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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Table S1 Hepatitis B virus DNA-specific oligonucleotide primers used in the study.

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Special Report

Etiology and prognosis of fulminant hepatitis and late-onset hepatic failure in Japan: Summary of the annual nationwide survey between 2004 and 2009

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Aim: To summarize the annual nationwide survey on fulminant hepatitis (FH) and late-onset hepatic failure (LOHF) between 2004 and 2009 in Japan.

Methods: The annual survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients in special hospitals.

Results: Four hundred and sixty ($n = 227$ acute type; $n = 233$ subacute type) patients had FH and 28 patients had LOHF. The mean age of patients with FH and LOHF were 51.1 ± 17.0 and 58.0 ± 14.4 years, respectively. The causes of FH were hepatitis A virus in 3.0%, hepatitis B virus (HBV) in 40.2%, other viruses in 2.0%, autoimmune hepatitis in 8.3%, drug allergy-induced in 14.6% and indeterminate etiology in 29.6% of patients. HBV reactivation due to immunosuppressive therapy was observed in 6.8% of FH patients. The short-term survival rates of patients without liver transplantation (LT) were 48.7% and 24.2% for the acute and subacute type, respectively, and 13.0% for LOHF. The prognosis was poor in patients with HBV reactivation. The implementation rate for LT in FH patients was equivalent to that in the previous survey. The short-term survival rates of total patients, including LT patients, were 54.2% and 40.8% for the acute and subacute type, respectively, and 28.6% for LOHF.

Conclusion: The demographic features and etiology of FH patients has gradually changed. HBV reactivation due to immunosuppressive therapy is problematic. Despite advances in therapeutic approaches, the prognosis of patients without LT has not improved.

Key words: acute liver failure, fulminant hepatitis, Japan, liver transplantation, viral hepatitis

INTRODUCTION

IN JAPAN, FULMINANT hepatitis (FH) is defined as having hepatitis, when grade II or worse hepatic encephalopathy develops within 8 weeks of the onset of disease symptoms, with a prothrombin time of 40% or less.[1,2] FH is further classified into two subtypes, acute and subacute types, in which encephalopathy occurs within 10 days and later than 11 days, respectively, of the onset of the disease symptoms. Patients showing a prothrombin time of 40% or less, with hepatic encephalopathy developing between 8 and 24 weeks of disease onset are classified as having late-onset hepatic failure (LOHF).[3] Etiologies with hepatitis present in the histology, such as viral infection, autoimmune hepatitis and drug allergy-induced liver injury are defined as causes of

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FH and LOHF. In contrast, acute liver failure due to other causes with the absence of hepatitis in the histology, such as drug toxicity, circulatory disturbance and metabolic disease, are excluded as causes of FH and LOHF. Recently, a novel diagnostic criteria for acute liver failure in Japan was established by the Intractable Hepato-Biliary Disease Study Group.[4,5] These criteria included other causes with liver damage without the absence of hepatitis in the histology in addition to the present criteria.

Among viral infection, hepatitis B virus (HBV) is a major cause of FH in Japan.[6,7] HBV infection is classified into transient HBV infection type and acute exacerbation in an HBV inactive carrier. With advances in cytotoxic chemotherapy and immunosuppressive therapy, reactivation of hepatitis B is becoming a clinical problem.[8] Moreover, recent introduction of rituximab plus steroid combination therapy for non-Hodgkin's lymphoma has been associated with HBV reactivation in transiently infected patients, namely, de novo hepatitis. However, the prevalence of HBV reactivation in patients with FH and LOHF is unknown.

Advances in therapeutic strategies for FH and LOHF have improved the prognosis. Since 1988, living-donor liver transplantation (LT) has been adopted in patients who are beyond the supportive care of a critical unit.[6] Recently, artificial liver support with high-flow or on-line hemodiafiltration (HDF) has been used. Since 2006, a nucleoside analog, entecavir, has been used as a substitute for lamivudine, as an antiviral agent for HBV. However, it is unknown whether these new treatments improve the prognosis of FH.

The Intractable Hepato-Biliary Diseases Study Group has annually performed a nationwide survey of patients with FH and LOHF since 1983.[6] Since 2000, approximately 600 hospitals have been enrolled in the survey. This report summarizes the results of the survey between 2004 and 2009 to addresses the trends in the etiology and prognosis of patients with FH and LOHF and compares them with the previous survey.[7]

METHODS

THE NATIONWIDE SURVEY was performed annually. The number of hospitals for survey has changed in each year. Maximum (608) was in 2007 and minimum (544) was in 2006, with active members of the Japan Society of Hepatology and the Japanese Society of Gastroenterology between 2005 and 2010. The survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients who were diagnosed as FH or LOHF in the previous year. The recovery rate of the first and second questionnaire was 39–59% and 60–100%, respectively. Patients who met the diagnostic criteria for FH or LOHF were entered into the survey. Patients under 1 year of age, those with alcoholic hepatitis, those with chronic liver diseases and those with acute liver failure with no histological features of hepatitis were excluded from the analysis.

According to criteria described in previous reports,[7,9] the etiology of FH and LOHF was classified into five categories: (i) viral infection; (ii) autoimmune hepatitis; (iii) drug allergy-induced liver injury; (iv) indeterminate etiology despite sufficient examinations; and (v) unclassified due to insufficient examinations. Patients with viral infection consisted of those with hepatitis A virus (HAV), HBV, hepatitis C virus (HCV), hepatitis E virus (HEV) and other viruses. The patients with HBV infection were classified into three subgroups according to serum markers of HBV, hepatitis B core antibody (HBcAb) and immunoglobulin (Ig)M-HBcAb: (i) transient HBV infection; (ii) acute exacerbation in HBV carriers; and (iii) indeterminate infection patterns. In the present study, we classified acute exacerbation in HBV carriers into three subgroups according to the new criteria:[4,5] (i) inactive carriers, without drug exposure; (ii) reactivation in inactive carriers by immunosuppressant and/or anticancer drugs; and (iii) reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (i.e. de novo hepatitis). Because not every patient was examined for serological markers of transient HBV infection before the onset of FH and LOHF (with HBcAb and/or hepatitis B surface antigen [HBsAg] in serum), we defined HBV reactivation as that occurring in transiently infected patients when they developed HBV-related hepatitis due to immunosuppressive therapy or cytotoxic chemotherapy with reappearance of HBsAg in the serum and did not conform to the criteria of transient HBV infection.

The statistical significance of differences between groups was assessed using Student's *t*-test, Fisher's exact test or Kruskal–Wallis one-way ANOVA. Data are shown as mean ± standard deviation. The study was conducted with the approval of the Ethical Committee of Kagoshima University Graduate School of Medical and Dental Sciences.

RESULTS

Demographic features and survival rates

FROM 2004–2009, 582 PATIENTS were enrolled in the survey. Ninety-four patients were excluded from

the survey according to the exclusion criteria. Consequently, 460 patients ($n = 227$ acute type; $n = 233$ subacute type) were classified as having FH and 28 as having LOHF (Table 1). The incidence of the acute and subacute types of FH was similar and the incidence of LOHF was one-sixteenth of FH. The male : female ratio was higher for the acute type and lower for the subacute type of FH and LOHF. The mean age of patients was significantly higher for the subacute type of FH and LOHF than that for the acute type of FH. Almost half of the patients with FH and LOHF had complications which preceded the onset of acute liver failure. Furthermore, approximately 60% of patients with FH had received daily medication. This tendency for receiving medication was more obvious in patients with the subacute type of FH and LOHF.

The survival rates of patients without LT were 48.7% for the acute type and 24.2% for the subacute type of FH, and 13.0% for LOHF. The survival rates of the subacute type of FH and LOHF was worse than that of the acute type. The prognosis of both the acute type and the subacute type of FH appeared to be equivalent annually. The survival rates of patients with LT were 79.6% for FH and 100% for LOHF, with no difference in these rates among the disease types.

Clinical profile

Symptoms, imaging findings and complications are shown in Table 2. Since 2006, diagnostic criteria of systemic inflammatory response syndrome (SIRS) for fever, tachycardia and tachypnea have been adopted in the survey.[10] Icterus, flapping tremor, ascites, hepatic fetor, tachycardia, tachypnea and pretibial edema were frequently found. The frequency of patients with ascites and pretibial edema was significantly greater in the subacute type of FH and LOHF than in the acute type of FH. In contrast, fever appeared more frequently in patients with the acute type of FH. The frequency of liver atrophy was greater in the subacute type of FH, and even higher in LOHF, than in the acute type of FH.

With regard to complications, disseminated intravascular coagulation, renal failure and bacterial infection were found in more than 30% of patients with FH and LOHF. Brain edema was less frequent in the subacute type than in the acute type of FH.

Causes of FH and LOHF

The cause of FH was identified as viral infection in 46.1% of the patients (Table 3). The frequencies of viral infection were highest for the acute type of FH. HAV infection was found in 3% of patients with FH. HBV infection was found in 40.2% of patients with FH and 32.1% of patients with LOHF. Transient HBV infection was more frequent in the acute type than in the subacute type of FH, whereas the frequency of acute exacerbation in HBV carriers was greater in the subacute type than in the acute type of FH. HBV reactivation in inactive carriers and in transiently infected patients were found in 3.3% and 3.5% of patients with FH, respectively. With regard to underlying diseases in patients with HBV reactivation, non-Hodgkin's lymphoma/mucosa-associated lymphoid tissue lymphoma was most prevalent in 50% of inactive carriers and in 76% of those with transiently infected patients. Among patients with HBV

Table 1 Demographic features and survival rates of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Male/female	227/233	127/100	100/133**	9/19*
Age (years; mean ± SD)	51.1 ± 17.0	48.8 ± 16.9	53.4 ± 16.7**	58.0 ± 14.4**
HBV carrier (%)	13.1 (52/397)	10.5 (19/181)	15.3 (33/216)	22.2 (6/27)
Complications preceding acute liver failure (%)†	46.4 (208/448)	40.0 (88/220)	52.6 (120/228)**	50.0 (14/28)
History of medication (%)	59.9 (260/434)	51.2 (108/211)	68.2 (152/223)**	71.4 (20/28)*
Survival rates				
All patients	47.4 (218/460)	54.2 (123/227)	40.8 (95/233)**	28.6 (8/28)*
No LT	37.5 (132/352)	48.7 (93/191)	24.2 (39/161)**	13.0 (3/23)**
LT	79.6 (86/108)	83.3 (30/36)	77.8 (56/72)	100 (5/5)

*$P < 0.05$, **$P < 0.01$ vs acute type.
†Diseases such as metabolic syndrome, malignancy and psychiatric disorders.
Data in parenthesis indicate patient numbers.
HBV, hepatitis B virus; LT, liver transplantation; SD, standard deviation.

Table 2 Symptoms, imaging findings and complications of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
(a) Symptoms at diagnosis				
Fever†	13.0 (42/322)	17.5 (28/160)	8.6 (14/162)*	0 (0/23)*
Icterus	96.8 (427/441)	95.0 (208/219)	98.6 (219/222)*	96.4 (27/28)
Ascites	57.2 (237/414)	45.2 (88/204)	71.0 (149/210)**	81.5 (22/27)**
Convulsion	5.2 (22/422)	6.7 (14/210)	3.8 (8/212)	0 (0/27)
Tachycardia‡	36.7 (117/319)	39.5 (62/157)	34.0 (55/162)	47.8 (11/23)
Tachypnea§	34.5 (87/252)	39.1 (52/133)	29.4 (35/119)	31.6 (6/19)
Flapping tremor	79.0 (309/391)	75.8 (144/190)	82.1 (165/201)	80.8 (21/26)
Hepatic fetor	46.6 (146/313)	49.0 (73/149)	44.5 (73/164)	42.1 (8/19)
Pretibial edema	35.5 (127/358)	24.1 (42/174)	46.2 (85/184)**	75.0 (15/20)**,***
(b) Imaging findings				
Liver atrophy¶	58.8 (255/434)	45.6 (98/215)	71.7 (157/219)**	92.6 (25/27)**,***
(c) Complications				
Infection	34.8 (149/428)	32.9 (68/207)	36.7 (81/221)	51.9 (14/27)
Brain edema	18.5 (71/384)	24.1 (46/191)	13.0 (25/193)**	22.7 (5/22)
Gastrointestinal bleeding	13.2 (59/446)	11.0 (24/219)	15.4 (35/227)	20.0 (5/25)
Renal failure	38.9 (177/455)	40.9 (92/225)	37.0 (85/230)	39.3 (11/28)
DIC	34.6 (150/433)	35.7 (76/213)	33.6 (74/220)	53.8 (14/26)
Congestive heart failure	7.3 (31/427)	8.9 (19/214)	5.6 (12/213)	12.0 (3/25)

*$P < 0.05$, **$P < 0.01$ vs acute type, ***$P < 0.05$ vs subacute type.
†Temperature: >38°C or <36°C.
‡Heart rate: >90 beats/min.
§Respiratory rate: >20 breaths/min or $PaCO_2$: <32 Torr.
† ‡ § Cases between 2005 and 2009.
¶Liver atrophy detected by ultrasound and/or computed tomography imaging.
Data in parentheses indicate patient numbers.
DIC, disseminated intravascular coagulation.

reactivation, rituximab plus steroid combination chemotherapy was administrated to 35% of patients in inactive carriers and to 59% of those with transiently infected patients. HCV and HEV infection were less frequently found. In the survey, Epstein–Barr virus, herpes simplex virus and human herpes virus type-6 were found as other causes of viral hepatitis.

Autoimmune hepatitis was frequently observed in patients with the subacute type of FH and LOHF. Drug allergy-induced liver injury was observed in approximately 10–20% of patients irrespective of disease types. Anti-tuberculosis agents, non-steroidal anti-inflammatory drugs, anticancer agents, drugs for metabolic syndrome, and various herbal and natural remedies were the probable causative agents for this liver injury in the survey. Notably, the etiology was indeterminate in approximately 40% of patients with the subacute type of FH.

Therapies

For artificial liver support, plasma exchange and HDF were performed in most patients with FH (Table 4). Conventional HDF and continuous HDF (CHDF) were performed in 22.5% and 51.8% of patients with FH, respectively. A more powerful method, high-flow HDF (HF-HDF), high-flow CHDF (HF-CHDF) and on-line HDF were performed in 2.6%, 11.7% and 1.8% of the patients, respectively. The nucleoside analogs lamivudine and entecavir were used in approximately a quarter of patients with FH. Entecavir were used more frequently than lamivudine since 2007. Glucocorticosteroid, mainly as steroid pulse therapy, were administrated in more than 70% of patients with FH and LOHF. Anticoagulation therapy were performed in approximately 40–50% of patients with FH and LOHF. Glucagon/insulin, branched-chain amino acid-rich solution,

Table 3 Causes of fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Viral infection	46.1 (212)	62.6 (142)	30.0 (70)	32.1 (9)
HAV	3.0 (14)	5.7 (13)	0.4 (1)	0 (0)
HBV	40.2 (185)	54.2 (123)	26.6 (62)	32.1 (9)
(1) Transient infection	19.6 (90)	35.2 (80)	4.3 (10)	3.6 (1)
(2) Acute exacerbation in HBV carrier	14.1 (65)	7.9 (18)	20.2 (47)	25.0 (7)
(i) Inactive carrier, without drug exposure	7.4 (34)	6.2 (14)	8.6 (20)	3.6 (1)
(ii) Reactivation in inactive carrier†	3.3 (15)	1.8 (4)	4.7 (11)	17.9 (5)
(iii) Reactivation in transiently infected patient‡	3.5 (16)	0 (0)	6.9 (16)	3.6 (1)
(3) Indeterminate infection patterns	6.5 (30)	11.0 (25)	2.1 (5)	3.6 (1)
HCV	1.1 (5)	0.9 (2)	1.3 (3)	0 (0)
HEV	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Other viruses	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Autoimmune hepatitis	8.3 (38)	2.2 (5)	14.2 (33)	32.1 (9)
Drug allergy-induced liver injury	14.6 (67)	13.7 (31)	15.5 (36)	17.9 (5)
Indeterminate§	29.6 (136)	19.4 (44)	39.5 (92)	17.9 (5)
Unclassified¶	1.5 (7)	2.2 (5)	0.9 (2)	0 (0)

†Reactivation in inactive carrier by immunosuppressant and/or anticancer drugs.
‡Reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (de novo hepatitis).
§Indeterminate etiology despite sufficient examinations.
¶Unclassified due to insufficient examinations.
Data in parentheses indicate patient numbers.
HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

cyclosporin A and prostaglandin E_1 therapy were administrated less frequently compared with the previous survey.

Liver transplantation was performed in 23.5% and 17.9% of patients with FH and LOHF, respectively. Two patients received deceased-donor LT and 111 patients received living-donor LT. The frequency of LT was significantly greater in the subacute type than in the acute type of FH.

Prognosis

The prognosis of patients with FH and LOHF differed depending on the etiology (Table 5). Prognosis was good in patients with HAV infection. The prognosis was fair in patients with transient HBV infection. In contrast, the prognosis was poor in acute exacerbation in HBV carriers. The prognosis was extremely poor in patients with HBV reactivation, either from inactive carriers or transiently infected patients. Patients with the subacute type of FH and LOHF caused by autoimmune hepatitis, drug allergy-induced liver injury and indeterminate etiology also showed a poor prognosis.

The clinical features of the patients appeared to be associated with the prognosis. In the acute type of FH with no LT, the frequency of patients with SIRS (tachycardia or tachypnea) was greater in patients who died than in surviving patients ($P < 0.05$). Liver atrophy on ultrasound and/or computed tomography imaging was an important factor in predicting the prognosis of FH and LOHF with no LT. The frequencies were 25.0% and 64.5% in patients with the acute type ($P < 0.01$) and 55.6% and 78.1% in those with the subacute type of FH in surviving patients and those who died, respectively ($P < 0.05$).

Prognosis also appeared to be affected by complications. Any of the complications significantly decreased survival rate (data not shown). Furthermore, the number of these complications affected the prognosis. The survival rate of patients with the acute type of FH was greater than 80% in those with no complications, while it was less than 30% in those with two or more complications. The survival rate of patients with the subacute type of FH was decreased in proportion to the number of complications.

Table 4 Therapies for patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Plasma exchange	90.9 (418/460)	92.5 (210/227)	89.3 (208/233)	71.4 (20/28)**,***
Hemodiafiltration	75.0 (342/456)	75.1 (169/225)	74.9 (173/231)	57.1 (16/28)
Glucocorticosteroids	72.4 (333/460)	68.3 (155/227)	76.4 (178/233)	89.3 (25/28)*
Glucagon/insulin	14.6 (67/459)	13.7 (31/227)	14.7 (34/232)	17.9 (5/28)
BCAA-rich solution	19.1 (87/456)	14.3 (32/223)	23.6 (55/233)*	39.3 (11/28)**
Prostaglandin E_1	7.0 (32/458)	6.7 (15/225)	7.3 (17/233)	3.6 (1/28)
Cyclosporin A	10.0 (46/460)	7.0 (16/227)	12.9 (30/233)*	10.7 (3/28)
Interferon	14.1 (65/460)	15.4 (35/227)	12.9 (30/233)	10.7 (3/28)
Nucleoside analog	38.9 (179/460)	50.9 (115/226)	27.5 (64/233)**	32.1 (9/28)
Lamivudine	25.5 (116/455)	40.0 (76/224)	30.4 (40/231)	12.5 (6/28)
Entecavir†	22.4 (70/312)	27.7 (41/148)	17.7 (29/164)	33.3 (5/15)
Anticoagulation therapy‡	47.2 (216/458)	43.2 (98/227)	51.1 (118/231)	39.3 (11/28)
Liver transplantation	23.5 (108/460)	15.9 (36/227)	30.9 (72/233)	17.9 (5/28)

*$P < 0.05$, **$P < 0.01$ vs acute type, ***$P < 0.05$ vs subacute type.
†Cases between 2006 and 2009.
‡Drugs such as antithrombin III concentrate and protease inhibitor compounds, gabexate mesylate and nafamostat mesilate.
Data in parentheses indicate patient numbers.
BCAA, branched-chain amino acid.

DISCUSSION

IN THIS SURVEY, 488 patients were enrolled over 6 years. In the previous 6-year survey, 697 patients (634 for FH and 64 for LOHF) were enrolled.[7] The incidence ratio of LOHF to FH was decreased from 9:1 to 16:1. In national epidemiology research, the annual incidence of FH was estimated at 1050 cases in 1996 and 429 cases in 2004.[11] Therefore, the incidence of FH and LOHF could be decreasing longitudinally. In this survey, the mean age of patients with FH and LOHF was older than that in the previous survey. More patients with complications received daily medication compared with the previous survey. Changes in demographic features of the patients may affect the etiology and prognosis of FH. A relationship between daily dose of oral medication and idiosyncratic drug-induced liver injury has been reported.[12] Additionally, older age is considered a poor prognostic factor in acute liver failure and may be considered a relative contraindication for LT.[13,14]

The current study showed that HBV still remains a major cause of FH and LOHF. Notably, almost half of acute exacerbations in HBV carriers occurred in patients with HBV reactivation owing to immunosuppressive or cytotoxic therapy. Approximately 80% of patients with transiently infected patients had received rituximab plus steroid combination therapy for non-Hodgkin's lymphoma. This combination therapy has been identified as a risk factor for HBV reactivation in HBsAg positive/negative patients with non-Hodgkin's lymphoma.[15,16] Our survey revealed that careful attention is necessary for transiently infected patients, as well as for inactive HBV carriers using intensive immunosuppressive agents.

The frequency of HAV infection in patients with FH was decreased compared with the previous survey. This result is compatible with no occurrence of outbreak of acute hepatitis A during this period. In Japan, zoonotic transmission from pigs, wild boar and deer, either foodborne or otherwise, is the cause of HEV infection.[17,18] In the currently studied survey, two-thirds of the patients were from endemic areas (Hokkaido Island and the northern part of mainland Honshu) in Japan.

The other principal finding in this survey was that the etiology was indeterminate in approximately 40% of patients with FH. One of the reasons for this result may be the failure of diagnosis for autoimmune hepatitis or drug-induced liver injury. Although the diagnosis of autoimmune hepatitis relies on the presence of serum autoantibodies, with higher IgG levels (>2 g/dL), acute-onset autoimmune hepatitis does not always show typical clinical features.[19–21] Additionally, the sensitivity of the drug-induced lymphocyte stimulation test for diagnosis is not completely reliable.

Table 5 Survival rates and etiology of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF) who did not have liver transplantation

	FH			LOHF ($n = 23$)
	Total ($n = 352$)	Acute type ($n = 191$)	Subacute type ($n = 161$)	
Viral infection	39.8 (70/176)	49.2 (58/118)	20.7 (12/58)**	14.3 (1/7)
HAV	57.1 (8/14)	61.5 (8/13)	0 (0/1)	–
HBV	36.2 (55/152)	46.1 (47/102)	16.0 (8/50)**	14.3 (1/7)
(1) Transient infection	52.6 (40/76)	54.4 (37/68)	37.5 (3/8)	–
(2) Acute exacerbation in HBV carrier	15.1 (8/53)	21.4 (3/14)	12.8 (5/39)	14.3 (1/7)
(i) Inactive carrier, without drug exposure	29.2 (7/24)	27.3 (3/11)	30.8 (4/13)	0 (0/1)
(ii) Reactivation in inactive carrier†	7.7 (1/13)	0 (0/3)	10.0 (1/10)	20.0 (1/5)
(iii) Reactivation in transiently infected patients‡	0 (0/16)	–	0 (0/16)	0 (0/1)
(3) Indeterminate infection patterns	30.4 (7/23)	35.0 (7/20)	0 (0/3)	–
HCV	50.0 (2/4)	100 (1/1)	33.3 (1/3)	–
HEV	75.0 (3/4)	100 (2/2)	50 (1/2)	–
Other viruses	100 (2/2)	–	100 (2/2)	–
Autoimmune hepatitis	32.4 (9/28)	40.0 (2/5)	30.4 (7/23)	12.5 (1/8)
Drug allergy-induced	32.8 (19/58)	43.3 (13/30)	21.4 (6/28)	0 (0/3)
Indeterminate§	37.6 (32/85)	54.5 (18/33)	26.9 (14/52)*	20.0 (1/5)
Unclassified¶	1.5 (7)	40.0 (2/5)	–	–

**$P < 0.01$ vs acute type.
†Reactivation in inactive carrier by immunosuppressant and/or anticancer drugs.
‡Reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (de novo hepatitis).
§Indeterminate etiology despite sufficient examinations.
¶Unclassified due to insufficient examinations.
Data in parentheses indicate patient numbers.
HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

Recently, powerful HDF using large buffer volumes (HF-HDF or HF-CHDF), or on-line HDF has been used. HF-HDF or HF-CHDF has a high recovery rate from a coma.[22–24] On-line HDF has an excellent recovery rate from a coma and is useful as a liver support system.[25] However, only 16% of patients with FH received these powerful HDF in the survey examined in the current study. The frequency of brain edema, gastrointestinal bleeding and congestive heart failure was decreased compared with that in the previous survey. Advances in artificial liver support and management may contribute to prevent these complications. Further evaluation is required to determine whether a new powerful support system can improve the prognosis of FH. The survival rate for FH patients with autoimmune hepatitis improved 17.1% in the previous survey to 32.4% in the 2004–2009 survey. Early commencement of corticosteroids may improve the prognosis. However, the efficacy of these drugs has not been evaluated statistically.

Recently, in patients with acute liver failure due to HBV, entecavir has been used more frequently than lamivudine because of its high potency and extremely low rates of drug resistance.[26] Entecavir beneficially affects the course of acute liver failure as lamivudine.[27,28] Despite the use of entecavir, the prognosis of HBV-infected patients, especially in HBV carriers, has not improved. In the case of HBV reactivation, it is difficult to prevent development of liver failure, even when nucleoside analogs are administrated after the onset of hepatitis. Because these agents require a certain amount of time to decrease HBV DNA in serum, they need to be administrated in the early phase of hepatitis. Guidelines for preventing HBV reactivation recommend the administration of nucleoside analogs before the start of immunosuppressive therapy in inactive carriers and at an early stage of HBV reactivation during or after immunosuppressive therapy in transiently infected patients.[29]

Despite new therapeutic approaches and intensive care, the prognosis of patients without LT with both types of FH and LOHF appeared similar to that in the previous survey. In contrast, the prognosis of patients receiving LT was good in the present survey. Yamashiki

et al. reported that the short-term and long-term outcomes of living-donor LT for acute liver failure were good, irrespective of the etiology and disease types.[30] In the current survey, the implementation rate of receiving LT was almost equivalent to that in the previous survey, irrespective of disease type. Notably, only two patients received deceased-donor LT in the current survey. Recently, patients with FH who received deceased-donor LT have been increasing since the new organ transplant bill passed in 2009. Hepatologists should realize that more donor action to increase deceased-donor LT is necessary to improve the prognosis of patients with FH or LOHF. Determining appropriate judgment to move forward to LT is the most important step. The indications for LT in cases of FH are determined according to the 1996 Guidelines of the Acute Liver Failure Study Group of Japan.[31] To improve the low sensitivity and specificity of assessment in patients with acute and subacute types,[32] new guidelines for using a scoring system have been established by the Intractable Hepato-Biliary Disease Study Group of Japan.[33] This novel scoring system showed sensitivity and specificity of 0.80 and 0.76, respectively, and greater than those in the previous guideline.[33] Recently, new prediction methods using data-mining analysis has been established.[34,35]

In conclusion, the demographic features and etiology of FH and LOHF have been gradually changing. HBV reactivation due to immunosuppressive therapy is a particular problem because of poor prognosis. The subacute types of FH and LOHF have a poor prognosis, irrespective of the etiology. Despite recent advances in therapeutic approaches, the implementation rate for LT and survival rates of patients without LT are similar to those in the previous survey.

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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.

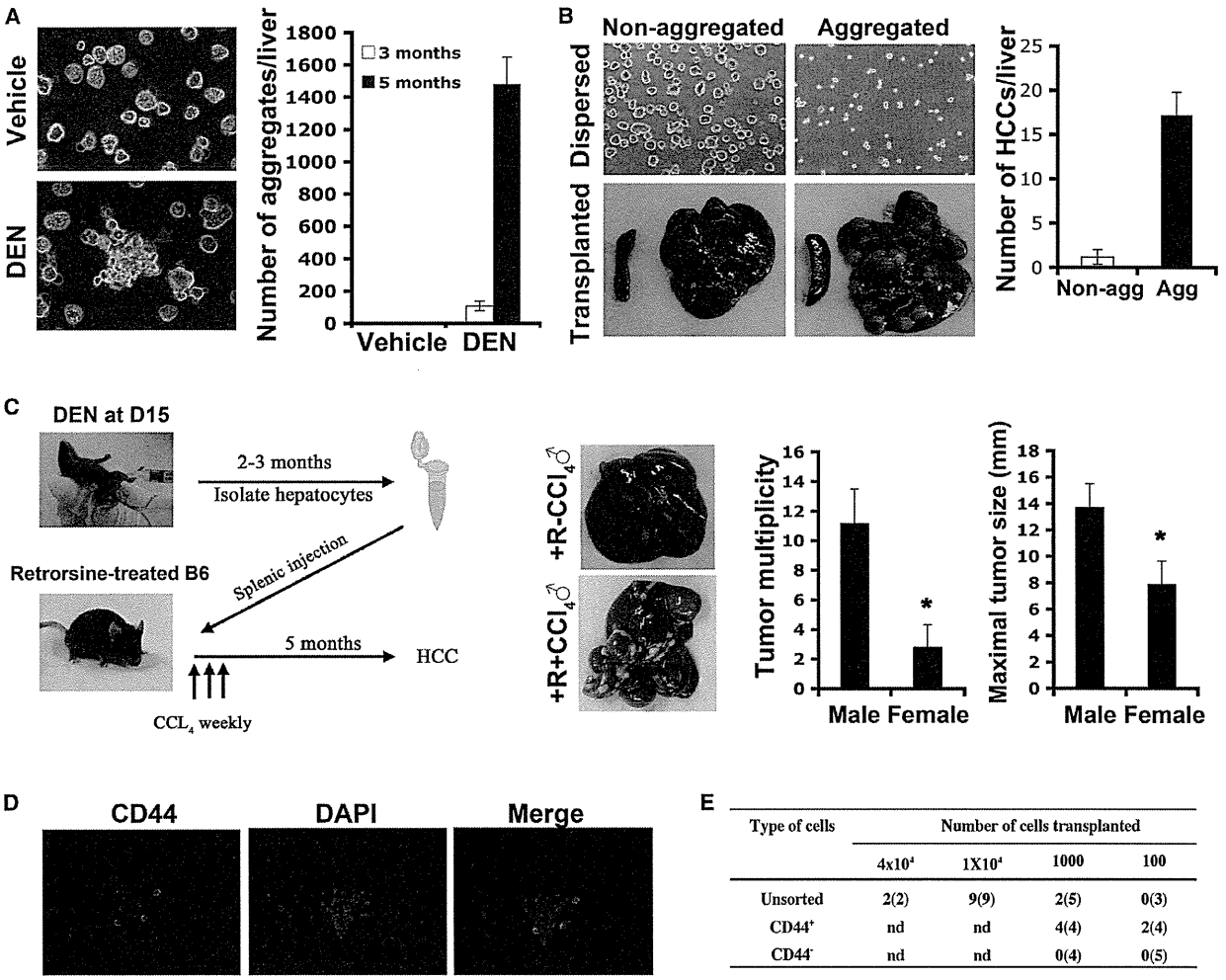
We now describe HcPC isolation from mice treated with the procarcinogen diethyl nitrosamine (DEN), which induces poorly differentiated HCC nodules within 8 to 9 months (Verna et al., 1996). Although these tumors do not evolve in the context of cirrhosis, the use of a chemical carcinogen is justified 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			
	4x10⁴	1X10⁴	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

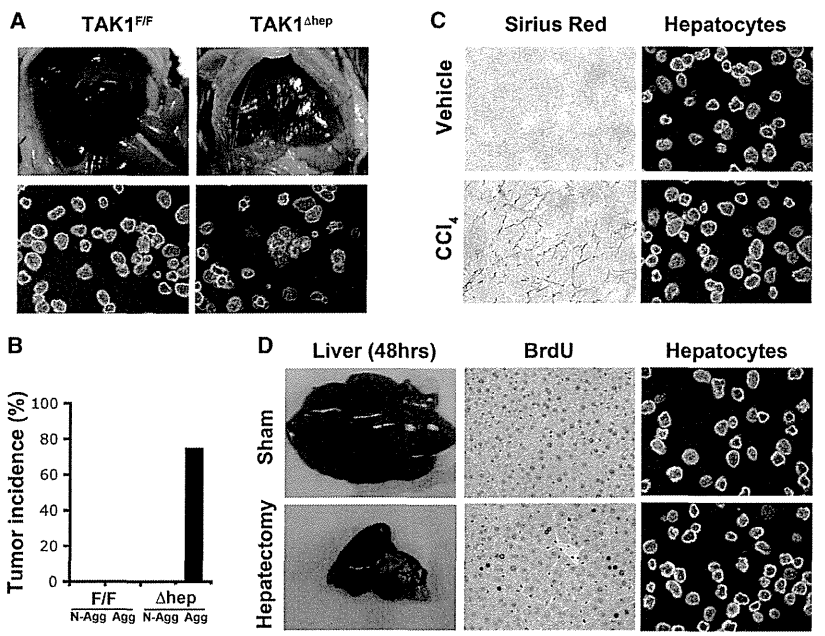


Figure 2. *Tak1*$^{\Delta hep}$ Livers Contain Collagenase-Resistant HcPC Aggregates

(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

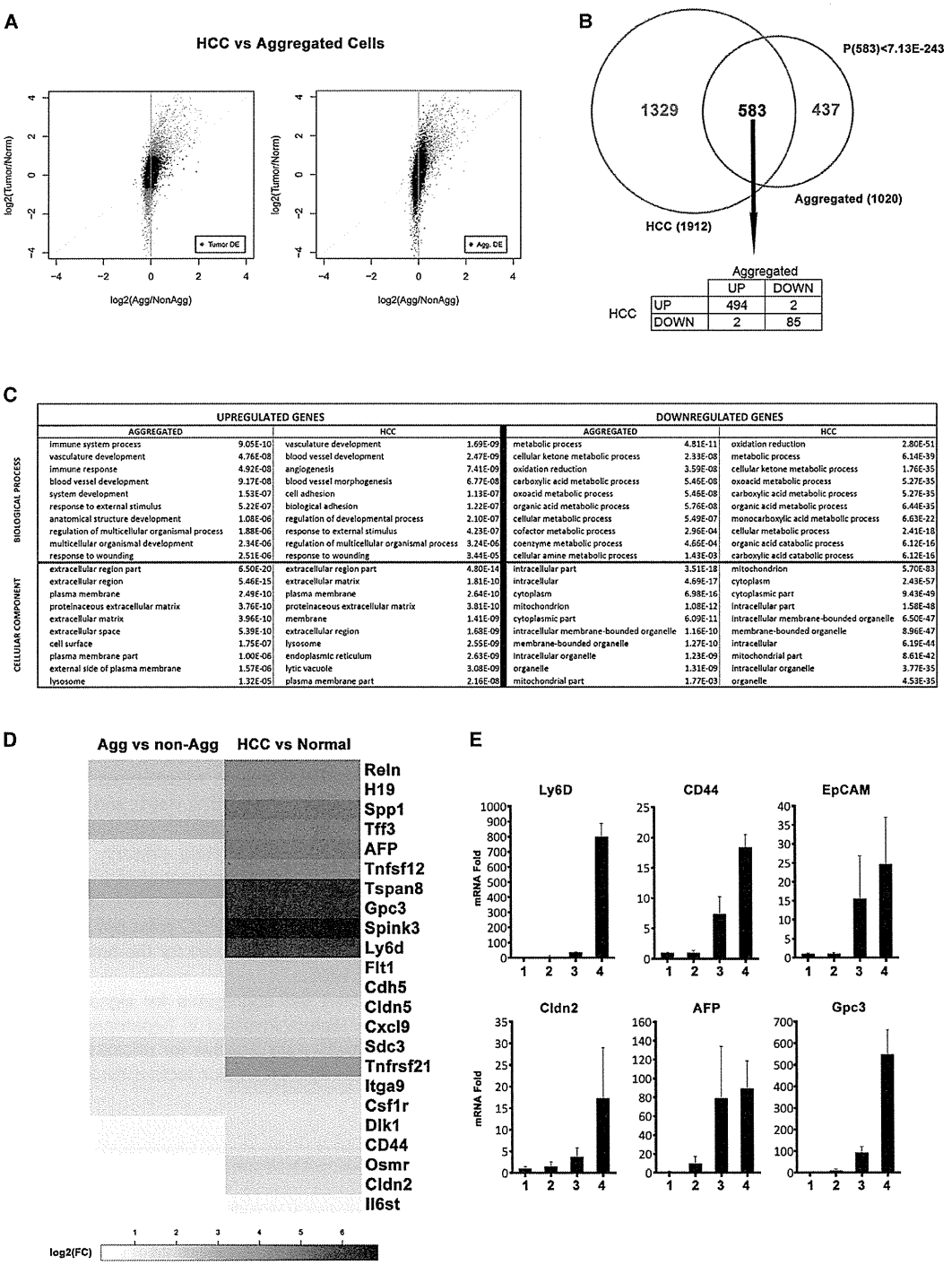


Figure 3. Aggregated Hepatocytes Exhibit an Altered Transcriptome Similar to that of HCC Cells

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.

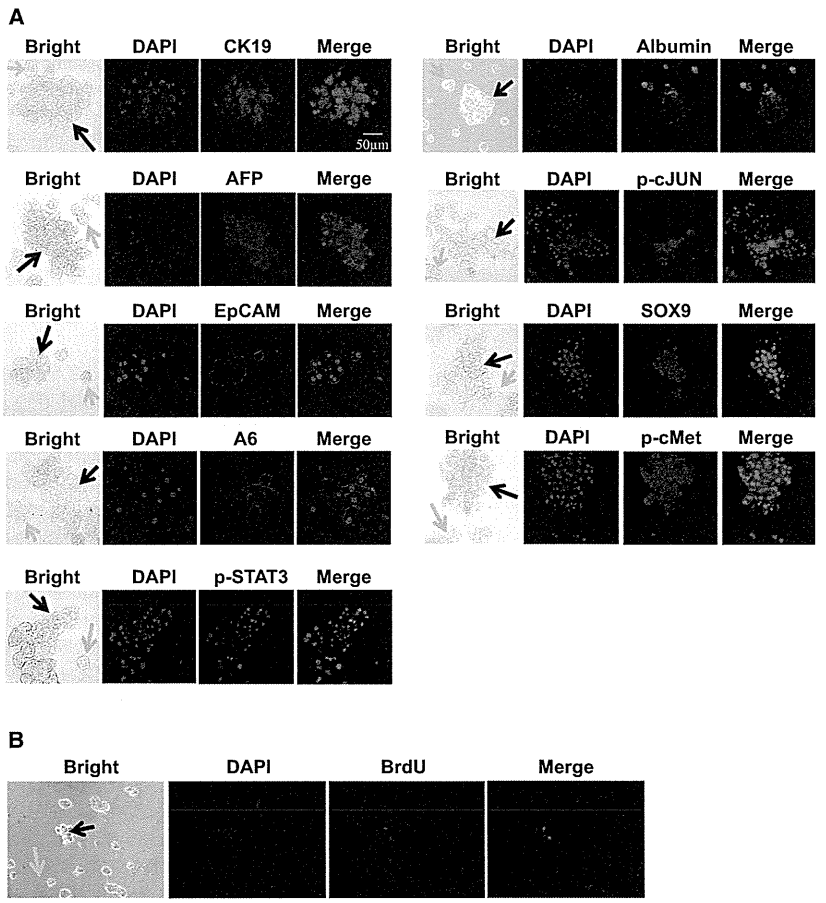


Figure 4. DEN-Induced HcPC Aggregates Express Pathways and Markers Characteristic of HCC and Hepatobiliary Stem Cells

(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

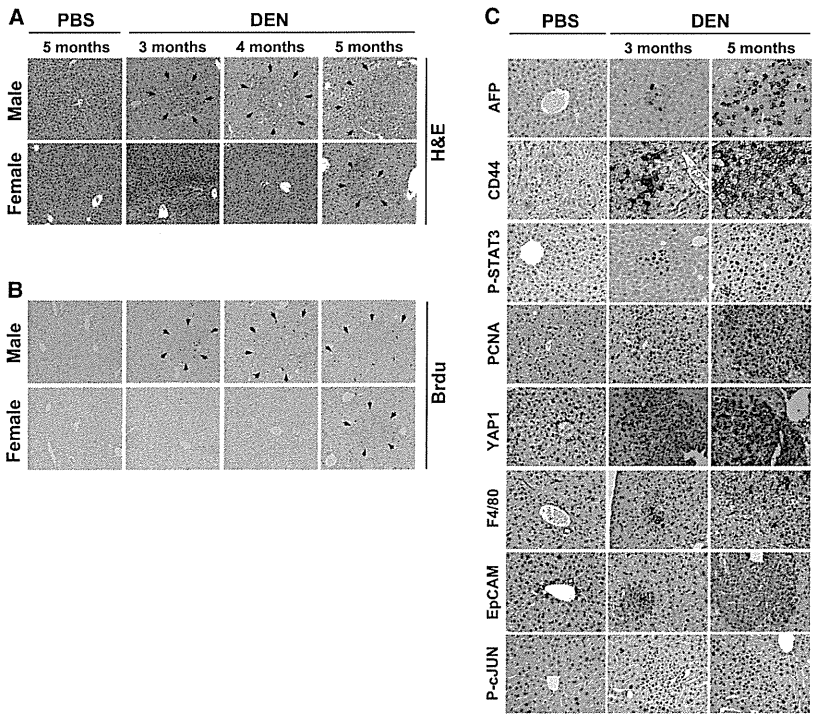


Figure 5. HcPC-Containing Aggregates May Originate from Liver Premalignant Lesions

(A and B) Male and female mice were injected with PBS or DEN at 15 days. At the indicated time points, BrdU was administrated, and livers were collected 2 hr later and stained with H&E (A) or a BrdU-specific antibody (B). Arrows indicate borders of FAH (magnification: 200×).

(C) Sections of male livers treated as above were subjected to IHC with the indicated antibodies (400×).

factors that control IL-6 expression and found that LIN28A and B were significantly upregulated in HcPCs and HCC (Figures 6D and 6E). LIN28-expressing cells were also detected within FAH (Figure 6F). As reported (Iliopoulos et al., 2009), knockdown of LIN28B in cultured HcPC or HCC cell lines decreased IL-6 expression (Figure 6G). LIN28 exerts its effects through downregulation of the microRNA (miRNA) Let-7 (Iliopoulos et al., 2009). Accordingly, miRNA array analysis of aggregated and nonaggregated hepatocytes from DEN-treated mice indicated that the amount of Let-7, along with other miRNAs that also inhibit IL-6 expression (miR194 and miR872), was lower in aggregated cells than in nonaggregated cells (Table S2).

To determine whether autocrine IL-6 production is needed for HCC growth, we silenced IL-6 expression with small hairpin RNA (shRNA) in dih10 HCC cells (He et al., 2010). This resulted in nearly a 75% decrease in IL-6 mRNA (Figure 7A) but had little effect on cell growth in the presence of growth factors, including EGF and insulin (Figure S6A). IL-6 mRNA silencing, however, diminished the ability of dih10 cells to form s.c. tumors (Figures S6B and S6C) and inhibited their ability to form HCCs and proliferate after transplantation into MUP-uPA mice (Figures 7B and S6D). To investigate the importance of autocrine IL-6 production at an earlier step, we isolated HcPC from DEN-treated WT and *Il6*$^{-/-}$ mice. Although IL-6 ablation attenuates HCC induction (Naugler et al., 2007), we still could isolate collagenase-resistant aggregates from livers of DEN-injected *Il6*$^{-/-}$ mice. Notably, IL-6 ablation did not reduce the proportion of CD44^{+} cells in the aggregates (Figures S7A and S7B). We introduced an identical number of WT and *Il6*$^{-/-}$ aggregated hepatocytes into MUP-uPA mice and scored HCC development 5 months later. The IL-6 deficiency resulted in a 2.5-fold decrease in tumorigenic potential (Figure 7C), suggesting that autocrine IL-6 contributes to HcPC to HCC progression. To confirm this point, we dispersed freshly isolated DEN-induced aggregates and transduced them with bicistronic lentiviruses encoding either scrambled or IL-6-specific shRNAs and a GFP marker. After a few days in culture, the transduced cells were introduced into MUP-uPA mice that were examined for HCC development 6 months later. Silencing of IL-6 reduced HCC generation (Figure 7D) and inhibited formation of GFP^{+} colonies within the MUP-uPA liver (Figure 7E). We also ablated IL-6 expression in mouse hepatocytes and found that this led to a marked reduction in DEN-induced tumorigenesis (Figure 7F). Thus, autocrine IL-6 production by DEN-initiated HcPC is important for HCC development. To investigate whether autocrine IL-6 signaling also occurs in human premalignant lesions, we examined needle biopsies of normal liver tissue and HCV-infected livers with dysplastic lesions. We found that 16% of all (n = 25) dysplastic lesions exhibited coexpression of LIN28 and IL-6 and contained activated STAT3 (Figure 7G). These markers were hardly detected in normal liver or nontumor portion of HCV-infected livers.

DISCUSSION

The isolation and characterization of cells that can give rise to HCC only after transplantation into an appropriate host liver undergoing chronic injury demonstrates that cancer arises from progenitor cells that are yet to become fully malignant. Importantly, unlike fully malignant HCC cells, the HcPCs we isolated cannot form s.c. tumors or even liver tumors when introduced into a nondamaged liver. Liver damage induced by uPA expression or CCl_4 treatment provides HcPCs with the proper cytokine and growth factor milieu needed for their proliferation. Although HcPCs produce IL-6, they may also depend on other cytokines such as TNF, which is produced by macrophages that are recruited to the damaged liver. In addition, uPA expression and CCl_4 treatment may enhance HcPC growth and progression through their fibrogenic effect on hepatic stellate cells. Although HCC and other cancers have been suspected to arise from

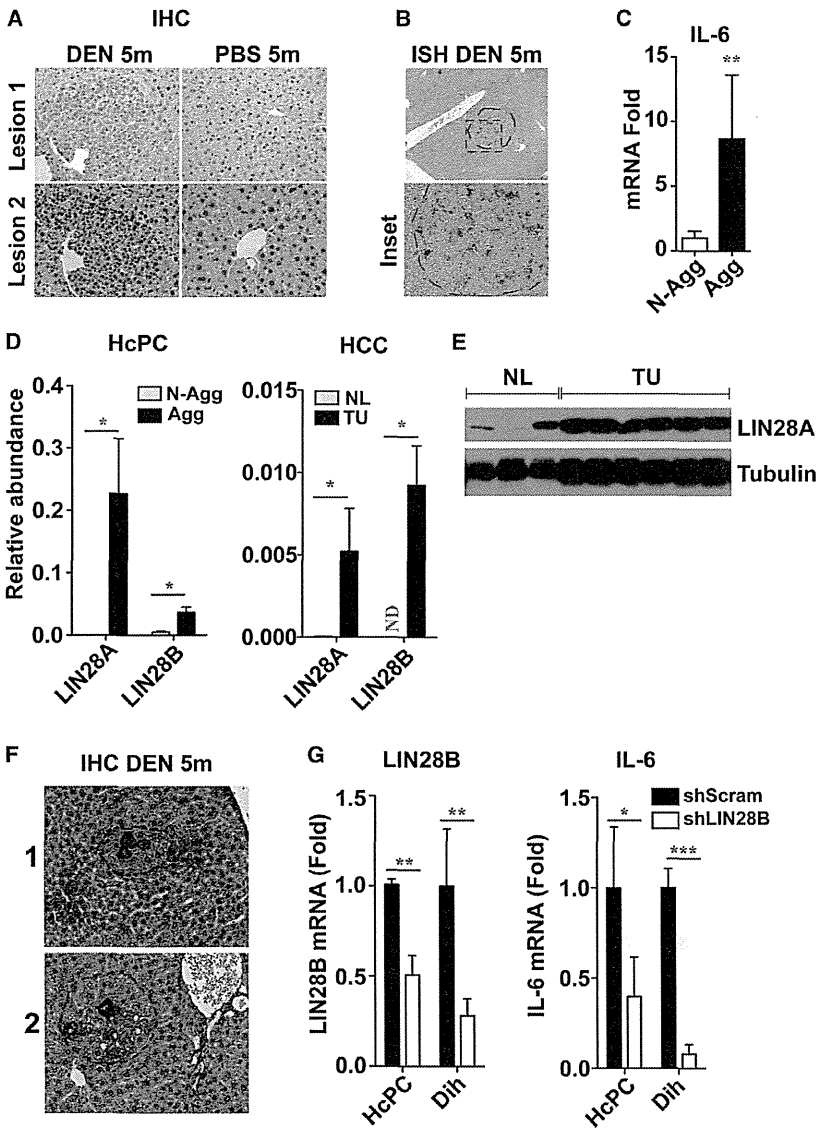


Figure 6. Liver Premalignant Lesions and HcPCs Exhibit Elevated IL-6 and LIN28 Expression

(A and B) Livers of 5-month-old DEN injected mice were analyzed for IL-6 expression by IHC (magnification: 400×) (A) and ISH (magnification: 100×, top; 400×, bottom) (B).

(C and D) Quantification of IL-6 (C) and LIN28 (D) mRNA in aggregated versus nonaggregated hepatocytes from 5-month-old DEN-treated livers and in normal versus tumor-bearing livers ($n = 6$; ± SEM) (ND, not detected).

(E) Immunoblot analyses of LIN28A in normal (NL) and tumor-bearing (TU) livers.

(F) DEN-treated livers were subjected to IHC with a LIN28A antibody. Broken lines indicate borders of FAH (400×).

(G) LIN28B was silenced with shRNA in HCC (dih) cells and cultured HcPCs, and LIN28B and IL-6 mRNAs were quantitated by qRT-PCR ($n = 3$; ± SEM).

See also Figure S5 and Table S2.

premalignant/dysplastic lesions (Hruban et al., 2007; Hytiroglou et al., 2007), a direct demonstration that such lesions progress into malignant tumors has been lacking. Based on expression of common markers—EpCAM, CD44, AFP, activated STAT3, and IL-6—that are not expressed in normal hepatocytes, we postulate that HcPCs originate from FAH or dysplastic foci, which are first observed in male mice within 3 months of DEN exposure. Indeed, the cells that are contained within the FAH are smaller than the surrounding parenchyma and are similar in size to isolated HcPCs. Importantly, FAH or premalignant dysplastic foci are not unique to DEN-treated rodents (Bannasch, 1984; Rabes, 1983), and similar lesions were detected in human cirrhotic livers (Hytiroglou et al., 2007; Seki et al., 2000; Takayama et al., 1990) in which the rate of HCC progression is 3%–5% per year (El-Serag, 2011). We found that human dysplastic lesions and mouse FAH and HcPC exhibit autocrine IL-6 signaling. HcPC are not unique to DEN-treated mice, and similar cells were isolated from *Tak1*$^{\Delta hep}$ mice in which HCC development resembles cirrhosis-associated human HCC (Inokuchi et al., 2010).

HcPC Origin and Relationship to Liver and HCC Stem Cells

Transcriptomic analysis indicates that DEN-induced HcPCs are related to both normal hepatobiliary bipotential stem cells/oval cells and HCC cells. Although HcPCs are not fully transformed, they express several markers—CD44, EpCAM, AFP, SOX9, OV6, and CK19—found to be expressed by HCC stem cells and oval cells (Guo et al., 2012; Mikhail and He, 2011; Terris et al., 2010; Yamashita et al., 2008; Zhu et al., 2010). However, unlike oval cells, which do not express albumin or AFP and do not give rise to liver tumors upon transplantation into MUP-uPA mice, HcPCs give rise to HCC after intrasplenic transplantation. Yet, unlike dih10 HCC cells, which express high levels of the HCC stem cell markers AFP, CD44, and EpCAM, HcPCs do not form s.c. tumors.

At this point, it is not clear whether HcPCs arise from oval cells or from dedifferentiated hepatocytes. Given that DEN is metabolically activated by Cyp2E1 that is expressed only in fully differentiated zone 3 hepatocytes (Tsutsumi et al., 1989) and that *Cyp2E1*$^{-/-}$ mice are refractory to DEN (Kang et al., 2007), DEN-induced HcPC are most likely derived from dedifferentiated hepatocytes. Consistent with this hypothesis, DEN-induced FAH and proliferating cells were found in zone 3 and not near bile ducts or the canals of Hering, sites at which oval cells reside