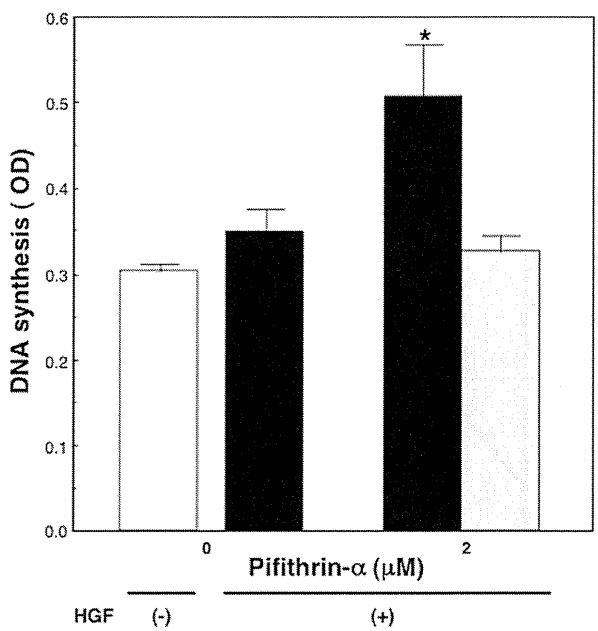

Figure 7. The effect of pifithrin-α on DNA synthesis of hepatocytes in the presence of HGF. Rat hepatocytes were cultured at high density in the same medium as described in the legend of Figure 6, except that the medium contained 1 mmol/L BrdU, for 24 hours. An open bar denotes hepatocytes cultured in the absence of HGF. Closed bars denote hepatocytes cultured with pifithrin-α in the presence of 10 ng/mL HGF. A dotted bar denotes hepatocytes cultured with DMSO in the presence of 10 ng/mL HGF. Data are mean ± SEM of eight dishes. *$p<0.01$ compared with the values treated only with HGF or values treated with HGF and DMSO.
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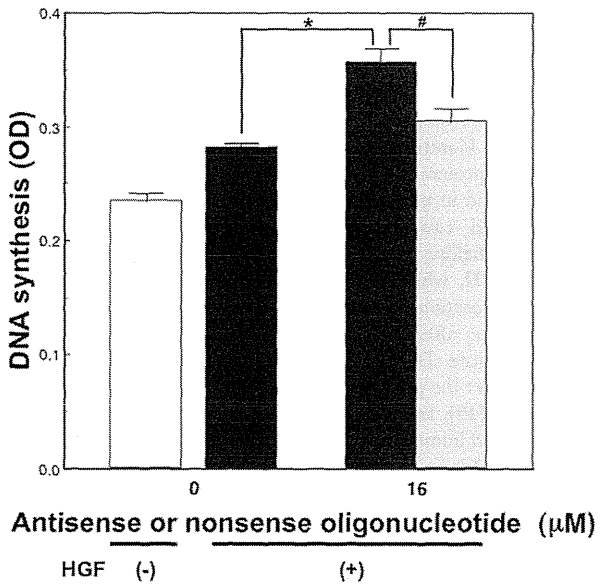

Figure 8. The effect of p21 antisense on DNA synthesis of hepatocytes in the presence of HGF. Rat hepatocytes were cultured at high density in WE containing 10% FCS, 10 ng/mL HGF, 1 mmol/L BrdU, along with various concentrations of either p21 antisense or nonsense oligonucleotide for 24 hours. An open bar denotes hepatocytes cultured in the absence of HGF. Closed bars denote hepatocytes cultured with p21 antisense oligonucleotide in the presence of 10 ng/mL HGF. A dotted bar denotes hepatocytes cultured with nonsense oligonucleotide in the presence of 10 ng/mL HGF. Data are mean ± SEM of eight dishes. *p<0.05 compared with the values treated only with HGF, and #p<0.01 compared with the values treated with HGF and nonsense oligonucleotide.
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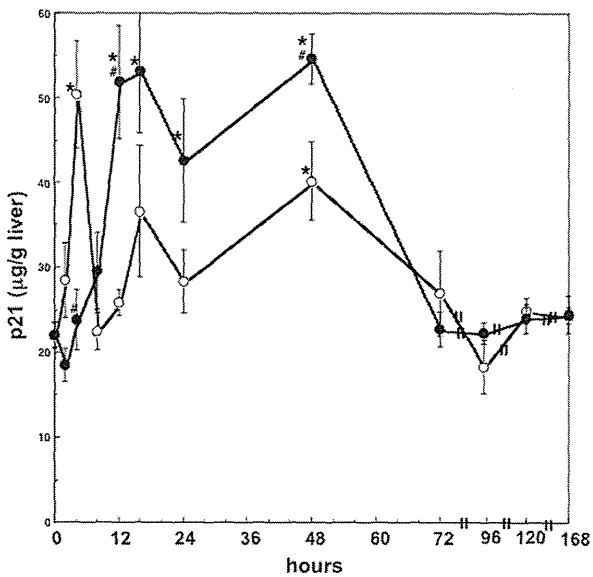

Figure 9. Changes in hepatic p21 levels after two thirds partial hepatectomy in rats. Data are mean ± SEM of four rats. Open and closed circles indicate the hepatic p21 levels in partially hepatectomized rats and in sham-operated rats, respectively. *p<0.05 compared with the values at 0 hour, and #p<0.05 compared with the values of partially hepatectomized rats.
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preoperative level at 72 hours. Hepatic p21 levels were significantly higher in sham-operated rats than in rats after PH except within 4 hours after surgery (Figure 9). In sham-operated rats, hepatic p21 levels were increased on sustained time scales while only transient elevation was observed in partial hepatectomized rats.

Discussion

We confirmed that DNA synthesis was not induced significantly in hepatocytes cultured at high density even in the presence of HGF, as we previously reported [2]. HGF seems to increase p53 and p21, and maintain mitotically quiescent in the hepatocytes.

We determined p21 protein levels *in vitro* and *in vivo* using newly developed ELISA system. Although p21 expression has been extensively studied in proliferating and non-proliferating hepatocytes and in the liver with or without regenerative stimuli [33–36], the results are inconsistent. In most of these reports, p21 mRNA levels were studied, or p21 protein levels were determined by Western blotting. When p21 synthesis is induced, p21 mRNA levels are generally up-regulated. Furthermore, p21 synthesis is further increased at the post-transcriptional level in hepatocytes [37]. Thus, in this study, we determined p21 protein expressions which may function as transcription factor. In addition, to avoid the unstable results, we determined p21 protein levels utilizing a quantitative method which was sensitive enough to detect p21 during observation periods *in vitro* and *in vivo*.

We suppressed p53 activity and p21 expression using pifithrin-α and a p21 antisense oligonucleotide, respectively. Pifithrin-α disturbs the nuclear transport of p53 leading to the inhibition of the function of p53 *in vitro* and *in vivo* [31,38]. We used pifithrin-α at the same concentration as previously reported in hepatocytes [14,38]. The efficacy of the p21 antisense oligonucleotide was also previously reported in other cell culture systems [32].

Previously, we reported that the addition of HGF to the medium increased p53 contents in hepatocytes cultured at low density followed by the increase of DNA synthesis by hepatocytes [14]. In this present paper, we showed that the p53 levels were also increased by HGF treatment in hepatocytes cultured at high density which did not show apparent burst of DNA synthesis. The mechanisms responsible for the increase of p53 in hepatocytes by HGF are still undefined. However, it has been reported that activation of mitogen activated protein (MAP) kinase influences transcription factors including p53 [39]. Considering that MAP kinase is thought to mediate the intracellular effects of HGF [40], HGF might increase p53 through the interaction of MAP kinase and p53. Recently, the relationship between growth factors and p53 has been shown in a couple of reports. HGF was shown to increase p53 expression in a rat epithelial cell line, and insulin-like growth factor I was reported to induce p53 expression in cardiac muscle cells [41,42]. In primary cultured rat hepatocytes, epidermal growth factor (EGF) was shown to induce p53 expression in a phosphatidylinositol-3 kinase-dependent way [36]. In addition, p53 null hepatocytes were reported to be refractory to the stimulation of EGF [43].

p21 is known to be induced by p53-dependent and -independent mechanisms according to the cell types and situations [18]. Following DNA damage, p53 appears to be necessary for p21 induction in various kinds of cell types [18]. Many experiments showed that p21 was the major effector of p53 in inducing growth arrest in malignant cells [17,18]. Furthermore,

p21 was reported to be induced by p53 and negatively regulate cell proliferation in normal fibroblasts without DNA damage [44]. In addition, expression of the p53-induced p21 was greatly diminished by targeting p53 with anti-p53 antibody, and the cells reentered S-phase in fibroblasts [45]. We showed that cellular levels of p21 correlated with those of p53 and suppression of p53 activity by pifithrin-α resulted in the decrease of p21 levels followed by an increase of DNA synthesis in hepatocytes cultured at high density. p21 seems to be induced by p53 dependent mechanism in the present culture system of hepatocytes leading to suppression of proliferation.

Previous several reports have shown that growth factors can induce p21 production and suppress cell proliferation. Transient induction of p21 mRNA following stimulation of growth factors such as EGF, platelet-derived growth factor and fibroblast growth factor is reported in several cell lines, leading to cell cycle arrest [18,26–28,46]. p21 was up-regulated by HGF addition, and mediated growth inhibition in a hepatoma cell-line [23–25]. In primary cultured rat and mouse hepatocytes, p21 is reported to be induced by EGF and to have a role in the blockage of hepatocyte replication of the second round but not of the first round [33]. In addition, Wierod et.al reported that EGF induced p21 through the activation of p53 in primary cultured rat hepatocytes [36]. However, they noted that EGF-induced p21 might positively regulate DNA synthesis, since stimulatory effect of EGF on DNA synthesis was abrogated when p53 was inhibited, and rescued by ectopical p21 addition [36]. It was reported that the role of p21 might differ by its concentrations [47]. To examine the role of intrinsic p21 in regulating DNA synthesis of hepatocytes, the effect of p21 inhibition in the presence of EGF should be studied. In our culture system using primary cultured rat hepatocytes and HGF, we showed that suppression of HGF-induced increase of p21 production positively regulated DNA synthesis by hepatocytes cultured at high density, suggesting that up-regulation of p21 maintained mitotically quiescent in hepatocytes in the presence of HGF. No apparent change of p21 expression was caused by pifithrin-α in hepatocytes cultured without HGF treatment, suggesting the effect of p53 inhibition on baseline p21 seemed to be minimal. In the high density cultured hepatocytes, protein production such as albumin is increased by HGF addition [2,11]. In contrast, in hepatocytes cultured at low density, we previously reported that HGF induced TGF-α production through the induction of p53, and hepatocyte proliferation occurred [14]. It is possible to speculate that induction of diverse effector genes of p53 plays a role in the expression of different activities of HGF. Further investigations would be required to clarify the mechanism(s) of the selective expression of p53 related genes in hepatocytes in different conditions stimulated by HGF.

Previous experiments in rats showed that hepatic levels of p21 protein after PH determined by Western blot analysis were increased at two time points, first immediately after resection and second after the peak of DNA synthesis. In sham operated rats, the p21 levels were almost undetectable throughout the 7-day time course [34]. Controversially, some reports demonstrated that hepatic p21 protein levels determined by Western blot analysis were decreased in partially hepatectomized rats and increased in sham-operated rats. The maximum decrease was observed immediately before the peak DNA synthesis, with increased p53 levels after PH, while the levels began to increase immediately after sham operation, and continued up to 48 hours of observation [48]. In mice after PH, p21 expression was reported to be increased before DNA synthesis of hepatocytes and reached maximum after the peak of hepatocyte DNA synthesis on Western blot analysis [49]. In the present study, hepatic p21 protein levels were increased immediately after PH, but returned to preoperative levels within 8 hours, while a sustained increase of p21 levels was observed after sham operation up to 48 hours. The reason for the discrepancy of the data is unclear. The difference of the assay system might influence the observations. Previously, we reported that hepatic p53 levels as well as hepatic and circulating HGF levels were increased after sham operation without the increase of hepatocyte proliferation [12,14], while, in rats after PH, hepatic p53 levels were increased and reached maximal levels, when hepatic HGF levels have been shown to reach maximum prior to an increase in hepatocyte proliferation [13,14]. These observations raise the possibility that the increase of p21 is related to keep quiescent when hepatocyte proliferation is not physiologically required even though HGF levels are increased. Previous studies described that c-Jun controlled hepatocyte proliferation by a p53/p21-dependent mechanism in mice [50]. p21 knock out mice demonstrated markedly accelerated hepatocyte proliferation compared to cogenic wild-type mice after PH [49]. These reports support our hypothesis. Significance of the transient increase of hepatic p21 levels after PH is still to be elucidated. The role of p21 may change depending on its concentration; p21 promotes cyclin/cdk complex assembly at low concentration, whereas, at higher concentrations, p21 is inhibitory to cdk in human normal fibroblast and human osteosarcoma cell [47]. The levels and the timing of the increase might influence the activity of p21.

Author Contributions

Conceived and designed the experiments: YI TT. Performed the experiments: YI TT TN YT. Analyzed the data: YI TT NO. Contributed reagents/materials/analysis tools: TT KK. Wrote the paper: YI TT HI.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Perihepatic lymph node enlargement is a negative predictor of liver cancer development in chronic hepatitis C patients

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Abstract
Background Perihepatic lymph node enlargement (PLNE) is a common ultrasound finding in chronic hepatitis C patients. Although PLNE is considered to reflect the inflammatory response to hepatitis C virus (HCV), its clinical significance remains unclear.
Methods Between December 2004 and June 2005, we enrolled 846 chronic hepatitis C patients in whom adequate ultrasound examinations had been performed. PLNE was defined as a perihepatic lymph node that was at least 1 cm in the longest axis by ultrasonography. We analyzed the clinical features of patients with PLNE and prospectively investigated the association between PLNE and hepatocellular carcinoma (HCC) development.
Results We detected PLNE in 169 (20.0 %) patients. Female sex, lower body mass index (BMI), and HCV serotype 1 were independently associated with the presence of PLNE. However, there were no significant differences in liver function tests, liver stiffness, and hepatitis C viral loads between patients with and without PLNE. During the follow-up period (mean 4.8 years), HCC developed in 121 patients. Unexpectedly, patients with PLNE revealed a significantly lower risk of HCC development than those without PLNE ($p = 0.019$, log rank test). Multivariate analysis revealed that the presence of PLNE was an independent negative predictor of HCC development (hazard ratio 0.551, $p = 0.042$). In addition, the sustained viral response rate in patients who received interferon (IFN) therapy was significantly lower in patients with PLNE than in patients without PLNE.
Conclusions Patients with PLNE had a lower risk of HCC development than those without PLNE. This study may provide new insights into daily clinical practice and the pathophysiology of HCV-induced hepatitis and hepatocarcinogenesis.

Keywords Perihepatic lymph node enlargement · Chronic hepatitis C · Hepatocarcinogenesis

Abbreviations

ALT	Alanine aminotransferase
AFP	α-Fetoprotein
PLNE	Perihepatic lymph node enlargement
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
IFN	Interferon
LN	Lymph node
US	Ultrasonography

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and chronic hepatitis C virus (HCV)

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infection is a major cause of HCC in the United States, southern European countries, and Japan [1, 2]. The host immune responses to HCV are often not strong enough to completely clear the infection, resulting in chronic stimulation of the antigen-specific immune response. Accumulating basic and clinical lines of evidence indicate that a sustained inflammatory reaction in the liver is the major contributing factor to HCC development [3–5].

Inflammatory processes in organs frequently lead to hyperplasia of regional lymph nodes (LNs). Perihepatic LN enlargement (PLNE) is a common finding in patients with chronic hepatitis, especially in patients with hepatitis C [6, 7]. Some studies have revealed that PLNE in chronic hepatitis C patients was associated with a higher HCV viral load, [7] higher histological grade of hepatic inflammation and fibrosis [8, 9], and higher CD8 lymphocyte level in the blood [10]; therefore, PLNE is considered to reflect an inflammatory response to HCV. However, such associations as those noted above are inconsistent among studies [7–15], and the precise mechanism and clinical relevance of PLNE are not fully understood. Furthermore, to our knowledge, there have been no studies designed to investigate the association between PLNE and hepatocarcinogenesis. To clarify whether we should pay attention to the risk of HCC development in patients with PLNE is very important, because we encounter such situations very often.

The purpose of this study was to reevaluate the clinical relevance of PLNE and to carry out a prospective assessment of the association between PLNE and HCC development. To elucidate these matters, we investigated a well-characterized chronic hepatitis C cohort in which we previously reported the utility of performing transient elastography for risk assessment of HCC development [16]. In the present study, we prospectively assessed the association between PLNE and HCC development in chronic hepatitis C patients in that cohort.

Patients and methods

Patients and screening for perihepatic LN enlargement

As described previously [16], we enrolled 866 chronic hepatitis C patients, excluding those with HCC or a past history of it, who visited the University of Tokyo Hospital between December 2004 and June 2005. All patients were positive for HCV-RNA and showed at least a transiently elevated serum alanine aminotransferase (ALT) level. Patients with concomitant hepatitis B virus surface antigen positivity, patients with uncontrollable ascites, patients on interferon (IFN) therapy, and patients who visited only for consultation purposes were excluded from this study.

Each subject was screened for HCC with ultrasonography (US) at or immediately after the first visit. At the same time, we surveyed the presence of PLNE with US. The US examination was performed using the SSD-2000 (Aloka, Tokyo, Japan). To identify LNs the following criteria were used, according to a previous report [17]: one or more masses with an ovoid shape and less echogenic than liver parenchyma, separated from adjacent organs and vessels by a clear-cut cleavage on repeated transverse, sagittal, and oblique scans. LNs were searched for near the trunk of the portal vein, hepatic artery, celiac axis, superior mesenteric vein, and pancreas head. Furthermore, we used Doppler US to differentiate LNs from vessels.

We defined PLNE as an LN that was at least 1 cm in the longest axis. There were two reasons for this definition. First, we preliminarily investigated the prevalence of PLNE with US in 465 healthy subjects who had had medical check-ups and did not have liver disease or other underlying causes of LN enlargement. We found that only 15 (3.2 %) of these subjects had a perihepatic LN larger than 1 cm in the longest axis (unpublished data). Second, when an LN was smaller than 1 cm, it was sometimes difficult to distinguish the LN from other structures. If two or more LNs were detected with US, we determined PLNE to be present based on the length of the largest LN. In 20 of the 866 patients in the present study, adequate visualization of the liver hilum was not achieved with US because of severe obesity or excessive meteorism. Therefore, we analyzed the association between PLNE and the subsequent incidence of HCC in 846 patients.

HCV RNA was measured using Amplicore HCV version 2.0 (Roche, Tokyo, Japan), and the HCV serotype was examined using a serotyping assay (SRL, Tokyo, Japan). We also monitored IFN therapy and responses during the follow-up period. A sustained virological response (SVR) was defined as undetectable HCV-RNA at least 24 weeks after the end of therapy. All blood tests were performed at the time of US examination.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Patient follow up

Patients were followed up every 3–6 months at the outpatient clinic, when blood tests (including tumor markers) and US were carried out. Contrast-enhanced computed tomography (CT) was performed when HCC was suspected, based on US, and/or if the serum α-fetoprotein (AFP) level showed an abnormal increase. HCC was diagnosed by dynamic CT, and hyperattenuation in the arterial phase with washout in the late phase was considered a definite sign of HCC [18]. When a diagnosis of HCC was ambiguous, ultrasound-guided tumor biopsy was

performed and a pathologic diagnosis was made based on the Edmondson and Steiner criteria. Time to HCC occurrence was defined as the interval between the date of the first US screening and the diagnosis of HCC. Patients were censored at the time of death without HCC development, the last visit when lost to follow up, or the end of the study period. The last observation in this study was made on December 31, 2010. Thus, the time of observation was extended from that of our previous study, which was censored on May 31, 2008 [16].

Transient elastography

Transient elastography was performed using Fibroscan (Echosens, Paris, France) as described previously [16].

Statistical analysis

Data were expressed as means ± standard deviation (SD) unless otherwise indicated. Categorical variables were compared by χ^2tests, whereas continuous variables were compared by the unpaired Student's *t*-test (parametric) or the Mann–Whitney *U*-test (non-parametric). Multivariate logistic regression analysis was used to identify factors that were independently associated with the presence of PLNE. Cumulative HCC incidence was estimated using the Kaplan–Meier method, and the difference between groups was assessed with the log-rank test. In the analysis of risk factors for hepatocarcinogenesis, we tested the following variables in univariate analysis and multivariate Cox proportional hazard regression analysis: age, sex, platelet count, serum albumin concentration, total bilirubin concentration, ALT and aspartate aminotransferase (AST) levels, higher AFP concentration (>10 ng/ml), prothrombin activity, heavy alcohol drinking (alcohol intake >80 g/day), BMI, higher liver stiffness measurement (LSM) (>10 kPa), HCV serotype, HCV viral load (>100 kIU/ml), IFN treatment, achievement of SVR, and presence of PLNE. A *p* value of less than 0.05 on a two-tailed test was considered significant. Data processing and analysis were performed using StatView (ver. 5.0; SAS Institute, Cary, NC, USA) and SPSS (ver. 14.0; SPSS, Chicago, IL, USA) software.

Results

Patient profiles

We detected PLNE in 169 of 846 (20.0 %) patients with chronic hepatitis C. A representative ultrasound image is shown in Fig. 1. The mean (±SD) length of the longest axis was 1.7 (±0.5) cm (range 1.0–3.5 cm). The clinical features of patients with and without PLNE are summarized in Table 1. The proportion of females was significantly higher in the PLNE-positive group than in the PLNE-negative group (63.3 vs. 52.9 %), and BMI was slightly but significantly lower in the PLNE-positive group than in the PLNE-negative group (21.9 ± 2.6 vs. 22.5 ± 2.9). The proportion of HCV serotype 1 patients was higher in the PLNE-positive group than in the PLNE-negative group, with borderline significance. There was a tendency of a higher serum ALT level in the PLNE-positive group, but the difference was without statistical significance. There were no significant differences in other liver function test results, or in liver stiffness and hepatitis C viral load between the two groups. Multivariate logistic regression analysis using the factors of sex, serum ALT, BMI, and HCV serotype revealed that female sex, lower BMI, and HCV serotype 1 were independently associated with the presence of PLNE (Table 2).

Incidence of HCC

The mean follow-up period was 4.8 years, constituting a total observation of 4,021 person-years. During the observation period, 70 (8.3 %) patients were lost to follow up: 15 (8.8 %) patients in the PLNE-positive group and 55 (8.1 %) patients in the PLNE-negative group. There were no patients in whom an enlarged perihepatic LN turned out to be caused by other underlying diseases including metastasis of HCC. The SVR rate in patients who received IFN therapy during the follow-up period was significantly lower in the PLNE-positive group compared with that in the PLNE-negative group [7/34 (20.6 %) vs. 93/172

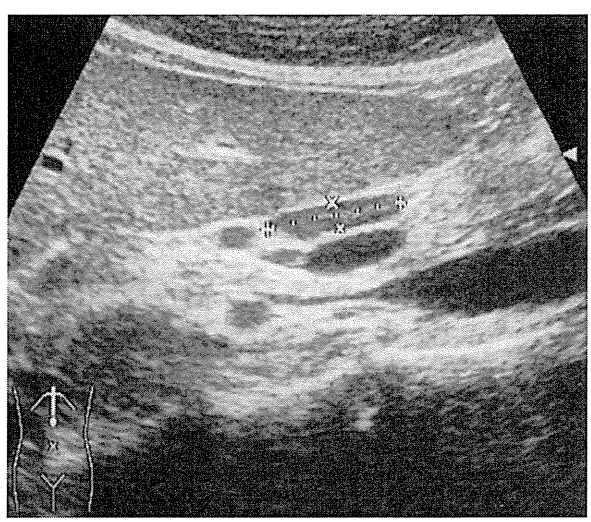

Fig. 1 Representative ultrasound image of enlarged perihepatic lymph node (LN) in a patient with chronic hepatitis C

Table 1 Clinical features of patients with and without PLNE

Variable	PLNE-positive group ($n = 169$)	PLNE-negative group ($n = 677$)	p value
Age (years)	62.4 ± 10.1 (29–83)	62.4 ± 11.5 (17–89)	0.58
Male, n (%)	62 (36.7)	319 (47.1)	0.018
Serum albumin (g/dl)	4.0 ± 0.4 (2.8–4.8)	4.0 ± 0.4 (2.5–5.0)	0.93
Total bilirubin (mg/dl)	0.8 ± 0.3 (0.3–2.1)	0.9 ± 0.5 (0.3–4.6)	0.23
AST (IU/l)	52 ± 34.1 (17–223)	50 ± 33.8 (9–286)	0.16
ALT (IU/l)	57 ± 48.7 (4–374)	53 ± 45.2 (2–503)	0.10
Platelet count ($\times 10^4/\mu l$)	16.1 ± 6.6 (2.1–42.2)	16.1 ± 6.7 (3.2–43.6)	0.89
Prothrombin time (%)	86.0 ± 12.1 (50.3–100.0)	85.7 ± 12.4 (38.9–100.0)	0.88
AFP (ng/ml)	22.0 ± 67.9 (1–592)	13.4 ± 37.1 (1–563)	0.61
BMI (kg/m^2)	21.9 ± 2.6 (14.4–28.7)	22.5 ± 2.9 (15.1–29.8)	0.007
Liver stiffness (kPa)	10.9 ± 7.8 (2.8–42.2)	12.0 ± 10.0 (2.5–75.0)	0.59
Alcohol consumption >80 g/day, n (%)	6 (3.6)	25 (3.7)	0.82
HCV viral load (kIU/ml)	549 ± 646 (5–5000)	651 ± 842 (5–5000)	0.48
HCV serotype 1, n (%)	146 (86.3)	538 (79.5)	0.053
Patients who received IFN, n (%)	34 (20.1)	172 (25.4)	0.18
Patients who achieved SVR, n (%)	7 (4.1)	93 (13.7)	0.0009

PLNE perihepatic lymph node enlargement, *AST* aspartate aminotransferase. *ALT* alanine aminotransferase, *AFP* α-fetoprotein, *BMI* body mass index, *HCV* hepatitis C virus

Table 2 Factors associated with the presence of PLNE: multivariate analysis

Variable	Odds ratio (95 % confidence interval [CI])	p value
Male sex	0.667 (0.464–0.936)	0.024
ALT level (per 1 IU/l)	1.003 (0.999–1.006)	0.10
BMI (per 1 kg/m^2)	0.919 (0.864–0.978)	0.017
HCV serotype 1	1.64 (1.02–2.66)	0.043

(54.1 %), $p = 0.0005$]. This finding was consistent with previous reports [12, 19].

By the end of the follow-up period, HCC had developed in 121 patients (3.0 % per 1 person-year). The cumulative incidence rates of HCC at 3 and 5 years estimated by the Kaplan–Meier method were 8.9 and 13.7 %, respectively. We then assessed the incidence of HCC stratified by the presence of PLNE. Unexpectedly, the PLNE-positive group revealed a significantly lower incidence of HCC than the PLNE-negative group ($p = 0.019$, log-rank test) (Fig. 2). The cumulative incidence rates at 3 and 5 years were 3.6 and 8.2 %, respectively, in the PLNE-positive group, and 10.1 and 15.1 % in the PLNE-negative group. These results indicate that patients with PLNE have a lower risk of HCC development despite having a lower SVR rate with IFN therapy.

Risk analyses

We analyzed the risk factors for HCC development. In the univariate analyses, older age, male sex, lower serum albumin concentration, higher total bilirubin concentration, higher AST level, higher ALT level, lower prothrombin activity, lower platelet count, heavy alcohol drinking, higher BMI, LSM greater than 10 kPa, AFP level greater than 10 ng/ml, HCV serotype 1, not receiving IFN, not achieving SVR, and absence of PLNE were significant risk factors for HCC (Table 3). As we had reported previously, a higher LSM (i.e., greater than 10 kPa) was a strong predictor of HCC development [hazard ratio (HR) 15.4, 95 % confidence interval (CI) 8.6–27.0, $p < 0.0001$)]. Multivariate proportional hazard regression analyses

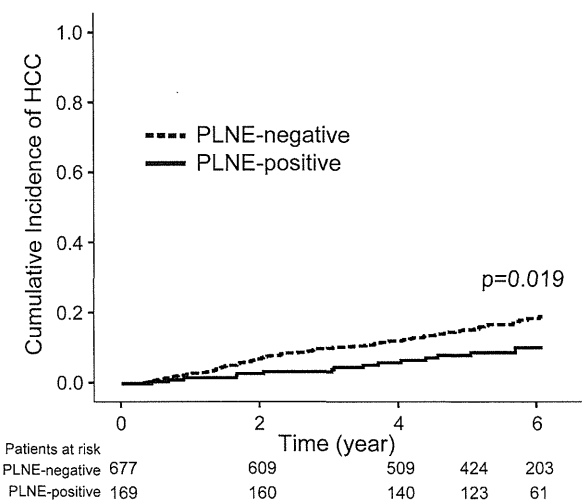

Fig. 2 Cumulative incidence of hepatocellular carcinoma (*HCC*) development stratified by the presence of perihepatic lymph node enlargement (*PLNE*)

Table 3 Risk factors for HCC development: univariate and multivariate analyses

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Age (per 1 year age)	1.07 (1.05–1.09)	<0.0001	1.04 (1.01–1.06)	0.002
Male sex	1.45 (1.02–2.08)	0.041	1.49 (1.02–2.17)	0.039
Platelet count (per 10^4/μl)	0.852 (0.823–0.882)	<0.0001	0.965 (0.926–1.005)	0.089
Total bilirubin (per 1 mg/dl)	1.88 (1.45–2.45)	<0.0001	0.825 (0.567–1.2)	0.32
Serum albumin level (per 1 g/dl)	0.12 (0.084–0.17)	<0.0001	0.441 (0.263–0.739)	0.002
AST level (per 1 IU/l)	1.01 (1.007–1.014)	<0.0001	1.002 (0.991–1.013)	0.71
ALT level (per 1 IU/l)	1.004 (1.002–1.007)	0.002	1.0 (0.991–1.013)	0.94
AFP level >10 ng/ml	6.76 (4.69–9.8)	<0.0001	1.9 (1.22–2.97)	0.005
Prothrombin time (per 1 %)	0.973 (0.966–0.979)	<0.0001	0.989 (0.976–1.001)	0.072
Alcohol consumption >80 g/day	2.73 (1.43–5.24)	0.002	3.53 (1.76–7.09)	0.0004
BMI (per 1 kg/m^2)	1.09 (1.03–1.16)	0.006	1.09 (1.01–1.17)	0.025
Liver stiffness >10 kPa	15.4 (8.6–27.0)	<0.0001	4.41 (2.24–8.7)	<0.0001
HCV serotype 1	1.76 (1.03–3.03)	0.04	1.36 (0.774–2.38)	0.29
HCV-RNA >100 kIU/ml	1.36 (0.87–2.13)	0.18	1.24 (0.781–1.97)	0.36
Patients treated with IFN	0.44 (0.262–0.75)	0.002	0.59 (0.315–1.11)	0.1
Patients with SVR	0.175 (0.055–0.549)	0.003	0.621 (0.169–2.28)	0.47
Presence of PLNE	0.53 (0.31–0.91)	0.02	0.551 (0.31–0.978)	0.042

HCC hepatocellular carcinoma, *IFN* interferon, *SVR* sustained viral response

revealed that older age, male sex, lower serum albumin concentration, AFP level greater than 10 ng/ml, heavy alcohol drinking, higher BMI, LSM greater than 10 kPa, and absence of PLNE were independent risk factors for HCC (Table 3). These results suggest that the presence of PLNE is an independent negative predictor of HCC development in chronic hepatitis C patients.

Subgroup analysis of non-obese patients

To further rule out the possibility that obesity acted as a confounder in the association between the presence of PLNE and HCC development, we reanalyzed the contribution of PLNE to HCC development in a subgroup of non-obese patients, defined as those with BMI <25 kg/m^2 ($n = 695$), because we could clearly visualize the liver hilum in such individuals. As shown in Fig. 3, the PLNE-positive group had a significantly lower incidence of HCC than the PLNE-negative group even in the non-obese subgroup ($p = 0.02$). Thus, we further confirmed that the presence of PLNE was negatively associated with HCC development independently of obesity.

Significance of the size of perihepatic LNs

To examine the significance of the size of perihepatic LNs, we divided patients with PLNE into two groups: a smaller LN group (longest axis of LN 1 cm to 2 cm, $n = 122$) and a larger LN group (longest axis of LN ≥2 cm, $n = 47$).

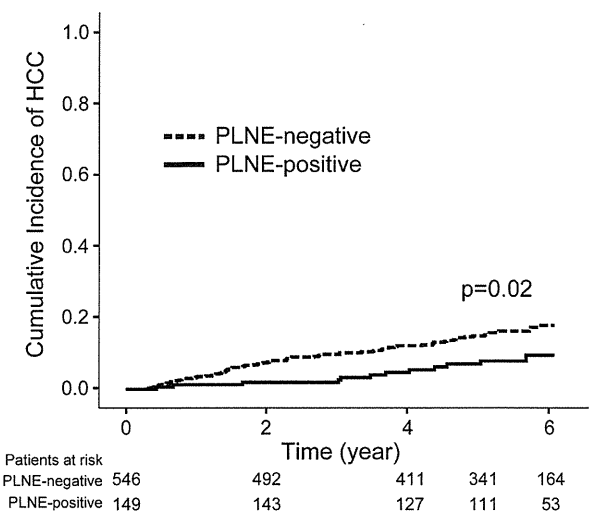

Fig. 3 Cumulative incidence of HCC development stratified by the presence of PLNE: subgroup analysis of non-obese patients (body mass index [BMI] <25 kg/m^2)

The characteristics of each group are summarized in Table 4. The proportion of male patients and the LSM value tended to be higher in the larger LN group than in the smaller LN group, but the difference was not statistically significant for either factor. There were no significant differences in other factors between the two groups. Furthermore, there was no significant difference in HCC incidence rates between the two groups (Fig. 4), although the larger LN group revealed a slightly higher incidence of

Table 4 Comparison of clinical features between patients with small perihepatic lymph nodes (LNs) and those with large perihepatic LNs

Variable	LN size 1 cm to <2 cm, (n = 122)	LN size ≥2 cm (n = 47)	p value
Age (years)	62.7 ± 10.4 (29–83)	61.5 ± 9.2 (32–77)	0.32
Male, n (%)	40 (32.8)	22 (46.8)	0.092
Serum albumin (g/dl)	4.0 ± 0.3 (3.0–4.8)	4.0 ± 0.4 (2.8–4.8)	0.96
Total bilirubin (mg/dl)	0.8 ± 0.3 (0.3–1.6)	0.8 ± 0.4 (0.3–2.1)	0.98
AST (IU/l)	53 ± 36.0 (17–223)	50 ± 28.7 (17–181)	0.83
ALT (IU/l)	56 ± 49.0 (4–374)	59 ± 48.2 (6–308)	0.48
Platelet count ($\times 10^4/\mu l$)	16.3 ± 6.2 (2.1–36.4)	15.4 ± 7.4 (4.8–42.2)	0.25
Prothrombin time (%)	86.7 ± 11.7 (57.4–100.0)	84.2 ± 13.0 (50.3–100.0)	0.27
AFP (ng/ml)	13.0 ± 27.7 (1–168)	45.3 ± 118.6 (1–592)	0.19
BMI (kg/m^2)	21.8 ± 2.6 (16.8–28.0)	22.0 ± 2.6 (14.4–28.7)	0.75
Liver stiffness (kPa)	10.2 ± 7.1 (2.8–37.4)	12.5 ± 9.3 (4.2–42.2)	0.064
Alcohol consumption >80 g/day, n (%)	5 (4.1)	1 (2.1)	0.54
HCV viral load (kIU/ml)	658 ± 788	504 ± 582	0.17
HCV serotype 1, n (%)	107 (87.7)	39 (83.0)	0.58
Patients who received IFN, n (%)	24 (19.6)	10 (21.2)	0.98
Patients who achieved SVR, n (%)	5 (4.1)	2 (4.3)	0.99

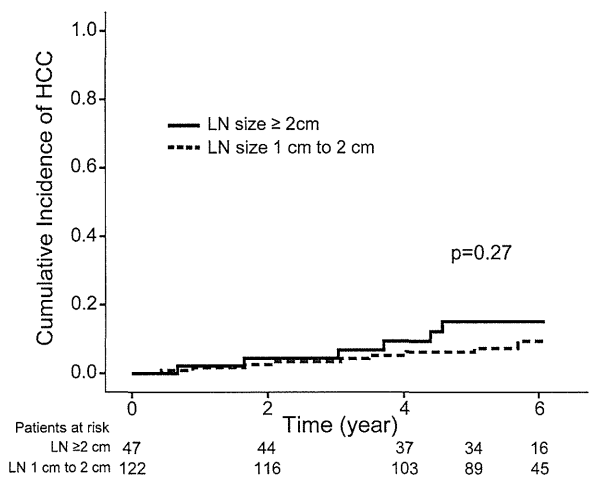

Fig. 4 Cumulative incidence of HCC development in patients with PLNE stratified by the size of perihepatic LNs: i.e., smaller (longest axis of LN 1 to <2 cm) and larger (longest axis of LN ≥2 cm)

HCC. These results suggest that the size of perihepatic LNs in chronic hepatitis C patients may not be clinically as important as the presence of PLNE itself.

Discussion

Although PLNE is a common finding in patients with chronic hepatitis C, its clinical significance has remained unclear. In the present study, we reevaluated the clinical relevance of PLNE in a large cohort of chronic hepatitis C patients. We found, by prospective analysis, that patients with PLNE had a lower risk of HCC development than those without PLNE. To our knowledge, this is the first study reporting a negative association between the presence of PLNE and HCC development.

Before we started this study, we expected that patients with PLNE would have a higher risk of HCC development, based on previous reports showing positive associations between PLNE and liver inflammation and fibrosis [8, 13–15]. However, in our study, neither inflammatory markers, such as serum AST and ALT levels, nor fibrosis markers, such as the platelet count and LSM, had statistically significant associations with the presence of PLNE. On the contrary, patients with PLNE revealed a significantly lower risk of HCC development. One possible explanation for this result is that obesity may affect the ability of US to detect perihepatic LNs, although patients with severe obesity were excluded from the study. To rule out the effect of confounders, especially obesity, we performed multivariate analysis and subgroup analysis of non-obese patients, and the results showed that the presence of PLNE was an independent negative predictor of HCC development. Additionally, of the 846 patients enrolled in this study, 175 patients underwent abdominal computed tomography (CT) within one year from the date of the US examination. The concordance rate for the diagnosis of PLNE between CT and US in these patients was 91.4 % (160/175). Therefore, we consider that the diagnostic accuracy of US for PLNE was acceptable in this study.

Although the mechanism of PLNE in patients with hepatitis C is still unknown, hyperplasia of regional LNs is generally considered to reflect inflammatory responses in the adjacent organs. The volume of perihepatic LNs has been reported to significantly decrease after antiviral

therapy, especially in patients with an SVR, supporting the hypothesis that PLNE reflects the inflammatory response to HCV [19–21]. In fact, PLNE was reported to be associated with CD8 lymphocyte counts in the peripheral blood [10]. Furthermore, HCV-specific IFN-γ production and proliferative responses of T cells were found most commonly in perihepatic LNs rather than in liver tissue or in the peripheral blood, indicating that there was ongoing T-cell activation in perihepatic LNs [22]. Our results, taken together with these previous reports, suggest that the presence of PLNE may reflect an adequate host immune response to HCV. T-cell immunity is very important in the control of HCV infection and in the prevention of hepatocarcinogenesis [23–25], and a T-cell response that is too weak may accelerate hepatocarcinogenesis, as seen in patients co-infected with HCV and human immunodeficiency virus [26, 27]. Thus, a weak T-cell response may be one explanation of the higher risk of HCC development in patients without PLNE. On the other hand, too strong an anti-HCV T-cell response may induce hepatocellular damage and lead to subsequent hepatocarcinogenesis [28], so patients with larger perihepatic LNs may have a slightly higher tendency to develop HCC. However, from the present type of observational study, we cannot evaluate a causal relationship between PLNE and hepatocarcinogenesis, so further studies are needed to clarify this point.

As mentioned above, several studies have shown that PLNE was positively associated with the degree of liver inflammation or fibrosis [8, 13–15], but, in the present study we could not find such associations, except for slight serum ALT elevation. However, because of ethical concerns regarding the performance of liver biopsy, we did not assess liver histology, so we cannot conclude whether or not PLNE is really associated with liver inflammation and fibrosis. Of note, the reported relationships of PLNE to liver function tests and liver inflammation and fibrosis are inconsistent among studies [7–15]. One reason may be that these findings were based on relatively small samples. Another reason is that there is a lack of established criteria for the diagnosis of PLNE. The lack of definite criteria may also contribute to the wide variation in the prevalence of PLNE among studies (from 20 to 100 %) [8–10, 21]. We defined PLNE as an LN that was at least 1 cm in the longest axis, and this definition was based on the report by Grier et al. [21] and our preliminary investigation in healthy subjects. Some studies have used more detailed measurements of LNs with calculations of node volume and shape [8, 10, 20]. These methods are certainly more accurate in terms of the assessment of nodal volume, but may be too complicated in the clinical setting, as discussed by Grier et al. [21]. We used a simpler method, because our study included a large number of patients and was conducted to examine the significance of PLNE in daily clinical practice. Admittedly, a more detailed method would be appropriate to elucidate more clearly the involvement of PLNE in the pathophysiology of hepatitis and hepatocarcinogenesis.

In the present study, female sex, lower BMI, and HCV serotype 1 were independently associated with the presence of PLNE. Soresi et al. [29] also reported that PLNE was observed significantly more often in female patients than in male patients with chronic hepatitis C. Although we cannot clarify the mechanism underlying this association, this finding may be interesting from the point of view of gender differences in immune systems and hepatocarcinogenesis. In the study by Soresi et al., BMI in patients with PLNE tended to be lower than that in patients without PLNE, although the difference was not statistically significant [29], and this finding may be in line with our present results. Regarding BMI in patients with chronic HCV infection, an anti-HCV specific immune response was reportedly associated with lower BMI through the expression of adiponectin, one of the major adipokines [30]. Thus, the active immune response to HCV in patients with lower BMI might cause PLNE. Recent studies have reported that obesity and obesity-induced dysregulation of adipokines play important roles in hepatocarcinogenesis [31–33], so the examination of adipokine expression may help to explain the relationship of PLNE to BMI and hepatocarcinogenesis.

Another important finding in our study was that the SVR rate in patients who received IFN therapy was significantly lower in patients with PLNE than in patients without PLNE. This finding is consistent with previous reports [12, 19]. Although the proportion of individuals with HCV serotype 1 was higher in our patients with PLNE than in patients without PLNE, subgroup analysis of the patients with HCV serotype 1 also revealed a significantly lower SVR rate in patients with PLNE than in patients without PLNE (data not shown). Therefore, further analyses are planned to clarify the relationship of PLNE to HCV serotype and response to IFN therapy.

In conclusion, the presence of PLNE is an independent negative predictor of HCC development in chronic hepatitis C patients. This study may provide new insights into daily clinical practice and the pathophysiology of HCV-induced hepatitis and hepatocarcinogenesis.

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Conflict of interest The authors have no conflicts of interest regarding this study.

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Cell

Identification of Liver Cancer Progenitors Whose Malignant Progression Depends on Autocrine IL-6 Signaling

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SUMMARY

Hepatocellular carcinoma (HCC) is a slowly developing malignancy postulated to evolve from premalignant lesions in chronically damaged livers. However, it was never established that premalignant lesions actually contain tumor progenitors that give rise to cancer. Here, we describe isolation and characterization of HCC progenitor cells (HcPCs) from different mouse HCC models. Unlike fully malignant HCC, HcPCs give rise to cancer only when introduced into a liver undergoing chronic damage and compensatory proliferation. Although HcPCs exhibit a similar transcriptomic profile to bipotential hepatobiliary progenitors, the latter do not give rise to tumors. Cells resembling HcPCs reside within dysplastic lesions that appear several months before HCC nodules. Unlike early hepatocarcinogenesis, which depends on paracrine IL-6 production by inflammatory cells, due to upregulation of LIN28 expression, HcPCs had acquired autocrine IL-6 signaling that stimulates their in vivo growth and malignant progression. This may be a general mechanism that drives other IL-6-producing malignancies.

INTRODUCTION

Every malignant tumor is probably derived from a single progenitor that had acquired growth and survival advantages through genetic and epigenetic changes, allowing clonal expansion (Nowell, 1976). Tumor progenitors are not necessarily identical to cancer stem cells (CSCs), which maintain and renew fully established malignancies (Nguyen et al., 2012). However, clonal evolution and selective pressure may cause some descendants of the initial progenitor to cross the bridge of no return and form a premalignant lesion. Cancer genome sequencing indicates that most cancers require at least five genetic changes to evolve (Wood et al., 2007). How these changes affect the properties of tumor progenitors and control their evolution into a CSC is not entirely clear, as it has been difficult to isolate and propagate cancer progenitors prior to detection of tumor masses. Given these difficulties, it is also not clear whether cancer progenitors are the precursors for the more malignant CSC isolated from fully established cancers. An answer to these critical questions depends on identification and isolation of cancer progenitors, which may also enable definition of molecular markers and signaling pathways suitable for early detection and treatment. This is especially important in cancers of the liver and pancreas, which evolve over the course of many years but, once detected, are extremely difficult to treat (El-Serag, 2011; Hruban et al., 2007).

Hepatocellular carcinoma (HCC), the most common liver cancer, is the end product of chronic liver diseases, requiring

several decades to evolve (El-Serag, 2011). Currently, HCC is the third most deadly and fifth most common cancer worldwide, and in the United States its incidence has doubled in the past two decades. Furthermore, 8% of the world's population are chronically infected with hepatitis B or C viruses (HBV and HCV) and are at a high risk of new HCC development (El-Serag, 2011). Up to 5% of HCV patients will develop HCC in their lifetime, and the yearly HCC incidence in patients with cirrhosis is 3%–5%. These tumors may arise from premalignant lesions, ranging from dysplastic foci to dysplastic hepatocyte nodules that are often seen in damaged and cirrhotic livers and are more proliferative than the surrounding parenchyma (Hytiroglou et al., 2007). However, the tumorigenic potential of these lesions was never examined, and it is unknown whether they contain any genetic alterations. Given that there is no effective treatment for HCC and, upon diagnosis, most patients with advanced disease have a remaining lifespan of 4–6 months, it is important to detect HCC early, while it is still amenable to surgical resection or chemotherapy. Premalignant lesions, called foci of altered hepatocytes (FAH), were also described in chemically induced HCC models (Pitot, 1990), but it was questioned whether these lesions harbor tumor progenitors or result from compensatory proliferation (Sell and Leffert, 2008). The aim of this study was to determine whether HCC progenitor cells (HcPCs) exist and if so, to isolate these cells and identify some of the signaling networks that are involved in their maintenance and progression.

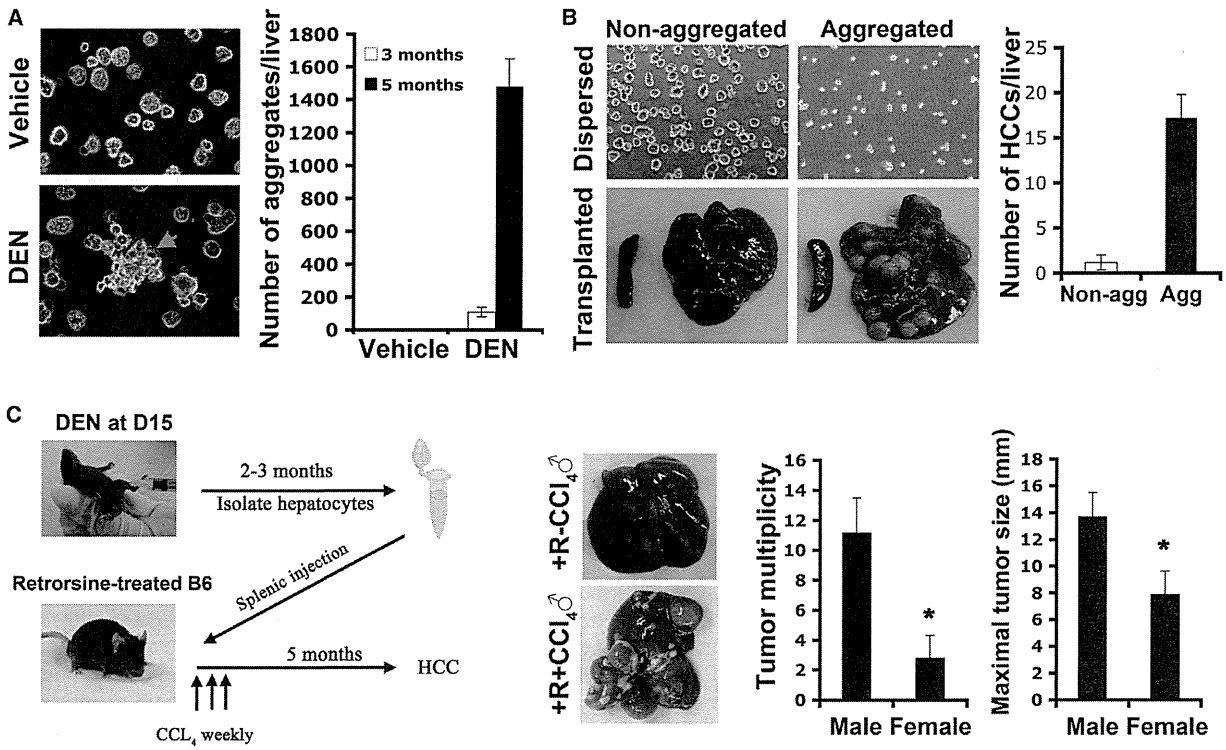
We now describe HcPC isolation from mice treated with the procarcinogen diethyl nitrosamine (DEN), which induces poorly differentiated HCC nodules within 8 to 9 months (Verna et al., 1996). Although these tumors do not evolve in the context of cirrhosis, the use of a chemical carcinogen is justified because the finding of up to 121 mutations per HCC genome suggests that carcinogens may be responsible for human HCC induction (Guichard et al., 2012). Furthermore, 20%–30% of HCC, especially in HBV-infected individuals, evolve in noncirrhotic livers (El-Serag, 2011). Nonetheless, we also isolated HcPCs from *Tak1*$^{\Delta hep}$ mice, which develop spontaneous HCC as a result of progressive liver damage, inflammation, and fibrosis caused by ablation of TAK1 (Inokuchi et al., 2010). Although the etiology of each model is distinct, both contain HcPCs that express marker genes and signaling pathways previously identified in human HCC stem cells (Marquardt and Thorgeirsson, 2010) long before visible tumors are detected. Furthermore, DEN-induced premalignant lesions and HcPCs exhibit autocrine IL-6 production that is critical for tumorigenic progression. Circulating IL-6 is a risk indicator in several human pathologies and is strongly correlated with adverse prognosis in HCC and cholangiocarcinoma (Porta et al., 2008; Soresi et al., 2006). IL-6 produced by in-vitro-induced CSCs was suggested to be important for their maintenance (Iliopoulos et al., 2009). Furthermore, autocrine IL-6 was detected in several cancers, but its origin is poorly understood (Grivennikov and Karin, 2008). In particular, little is known about the source of IL-6 in HCC. In early stages of hepatocarcinogenesis, IL-6 is produced by Kupffer cells or macrophages (Maeda et al., 2005; Naugler et al., 2007). However, paracrine IL-6 production is transient and does not explain its expression by HCC cells.

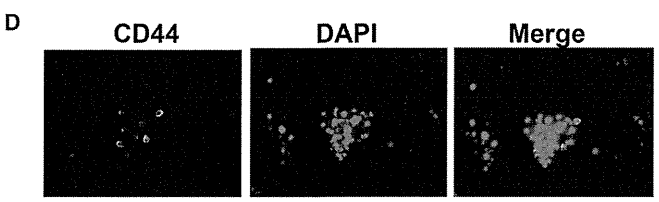
RESULTS

DEN-Induced Collagenase-Resistant Aggregates of HCC Progenitors

A single intraperitoneal (i.p.) injection of DEN into 15-day-old BL/6 mice induces HCC nodules first detected 8 to 9 months later. However, hepatocytes prepared from macroscopically normal livers 3 months after DEN administration already contain cells that progress to HCC when transplanted into the permissive liver environment of MUP-uPA mice (He et al., 2010), which express urokinase plasminogen activator (uPA) from a mouse liver-specific major urinary protein (MUP) promoter and undergo chronic liver damage and compensatory proliferation (Rhim et al., 1994). Collagenase digestion of DEN-treated livers generated a mixture of monodisperse hepatocytes and aggregates of tightly packed small hepatocytic cells (Figure 1A). Aggregated cells were also present—but in lower abundance—in digests of control livers (Figure S1A available online). HCC markers such as α fetoprotein (AFP), glypican 3 (Gpc3), and Ly6D, whose expression in mouse liver cancer was reported (Meyer et al., 2003), were upregulated in aggregates from DEN-treated livers, but not in nonaggregated hepatocytes or aggregates from control livers (Figure S1A). Thus, control liver aggregates may result from incomplete collagenase digestion, whereas aggregates from DEN-treated livers may contain HcPC. DEN-induced aggregates became larger and more abundant 5 months after carcinogen exposure, when they consisted of 10–50 cells that were smaller than nonaggregated hepatocytes. Using 70 μm and 40 μm sieves, we separated aggregated from nonaggregated hepatocytes (Figure 1A) and tested their tumorigenic potential by transplantation into MUP-uPA mice (Figure 1B). To facilitate transplantation, the aggregates were mechanically dispersed and suspended in Dulbecco's modified Eagle's medium (DMEM). Five months after intrasplenic (i.s.) injection of 10^4 viable cells, mice receiving cells from aggregates developed about 18 liver tumors per mouse, whereas mice receiving nonaggregated hepatocytes developed less than 1 tumor each (Figure 1B). The tumors exhibited typical trabecular HCC morphology and contained cells that abundantly express AFP (Figure S1B). To confirm that the HCCs were derived from transplanted cells, we measured their relative MUP-uPA DNA copy number and found that they contained much less MUP-uPA transgene DNA than the surrounding parenchyma (Figure S1C). Transplantation of aggregated cells from livers of DEN-treated actin-GFP transgenic mice resulted in GFP-positive HCCs (Figure S1D). Both experiments strongly suggest that the HCCs were derived from the transplanted cells. No tumors were ever observed after transplantation of control hepatocytes (nonaggregated or aggregated).

Only liver tumors were formed by the transplanted cells. Other organs, including the spleen into which the cells were injected, remained tumor free (Figure 1B), suggesting that HcPCs progress to cancer only in the proper microenvironment. Indeed, no tumors appeared after HcPC transplantation into normal BL/6 mice. But, if BL/6 mice were first treated with retrorsine (a chemical that permanently inhibits hepatocyte proliferation [Laconi et al., 1998]), intrasplenically transplanted with HcPC-containing aggregates, and challenged with CCl_4 to induce liver injury and compensatory proliferation (Guo et al., 2002), HCCs readily





E

Type of cells	Number of cells transplanted			
	$4x10^4$	$1X10^4$	1000	100
Unsorted	2(2)	9(9)	2(5)	0(3)
$CD44^+$	nd	nd	4(4)	2(4)
$CD44^-$	nd	nd	0(4)	0(5)

Figure 1. DEN-Induced Hepatocytic Aggregates Contain $CD44^+$ HCC Progenitors

(A) Fifteen-day-old BL/6 males were given DEN or vehicle. After 3 or 5 months, their livers were removed and collagenase digested. Left: typical digest appearance (magnification: 400×; 3 months after DEN). Red arrow indicates a collagenase-resistant aggregate. Right: aggregates per liver (n = 5; ± SD for each point).

(B) Livers were collagenase digested 5 months after DEN administration. Aggregates were separated from nonaggregated cells and mechanically dispersed into a single-cell suspension (left upper panels; 200×). 10^4 viable aggregated or nonaggregated cells were i.s. injected into MUP-uPA mice whose livers and spleens were analyzed for tumors 5 months later (left lower panels). The number of HCC nodules per liver was determined (n = 5; ± SD).

(C) Adult BL/6 mice were given retrorsine twice with a 2 week interval to inhibit hepatocyte proliferation. After 1 month, mice were i.s. transplanted with dispersed hepatocyte aggregates (10^4 cells) from DEN-treated mice and, 2 weeks later, were given three weekly i.p. injections of CCl_4 or vehicle. Tumor multiplicity and size were evaluated 5 months later (n = 5; ± SD).

(D) Hepatocyte aggregates were prepared as in (A), stained with CD44 antibody and DAPI, and examined by fluorescent microscopy (400×).

(E) Hepatocyte aggregates were dispersed as above, and $CD44^+$ cells were separated from $CD44^-$ cells. The indicated cell numbers were injected into MUP-uPA mice, and HCC development was evaluated 5 months later. n values are in parentheses (n.d., not done).

See also Figure S1.

appeared (Figure 1C). CCl_4 omission prevented tumor development. Notably, MUP-uPA or CCl_4-treated livers are fragile, rendering direct intrahepatic transplantation difficult. The transplanted HcPC-containing aggregates formed more numerous and larger HCC nodules in male recipients than in females (Figure 1C), as observed in MUP-uPA mice transplanted with unfractionated DEN-exposed hepatocytes (He et al., 2010). Thus, CCl_4-induced liver damage, especially within a male liver, generates a microenvironment that drives HcPC proliferation and malignant progression. To examine this point, we transplanted GFP-labeled HcPC-containing aggregates into retrorsine-treated BL/6 mice and examined their ability to proliferate with or without subsequent CCl_4 treatment. Indeed, the GFP^+ cells formed clusters that grew in size only in CCl_4-treated host livers

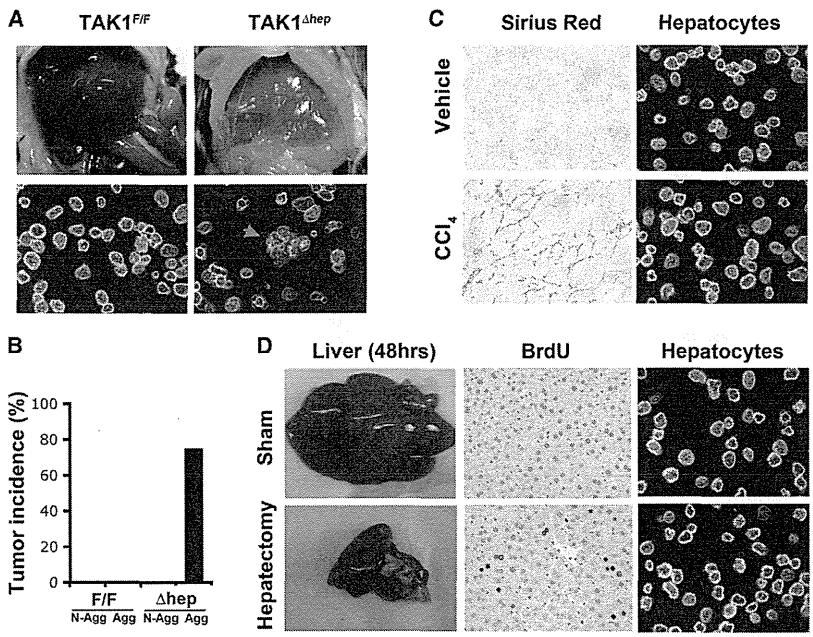


Figure 2. *Tak1*$^{\Delta hep}$ Livers Contain Collagenase-Resistant HcPC Aggregates

(A) Livers, free of tumors (upper panels), were removed from 1-month-old *Tak1*$^{F/F}$ and *Tak1*$^{\Delta hep}$ males and collagenase digested (lower panels; red arrow indicates collagenase-resistant aggregate).

(B) 10^4 nonaggregated or dispersed aggregated hepatocytes from (A) were i.s. injected into MUP-uPA mice that were analyzed 6 months later to identify mice with at least one liver tumor (n = 5–8 mice per genotype).

(C) BL/6 males were injected with vehicle or CCl_4 twice weekly for 2 weeks. Hepatocytes were isolated by collagenase digestion and photographed (right panels; 400×). Liver sections were stained with Sirius red to reveal collagen deposits (left panels).

(D) 8-week-old BL/6 males were subjected to 70% partial hepatectomy, pulsed with BrdU at 46 and 70 hr, and sacrificed 2 hr later. Isolated hepatocytes were photographed. Liver sections were analyzed for BrdU incorporation (400×). See also Figure S2 and Table S1.

(Figure S1E). Omission of $CC1_4$ prevented their expansion. Unlike HCC-derived cancer cells (dih10 cells), which form subcutaneous (s.c.) tumors with HCC morphology (He et al., 2010; Park et al., 2010), the HcPC-containing aggregates did not generate s.c. tumors in BL/6 mice (Figure S1F).

Despite their homogeneous appearance, the HcPC-containing aggregates contained both $CD44^+$ and $CD44^-$ cells (Figure 1D). Because CD44 is expressed by HCC stem cells (Yang et al., 2008; Zhu et al., 2010), we dispersed the aggregates and separated $CD44^+$ from $CD44^-$ cells and transplanted both into MUP-uPA mice. Whereas as few as 10^3 $CD44^+$ cells gave rise to HCCs in 100% of recipients, no tumors were detected after transplantation of $CD44^-$ cells (Figure 1E). Remarkably, 50% of recipients developed at least one HCC after receiving as few as 10^2 $CD44^+$ cells. Mature $CD44^-$ hepatocytes were found to engraft as well as or better than $CD44^+$ small hepatocytic cells (Haridass et al., 2009; Ichinohe et al., 2012). Hence, livers of DEN-treated mice contain $CD44^+$ HcPC that can be successfully isolated and purified and give rise to HCCs after transplantation into appropriate hosts. Unlike fully transformed HCC cells, HcPCs only give rise to tumors within the liver.

HcPC-Containing Aggregates in *Tak1*$^{\Delta hep}$ Mice

We applied the same HcPC isolation protocol to *Tak1*$^{\Delta hep}$ mice, which develop HCC of different etiology from DEN-induced HCC. Importantly, *Tak1*$^{\Delta hep}$ mice develop HCC as a consequence of chronic liver injury and fibrosis without carcinogen or toxicant exposure (Inokuchi et al., 2010). Indeed, whole-tumor exome sequencing revealed that DEN-induced HCC contained about 24 mutations per 10^6 bases (Mb) sequenced, with *B-Raf*^{V637E} being the most recurrent, whereas 1.4 mutations per Mb were detected in *Tak1*$^{\Delta hep}$ HCC's exome (Table S1). By contrast, *Tak1*$^{\Delta hep}$ HCC exhibited gene copy number changes. Collagenase digests of 1-month-old *Tak1*$^{\Delta hep}$ livers contained much more hepatocytic aggregates than *Tak1*$^{f/f}$ liver digests (Figure 2A). Notably, HCC developed in 75% of MUP-uPA mice that received dispersed *Tak1*$^{\Delta hep}$ aggregates, but no tumors appeared in mice receiving nonaggregated *Tak1*$^{\Delta hep}$ or total *Tak1*$^{f/f}$ hepatocytes (Figure 2B). Because *Tak1*$^{\Delta hep}$ mice are subject to chronic liver damage and consequent compensatory proliferation, we wanted to ascertain that the HcPCs are not simply proliferating hepatocytes or expanding bipotential hepatobiliary progenitors using CCl_4 to induce liver injury and compensatory proliferation in WT mice. Although this treatment caused acute liver fibrosis, it did not augment formation of collagenase-resistant aggregates (Figure 2C). Similarly, few aggregates were detected in collagenase digests of livers after partial hepatectomy (Figure 2D). However, bile duct ligation (BDL) or feeding with 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC), treatments that cause cholestatic liver injuries and oval cell expansion (Dorrell et al., 2011), did increase the number of small hepatocytic cell aggregates (Figure S2A). Nonetheless, no tumors were observed 5 months after injection of such aggregates into MUP-uPA mice (Figure S2B). Thus, not all hepatocytic aggregates contain HcPCs, and HcPCs only appear under tumorigenic conditions.

The HcPC Transcriptome Is Similar to that of HCC and Oval Cells

To determine the relationship between DEN-induced HcPCs, normal hepatocytes, and fully transformed HCC cells, we analyzed the transcriptomes of aggregated and nonaggregated hepatocytes from male littermates 5 months after DEN administration, HCC epithelial cells from DEN-induced tumors, and normal hepatocytes from age- and gender-matched littermate controls. Clustering analysis distinguished the HCC samples from other samples and revealed that the aggregated

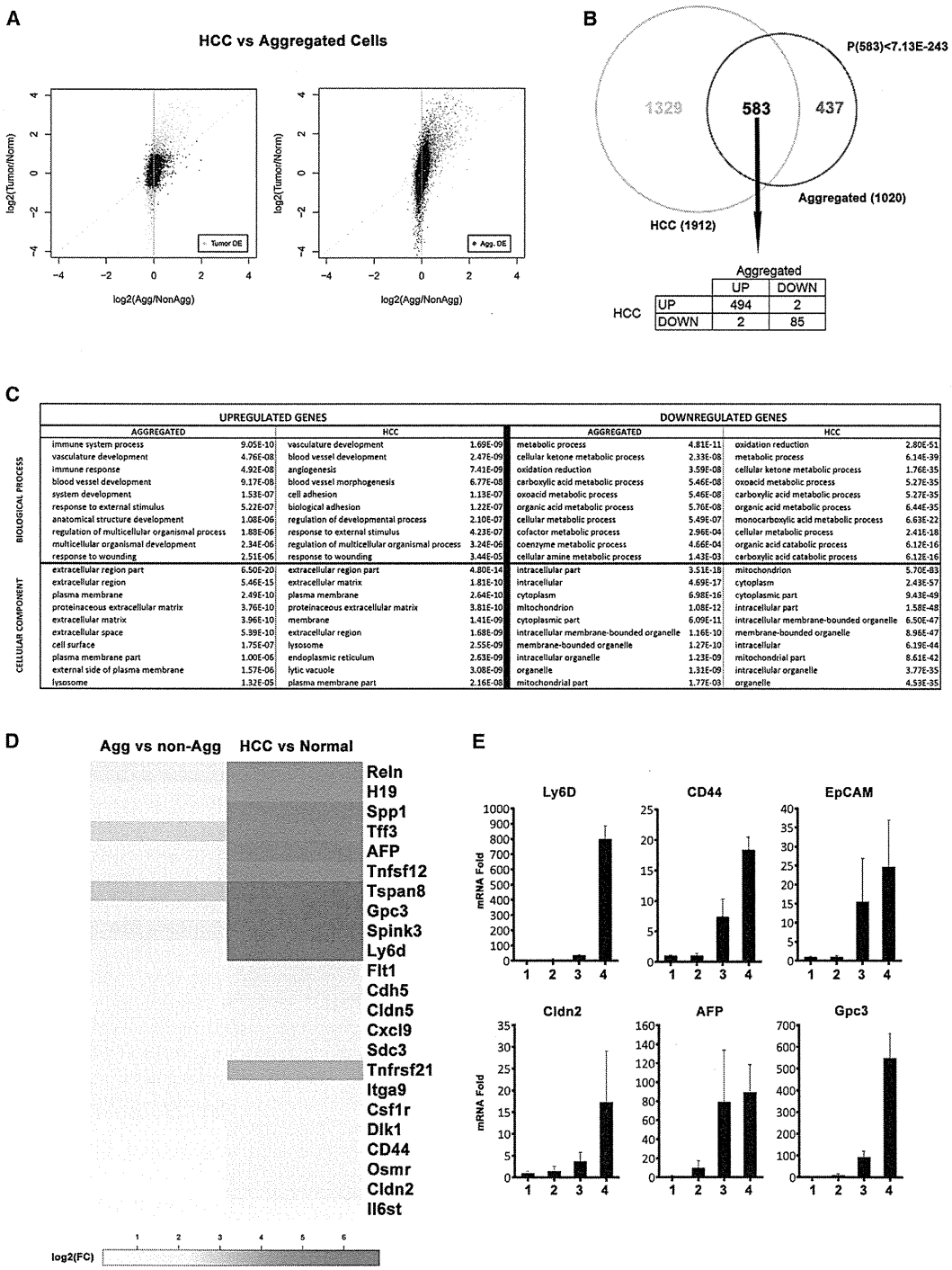


Figure 3. Aggregated Hepatocytes Exhibit an Altered Transcriptome Similar to that of HCC Cells

Aggregated and matched nonaggregated hepatocytes were isolated 5 months after DEN treatment. HCC cells were isolated from DEN-induced tumors, and normal hepatocytes were from age- and gender-matched control mice. RNA was extracted and subjected to microarray analysis (n = 3 for each sample).

(A) Scatterplot representing fold changes (log 2 of expression ratio) in gene expression for HCC versus normal (y axis) and aggregated versus nonaggregated (x axis) pairwise transcriptome comparisons. The plot is displayed twice: in the left panel, genes with an FDR < 0.01 in the aggregated versus nonaggregated

(legend continued on next page)

hepatocyte samples did not cluster with each other but rather with nonaggregated hepatocytes derived from the same mouse (Figure S3A). Interestingly, the aggregated cell transcriptome appeared closer to that of normal hepatocytes than to the HCC profile. This similarity may be due to the presence of ~70% nontumorigenic (or $CD44^-$) hepatocytes within the purified aggregates (Figure 1D). Comparison of the HCC and normal hepatocyte transcriptomes revealed 1,912 differentially expressed genes (false discovery rate [FDR] < 0.01; Figure 3A, left, cyan dots). A similar comparison revealed 1,020 genes that are differentially expressed between aggregated and nonaggregated hepatocytes (FDR < 0.01; Figure 3A, right, red dots). The range of differential expression is wider for the HCC and normal hepatocyte pair than the aggregate versus nonaggregate pair, reflecting presence of normal, nontransformed hepatocytes within the aggregates, resulting in signal dilution. Interestingly, 57% (583/1,020) of genes differentially expressed in aggregated relative to nonaggregated hepatocytes are also differentially expressed in HCC relative to normal hepatocytes (Figure 3B, top), a value that is highly significant ($p < 7.13 \times 10^{-243}$). More specifically, 85% (494/583) of these genes are overexpressed in both HCC and HcPC-containing aggregates (Figure 3B, bottom table). Thus, hepatocyte aggregates isolated 5 months after DEN injection contain cells that are related in their gene expression profile to HCC cells isolated from fully developed tumor nodules.

To gain insight into the functional differences between the transcriptomes of the four populations, we examined which biological processes or cellular compartments were significantly overrepresented in the induced or repressed genes in both pairwise comparisons (Gene Ontology Analysis). As expected, processes and compartments that were enriched in aggregated hepatocytes relative to nonaggregated hepatocytes were almost identical to those that were enriched in HCC relative to normal hepatocytes (Figure 3C). Upregulated genes were related to immune response, angiogenesis, development, and wound healing, and many encoded plasma membrane or secreted proteins. By contrast, downregulated genes were highly enriched for metabolic processes, and many of them encoded mitochondrial proteins or had functions associated with differentiated hepatocytes (Figure 3C). Several human HCC markers, including AFP, Gpc3 and H19, were upregulated in aggregated hepatocytes (Figures 3D and 3E). Aggregated hepatocytes also expressed more Tetraspanin 8 (Tspan8), a cell-surface glycoprotein that complexes with integrins and is overexpressed in human carcinomas (Zöller, 2009). Another cell-surface molecule highly expressed in aggregated cells is Ly6D (Figures 3D and 3E). Immunofluorescence (IF) analysis revealed that Ly6D was undetectable in normal liver but was elevated in FAH and ubiquitously expressed in most HCC cells (Figure S3C). A fluorescent-labeled Ly6D antibody injected into HCC-bearing mice specifically stained tumor nodules (Figure S3D). Other cell-surface molecules that were upregulated in aggregated cells included syndecan 3 (Sdc3), integrin α 9 (Itga9), claudin 5 (Cldn5), and cadherin 5 (Cdh5) (Figure 3D). Aggregated hepatocytes also exhibited elevated expression of extracellular matrix proteins (TIF3 and Reln1) and a serine protease inhibitor (Spink3). Elevated expression of such proteins may explain aggregate formation. Aggregated hepatocytes also expressed progenitor cell markers, including the epithelial cell adhesion molecule (EpCAM) (Figure 3E) and Dlk1 (Figure 3D). Elevated expression of cytokines and cytokine receptors was also detected, including tumor necrosis factor superfamily members 12 and 21, colony-stimulating factor 1 receptor, FMS-like tyrosine kinase 1, chemokine (C-X-C motif) ligand 9, the STAT3-activating cytokine osteopontin, IL-6 receptor (IL-6R) signal transducing subunit (gp130), and oncostatin M (OSM) receptor, which also activates STAT3 (Figure 3D).

Aggregated hepatocytes expressed albumin, albeit less than nonaggregated hepatocytes (Figure 4A). Some aggregated cells were positive for cytokeratin 19 (CK19) and A6, markers for bile duct epithelium and oval cells (Figure 4A). Most cells in the DEN-induced aggregates were AFP positive, and some of them expressed EpCAM (Figure 4A). However, not all markers were expressed by every cell within a given aggregate, suggesting that the aggregates contain liver cells that are related to bipotential hepatobiliary progenitors/oval cells as well as more differentiated progeny and normal hepatocytes. To confirm these observations, we compared the HcPC and HCC (Figure 3A) to the transcriptome of DDC-induced oval cells (Shin et al., 2011). This analysis revealed a striking similarity between the HCC, HcPC, and the oval cell transcriptomes (Figure S3B). Despite these similarities, some genes that were upregulated in HcPC-containing aggregates and HCC were not upregulated in oval cells. Such genes may account for the tumorigenic properties of HcPC and HCC.

We examined the aggregates for signaling pathways and transcription factors involved in hepatocarcinogenesis. Many aggregated cells were positive for phosphorylated c-Jun and STAT3 (Figure 4A), transcription factors involved in DEN-induced

comparison are highlighted in red, and in the right panel, genes with an FDR < 0.01 in the HCC versus normal comparison are highlighted in cyan. DE, differentially expressed.

(B) Venn diagram showing overlap between genes that are differentially expressed between aggregated and nonaggregated hepatocytes and between HCC cells and normal hepatocytes with an FDR < 0.01 (cyan and red dots from A). The probability to find 583 overlapping genes is $<7.13 \times 10^{-243}$. From these 583 common genes, only 4 behaved differently.

(C) The ten most enriched biological processes (upper table) and cellular compartments (lower panel) represented by genes that are significantly upregulated (left panel) or downregulated (right panel) in HCC relative to normal hepatocytes (HCC) or in aggregated relative to nonaggregated hepatocytes (aggregated).

(D) Heatmap displaying positive fold changes (FC) in expression of genes of interest in aggregated versus nonaggregated HcPCs (left) and in HCC versus normal hepatocytes (right).

(E) Expression of selected genes was examined by real-time PCR and is depicted as fold change relative to normal hepatocytes given an arbitrary value of 1.0 (n = 3; ± SD). (1) Normal hepatocytes; (2) nonaggregated hepatocytes from DEN-treated liver; (3) HcPC aggregates from DEN-treated liver; and (4) DEN-induced HCCs.

See also Figure S3.

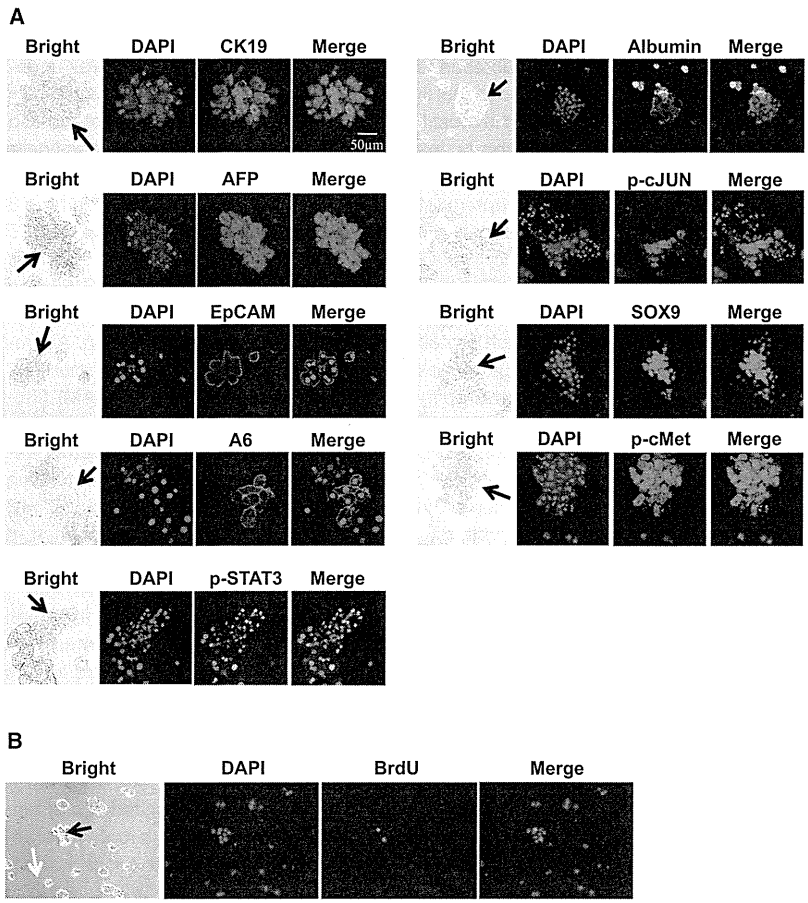


Figure 4. DEN-Induced HcPC Aggregates Express Pathways and Markers Characteristic of HCC and Hepatobiliary Stem Cells

(A) Cytospin preps of collagenase-resistant aggregates from 5-month-old DEN-injected mice were stained with antibodies to CK19, AFP, EpCAM, A6, phospho-Y-STAT3 (Tyr705), albumin, phospho-c-Jun, Sox9, and phospho-c-Met. Black arrows indicate aggregates, and yellow arrows indicate nonaggregated cells (magnification: 400×). (B) 5-month-old DEN-treated mice were injected with BrdU, and 2 hr later, collagenase-resistant aggregates were isolated and analyzed for BrdU incorporation (400×).

See also Figure S4.

hepatocarcinogenesis (Eferl et al., 2003; He et al., 2010). Sox9, a transcription factor that marks hepatobiliary progenitors (Dorrell et al., 2011), was also expressed by many of the aggregated cells, which were also positive for phosphorylated c-Met (Figure 4A), a receptor tyrosine kinase that is activated by hepatocyte growth factor (HGF) and is essential for liver development (Bladt et al., 1995) and hepatocarcinogenesis (Wang et al., 2001). Few of the nonaggregated hepatocytes exhibited activation of these signaling pathways. Aggregates from bromodeoxyuridine (BrdU)-pulsed DEN-treated mice contained BrdU-positive cells (Figure 4B), indicating that they were actively proliferating prior to isolation. Hepatocyte aggregates from 1-month-old *Tak1*$^{\Delta hep}$ mice also contained cells positive for AFP, Sox9, phosphorylated c-Met, and EpCAM, but not A6-positive cells (Figure S4A). Many of the cells also exhibited partially activated β-catenin, phosphorylated STAT3, and phosphorylated c-Jun. Thus, despite different etiology, HcPC-containing aggregates from *Tak1*$^{\Delta hep}$ mice exhibit upregulation of many of the same markers and pathways that are upregulated in DEN-induced HcPC-containing aggregates. Flow cytometry confirmed enrichment of CD44$^+$ cells as well as CD44$^+$/CD90$^+$ and CD44$^+$/EpCAM$^+$ double-positive cells in the HcPC-containing aggregates from either DEN-treated or *Tak1*$^{\Delta hep}$ livers (Figure S4B).

HcPC-Containing Aggregates Originate from Premalignant Dysplastic Lesions

FAH are dysplastic lesions occurring in rodent livers exposed to hepatic carcinogens (Su et al., 1990). Similar lesions are present in premalignant human livers (Su et al., 1997). Yet, it is still debated whether FAH correspond to premalignant lesions or are a reaction to liver injury that does not lead to cancer (Sell and Leffert, 2008). In DEN-treated males, FAH were detected as early as 3 months after DEN administration (Figure 5A), concomitant with the time at which HcPC-containing aggregates were detected. In females, FAH development was delayed. In both genders, FAH were confined to zone 3 and consisted of tightly packed small hepatocytic cells, some of which were proliferative based on BrdU incorporation (Figure 5B). BrdU$^+$ cells were first detected in DEN-treated males and were confined to FAH and rarely detected in age-matched control mice. FAH contained cells positive for the same progenitor cell markers and activated signaling pathways present in HcPC-containing aggregates, including AFP, CD44, and EpCAM (Figure 5C). FAH also contained cells positive for activated STAT3, c-Jun, and PCNA (Figure 5C). Many cells within FAH exhibited strong upregulation of YAP (Figure 5C), a transcriptional coactivator that is negatively regulated by the Hippo pathway and a liver cancer oncoprotein (Zheng et al., 2011). FAH were also enriched in F4/80$^+$ macrophages (Figure 5C). These results suggest that the HcPC-containing aggregates may be derived from FAH.

HcPCs Exhibit Autocrine IL-6 Expression Necessary for HCC Progression

In situ hybridization (ISH) and immunohistochemistry (IHC) revealed that DEN-induced FAH contained IL-6-expressing cells (Figures 6A, 6B, and S5), and freshly isolated DEN-induced aggregates contained more IL-6 messenger RNA (mRNA) than nonaggregated hepatocytes (Figure 6C). We examined several