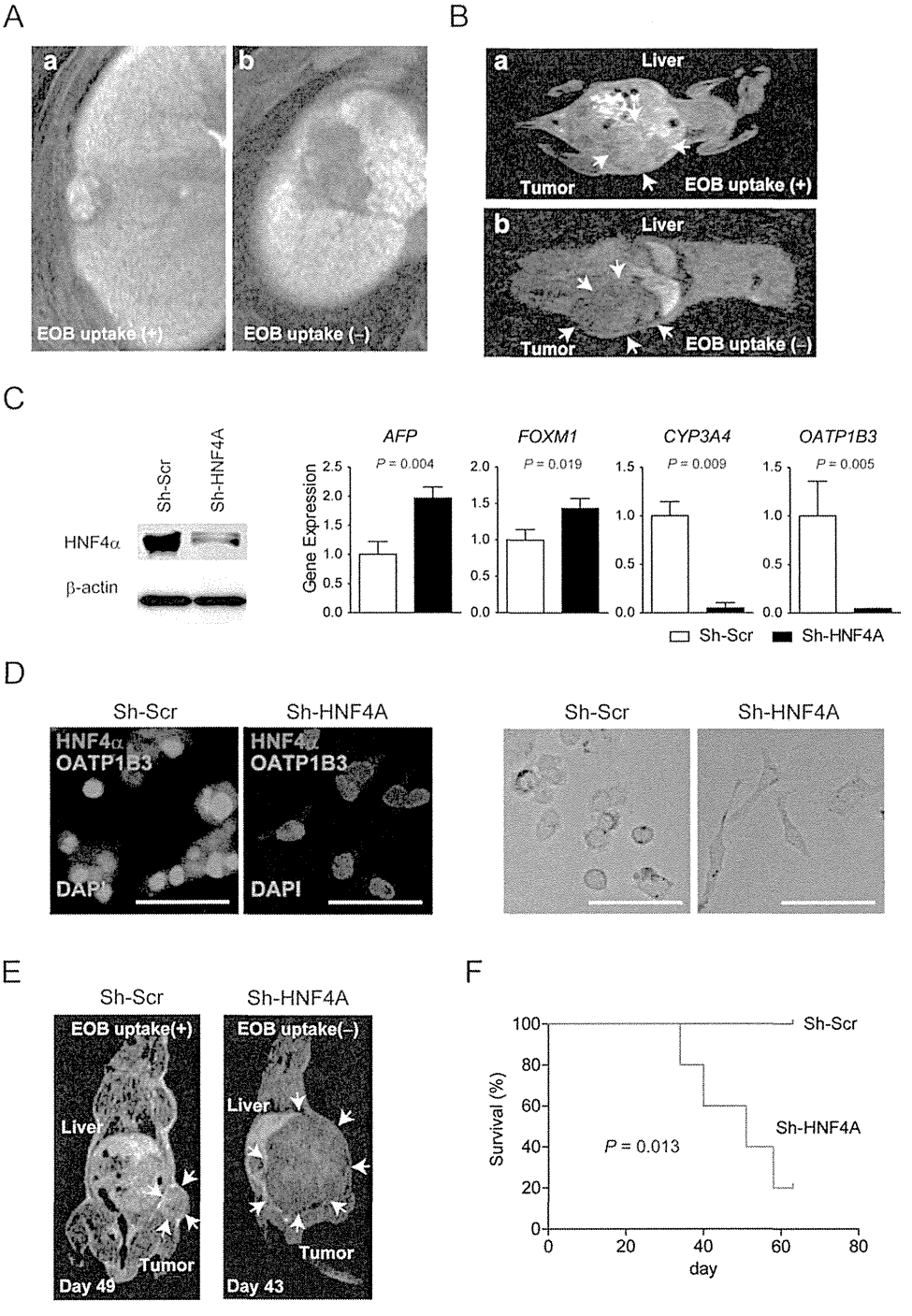

Fig. 3. HNF4α regulates a mature hepatocyte-like, less aggressive HCC phenotype coupled with Gd-EOB-DTPA uptake in hyperintense HCC. (A) MRI scans of hyperintense (a) and hypointense (b) HCCs in the hepatobiliary phase before surgery. The T/N signal intensity ratios of the images in the hepatobiliary phase were 1.02 (left panel) and 0.49 (right panel). Surgically resected specimens were subsequently used for mouse xenotransplantation. (B) MRI scans of NOD/SCID mouse xenotransplanted with hyperintense (a) and hypointense (b) HCCs in the hepatobiliary phase. The T/N signal intensity ratios of the images were 0.82 (upper panel) and 0.45 (lower panel). (C) Left panel: Expression of HNF4α protein by western blotting. Hyperintense HCC cells were harvested in dishes and treated with retroviruses encoding an expression cassette against *HNF4A* (Sh-HNF4A) or scramble sequence (Sh-Scr). Right panel: qRT-PCR of *AFP, FOXM1, CYP3A4*, and *OATP1B3* in hyperintense HCC cells transfected with Sh-Scr or Sh-HNF4A. (D) Left panel: Immunofluorescence analysis of HNF4α (red) and OATP1B3 (green) in hyperintense HCC cells transfected with Sh-Scr or Sh-HNF4A (scale bar = 100 μm). Right panel: Representative photomicrographs of hyperintense HCC cells transfected with Sh-Scr or Sh-HNF4A (scale bar = 100 μm). (E) MRI scans of NOD/SCID mouse xenotransplanted with hyperintense HCC cells transfected with Sh-Scr (day 49 after transplantation) or Sh-HNF4A (day 43 after transplantation). The T/N signal intensity ratios of the images in the hepatobiliary phase were 0.65 (left panel) and 0.34 (right panel). (F) Survival of NOD/SCID mice xenotransplanted with hyperintense HCC cells transfected with Sh-Scr (n = 5) or Sh-HNF4A (n = 5).

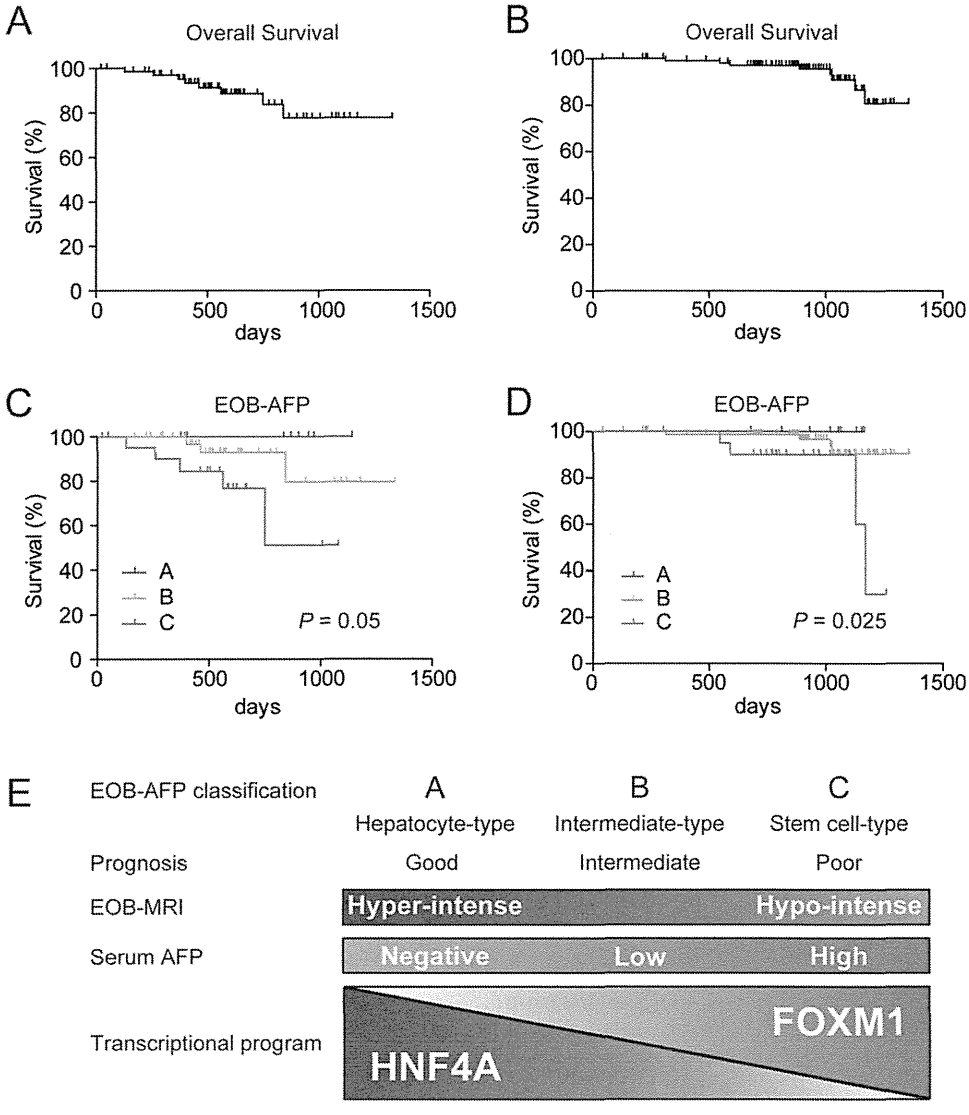

Fig. 4. Prognostic utility of the EOB-AFP classification. (A,B) Overall survival curves of Cohorts 1 (A) and 2 (B). (C,D) Overall survival curves of Cohorts 1 (C) and 2 (D) according to the EOB-AFP classification. (E) The EOB-AFP classification system and its molecular basis.

observed in Sh-HNF4A-transfected cells, whereas Sh-Scr-transfected cells still showed Gd-EOB-DTPA uptake with less tumorigenic capacity (Fig. 3E). Mice xenotransplanted with Sh-HNF4A-transfected cells had a worse prognosis compared with those xenotransplanted with Sh-Scr-transfected cells (Fig. 3F), indicating a crucial role for HNF4α in the maintenance of a mature hepatocyte-like, less aggressive HCC phenotype coupled with Gd-EOB-DTPA uptake capacity.

Prognosis of Early-Stage HCC by EOB-AFP Classification. Finally, we evaluated the prognosis of patients with HCC diagnosed by EOB-MRI and serum AFP. To exclude the potential effect of lead-time bias on survival analysis for HCCs at different stages, we evaluated the power of the EOB-AFP classification system to predict the prognosis of patients with early-stage BCLC stage 0 or A HCCs diagnosed by EOB-MRI in an independent multicenter cohort (Cohort 2). Nine of the 109 HCC cases (8.3%) were diagnosed with hyperintense HCCs and were found to be significantly associated with low serum AFP levels (Table 1). The clinicopathologic characteristics of the patients defined by the EOB-AFP classification are shown in Supporting Table 5. The median follow-up times in Cohorts 1 and 2 were 569 and 932 days, respectively. The 3-year overall survival rates in Cohorts 1 and 2 were 77.7% and 90.9%, respectively (Fig. 4A,B). The prognosis of HCC patients was not separated by TNM or BCLC stages because most of these patients were diagnosed at early stages (Fig. S4A-D); nevertheless, the EOB-AFP classification system robustly stratified HCCs according to survival with statistically significant differences between the classes (Fig. 4C,D). EOB-AFP class A patients had 100% overall survival, whereas class C patients had 30% overall survival at 1,200 days after radical resection in Cohort 2.

The prognosis of HCC patients stratified by the EOB-AFP classification was most likely affected by the malignant nature of the tumor at surgical resection, because EOB-AFP class C patients showed a 40-60% recurrence-free survival rate, whereas class A patients had a 88-100% recurrence-free survival rate at 1 year after radical resection in both cohorts (Fig. S5).

Altogether, our data, for the first time, revealed that the prognosis of early-stage HCC patients is heterogeneous and related to the malignant phenotypes of the tumors, even after successful treatment by radical resection. The EOB-AFP classification system reflects the malignant nature of the tumor and predicts the survival of early-stage HCC patients prior to surgery.

Discussion

Among several HCC staging systems currently used,[2] the BCLC system is recommended because it is linked to treatment strategy.[22] The assessment of the malignant nature of tumors coupled with current staging systems will supplement the management of early-stage HCC[23] because early recurrence after potentially curative treatment may be associated with the characteristics of the resected tumor rather than the development of a *de novo* HCC in the background liver.[24] Molecular profiling approaches have tried to evaluate the malignant features of HCCs and the surrounding noncancerous liver tissue,[3-6,12,18] although the evaluation of the potential clinical application of these approaches is ongoing. Our EOB-AFP classification system is molecularly related to the *OATP1B3* gene signature, which can be used to classify HCCs according to their stem/maturational status. Interestingly, the differential expression of *OATP1B3* was also noted in two HCC subtypes associated with the stem/maturational status, as reported recently by our group (hepatic stem cell-like and mature hepatocyte-like HCC)[12] and others (hepatoblast-type and hepatocyte type)[4] (Fig. S6). As expected, all class A HCCs were categorized as mature hepatocyte-like HCC in Cohort 1 (data not shown). The stem/maturational status defined by the EOB-AFP classification is most likely regulated by at least two transcription factors: HNF4α and FOXM1 (Fig. 4E).

HNF4α was first discovered as a liver-enriched nuclear orphan receptor activating the transcription of transthyretin genes, and it is known to regulate bile acid and cholesterol metabolism.[25] The liver-specific loss of *HNF4A* in adult mice results in hepatocyte proliferation,[26] whereas the introduction of *HNF4A* suppresses HCC growth.[27,28] Furthermore, a recent study suggested a role for *HNF4A* as a tumor suppressor in inflammation-related hepatocarcinogenesis through the regulation of microRNAs.[29] The present study demonstrated a crucial role for HNF4α in maintaining a hepatocyte-like, less aggressive phenotype coupled with Gd-EOB-DTPA uptake in a class A HCC by directly modifying *HNF4A* gene expression. Thus, *HNF4A* may work as a tumor suppressor gene and inhibit the progression of HCC, which may be related to the good prognosis of class A HCCs.

FOXM1 belongs to the forkhead superfamily of transcription factors and regulates a myriad of biologic processes including cell proliferation and differentiation.[30] The pivotal role of FOXM1 in liver development and regeneration has been reported previously.[17] FOXM1 was also required for HCC development in a mouse hepatocarcinogenesis model[31] and acted as an oncogene in a transgenic mouse model.[32] It was recently shown that FOXM1 levels are elevated in various cancers including HCC.[32,33] A prognostic role for FOXM1 in HCC patients after liver transplantation was also reported[34]; this may be associated with the metastatic capacity of tumors regulated by FOXM1.[35] As FOXM1 and AFP are known to be activated during liver regeneration and hepatocarcinogenesis, serum AFP levels may be a surrogate marker for the expression status of FOXM1 and thus facilitate the prognostic stratification of HCCs by the EOB-AFP classification.

Among the molecular markers reported to be differentially expressed between dysplastic nodule and well-differentiated HCC, we found preferential overexpression of GS in EOB-AFP class A and GPC-3 in class C HCCs. Our data suggest that class A and class C HCCs may follow different processes of early hepatocarcinogenesis events that might be associated with the differential activation of HNF4α and FOXM1, and further studies are required to obtain molecular insights into these processes.

Our overall survival data in Cohort 2 indicated that EOB-AFP class A patients had 100% overall survival, whereas class C patients had 30% overall survival at 1,200 days after radical resection. This suggests that the micro-dissemination of tumor cells in EOB-AFP class C HCC patients has already occurred by the time they are diagnosed with early-stage disease. Indeed, 50% of all class C patients showed tumor recurrence, whereas 88-100% of class A patients showed no recurrence within 1 year of resection; this is consistent with a recent study evaluating the clinical features of hyperintense HCCs[36] and may be due to

the overexpression of FOXM1, which results in the activation of metastatic programs. Therefore, these patients might have survival benefits if they receive adjuvant therapies. As several adjuvant therapies might be beneficial for HCC patients after surgical resection,[37] integration of the EOB-AFP classification system into current staging practices may provide additional therapeutic options for early-stage HCC patients who will receive surgery.

A limitation of the present study is that we used three different cohorts to reveal the molecular portraits associated with clinical imaging and prognosis (i.e., the microarray cohort of 238 HCCs of various stages for the evaluation of molecular profiling; Cohort 1 for the validation of molecular profiling and EOB-MRI findings in various stages of HCC; and Cohort 2 for evaluating the utility of EOB-MRI and serum AFP in predicting the prognosis of early-stage HCCs), which made the molecular and prognostic analyses complex. Another limitation of this study was in the evaluation of prognostic utility because it uses small retrospective cohorts. Direct evaluation of the molecular profiles and prognostic values of hyperintense HCCs should be performed in a prospective study using a large-scale HCC cohort.

Taken together, the present study demonstrates for the first time that the combined approach of noninvasive Gd-EOB-DTPA-enhanced MRI and serum AFP levels can be used preoperatively to classify resectable HCCs into three subgroups with distinct prognoses. This classification is molecularly related to the stem/maturation status of HCCs regulated by HNF4α and FOXM1. The multicenter early-stage HCC cohort that received radical resection revealed that the EOB-AFP classification is clinically useful to determine the prognosis of early-stage HCC patients. On the basis of these observations, we propose that the EOB-AFP classification system be incorporated into current HCC staging practices, especially for the management of early-stage HCCs.

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Supporting Information

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HEPATOLOGY

Efficacy of continuous plasma diafiltration therapy in critical patients with acute liver failure

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Key words
acute kidney disease, acute liver failure, blood purification therapy, plasma exchange.

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Abstract

Background and Aims: Acute liver failure (ALF) is a critical illness with high mortality. Plasma diafiltration (PDF) is a blood purification therapy that is useful for ALF patients, but it is difficult to use when those patients have multiple organ failure or unstable hemodynamics. In these patients, symptoms are also likely to exacerbate immediately after PDF therapy. We developed continuous PDF (CPDF) as a new concept in PDF therapy, and assessed its efficacy and safety in ALF patients.

Methods: Ten ALF patients (gender: M/F 6/4, Age: 47 ± 14) were employed CPDF therapy. The primary outcomes were altered liver function, measured by the model for end-stage liver disease (MELD) score, and total bilirubin and prothrombin time international normalized ratios (PT-INR), 5 days after CPDF therapy. Secondary outcomes included sequential organ failure assessment (SOFA) scores, 5 days after CPDF therapy, and the survival rate 14 days after this therapy.

Results: The MELD score (34.5–28.0; $P = 0.005$), total bilirubin (10.9–7.25 mg/dL; $P = 0.048$), PT-INR (1.89–1.31; $P = 0.084$), and SOFA score (10.0–7.5; $P < 0.039$) were improved 5 days after CPDF therapy. Nine patients were alive, and one patient died because of acute pancreatitis, complicated by ALF. There were no major adverse events related to this therapy under hemodynamic stability.

Conclusion: In the present study, CPDF therapy safely supported liver function and generally improved the condition of critically ill patients with ALF.

Introduction

Acute liver failure (ALF) is a rapidly progressing critical illness associated with a high mortality rate, and characterized by jaundice, ascites, hepatic encephalopathy, and bleeding due to severe impairment of liver function caused by massive liver necrosis.[1,2] Acute, severe hepatic necrosis releases toxic metabolites such as ammonia from the splanchnic circulation.[3]

The therapeutic strategy for ALF differs between Western countries and Japan. In Japan, an artificial liver support system (ALSS) is commonly the first-line therapy because liver transplantation is usually accomplished with living donors.[4–7] In Western countries, deceased donor liver transplantation is the first-line therapy for ALF.[8]

Plasma exchange (PE) is a fundamental and simple ALSS. However, the procedure is accompanied by adverse effects such as hypernatremia, metabolic alkalosis, citrate poisoning, and abrupt changes in colloid osmotic pressure.[9,10] In Japan, PE is often combined with continuous hemodiafiltration (CHDF), a process in which electrolyte imbalance is corrected and fluids are controlled, simultaneously. PE/CHDF therapy, however, can be expensive because it requires 3.2–4.8 L of fresh frozen plasma (FFP), along with the necessary equipment; a risk of infection is also associated with this therapy.

Plasma diafiltration (PDF or selective plasma filtration with dialysis) has been developed as an alternative to PE/CHDF therapy. PDF is a blood purification therapy in which simple PE is performed with a membrane plasma separator, while dialysate flows outside the hollow fibers.[11] In ALF patients, PDF is a useful bridge therapy for liver regeneration or transplantation.[12,13] However, PDF therapy is difficult to use in critically ill ALF patients with complicated multiple organ failure, especially in those with unstable hemodynamics. Symptoms can also be exacerbated immediately after PDF therapy because this conventional PDF therapy is used intermittently throughout an 8-h day.

In this study, we designed a new PDF therapy concept, termed continuous PDF (CPDF), and conducted an observational study to assess the efficacy and safety of this therapy for patients with ALF.

Materials and methods

The study protocols conformed to the ethical guidelines of the 2008 Declaration of Helsinki. This study was approved by the

Table 1 Demographics of ten ALF patients

No.	Age (years)	Gender (M/F)	Etiology	MELD score	PT-INR	Total bilirubin (mg/dL)	Encephalopathy grade	M.V.	SOFA score	Total duration (days)	Outcome
1	33	F	HELLP	29	1.81	5.4	—	+	13	9	Alive
2	37	M	Alcohol	21	1.57	11.3	—	–	5	14	Alive
3	60	F	AIH	40	1.90	34	III	–	11	5	Death
4	38	M	Drug	43	4.83	5.1	III	–	8	8	Alive
5	54	M	HBV	34	1.88	28	II	+	14	12	Alive
6	63	F	AIH	26	2.30	15.2	II	+	8	9	Alive
7	26	M	Unknown	36	1.96	10.5	IV	–	11	9	Alive
8	47	F	HBV	51	9.57	4.5	III	+	9	7	Alive
9	34	M	Drug	35	1.76	10.4	II	+	14	14	Alive
10	38	M	Unknown	27	1.57	65.3	—	–	12	11	Alive

—, not detected; AIH, autoimmune hepatitis; ALF, acute liver failure; HBV, hepatitis B virus; HELLP, hemolysis elevated liver enzymes; MELD, model for end-stage liver disease; M.V., mechanical ventilation; PT-INR, prothrombin time international normalized ratios; SOFA, sequential organ failure assessment.

institutional review board of the Kanazawa University Graduate School of Medicine.

Patients. Ten patients in the intensive care unit (ICU) at Kanazawa University Hospital from January 2011 to April 2013 received CPDF therapy. These patients fulfilled the Japanese diagnostic criteria for ALF,[14] which consists of prothrombin time international normalized ratios (PT-INR) of > 1.5 caused by severe liver damage within 8 weeks of onset of the symptoms when prior liver function was estimated as normal. Informed written consent of the patients or responsible family members was obtained prior to enrollment.

CPDF decision process and implementation. The decision to employ CPDF for each patient included fulfilling the diagnostic criteria of ALF; complicated renal dysfunction that compromised fluid management; and ongoing fluid management concerns including significant ascites, edema, and/or fluid overload. The patient characteristics are listed in Table 1. CPDF therapy was performed using an Evacure EC-2A plasma separator (Kuraray, Tokyo, Japan) at a blood flow rate of 80 mL/min. Filtered replacement fluid for artificial kidneys (Sublood-BS; Fuso Pharmaceutical, Osaka, Japan) was infused at a dialysate flow rate of 400 mL/h and a replacement flow rate of 280 mL/h. FFP was infused intravenously at 120 mL/h, and nafamostat mesilate (Futhan; Torii Pharmaceutical, Tokyo, Japan) was used as an anticoagulant.

The CPDF column was replaced every 24–48 h unless disabled. Patients were monitored closely for signs and symptoms of adverse effects or complications during this therapy. We decided that the criteria for discontinuation of CPDF was a total bilirubin of < 5.0 mg/dL and PT-INR < 1.2, and the point in time at which the survival rate improved.

The primary outcomes were altered liver function measured by the model for end-stage liver disease (MELD) score, total bilirubin, and PT-INR 5 days after CPDF therapy. This time point was chosen because patients with ALF are generally re-assessed for liver transplantation every 5 days. Secondary outcomes included sequential organ failure assessment (SOFA) scores 5 days after CPDF therapy and survival rate 14 days after CPDF therapy. SOFA is a scoring system to determine the extent of a person's multiple organ function or rate of failure based on six different scores, such as respiratory, cardiovascular, hepatic, coagulation, renal, and neurological systems.[15]

Statistical analysis. Data are expressed as medians and interquartile ranges. Differences in variables before and after CPDF therapy were examined by paired Student's *t*-test after a symmetrical distribution was confirmed. $P < 0.05$ indicated statistical significance. We also considered clinical efficacy analyzed by effect size (ES) using Cohen's *d*, which measures the strength of the relationship before and after CPDF therapy due to the low number of patients in this study. We determined that ES > 0.2 = small, ES > 0.5 = moderate, and ES > 0.8 = large efficacy of this therapy based on Cohen's criteria.

Results

Demographics. We assessed 10 patients with ALF. All patients were diagnosed with ALF and received CPDF therapy. The characteristics of the patients are shown in Table 1. The etiology for ALF was variable, and the average age of patients was 47 ± 14 years (range, 26–64). Seven ALF patients had overt hepatic encephalopathy. Five ALF patients received mechanical ventilation therapy, while there was no patient with inotropic and vasopressor support.

Primary outcomes. The MELD score improved significantly from 34.5 to 28.0 ($P = 0.005$; Fig. 1a), resulting in high clinical effectiveness (ES = 0.78; Table 2) after CPDF therapy. Total bilirubin also significantly improved from 10.9 to 7.25 mg/dL ($P = 0.048$; Fig. 1b), resulting in moderate clinical effectiveness (ES = 0.65; Table 2) after CPDF therapy. PT-INR had a tendency to improve from 1.89 to 1.31 ($P = 0.084$; Fig. 1c), resulting in high clinical effectiveness (ES = 0.92; Table 2) after CPDF therapy.

Secondary outcomes. The SOFA score decreased from 10.0 to 7.5 ($P < 0.039$; Fig. 1d), resulting in moderate clinical

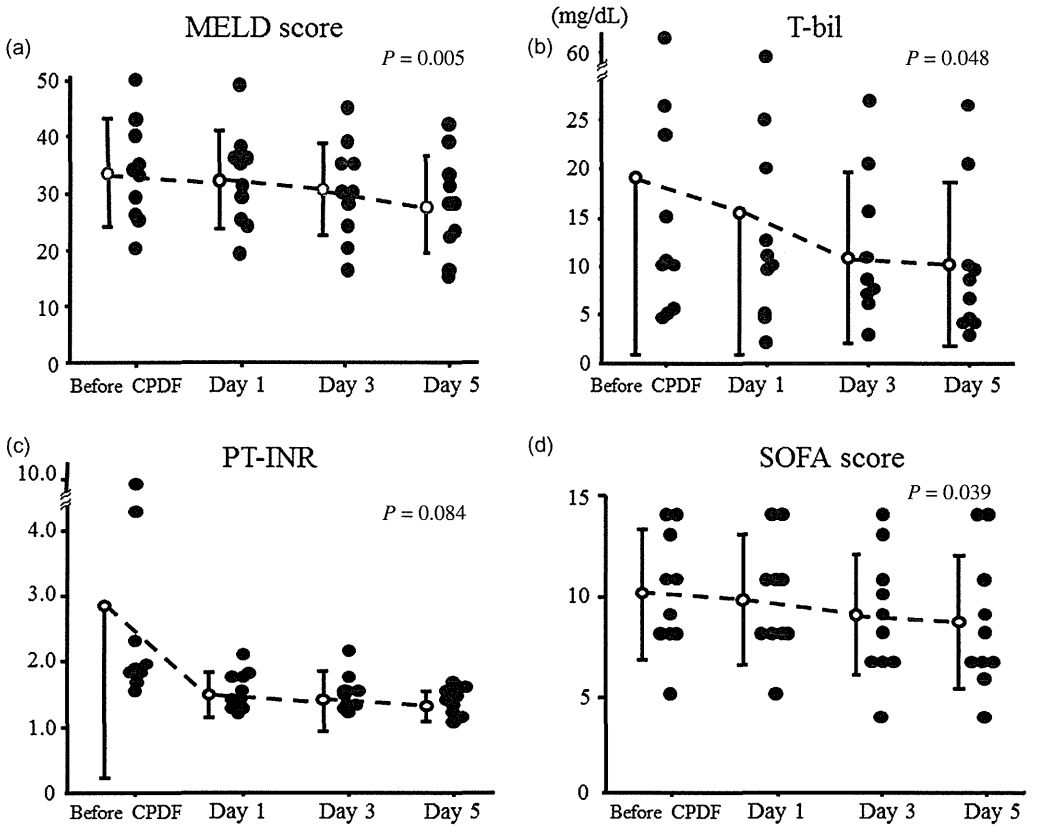

Figure 1 ALF patient parameters. (a–c) Primary study outcomes of this study. MELD score (a), total bilirubin value (b), and PT-INR (c) were improved 5 days after CPDF therapy. (d) Secondary study outcomes of this study. SOFA score (d) were improved after 5 days CPDF therapy. Each value are plotted, and also expressed as means ± SD.

Table 2 Parameter alteration 5 days after CPDF Treatment

Variables	Before CPDF	After CPDF	Effect size	95% CI	*P* value
SOFA score	10 (5–14)	7.5 (4–14)	0.47	0.085–2.715	0.039
MELD score	34.5 (21–51)	28 (14–42)	0.78	2.611–10.789	0.005
Total bilirubin (mg/dL)	10.9 (4.5–65.3)	7.25 (3.4–27.7)	0.65	0.076–17.82	0.048
Ammonia (mg/dL)	119 (60–316)	143 (58–407)	0.46	−115–24.2	0.174
Creatinine (mg/dL)	1.44 (0.47–4.79)	1.36 (0.27–3.12)	0.37	−0.176–1.198	0.127
PT-INR	1.89 (1.57–9.57)	1.31 (1.17)	0.92	−0.258–3.384	0.084
ALT (IU/L)	123 (21–10 892)	39 (17–482)	0.77	−743–495	0.129
AST (IU/L)	109 (60–15 183)	52.5 (28–436)	0.82	−759–6440	0.108
Albumin (g/dL)	3.1 (2.6–3.7)	2.7 (2.3–3.2)	1.08	−0.017–0.777	0.059
MAP (mm Hg)	82 (62–112)	89.5 (72–115)	0.23	−22.4–3.42	0.131
Heart rate (beat/min)	89.5 (76–122)	92 (57–135)	0.34	−5.49–17.49	0.268
PaO_2/FiO_2 ratio	330 (188–583)	383 (240–567)	0.38	−139–59.4	0.195

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; CPDF, continuous plasma diafiltration; MAP, mean arterial pressure; MELD, model for end-stage liver disease; PT-INR, prothrombin time international normalized ratios; SOFA, sequential organ failure assessment.

effectiveness (ES = 0.47; Table 2) after CPDF therapy. Nine patients were alive and discharged from the ICU, and one patient died due to acute pancreatitis complicated by ALF. CPDF therapy had no major adverse effect, including bleeding, and maintained hemodynamic stability.

Other outcomes. Parameters of hepatocyte injury such as aspartate aminotransferase/alanine aminotransferase also decreased (ES = 0.82 and 0.77, respectively) by CPDF therapy. The creatinine value (ES = 0.37) had a small clinical effectiveness (Table 2).

Five ALF patients with overt encephalopathy were controlled by CPDF therapy, while two patients with uncontrolled encephalopathy were treated with CPDF therapy combined with hemodialysis to control overt encephalopathy. Circulation parameters such as mean arterial pressure and heart rate were maintained (Table 2) without inotropic and vasopressor support during CPDF treatment period. Oxygenation index (PaO_2/FiO_2) as a measure of pulmonary function increased after this treatment (Table 2). In this study, mechanical ventilation was indicated not only by hepatic encephalopathy but also by impaired respiratory function because of massive ascites or pleural effusion, or an unstable hemodynamic state. Two patients were withdrawn from mechanical ventilation after pulmonary function improved.

Moreover, we could employ this treatment without any adverse events, such as infections and unstable hemodynamics.

Discussion

In the present study, we observed that CPDF therapy improved liver function in critical ALF patients with beneficial effects on renal, pulmonary, and hemodynamic function that led to an improved SOFA score which reflected the severity of critical illness without any adverse effects.

ALSS is the first-line therapy for ALF patients until liver regeneration or transplantation because only a small proportion of ALF patients can receive deceased donor liver transplants, in Japan.[4,6] Recently, several types of ALSS methodologies such as Prometheus or the Molecular Adsorbent Recirculating System have been developed, primarily to eliminate toxic substances. However, these therapies require complex equipment and are expensive.[16,17]

Conventional PDF therapy is simple, less expensive, and results in fewer adverse events than other therapies. Consequently, this therapy has been demonstrated to be one of the most useful blood purification therapies for ALF patients.[11,13] However, conventional PDF therapy, which is used intermittently throughout an 8-h day, does not usually maintain hemodynamic stability. Because we dealt with critical patients in ALF, a group in whom hemodynamic instability and a high SOFA score implying high mortality in this observational study, we employed CPDF therapy, which can maintain stable hemodynamics in most cases.

In this study, we showed that the efficacy of CPDF liver support. Moreover, CPDF therapy provides some of the characteristics of renal replacement treatment for patients with ALF; these patients frequently have renal functional impairment. CPDF therapy, as well as CHDF, avoid abrupt changes and successively remove toxic substances while managing fluid balance.[18] This reduces pulmonary edema and the exacerbation of impaired respiratory function and satisfactorily supports liver function.[19,20] In fact, CPDF therapy improved a pulmonary function in several patients of this study. Thus, CPDF therapy can improve the function of multiple organs, possibly making this therapy superior to conventional PDF therapy. Moreover, these benefits suggest that CPDF therapy is cost-effective and helps avoid the possibility of infection.

Whether CPDF therapy can effectively remove toxic substances (e.g. ammonia) during rapid disease progression, caused by hepatic encephalopathy, remains to be determined. Similarly, an assessment of how CPDF therapy maintains decreased plasma albumin value also remains to be determined, as we did not observe improvements in ammonia values. This study included two patients with high ammonia values and uncontrolled encephalopathy; these patients employed CPDF, combined with hemodialysis, to control the encephalopathy. The plasma albumin value was decreased during the CPDF therapy because the sieving coefficient of 0.3 for albumin selectively removed low- and intermediate-molecular weight, albumin-bound substances in the plasma separator (Evacure EC-2A). Albumin loss was managed by intravenously administering 12.5–25 g of albumin to maintain the plasma albumin level.

Some of the limitations of this study should also be considered. The first is that the therapeutic strategy for ALF is different, in Japan, from that in Western countries because few deceased donor liver transplants are performed. The second is that although our study was prospective, it was not a randomized controlled study (RCT), and the sample size was small. An RCT should be explored to determine the effects of CPDF in patients with ALF.

In summary, CPDF therapy, a new concept in ALSS, improved liver function in critically ill ALF patients and had beneficial effects on multiple organ functions, suggesting that it may be an alternative or at least one of the useful and desirable forms of ALSS for ALF patients.

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REVIEW

Orchestration of hepatocellular carcinoma development by diverse liver cancer stem cells

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Abstract Hepatocellular carcinoma (HCC) is one of the world's most aggressive diseases and carries a poor prognosis for patients. Recent evidence suggests that HCC is organized by cancer stem cells (CSCs), which are a subset of cells with stem cell-like features. CSCs are considered a pivotal target for the eradication of cancer, and liver CSCs have been investigated using various stem cell markers. Several hepatic stem/progenitor markers have been shown to be useful for isolating putative CSCs from HCC, although the expression patterns and phenotypic diversity of CSCs purified by these markers remain obscure. Recently, we found that liver CSCs defined by different markers show unique features of tumorigenicity and metastasis, with phenotypes closely associated with committed liver lineages. Furthermore, our data suggest that these distinct CSCs collaborate to orchestrate the tumorigenicity and metastasis of HCC. In this review article, we summarize the recent advances in understanding the pathogenesis and heterogeneity of liver CSCs.

Keywords Hepatocellular carcinoma · Cancer stem cell · Tumorigenicity · Metastasis

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide [1]. Its prevalence is mostly attributed to hepatitis B virus or hepatitis C virus infection, and high incidence is observed in Asia and Africa [2]. Increasing occurrences and mortality from HCC have also been observed in most industrialized countries [3]. Therefore, there is an urgent need to develop effective diagnostic and treatment strategies against this disease.

HCC is a heterogeneous disease in terms of morphology, biological behavior, response to treatment, and molecular profile [4]. This heterogeneity has traditionally been explained by the clonal evolution of tumor cells resulting from the progressive accumulation of multiple genetic and epigenetic changes [5, 6]. However, recent studies suggest that its heterogeneity may result from the hierarchical organization of tumor cells by a subset of cells with stem and progenitor cell features known as cancer stem cells (CSCs) [7]. CSCs are highly tumorigenic, metastatic, chemo- and radiotherapy resistant, responsible for tumor relapse after therapy, and able to divide symmetrically or asymmetrically to orchestrate the tumor mass [8]. Therefore, they are considered to be a pivotal target for eradicating HCC [9]. In this review, we summarize recent findings on liver CSCs in terms of heterogeneity and discuss an HCC treatment strategy that targets them.

CSC hypothesis

Cancer cells and stem cells have similar capabilities with respect to self-renewal, limitless division, and the generation of heterogeneous cell populations. The observation of these similarities many years ago led to the proposal that

cancer might be a type of abnormal stem cell disease [10], a concept which has recently been revisited [11]. The generally acknowledged definition of a CSC is a cell within a tumor that possesses the ability to self-renew and to give rise to heterogeneous lineages of cancer cells that comprise tumors in immunodeficient mice [11]. Experimentally, putative CSCs have been isolated using cell surface markers specific for normal stem cells. Stem cell-like features of CSCs have been confirmed by functional in vitro clonogenicity and in vivo tumorigenicity assays. Moreover, accumulating evidence suggests that CSCs play a role in perpetuating various cancers including leukemia and solid tumors [12–18].

In HCC, several markers are reported to enrich the CSC population, including the epithelial cell adhesion molecule (EpCAM), CD133, CD90, CD44, CD24, CD13, and oval cell marker OV6, as well as Hoechst dye efflux or aldehyde dehydrogenase activities [19–25]. Most of these markers are expressed in normal hepatic progenitors known as oncofetal markers [20–22, 26–35]. These marker-positive cells were experimentally confirmed to be more tumorigenic than marker-negative cells in immunodeficient mice using cell lines [9]. Among them, calcium channel α2δ1 isoform5, EpCAM, CD90, and CD133 are the markers confirmed thus far to enrich CSCs from primary HCCs [36, 37]. Recent studies have shown that some of these liver CSC markers are also functionally involved in the maintenance of CSC features (Table 1). EpCAM enhances Wnt signaling in ES cells and cancer [38, 39], and CD133 expression may maintain $CD133^+$ liver CSCs through the activation of neurotensin/IL-8/CXCL1 signaling [40]. CD44 regulates the redox status [41], while CD13 decreases cell damage induced by oxidative stress after exposure to genotoxic reagents [19]. Furthermore, a recent study demonstrated that the calcium channel α2δ1 isoform5, recognized by a monoclonal antibody 1B50-1, is expressed in liver CSCs and regulates calcium influx and ERK signaling [37]. Thus, the functional involvement of most liver CSC markers potentially makes them a good target for the eradication of liver CSCs. In particular, cell surface markers detected in liver CSCs may be good targets for immunotherapy.

Table 1 Cell surface markers in liver CSCs

Cell surface markers	Function in CSCs
Calcium channel α2δ1 isoform5	Calcium influx and activation of ERK signaling
CD13	ROS-induced DNA damage reduction
CD133	Neurotensin-interleukin-8-CXCL1 signaling
CD24	STAT3 mediated NANOG regulation
CD44	Regulation of redox status through xCT
CD90	Unknown
DLK1	Unknown
EpCAM	Activation of Wnt signaling
OV6	Unknown

Heterogeneity of liver CSCs

As described above, various hepatic progenitor markers have been detected in the population of liver CSCs. Purified cell populations using certain stem cell markers show CSC features such as high tumorigenicity, an invasive nature, and chemo- and radiotherapy resistance. However, it is unclear how these markers are expressed in primary HCC tissues or HCC cell lines. It is also unclear whether the CSCs expressing these markers exist in all HCCs or are restricted to a certain subtype. This is an especially important issue when treating HCC patients using molecularly targeted therapy against certain marker-positive CSCs.

In normal fetal livers, hepatoblasts express the biliary markers CK19 and EpCAM, as well as the hepatocyte markers albumin and alpha fetoprotein (AFP) [26, 27, 42, 43]. In addition, numerous studies have demonstrated that hepatic progenitor cells express a variety of markers putatively detected in various ectodermal or mesodermal lineages, including nestin, NCAM, CD34 and c-Kit, CD133, CD90, E-cadherin, and Dlk1 [44]. Hepatoblasts are also considered a heterogeneous population potentially organized in a hierarchical manner with various degrees of differentiation that may be related to their expression of stem cell markers [45]. Indeed, recent studies demonstrated that the characteristics of hepatic progenitors expressing different markers show distinct natures [32, 46]. Normal $EpCAM^+$ and $CD90^+$ oval cells represent two distinct populations: the former expresses classical oval cell markers such as AFP, OV-1, and CK19, and the latter expresses desmin and a-SMA but not AFP, OV-1, or CK19, which indicates that $CD90^+$ populations are more likely to be mesenchymal cells.

We explored the expression patterns of the representative liver CSC markers CD133, CD90, and EpCAM in primary HCC, and found that $EpCAM^+$ and $CD90^+$ CSCs show different gene expression patterns and cell morphology [36]. We further explored the tumorigenic capacity of sorted cells isolated from 15 primary HCCs and 7 liver cancer cell lines [36]. Although the number of samples analyzed was small, tumorigenic $EpCAM^+$, $CD133^+$, or $CD90^+$ CSCs were obtained in 26.6 % ($n = 4$), 20 % ($n = 3$), and 13.3 % ($n = 2$) of 15 HCCs, respectively, when xenotransplanted into NOD/SCID mice.

Interestingly, no EpCAM/CD90 double positive cells were detected in primary HCC, and $EpCAM^+$ and $CD90^+$ cells were distinctive with different tumorigenic/metastatic

capacities; that is, $EpCAM^+$ cells were associated with a high tumorigenic capacity and hepatic epithelial stem cell features, while $CD90^+$ cells had a metastatic propensity with mesenchymal vascular endothelial cell features. Importantly, the existence of $EpCAM^+$ cells correlated with high serum AFP values with a tendency for portal vein invasion, whereas the existence of $CD90^+$ cells was associated with a high incidence of distant organ metastasis. Furthermore, $CD90^+$ CSCs abundantly expressed c-Kit and showed chemosensitivity against the c-Kit inhibitor imatinib mesylate, whereas $EpCAM^+$ CSCs showed no such chemosensitivity. These data demonstrate that liver CSCs are not a single entity but exist heterogeneously with distinct CSC marker expression, suggesting that no common liver CSCs expressing particular stem cell markers exist in all HCCs. Our data also indicate that the presence of distinct CSCs is a key determinant of cancer phenotypes in terms of tumorigenicity and metastatic propensity, which may influence the clinical outcome of HCC.

The distinct nature of $EpCAM^+$ and $CD90^+$ liver CSCs raises the question whether these different types of CSCs originate from the same or different type of cells. This question remains elusive, but a recent study investigating three independent cell clones established from the same HCC specimen revealed that these clones maintain common karyotype abnormality but express EpCAM, CD90, and CD133 distinctively with different chemosensitivities against sunitinib [47], suggesting that distinct liver CSCs expressing different markers may originate from the same type of cells. In terms of liver CSC origin, a recent study demonstrated that acquisition of liver CSC properties is independent of the cell of origin, and liver CSCs can originate from hepatic progenitor cells, hepatoblasts, or adult hepatocytes in mice by forced H-Ras/SV40LT induction and subsequent oncogenic reprogramming [48]. In addition, another study has demonstrated the unexpected plasticity of normal mature hepatocytes to dedifferentiate into progenitor cells in rats [49], and this type of plasticity has also been reported in breast non-CSCs [50, 51]. Given the cellular plasticity reported in normal and cancer cells described above, it is reasonable to speculate that a similar plasticity may exist in $EpCAM^+$ and $CD90^+$ CSCs that can convert their tumorigenic/metastatic phenotypes and marker expression status. Further studies are required to clarify the role of cell plasticity on heterogeneity of HCC [36].

Interaction of distinct cell lineages in liver organogenesis and hepatocarcinogenesis

Embryogenesis is characterized by the ordered emergence of an organism made up of a multitude of stem and differentiated cells. Various signaling pathways play crucial roles in the dynamic cell proliferation and motility of organogenesis [52]. For example, in liver organogenesis, liver specification signaling is activated at the ventral endoderm (hepatic endoderm) by the paracrine secretion of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) from the cardiac mesoderm and septum transversum, respectively [53–55]. Wnt/beta-catenin signaling may also induce hepatic specification [56]. Activation of these signaling pathways results in the formation of the liver bud from the hepatic endoderm. The liver bud is considered to be the earliest developmental stage of liver organogenesis, which coincides with the expression of albumin and AFP [57].

Once the hepatic endoderm is specified and the liver bud begins to grow, the cells become hepatoblasts and have the ability to differentiate into hepatic and biliary lineages as bipotent progenitors. Epithelial and mesenchymal cells located in the endoderm and/or mesoderm collaborate to orchestrate liver organogenesis [58] (Fig 1a). The importance of this was elegantly demonstrated in a recent in vitro study generating liver buds using induced pluripotent stem cells, human umbilical vascular endothelial cells, and mesenchymal stem cells [59].

Embryogenesis and tumorigenesis share similar features including autonomous cell proliferation, motility, homing, dynamic morphologic changes, cellular heterogeneity, and interactions with the microenvironment. Liver cancer development may partially recapitulate fetal liver development in terms of the emergence of cells expressing certain stem cell markers and the activation of signaling pathways during liver development (Fig 1b). Indeed, signaling pathways activated in normal liver development are known to be activated and may be involved in the development and maintenance of liver CSCs. FGF and Wnt signaling has also been implicated in the development of HCC [60–63], with the latter shown to regulate the self-renewal of hepatoblasts and liver CSCs [20, 31, 64–68].

Moreover, as observed in the process of normal liver development, the collaboration of CSCs with epithelial or mesenchymal cell features may play an important role in the tumorigenicity and metastasis of HCC (Fig 1b). Our data indicate that $EpCAM^+$ CSCs have no metastatic capacity for distant sites when subcutaneously injected into NOD/SCID mice. However, when $CD90^+$ CSCs were co-injected with $EpCAM^+$ CSCs, $EpCAM^+$ cells could metastasize to the lung, whereas subcutaneous primary tumors showed no difference in size [36]. Furthermore, although imatinib mesylate treatment had little effect on the size of primary subcutaneous tumors, it significantly suppressed lung metastasis potentially through the suppression of $CD90^+$ CSCs.

We found that the effect of $CD90^+$ CSCs on the enhanced cell motility of $EpCAM^+$ cells was mediated, at least in part,

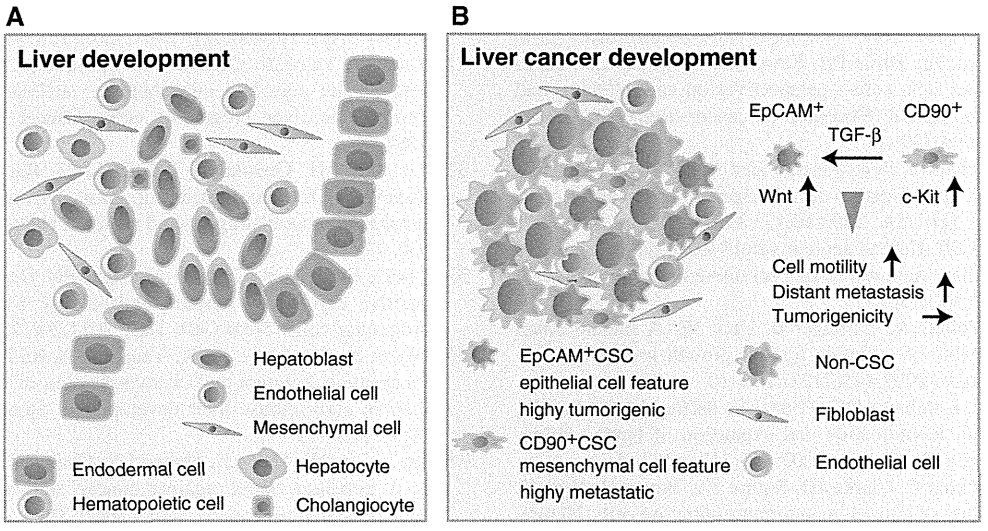

Fig. 1 Interaction of epithelial and mesenchymal cells in liver development and liver cancer development. **a** Liver bud formation is regulated by the activation of FGF, BMP, and Wnt signaling through the interaction of endodermal cells, endothelial cells, and mesenchymal cells. **b** Liver cancer development is regulated by the interaction of $EpCAM^+$ and $CD90^+$ CSCs. In primary HCC, $EpCAM^+$ and $CD90^+$ CSCs distinctively exist. $EpCAM^+$ CSCs show epithelial cell features with a high tumorigenic capacity and activated Wnt signaling, whereas $CD90^+$ CSCs show mesenchymal cell features with a highly metastatic capacity and activation of c-Kit signaling. In primary HCC where $EpCAM^+$ and $CD90^+$ CSCs co-exist, $CD90^+$ CSCs regulate distant organ metastasis through the activation of TGF-β signaling, but have no effect on tumorigenicity at primary sites which is mediated by $EpCAM^+$ CSCs

through the activation of TGF-β signaling by $CD90^+$ CSCs (Fig 1b) [36]. This suggests that $CD90^+$ cells are not only metastatic to the distant organ but also help the metastasis of $CD90^-$ cells, including $EpCAM^+$ cells, which have no distant metastatic capacity of their own. Our data further suggest that imatinib mesylate inhibits distant organ metastasis by suppressing $CD90^+$ metastatic CSCs, albeit with little effect on $EpCAM^+$ tumorigenic epithelial stem-like CSCs, which indicates the importance of $EpCAM^+$ and $CD90^+$ CSC interaction in the process of HCC development, especially in distant organ metastasis. These data suggest the limitations of a treatment strategy targeting only certain CSC marker-positive cells to eradicate HCC, as it is highly possible that marker-positive CSCs exist in each HCC patient with different chemosensitivities against molecularly targeted therapy. Interestingly, we have recently identified that EpCAM+ HCC cell lines show abundant expression of the transcription factor SALL4 and high histone deacetylase activity, and the histone deacetylase inhibitor successfully suppressed proliferation of EpCAM+ HCC cell lines but showed little effect on CD90+ HCC cell lines [69]. Further studies of liver CSC heterogeneity are required to provide better treatment strategies for HCC patients.

Conclusions

There is accumulating evidence that liver CSCs play a key role in the development and perpetuation of HCC, and the importance of targeting CSCs has become clearer. Understanding the diversity of liver CSCs will further the development of personalized medicine targeting patient-specific liver CSCs.

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

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Disulfiram Eradicates Tumor-Initiating Hepatocellular Carcinoma Cells in ROS-p38 MAPK Pathway-Dependent and -Independent Manners

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Abstract

Tumor-initiating cells (TICs) play a central role in tumor development, metastasis, and recurrence. In the present study, we investigated the effect of disulfiram (DSF), an inhibitor of aldehyde dehydrogenase, toward tumor-initiating hepatocellular carcinoma (HCC) cells. DSF treatment suppressed the anchorage-independent sphere formation of both HCC cells. Flow cytometric analyses showed that DSF but not 5-fluorouracil (5-FU) drastically reduces the number of tumor-initiating HCC cells. The sphere formation assays of epithelial cell adhesion molecule (EpCAM)$^+$ HCC cells co-treated with p38-specific inhibitor revealed that DSF suppresses self-renewal capability mainly through the activation of reactive oxygen species (ROS)-p38 MAPK pathway. Microarray experiments also revealed the enrichment of the gene set involved in p38 MAPK signaling in EpCAM$^+$ cells treated with DSF but not 5-FU. In addition, DSF appeared to downregulate *Glypican 3* (*GPC3*) in a manner independent of ROS-p38 MAPK pathway. GPC3 was co-expressed with EpCAM in HCC cell lines and primary HCC cells and *GPC3*-knockdown reduced the number of EpCAM$^+$ cells by compromising their self-renewal capability and inducing the apoptosis. These results indicate that DSF impaired the tumorigenicity of tumor-initiating HCC cells through activation of ROS-p38 pathway and in part through the downregulation of *GPC3*. DSF might be a promising therapeutic agent for the eradication of tumor-initiating HCC cells.

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Introduction

Accumulating evidence has revealed that a minor population of tumor cells, called cancer stem cells or tumor-initiating cells (TICs), organizes a cellular hierarchy in a similar fashion to normal stem cells and shows pronounced tumorigenic activity in xenograft transplantations [1]. Recent progress in stem cell biology and technologies has contributed to the identification and characterization of TICs in various cancers including hepatocellular carcinoma (HCC) [2]. In HCC, side population cells and cells expressing several surface molecules such as epithelial cell adhesion molecule (EpCAM), CD133, CD90, and CD13 have been reported to function as TICs [3]. Besides the identification of tumor-initiating HCC cells, cancer-related molecules and signaling pathways, such as the polycomb group proteins, NANOG, AKT/PKB signal, and Wnt/β-catenin, have been shown to play an important role in maintaining or augmenting of tumor-initiating capability of TICs [4]. Although inhibitors of these molecules and signaling pathways may be potent TIC-targeting drugs, no effective therapy targeting TICs has been developed.

Disulfiram (DSF) is an irreversible inhibitor of aldehyde dehydrogenase and has been clinically used in the treatment of alcohol dependence for roughly 70 years [5]. DSF is a potent therapeutic agent in a wide range of human cancers. In addition, recent reports showed that DSF reduced the number of tumor-initiating cells and attenuated their sphere-forming abilities in breast cancer and glioblastoma [6,7]. Although these findings

indicate that DSF could eradicate TICs, the molecular machinery of its effect against TICs still remains largely unknown.

In the present study, we examined the effects of DSF on tumor-initiating HCC cells *in vitro* and *in vivo*. We found that DSF impaired their tumor-initiating ability and induced apoptosis by activating the reactive oxygen species (ROS)-p38 pathway. Furthermore, the downregulation of *Glypican3 (GPC3)* expression, which is caused independently of the ROS-p38 pathway, appeared to also be responsible for the anti-TIC effect of DSF.

Results

DSF inhibited tumorigenicity of HCC cells *in vitro* and in a xenograft transplantation model

As shown in a variety of cancer cells [8–10], DSF treatment inhibited cell growth in both a time-dependent and dose-dependent manner in HCC cells (Figure S1A). Immunostaining of active caspase-3 (CASP3) showed that the DSF treatment induced apoptosis dose-dependently (Figure S1B). The percentage of apoptotic cells was roughly ten-fold higher among HCC cells treated with DSF (1 μM) than among control cells (Figure S1C). To examine whether DSF affected the tumorigenic ability of HCC cells, we conducted a non-adherent sphere assay, a standard assay for evaluating tumorigenic capacity. Sphere-forming ability was significantly impaired in DSF-treated HCC cell lines in a dose-dependent manner (Figure 1A and 1B). Subsequently, we determined the effects of DSF using a xenograft nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse model. After the implantation of 2×10^6 Huh1 and Huh7 cells into NOD/SCID mice, DSF was administered intraperitoneally every other day. Tumor initiation and growth were apparently suppressed by the DSF treatment in a dose-dependent manner (Figure 1C and 1D). Together, these results indicate that DSF reduced the tumorigenicity of HCC cells.

Loss-of-function assays of ALDH1 and ALDH2

DSF and its metabolites were shown to suppress ethanol metabolism mainly through the inhibition of cytosolic aldehyde dehydrogenase 1 (ALDH1) and mitochondrial ALDH2 [11]. It has been reported that *ALDH*-knockdown reduced proliferation and motility of lung cancer cells [12]. Because we previously showed that there was no association between the expression of ALDH1 and EpCAM or CD13 and that *ALDH1*-knockdown affected neither cell growth nor tumorigenicity in HCC cells [13], we conducted loss-of-function assays on ALDH2. We achieved the stable knockdown of *ALDH2* in Huh1 and Huh7 cells with lentivirus-mediated short hairpin RNA (shRNA) against *ALDH2* using enhanced red fluorescent protein (ERP) as a marker for infection (Figure S2A). No significant differences in cell growth and sphere formation were observed between *ALDH2*-knockdown cells and control cells expressing shRNA against *luciferase* (sh-*Luc*) (Figure S2B and S2C). Additionally, double-knockdown of *ALDH1* and *ALDH2* in the culture produced similar results to the single-knockdown of ALDH2 (Figure S2D-F). Taken together, the effects of DSF on HCC cells appeared to be independent of its inhibitory function toward ALDH1 and ALDH2.

Decrease in the number of tumor-initiating HCC cells after DSF exposure

We then examined the expression of various markers of tumor-initiating HCC cells such as CD13, epithelial cell adhesion molecule (EpCAM), and CD133 using flow cytometry. The DSF treatment appeared to decrease the number of HCC cells expressing these markers (Figure 2A). Among them, the EpCAM^{high} fraction markedly decreased from 44.4% to 9.8% in Huh1 cells and from 36.7% to 12.5% in Huh7 cells. Concordant with this, real-time RT-PCR analysis showed decreased expression of E-cadherin (CDH1) and alfa-fetoprotein (AFP), hepatic stem/progenitor cell markers, in DSF-treated cells (Figure 2B). In clear contrast, the 5-FU treatment resulted in the enrichment of TIC fractions (Figure S3). These results indicate that the biological effect of DSF differs from that of 5-FU, and is promising for the eradication of tumor-initiating HCC cells.

DSF activated p38 MAPK in response to increased intracellular ROS levels in tumor-initiating HCC cells

Consistent with previous reports [6,7], the present flow cytometric analyses showed that intracellular ROS levels were higher in DSF-treated HCC cells than in control cells (Figure 3A). However, co-treatment with NAC canceled this increase in ROS levels (Figure 3A). Western blotting showed increased levels of phosphorylated p38 after DSF exposure, which indicates p38 MAPK activation in HCC cells (Figure 3B). It has been well established that TICs maintain ROS at levels as low as normal stem cells [14,15]. ROS levels were higher in EpCAM$^-$ HCC cells than in EpCAM$^+$ cells (Figure 3C). Notably, the co-treatment of sorted EpCAM$^+$ cells with the antioxidant, NAC, canceled the phosphorylation of p38 induced by DSF (Figure 3D). Although EpCAM$^-$ HCC cells generated only a small number of spheres, DSF treatment further reduced the number of spheres (Figure S4A and S4B). Approximately 90% of EpCAM$^+$ cells treated with DSF was positive for phosphorylated p38 (Figure 3D), but the rate for EpCAM$^-$ cells positive for phosphorylated p38 was nearly 25% (Figure S4C). The cell growth of EpCAM$^+$ HCC cells was greatly restored by the additional NAC treatment (Figure 3E). Together, DSF caused activation of the ROS-p38 MAPK pathway in tumor-initiating HCC cells.

p38 MAPK activation impaired self-renewal capability of tumor-initiating HCC cells

To examine the impact of p38 MAPK activation on tumor-initiating HCC cells, we conducted sphere formation assays on EpCAM$^+$ HCC cells treated with DSF and/or SB203580, a specific inhibitor of p38 (Figure 4A). The co-treatment of cells with SB203580 largely abrogated the cell growth inhibition and apoptosis observed following the DSF treatment (Figure S5). Consistent with this, additional SB203580 treatment significantly restored the sphere-forming ability of EpCAM$^+$ HCC cells (Figure 4B). Additionally, subsequent analyses for secondary sphere formation after replating showed results similar to those for the primary spheres (Figure 4C). These results indicate that activated p38 MAPK restricts the self-renewal of tumor-initiating HCC cells. We then conducted immunocytochemical analyses of the spheres and examined the expression of EpCAM and α-fetoprotein (AFP), a hepatic stem/progenitor cell marker [16]. Although the DSF treatment decreased the number of cells positive for AFP or EpCAM, co-treatment with DSF and SB203580 restored the number of positive cells (Figure 4D and 4E). Taken together, DSF impaired the tumor-initiating capability of HCC cells in part in a p38-dependent manner.

Gene expression profiles of EpCAM$^+$ HCC cells treated with DSF

EpCAM$^+$ HCC cells treated with DSF or 5-FU for 48 hours were subjected to oligonucleotide microarray experiments. Concordant with the results presented in Figures 3 and 4, gene set enrichment analysis (GSEA) showed that EpCAM$^+$ HCC cells

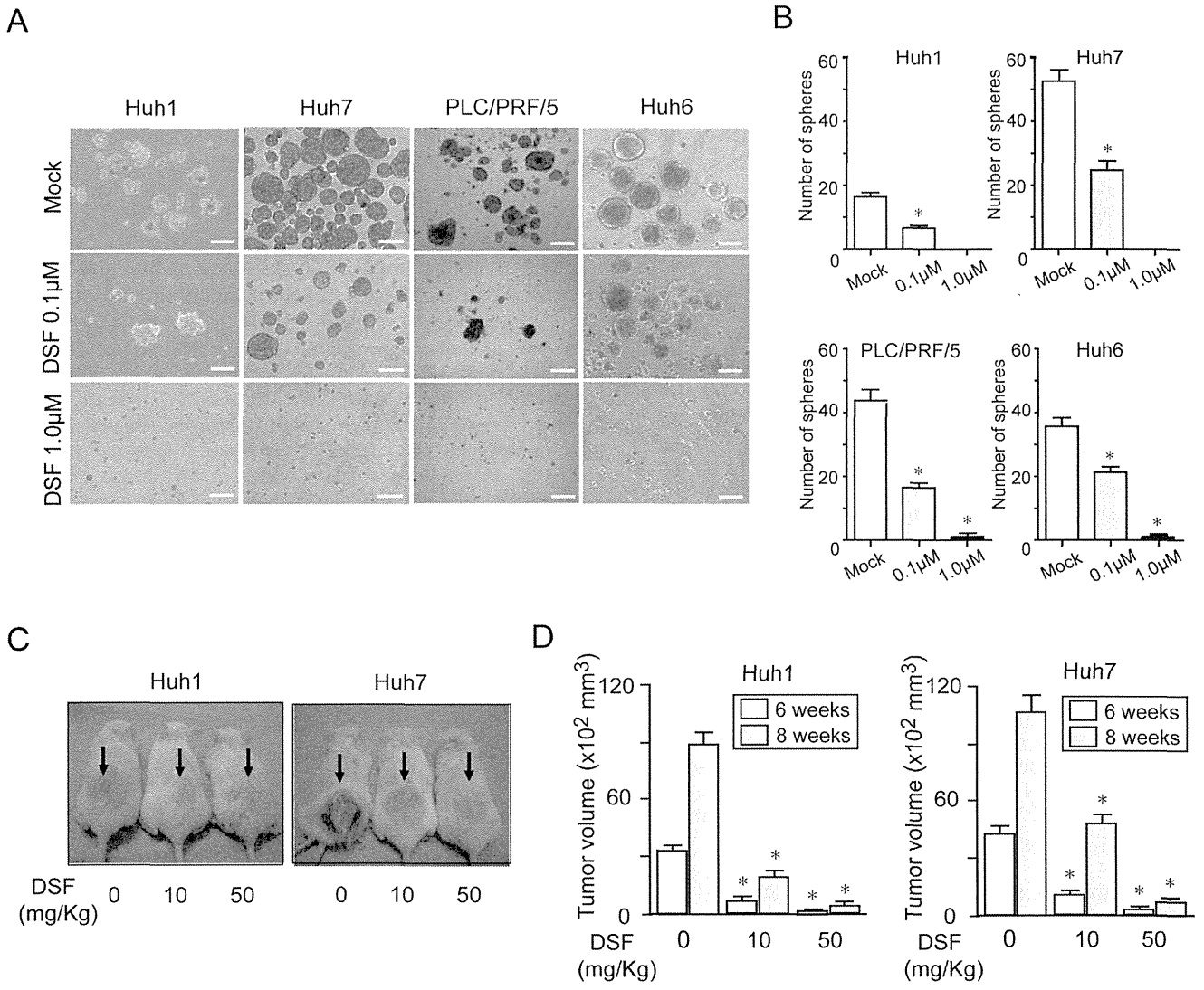

Figure 1. Sphere formation assays on HCC cells and xenograft transplantation. (A) Non-adherent sphere formation assay on HCC cell lines at day 14 of culture. Bright-field images are shown. Scale bar = 200 μm. (B) Number of large spheres generated from 1,000 HCC cells treated with DSF. *Statistically significant ($p<0.05$). (C) A total of 2×10^6 Huh1 or Huh7 cells were transplanted into the subcutaneous space of NOD/SCID mice. The growth of subcutaneous tumors (arrows) was apparently suppressed by the DSF treatment in a dose-dependent manner 8 weeks after transplantation. (D) Subcutaneous tumor volume was determined 6 and 8 weeks after transplantation. *Statistically significant ($p<0.05$).
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treated with DSF, but not 5-FU were significantly enriched for genes involved in p38-MAPK signaling (Figure 5A) [17,18]. The DSF treatment altered the expression of several genes involved in cell cycle regulation (Figure S6A and S6B). In particular, striking upregulation of *p57KIP2* was observed in Huh1 EpCAM$^+$ cells. The gene set for the proteasome pathway showed a higher enrichment score in DSF-treated EpCAM$^+$ HCC cells than in 5-FU-treated cells, although there was no significant difference (Figure S6C) [19].

We identified DSF-responsive genes (698 upregulated genes and 605 downregulated genes) and 5-FU-responsive genes (717 upregulated genes and 1,350 downregulated genes) (Figure 5B and 5C). Of interest, the DSF treatment causes no marked changes in the gene expression of the ROS scavenger pathway (Figure S6D). Furthermore, functional annotation analysis revealed different gene expression profiles between EpCAM$^+$ HCC cells treated with DSF and 5-FU (Table S1 and S2). In particular, gene ontology terms enriched for downregulated genes were different. Additionally, 23 genes categorized into "liver cancer" were downregulated after exposure to DSF, but not 5-FU (Figure 5D). Among them, Glypican3 (GPC3) was shown to be specifically overexpressed in human HCC and *GPC3*-knockdown induced apoptosis in HCC cells [20,21]. Quantitative RT-PCR showed that *GPC3* expression was downregulated in EpCAM$^+$ HCC cells treated with DSF as shown in the microarray analyses (Figure 5E). However, the downregulation of *GPC3* was not observed in EpCAM$^-$ HCC cells after DSF treatment (data not shown).

Regulation of *GPC3* gene expression

To examine whether activation of the ROS-p38 MAPK pathway was crucial to the downregulation of *GPC3* expression by DSF, we examined *GPC3* expression in EpCAM$^+$ HCC cells co-treated with NAC or SB203580. Neither NAC nor SB203580

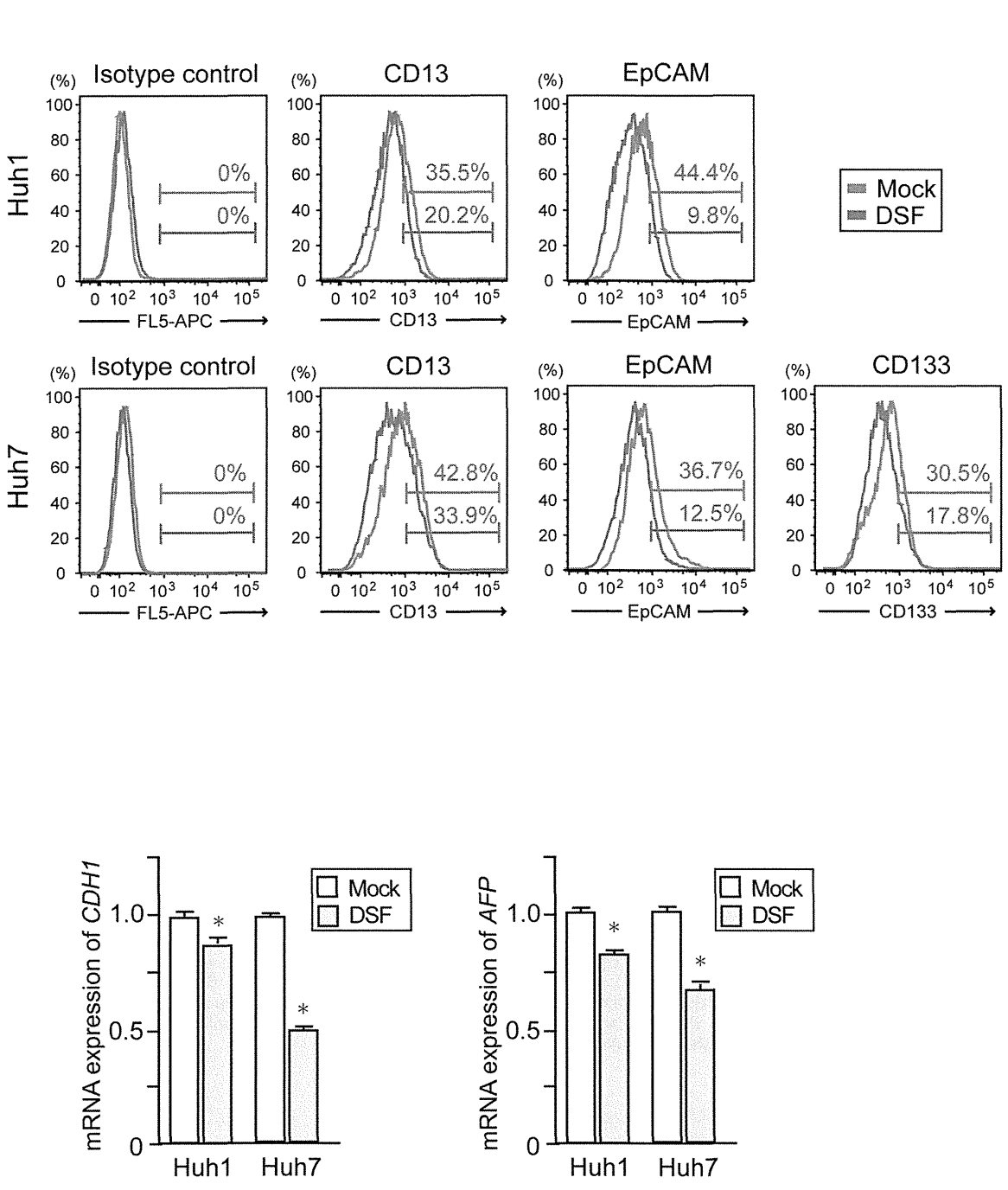

Figure 2. Flow cytometric analyses and quantitative RT-PCR analyses of HCC cells treated with DSF. (A) Flow cytometric profiles in Huh1 and Huh7 cells treated with DSF (0.1 μM) for 48 hours. The percentages of positive fractions for indicated markers are shown as the mean values for three independent analyses. (B) Real-time RT-PCR analyses of hepatic stem/progenitor cell marker genes. *Statistically significant ($p<0.05$).
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restored the expression of *GPC3* (Figure S7A). In addition, proteasome inhibition by the MG132 treatment had no effect on *GPC3* expression (Figure S7B). These findings indicate that neither ROS-p38 MAPK pathway activation nor proteasome inhibition contributed to the downregulation of *GPC3* expression.

Loss-of-function and gain-of-function assays of GPC3 in EpCAM$^+$ HCC cells

Dual immunostaining analyses showed that GPC3 and EpCAM were frequently co-expressed in HCC cells (Figure 6A). Moreover, quantitative RT-PCR revealed a higher level of GPC3 expression in the EpCAM$^+$ fraction than in the EpCAM$^-$ fraction (Figure 6B). Stable HCC cell lines expressing shRNA against *GPC3* or *luciferase* were successfully obtained by cell sorting with enhanced green fluorescent protein (EGFP) as a marker for viral infection. Western