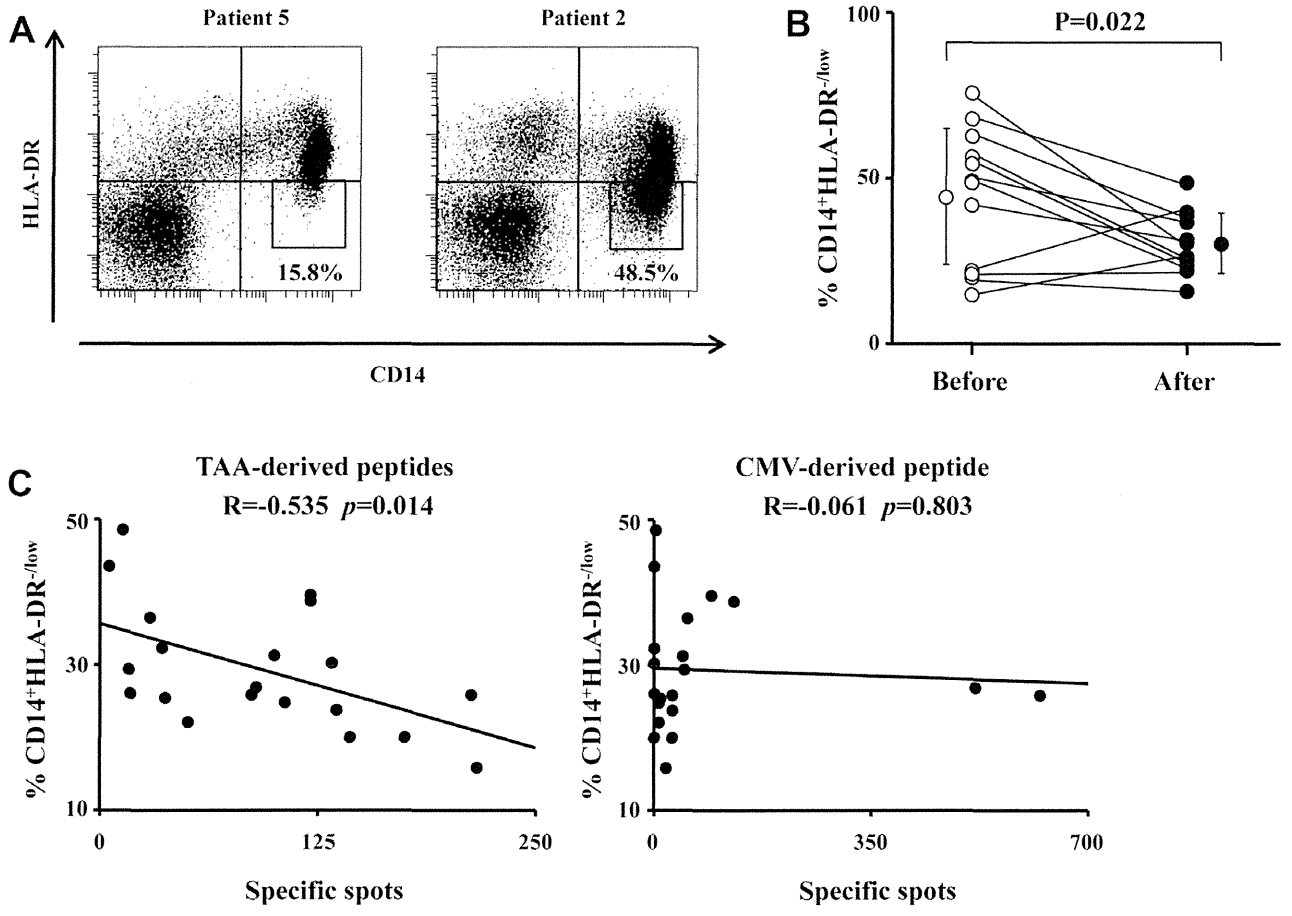


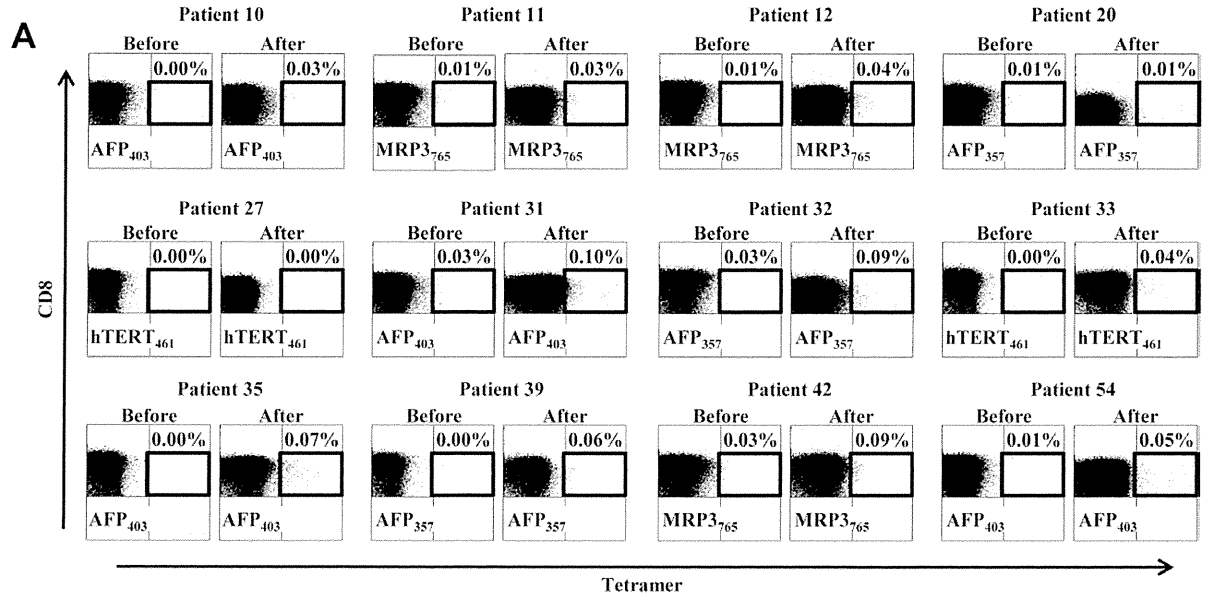
Fig. 3. Frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and its relationship with the frequency of TAA-specific T cells after RFA. (A) The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs was measured in 20 randomly selected patients by FACS analysis after RFA. The representative results of two patients are shown. (B) The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs was also measured in 12 of 20 randomly selected patients before RFA and compared with that after RFA. (C) Relationship between the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and the frequency of TAA- and CMV-derived peptide-specific T cells after RFA.

PBMCs is significantly increased in comparison with healthy controls and they exert immunosuppressive function via induction of regulatory T cells.[26] Taken together with our results, these reports suggest that an additional immunological approach to inhibit the function of MDSCs after RFA may enhance TAA-specific immune responses.

On the other hand, patients with a high number of TAA-specific T cells were not completely protected from HCC recurrence. To examine the mechanisms behind the failure to control HCC recurrence completely by RFA-induced TAA-specific immune responses, we performed phenotypic and kinetic analysis of T cells enhanced by RFA. The results showed that the frequency of T cells with each memory phenotype depended on the patient, and the ratio of these cells changed after RFA. The memory phenotype of T cells that showed a more than two-fold increase was the $CD45RA^{-}/CCR7^{+}$ (central memory) phenotype, which required secondary stimulation by antigen to exert stronger anti-tumor effects.[17] Interestingly, they were newly induced, suggesting that RFA may modify not only the frequency but also the phenotype of TAA-specific T cells.

The frequencies of TAA-derived peptide-specific T cells decreased in most of the patients at 24 weeks after RFA, suggesting that RFA could not induce long-lived T cells. In a previous study, it was reported that tumor-specific immune responses induced by RFA could not protect from HCC recurrence completely because of tumor immune escape.[27] In addition to this mechanism, our results suggest that one of the reasons that RFA-induced tumor-specific immune response is insufficient for controlling HCC recurrence is the weak induction of long-lived T cells.

Taken together with these results, the present study suggests that the antitumor effect of TAA-specific T cells induced by RFA should be enhanced by an additional immunological approach. In recent studies of cancer immunology, cancer vaccines consisting of TAA-derived protein or peptide, recombinant virus, and



B

	Patient 10		Patient 11		Patient 12		Patient 20		Patient 27		Patient 31	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CD45RA-CCR7+	ND	2.1	7.4	10.3	0.0	4.2	0.0	2.2	ND	ND	13.5	2.2
CD45RA-CCR7-	ND	27.1	14.8	17.6	47.8	41.7	7.4	13.0	ND	ND	18.9	51.7
CD45RA+CCR7-	ND	22.9	44.4	33.8	21.7	10.1	74.1	69.6	ND	ND	35.1	42.2

	Patient 32		Patient 33		Patient 35		Patient 39		Patient 42		Patient 54	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CD45RA-CCR7+	0.0	7.6	ND	6.2	ND	3.9	ND	6.2	0.0	6.9	0.0	3.1
CD45RA-CCR7-	29.6	38.6	ND	27.2	ND	4.5	ND	8.3	19.6	22.4	20.0	23.1
CD45RA+CCR7-	44.4	49.7	ND	11.1	ND	67.0	ND	41.7	80.4	63.8	60.0	51.5

Fig. 4. Phenotypic analysis of T cells induced by RFA. (A) Enhancement of TAA-specific T cell responses was also analyzed by tetramer assay. The results of all patients examined are shown. Peptide $MRP3_{765}$-, AFP_{357}-, AFP_{403}-, and $hTERT_{461}$- specific tetramers were used. The frequency of tetramer-positive cells is shown as the percentage in $CD8^+$ cells. (B) The memory phenotype of tetramer-positive cells was analyzed using the criterion of CD45RA/CCR7 expression. The box numbers show the percentage of cells in tetramer-positive cells. ND means that the experiments are not available because of the small number of tetramer-positive cells. Results with increased frequency after RFA are shown in gray boxes.

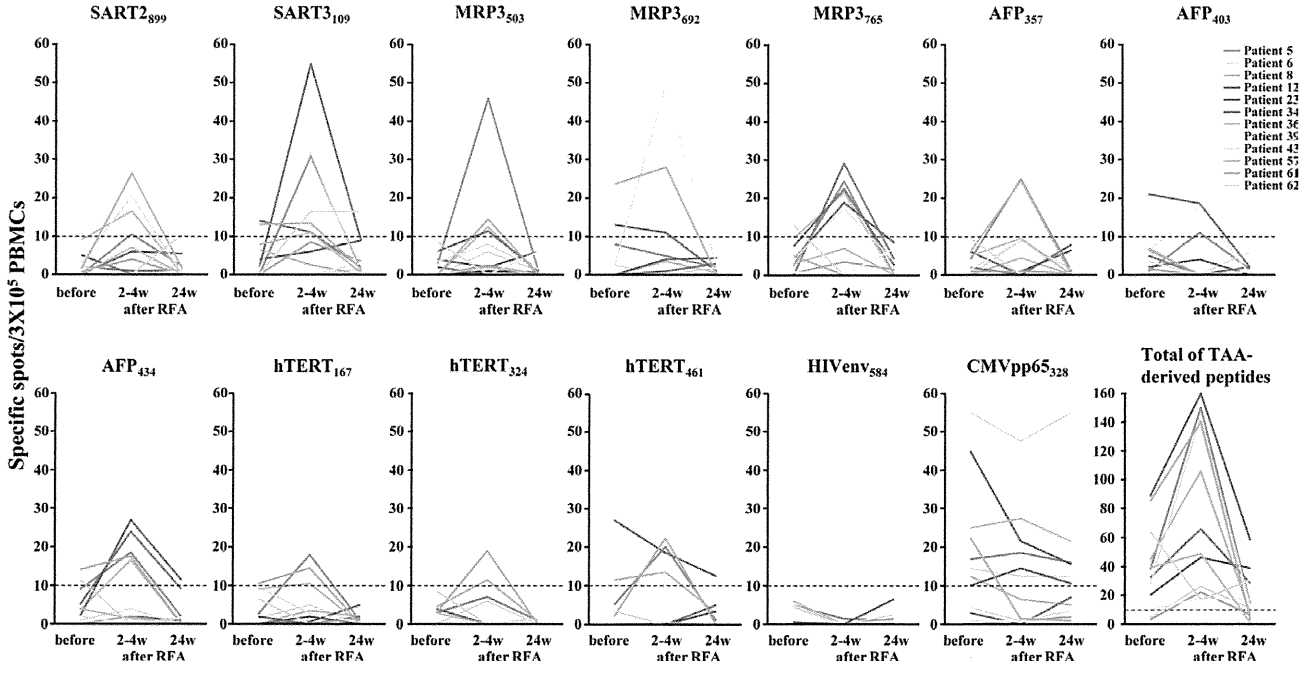


Fig. 5. Kinetics of TAA-specific T cell responses determined via IFN-γ ELISPOT assay. PBMCs were obtained at three different time points: (1) before RFA, (2) 2-4 weeks after RFA, and (3) 24 weeks after RFA. Each graph indicates the kinetics of T cells specific for each peptide in each patient.

engineered tumor cells have been considered as candidates to enhance host immune responses.[28] Alternatively, immunomodulating antibodies such as anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) and anti-programmed cell death 1 (PD-1) have been considered to reactivate T cell function.[28,29] These approaches may also be effective to enhance the antitumor effect induced by RFA.

In conclusion, the results of this study show that RFA can enhance various TAA-specific T cell responses and the number of T cells induced is associated with HCC recurrence-free survival. To maintain the TAA-specific T cell responses induced by RFA and to improve the immunological effect for HCC, additional treatment by vaccine or immunomodulatory drugs might be useful.

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ORIGINAL ARTICLE

Increase in $CD14^+HLA\text{-}DR^{-/low}$ myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis

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Abstract Myeloid-derived suppressor cells (MDSCs) are known as key immune regulators in various human malignancies, and it is reported that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs are increased in hepatocellular carcinoma (HCC) patients. However, the host factors that regulate the frequency and the effect on the prognosis of HCC patients are still unclear. We investigated these issues and clarified the relationships between a feature of MDSCs and host factors in HCC patients. We examined the frequency of MDSCs in 123 HCC patients, 30 chronic liver disease patients without HCC, and 13 healthy controls by flow cytometric analysis. The relationships between the clinical features and the frequency of MDSCs were analyzed. In 33 patients who received curative radiofrequency ablation (RFA) therapy, we examined the impact of MDSCs on HCC recurrence. The frequency of MDSCs in HCC patients was significantly increased. It was correlated with tumor progression, but not with the degree of liver fibrosis and inflammation. In terms of serum cytokines, the concentrations of IL-10, IL-13, and vascular endothelial growth factor were significantly correlated with the frequency of MDSCs. In HCC patients who received curative RFA therapy, the frequency of MDSCs after treatment showed various changes and was inversely correlated with recurrence-free survival time. The frequency of MDSCs is correlated with tumor progression, and this frequency after RFA is inversely correlated with the prognosis of HCC patients. Patients with a high frequency of MDSCs after RFA should be closely followed and the inhibition of MDSCs may improve the prognosis of patients.

Keywords Myeloid-derived suppressor cells · Hepatocellular carcinoma · Radiofrequency ablation · Recurrence · Cancer

Abbreviations

MDSCs	Myeloid-derived suppressor cells
HCC	Hepatocellular carcinoma
CLD	Chronic liver disease
RFA	Radiofrequency ablation
TACE	Transcatheter arterial chemoembolization
PBMC	Peripheral blood mononuclear cell
Tregs	Regulatory T cells
HLA	Human leukocyte antigen
FGF	Fibroblast growth factor
CCL	Chemokine C–C motif ligand
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
IP	Interferon gamma-induced protein
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
PDGF	Platelet-derived growth factor
RANTES	Regulated upon activation, normal T cell expressed and secreted
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

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JAK Janus kinase
STAT Signal transducer and activator of transcription

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading cause of cancer mortality globally [1, 2]. Current treatment options including surgical resection, radiofrequency ablation (RFA), liver transplantation, chemotherapy, transcatheter arterial chemoembolization (TACE), and sorafenib are reported to improve survival in HCC patients [3–7]. However, despite curative treatments for HCC, tumor recurrence rates remain high and the survival of those who have advanced HCC remains unsatisfactory [3–7]. Therefore, the development of new anti-tumor treatments for HCC remains an urgent and important field of research.

To overcome the limitations of these treatments, several immunotherapies have been developed as attractive strategies for HCC. In several studies of HCC immunotherapy, effective induction of immune-mediated cells by tumor antigen-derived peptides or antigen-presenting cells showed anti-tumor effects, but the population of patients who exhibited such effects was very small [8–12].

In previous studies, it was reported that many kinds of tumor generate a number of immune-suppressive mechanisms [13–15]. Recently, myeloid-derived suppressor cells (MDSCs) have been characterized as key immune regulators in various human cancers [15–24]. They show the capacity to inhibit T cell function and promote tumor development [15, 25]. Human MDSCs are a heterogeneous population that shows $CD11b^+$, $CD33^+$, $HLA\text{-}DR^{-/low}$ and can be divided into granulocytic $CD14^-$ and monocytic $CD14^+$ subtypes [26–28]. In most recent studies, it has been reported that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were increased in HCC patients and the cells inhibited the function of T cells through the induction of regulatory T cells (Tregs) [24]. Tregs represent 5–10 % of $CD4^+$ T cells and can suppress the activation and proliferation of $CD4^+$ and $CD8^+$ T cells [14, 29]. It was reported that an increased frequency of circulating Tregs was associated with poor survival of HCC patients [30]. Understanding the inhibitory mechanism of MDSCs and controlling their function are very important to develop more effective immunotherapy for HCC.

In this study, we investigate the host factors that are associated with the frequency of MDSCs in HCC patients and the effect of MDSCs on the prognosis of patients and clarify the relationships between a feature of MDSCs and host factors in HCC patients.

Materials and methods

Patients and healthy controls

Blood samples were obtained from a total of 123 HCC patients, 26 chronic liver disease (CLD) patients without HCC, and 13 healthy controls. The diagnosis of HCC was histologically confirmed in 68 patients. For the remaining 55 patients, diagnosis was made by dynamic CT or MRI. Patient characteristics and disease classification are shown in Suppl. table 1. All CLD patients without HCC underwent percutaneous liver biopsy to evaluate the disease severity according to the Metavir scoring system. In 33 patients treated with curative percutaneous RFA, blood samples were obtained on the day of treatment and 2–4 weeks after treatment, and we observed recurrence of these patients with periodic imaging studies. All subjects provided written informed consent to participate in this study in accordance with the Declaration of Helsinki. This study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University).

Cell isolation and flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were separated as described below; heparinized venous blood was diluted in phosphate-buffered saline (PBS) and loaded on Ficoll-Histopaque (Sigma, St. Louis, Mo.) in 50 ml tubes. After centrifugation at 2,000 rpm for 20 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS, centrifuged at 1,400 rpm for 10 min, and finally resuspended in complete culture medium consisting of RPMI (GibcoBRL, Grand Island, NY), 10 % heat inactivated FCS (Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). PBMCs were resuspended in RPMI 1,640 medium containing 80 % FCS and 10 % dimethyl sulfoxide and cryopreserved until use. The viability of cryopreserved PBMCs was 60–70 %. In some patients, fresh and cryopreserved PBMCs were obtained from the same sample. To determine the frequency and phenotype of MDSCs and Tregs, multicolor fluorescence-activated cell sorting analysis was carried out using the Becton–Dickinson FACSAria II system. The following anti-human monoclonal antibodies were used: anti-CD4 (Becton–Dickinson), anti-CD11b (Becton–Dickinson), anti-CD14 (Becton–Dickinson), anti-CD15 (Becton–Dickinson), anti-CD25 (Becton–Dickinson), anti-CD33 (Becton–Dickinson), anti-CD127 (Becton–Dickinson), and anti-HLA-DR (Becton–Dickinson).

Suppression assay

$CD14^+HLA\text{-}DR^{-/low}$ MDSCs and $CD14^+HLA\text{-}DR^+$ cells were sorted using the Becton–Dickinson FACSAria II

system. 2×10^4 PBMCs were cultured and stimulated with 1 μg/ml plate-bound anti-CD3 (eBioscience) and 1 μg/mL soluble anti-CD28 (eBioscience) in 96-well round-bottomed plates. 24 h later, to determine the suppressive ability of MDSCs, increasing concentrations of MDSCs were added to the stimulated PBMCs. Proliferation was measured by ^{3}Hincorporation after 72 h. [^{3}H] thymidine was added, and cell proliferation was measured by incorporation of radiolabeled thymidine for 24 h.

Cytokine and chemokine profiling

Blood samples were collected from patients at the same time of PBMC isolation. After centrifugation at 3,000 rpm for 10 min at 4 °C, serum fractions were obtained and stored at −20 °C until use. Serum levels of various cytokines and chemokines were measured using the Bio-Plex Protein Array System. Briefly, frozen serum samples were thawed at room temperature and diluted 1:4 in sample diluents; 50 μl aliquots of the diluted sample was added in duplicate to the wells of 96-well microtiter plates containing the coated beads for a validated panel of human cytokines and chemokines according to the manufacturer's instructions. The following 27 cytokines and chemokines were targeted: IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin (chemokine C–C motif ligand (CCL) 11), G-CSF, GM-CSF, IFN-γ, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated upon activation, normal T cell expressed and secreted (RANTES), TNF-α, and vascular endothelial growth factor (VEGF). Nine standards (ranging from 0.5 to 32,000 pg/ml) were used to generate calibration curves for each cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version 4.1.1.

Statistical analysis

Data are expressed as the mean ± SD. Chi-squared test with Yates' correction, unpaired *t* test, Mann–Whitney *U* test, and Kruskal–Wallis were used for univariate analysis of two groups that were classified according to the frequency of MDSCs. The probability of tumor recurrence-free survival was estimated using the Kaplan–Meier method. The Mantel-Cox log-rank test was used to compare curves between groups. The prognostic factors for tumor recurrence-free survival were analyzed for statistical significance by the Kaplan–Meier method (univariate) and the Cox proportional hazard model (multivariate). Variables with $p < 0.1$ were entered into multivariate logistic regression analysis. A level of $p < 0.05$ was considered significant.

Results

$CD14^+HLA\text{-}DR^{-/low}$ MDSCs are increased in the peripheral blood of HCC patients

We analyzed the peripheral blood of 123 patients with HCC, 26 CLD patients without HCC, and 13 healthy donors for the prevalence of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs. Because the PBMCs are tested after Ficoll, some cells may be lost. Therefore, we examined the population of MDSCs as a percentage of total $CD14^+$ cells by flow cytometry after cell surface labeling for the expression of HLA-DR (Fig. 1a). $CD14^+HLA\text{-}DR^{-/low}$ population in PBMCs of HCC patients represented 3.2–56.8 % of the $CD14^+$ cells. The frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs/$CD14^+$ cells in cryopreserved PBMCs correlated with that in fresh PBMCs (Fig. 1b). Therefore, we analyzed the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs/$CD14^+$ cells using cryopreserved PBMCs.

To confirm the function of these cells, sorted $CD14^+HLA\text{-}DR^{-/low}$ MDSCs and $CD14^+HLA\text{-}DR^+$ (control) cells were added at different ratios to autologous anti-CD3/CD28-stimulated PBMCs, and the proliferation was measured by ^{3}Hincorporation. $CD14^+HLA\text{-}DR^{-/low}$ MDSCs of HCC patients significantly decreased autologous PBMC proliferation (Fig. 1c). On the other hand, $CD14^+HLA\text{-}DR^+$ (control) cells could not suppress the autologous PBMC proliferation.

As shown in Fig. 1d, the frequency of MDSCs was significantly higher in HCC patients (19.0 %) than in healthy donors (9.4 %) ($p < 0.01$). Overall frequencies of $CD14^+$ cells did not differ significantly between the groups (Fig. 1e). Individual frequencies of MDSCs of all the patients and healthy donors are represented as scatter plots (Fig. 2a). The frequency of MDSCs was correlated with the stage of HCC (stage III and IV: 22.3 % ($n = 46$) vs. stage I and II: 17.0 % ($n = 77$), $p < 0.01$) and was significantly higher in HCC patients than CLD patients without HCC and healthy donors. Interestingly, there was no difference between CLD patients without HCC and healthy donors. Moreover, these numbers did not change depending on the degree of fibrosis or inflammatory activity of the liver (Fig. 2b, c).

In previous reports, granulocytic MDSCs were defined in combination with several surface markers including CD14, CD15, CD11b, CD33, CD66b, and HLA-DR in several cancers. Therefore, we examined the frequency of $CD15^+CD14^-CD11b^+CD33^+$ cells in 37 HCC patients and 11 healthy donors (Suppl. figure 1A). Although there was no statistical significant difference, the frequency of

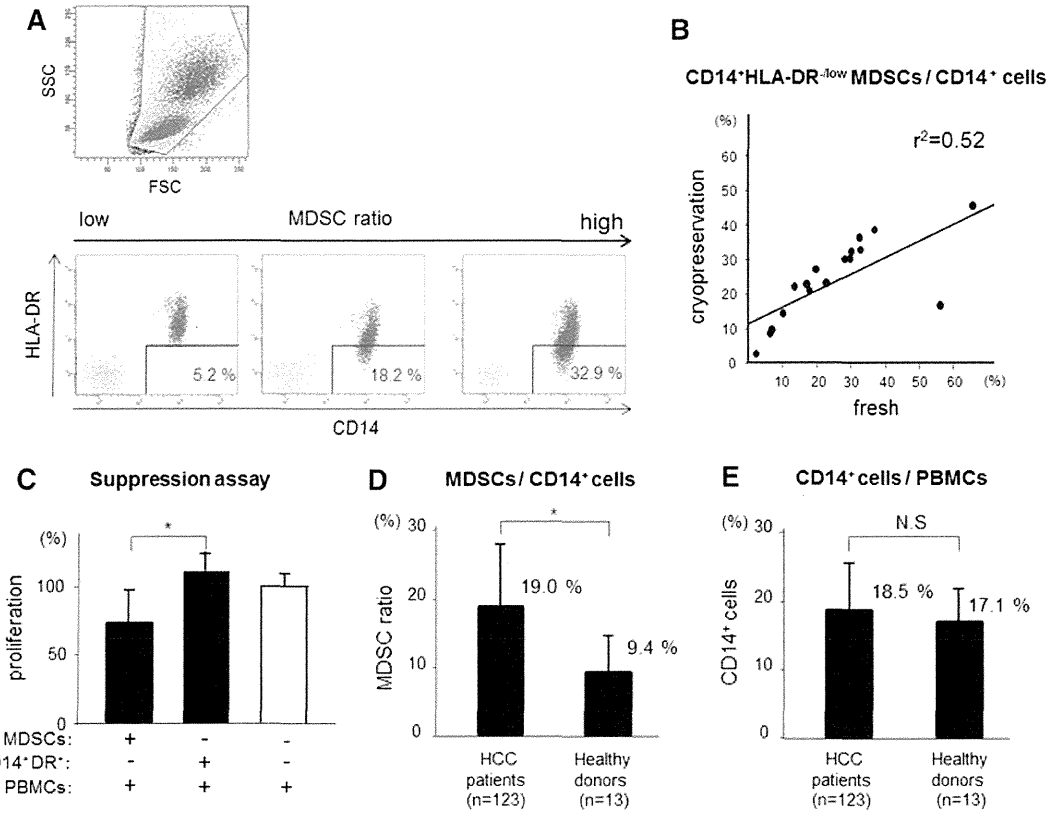

Fig. 1 **a** Flow cytometry shows $CD14^+HLA\text{-}DR^{-/low+}$ MDSCs. PBMCs from patients and healthy donors were labeled with anti-CD14 and HLA-DR. Three staining examples of HCC patients are shown in the order from a small number (*left*) to a large number (*right*). **b** The increase in $CD14^+HLA\text{-}DR^{-/low}$ MDSCs/$CD14^+$ cells in cryopreserved PBMC correlated with that in fresh PBMC ($r^2 = 0.52$). **c** Proliferation of PBMCs stimulated by anti-CD3/28 in the presence or absence of MDSCs was measured by ^{3}Hincorporation assay. $CD14^+HLA\text{-}DR^{-/low}$ MDSCs significantly decreased autologous PBMC proliferation ($n = 4$; *, $p < 0.05$). **d** The frequency of MDSCs was significantly higher in HCC patients than healthy donors (*, $p < 0.01$). **e** Overall frequencies of $CD14^+$ cells did not differ significantly

$CD15^+CD14^-CD11b^+CD33^+$ cells in HCC patients was higher than that in healthy donors (2.84 vs. 2.06 %, $p = 0.073$) (Suppl. figure 1B). The frequency was correlated with the stage of HCC (stage III and IV: 3.69 % ($n = 13$) vs. stage I and II: 2.39 % ($n = 24$), $p = 0.022$) (Suppl. figure 1C).

Relationship between the frequency of Tregs and MDSCs

It is well known that the frequency of circulating Tregs is increased and correlated with disease progression in HCC patients. The frequency of $CD4^+$ $CD25^+$ $CD127^{-/low}$ Tregs was significantly increased in HCC patients (Suppl. figure 2A) and associated with tumor progression (Suppl. figure 2B). However, there was not a strong correlation between the frequency of MDSCs and Tregs in our study (Suppl. figure 2C).

Identification of host factors related to the frequency of MDSCs in HCC patients

We divided the HCC patients into two groups using the threshold of an MDSC ratio of 22 %. This threshold is the average +2SD of the MDSC ratio in non-HCC patients. In the group with high frequency, the tumor factors including size, multiplicity, and stage were significantly worse (tumor size, 28.3 vs. 24.4 mm; tumor multiplicity (multiple/solitary), 27/12 vs. 42/42; TNM stage (I and II vs. III and IV), 17/22 vs. 60/24, $p < 0.05$) (Table 1). Moreover, hepatic reserve was also worse in the group with high frequency (Child-Pugh classification (A/B/C), 20/17/2 vs. 64/16/4, $p < 0.05$). In addition, overall survival was significantly shortened in the group with high frequency (hazard ratio 2.67, $p = 0.008$) (Suppl. figure 3A), and recurrence-free survival was also significantly shortened (hazard ratio 1.94, $p = 0.010$) (Suppl. figure 3B).

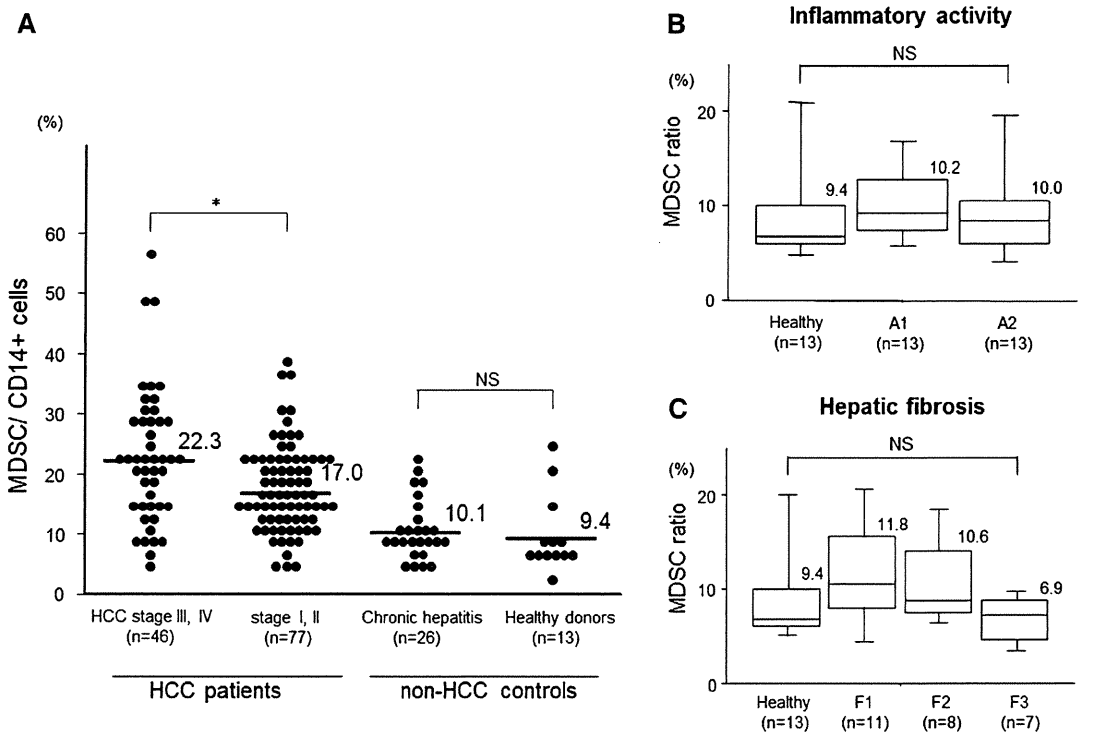

Fig. 2 **a** Scatter plots of MDSC ratio ($CD14^+HLA\text{-}DR^{-/low}$ MDSCs/ $CD14^+$ cells) in patients and healthy donors. The frequency of MDSCs was significantly increased in HCC patients compared with that in non-HCC controls. Moreover, the frequency of MDSCs was correlated with tumor progression (stage III and IV: 22.3 % ($n = 46$) vs. stage I and II: 17.0 % ($n = 77$); *, $p < 0.05$). In non-HCC controls, there was no significant difference in the frequency of MDSCs. **b**, **c** In non-HCC patients, the frequency of MDSCs did not change depending on the degree of fibrosis or inflammatory activity of the liver according to the Metavir scoring system

Relationship between serum cytokine levels and the frequency of MDSCs

In previous studies, the balance of circulating cytokines was thought to promote accumulation and activation of MDSCs [18, 31–34]. Therefore, we examined the relationship between serum cytokine levels and the frequency of MDSCs in HCC patients. In 54 HCC patients, serum levels of cytokines and chemokines were measured using the Bio-Plex Protein Array system. Serum concentrations of IL-10, IL-13, and VEGF were significantly increased in the group with a high frequency of MDSCs (Table 2). In addition, there was a positive correlation between these cytokine levels in serum and the frequency of MDSCs. We also examined the relationship between serum cytokine levels and the frequency of Tregs. We divided the HCC patients into two groups using the threshold of 7 %, which is the average +2SD of the % of Tregs among $CD4^+$ cells in non-HCC patients. Serum concentration of IL-10 was significantly increased in the group with a high frequency of Tregs (Suppl. table 2).

Kinetics of MDSCs before and after curative RFA therapy

We examined the frequency of MDSCs before and after curative RFA therapy in 33 patients. For this analysis, blood samples were obtained on the day of treatment (before) and 2–4 weeks after treatment (after). The frequency of MDSCs was significantly decreased after RFA therapy (18.0 to 15.5 %, $p < 0.05$) (Fig. 3a). However, in several patients, the frequency of MDSCs remained at a high level compared with that in non-HCC patients. The clinical parameters before RFA were not statistically different between the patients with and without a high frequency of MDSCs after RFA (Suppl. table 3).

Next, we followed up these patients for recurrence and analyzed the risk factors. If a high frequency of MDSC was observed after curative RFA therapy, the recurrence-free survival was significantly shortened (Fig. 3b). In contrast, the frequency of MDSCs before treatment did not affect the recurrence. In univariate analysis for recurrence, post-treatment MDSC ratio ≥22 % ($p = 0.023$) and tumor

Table 1 Clinical findings and MDSCs

Clinical characteristics	MDSC ratio ≥22 ($n = 39$)	MDSC ratio <22 ($n = 84$)	p value
Age (year)	68.5	70.1	0.646
Sex (M/F)	29/10	54/30	0.267
AST (IU/l)	62.0	61.5	0.543
ALT (IU/l)	47.1	53.9	0.759
LDH (IU/l)	225	218	0.832
γGTP (IU/l)	78.0	76.0	0.252
Platelet (10^4/μl)	10.9	10.6	0.884
Prothrombin time (%)	75.2	82.3	0.045
Serum albumin (g/dl)	3.53	3.68	0.120
Total bilirubin (mg/dl)	1.21	0.94	0.286
WBC (/μl)	3910	3610	0.235
Neutrophil (%)	63.2	59.4	0.093
Lymphocyte (%)	26.1	29.5	0.047
Total cholesterol (mg/dl)	151	149	0.926
HbA1c (%)	5.27	5.43	0.197
Type IV collagen 7S (ng/ml)	8.2	7.3	0.086
DCP (mAU/ml)	5157	432	0.561
AFP (ng/ml)	1301	934	0.240
Tumor size (mm)	28.3	24.4	0.014
Tumor multiplicity (multiple/solitary)	27/12	42/42	0.046
TNM stage (I plus II/III plus IV)	17/22	60/24	0.003
Child-Pugh (A/B/C)	20/17/2	64/16/4	0.015
Etiology (HCV/HBV/others)	21/11/7	61/11/12	0.081
$CD4^+$ $CD25^+$ $CD127^{-/low}$ Tregs/ $CD4^+$ cells (%)	7.04	6.70	0.281

AST aspirate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactic dehydrogenase, *γGTP* gamma glutamyltransferase, *WBC* white blood cell, *Hb* hemoglobin, *DCP* des-gamma-prothrombin, *AFP* alpha-fetoprotein, *HCV* hepatitis C virus, *HBV* hepatitis B virus, *Tregs* regulatory T cells

Chi-squared test with Yates' correction, unpaired t test, Mann–Whitney U test, and Kruskal–Wallis test were used for univariate analysis of two groups that were classified according to the frequency of MDSCs

multiplicity ($p = 0.010$) were significantly associated with HCC recurrence (Table 3). In multivariable analysis for recurrence, considering the variables in the univariate analysis with $p < 0.1$, only post-treatment MDSC ratio ≥22 % (HR 3.906, $p = 0.014$) was extracted as a significant risk factor for recurrence.

Discussion

MDSCs are expanded in pathological conditions such as malignancy, infection, or trauma and consist of a heterogeneous population of immature myeloid cells [15, 25]. In pathological conditions, immature myeloid cells are blocked to differentiate into mature macrophages, dendritic cells, or granulocytes; as a result, MDSCs are accumulated [15, 25]. MDSCs strongly inhibit anti-tumor immune response through a number of mechanisms [15, 25]. As monocytic subsets of MDSCs, $CD14^+HLA\text{-}DR^{-/low}$ MDSCs have been reported in various malignancies, including melanoma, multiple myeloma, prostate cancer, and bladder cancer [18, 20, 22, 35]. In the most recent study, Hoechst et al. [24] reported that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were significantly increased in HCC patients and they suppressed T cell functions through the induction of $CD4^+CD25^+Foxp3^+$ Treg.

In the present study, in addition to an increase in the number of MDSCs in HCC patients, we observed that the frequency was correlated with the progression of HCC. Consistent with our results, it has also been reported that the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs was correlated with tumor progression in patients with other cancers, such as melanoma, prostate cancer, and bladder cancer [22, 35, 36]. However, the mechanisms behind the increase in MDSCs in advanced cancer patients are still unclear. As is well known, there is a close relationship between hepatocarcinogenesis and histological status of underlying liver [37, 38]. Therefore, the advance of hepatic fibrosis and the increase in inflammatory cell infiltration into liver might result in an increase in MDSCs following the progression of HCC. However, there was no relationship between the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs and underlying liver status in our study. From our observations, increase in MDSCs was only correlated with tumor progression, but not with hepatic fibrosis or disease activity of CLD. This finding suggests that the expansion of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs was mostly derived from the tumor environment itself, but not from inflammation or fibrosis of liver tissue around the tumor. The finding that a significant decrease in the frequency of circulating $CD14^+HLA\text{-}DR^{-/low}$ MDSCs is observed in most patients with curative treatment in this study supports this hypothesis. On the other hand, Tregs were also increased in HCC patients and associated with the progression of HCC. Though it was reported that MDSCs suppressed T cell function through the induction of Tregs, there was not a strong correlation between the frequencies of these two immunosuppressive cells.

Regarding the mechanism of MDSC expansion, we also analyzed the relationship between the serum cytokine levels and the frequency of MDSCs. We observed that the serum concentrations of IL-10, IL-13, and VEGF were significantly increased in the group with high frequency of MDSCs and there was a positive correlation between these cytokine levels and the frequency of MDSCs. Moreover, although there was no significant difference, the serum

Table 2 Serum cytokines and MDSCs

Cytokine	Healthy donor (mean) ($n = 13$)	MDSC ratio ≥ 22 (mean) ($n = 21$)	Range	MDSC ratio < 22 (mean) ($n = 31$)	Range	p value
IL-1ra	34.2	97.0	(21.5–600)	40.3	(3.4–151)	0.057
IL-2	10.5	38.1	(4.3–54.3)	11.3	(0.9–49.7)	0.055
IL-4	2.6	5.75	(1.47–11.9)	5.03	(0.71–10.9)	0.159
IL-6	9.9	21.5	(1.2–130)	10.1	(0.2–97.2)	0.065
IL-8	24.5	64.7	(10.9–291)	35.1	(6.2–142)	0.156
IL-10	2.76	6.01	(0.8–11.5)	2.81	(0.1–12.0)	0.003
IL-12(p70)	14.6	33.3	(0.6–140)	17.6	(1.4–57)	0.058
IL-13	7.6	13.1	(1.2–33.6)	8.2	(2.7–22.9)	0.015
IL-17	15.7	23.5	(4.6–70)	20.8	(2.1–119)	0.115
Eotaxin	104	141	(51.9–493)	124	(26.3–331)	0.675
G-CSF	7.9	13.2	(2.7–41.3)	8.7	(0.5–17.9)	0.050
IFN-γ	52.6	95.4	(23.1–417)	69.9	(2.5–238)	0.136
MCP-1	20.2	26.8	(8.4–114)	23.8	(3.5–77)	0.744
MIP-1b	97.6	120	(58.3–490)	108	(39.7–263)	0.508
PDGF	4012	4375	(1,312–10,136)	4013	(831–13,557)	0.484
RANTES	2978	2890	(1,040–4,826)	3184	(599–6,165)	0.186
TNF-α	10.5	34.9	(0.1–175)	27.6	(2.9–105)	0.756
VEGF	34.6	101.7	(22.5–371)	59.5	(9.3–183)	0.045

IL interleukin, *G-CSF* granulocytic colony stimulating factor, *IFN* interferon, *MCP* monocyte chemoattractant protein, *MIP* macrophage inflammatory protein, *PDGF* platelet-derived growth factor, *RANTES* regulated upon activation, normal T cell expressed and secreted, *TNF* tumor necrosis factor, *VEGF* vascular endothelial growth factor

Mann–Whitney *U* test was used for univariate analysis of two groups that were classified according to the frequency of MDSCs

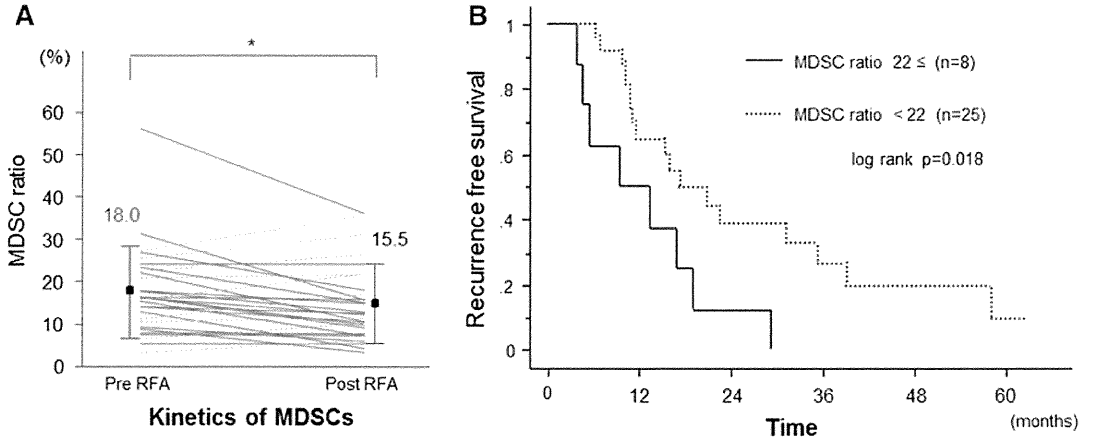

Fig. 3 **a** In 33 HCC patients who received curative RFA therapy, the frequency of MDSCs was significantly decreased after treatment. However, in several patients, the frequencies were increased after treatment (*dotted lines*) (*, $p < 0.05$). **b** Kaplan–Meier curve for recurrence-free survival after RFA therapy. The patients with high frequency of MDSCs (*solid line*) relapsed

concentrations of IL-1ra, IL-2, IL-6, IL-12(p70), and G-CSF tended to be increased in the group with high frequency of MDSCs. In accordance with our results, various cytokines, including IL-6, IL-10, IL-13, G-CSF, and VEGF, that trigger Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathways have been reported to be associated with the frequency of MDSCs [39]. In particular, the cytokines involved in the JAK2-STAT3 signaling pathway are considered to be the main regulators of the expansion of MDSCs, which leads to stimulation of myelopoiesis and inhibition of myeloid-cell differentiation [40–42].

Table 3 Cox proportional hazards regression for recurrence

Variant	Univariate HR (95 % CI)	*p* value	Multivariable HR (95 % CI)	*p* value
Sex: female	0.763 (0.446–1.308)	0.326		
Age: ≥70 years	1.111 (0.671–1.840)	0.683		
Pre-MDSC ratio: ≥22 %	1.210 (0.698–2.096)	0.497		
Pre-neutrophil	0.990 (0.969–1.012)	0.385		
Pre-lymphocyte	1.014 (0.987–1.043)	0.311		
Pre-neutrophil/lymphocyte	0.978 (0.787–1.216)	0.844		
Pre-ALT	1.001 (0993–1.008)	0.882		
Pre-serum albumin: <3.5 mg/dl	1.143 (0.665–1.982)	0.647		
Pre-prothrombin time: <70 %	1.662 (0.961–2.903)	0.073	1.881 (0.522–6.777)	0.101
Post-MDSC ratio: ≥22 %	2.795 (1.150–6.792)	0.023	3.906 (1.313–11.616)	0.014
Post-neutrophil	1.005 (0.975–1.035)	0.762		
Post-lymphocyte	0.993 (0.960–1.027)	0.678		
Post-neutrophil/lymphocyte	1.003 (0.810–1.242)	0.980		
Post-ALT	0.995 (0.981–1.010)	0.501		
Type IV collagen 7S	1.122 (0.992–1.268)	0.067	1.192 (0.907–1.566)	0.207
AFP: ≥100 ng/ml	1.357 (0.743–2.480)	0.321		
Tumor size: ≥20 mm	1.29 (0.78–2.12)	0.328		
Tumor multiplicity: multiple	2.00 (1.18–3.40)	0.010	1.851 (0.721–4.753)	0.201

HR hazard ratio, *CI* confidence interval, *ALT* alanine aminotransferase, *AFP* alpha-fetoprotein

Another important finding of our study is that the frequency of MDSCs showed various changes after curative RFA and this frequency is an independent risk factor of HCC recurrence. In most of the patients, the frequency of MDSCs decreased after RFA. A similar phenomenon has also been reported in other cancer treatments [19, 21, 36]. Liu et al. [21] reported that MDSCs were decreased in non-small cell lung cancer patients who had clinical benefit from chemotherapy or who received curative surgery. These results suggest that a decrease in the frequency of MDSCs is due to tumor eradication.

It is well known that tumor factors including multiplicity, tumor diameter, serum levels of tumor marker, and hepatic reserve are risk factors of HCC recurrence after RFA [43, 44], but it has not been reported that the frequency of circulating MDSCs is also a risk factor. From our findings, there was a clear inverse correlation between the frequency of MDSCs after RFA and recurrence-free survival. Consistent with our results, in the patients with pancreatic, esophageal, and gastric cancer, Gabitass et al. [23] reported that an increase in MDSCs was associated with an increased risk of death and that the frequency of MDSCs was an independent prognostic factor for patient survival. Taken together with these findings, our results suggest that the frequency of MDSCs might be one of the prognostic factors of patients after cancer treatments.

As we showed, the frequency of MDSCs is primarily correlated with tumor progression. However, between the patients with high and low frequency of MDSCs after RFA, there was no significant difference in hepatic reserve and tumor factors before treatment. Although an incomplete HCC eradication at a microscopic level may allow a high frequency of MDSCs after RFA, there may be other mechanisms such as subsequently tumor-specific immune responses after RFA. In addition, there is a limitation of the present study because we used cryopreserved PBMCs for phenotypic analysis of MDSCs. Further studies using fresh PBMCs are needed for precise phenotypic analysis of MDSCs and elucidation of the mechanism to regulate the frequency of MDSCs after HCC treatment.

In conclusion, the frequency of MDSCs in HCC patients is correlated with tumor progression, and the frequency after RFA is inversely correlated with the prognosis of HCC patients. HCC patients who show a high frequency of MDSCs after RFA should be closely followed, and the inhibition or elimination of MDSCs after HCC treatments may improve the prognosis of HCC patients.

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Conflict of interest The authors declare no conflict of interest.

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Research Article

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Carbamazepine promotes liver regeneration and survival in mice

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Background & Aims: Carbamazepine (CBZ), a widely used anticonvulsant and mood stabilizer, activates multiple proliferative and pro-survival pathways. Here, we hypothesize that CBZ may promote hepatocellular proliferation and ameliorate liver regeneration.

Methods: C57BL6/J mice were orally administered CBZ or vehicle and underwent a 70% partial hepatectomy (PHx), 85% PHx or treatment with carbon tetrachloride (CCl_4). Liver regeneration was determined by liver to body weight ratio, hepatocyte proliferation markers, and activation of intracellular signalling pathways.

Results: Two to 5 days after the 70% PHx, the liver to body weight ratio was significantly higher in the CBZ-treated mice than in the vehicle-treated mice. CBZ treatment upregulated the number of proliferative hepatocytes following PHx or CCl_4 treatment, as assessed by intrahepatic Ki-67 staining, BrdU uptake, and PCNA protein expression. PHx surgery induced the expression of several cyclins and activated Akt/mTOR signalling pathways, all of which were enhanced by CBZ treatment. The administration of the mTOR inhibitor temsirolimus abrogated the hepato-proliferative effect of CBZ. CBZ treatment significantly improved the survival rate of the mice that underwent lethal 85% massive hepatectomy.

Conclusions: CBZ demonstrated a novel hepato-proliferative effect through the activation of the mTOR signalling pathway in hepatectomised mice. CBZ has the potential to be a therapeutic option for facilitating efficient liver regeneration in patients subjected to liver surgery.

Keywords: Carbamazepine; Liver regeneration; Hepatocyte proliferation; Akt; mTOR.

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Abbreviations: CBZ, carbamazepine; PHx, partial hepatectomy; PI-3K, phosphotidylinositol-3 kinase; MAPK, ras-mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; DMSO, dimethyl sulfoxide; H&E, haematoxylin and eosin; IHC, immunohistochemistry; RT-PCR, reverse transcription PCR; JNK, c-jun N-terminal kinase; CCl_4, carbon tetrachloride; NPC, non-parenchymal cells; HGF, hepatocyte growth factor.

Introduction

Hepatocyte proliferation is critically important in liver regeneration after surgical resection or living donor transplantation. It involves the recovery from loss of volume and impaired liver function [1–3]. If this fundamental proliferative ability is not sufficient to compensate for the resected liver, postoperative liver failure will occur, which is a serious complication and remains an important clinical problem [4,5]. To overcome this issue, therapeutic methods that support liver regeneration must be explored. However, few treatment options are capable of enhancing liver regeneration in a clinical setting, despite widespread interest and numerous trials [6,7]. Carbamazepine (CBZ) is FDA-approved and widely used as an anticonvulsant or a mood stabiliser in clinical settings [8,9]. Mood stabilisers have been shown to exert prosurvival and cytoprotective effects on neuronal cells through the activation of intracellular signalling pathways that involve the phosphotidylinositol-3 kinase (PI-3K)-Akt pathway and the Ras-mitogen-activated protein kinase (MAPK) cascade [10–12]. In fact, CBZ induces a rapid and prolonged phosphorylation of extracellular signal regulated kinase (ERK) in human neuroblastoma cells [13]. In addition to the close relationship of CBZ to prosurvival signalling, a recent report revealed the therapeutic potential of CBZ in treating liver fibrosis caused by α1-antitrypsin deficiency, one of the chronic liver diseases leading to cirrhosis and liver failure [14]. These findings fascinated us enough to encourage the evaluation of the favourable effect of CBZ on liver regeneration after surgical resection. In the present study, we identified a novel hepatoproliferative effect of CBZ on hepatectomised mice that is mediated through the activation of the mTOR pathway. This effect could partially protect the mice against the high lethality associated with massive liver resection. These results imply the therapeutic potential of CBZ to support liver regeneration in patients who are subjected to liver resection or living donor transplantation.

Materials and methods

Mice

Six- to eight-week-old male C57BL/6J mice were purchased from Charles River Laboratories Japan (Tokyo). The mice were maintained in a specific pathogen-free facility with a 12-hour-dark/12-hour-light cycle and received humane treatment. All animal-related procedures were approved by the Animal Care and Use committee of Osaka University Medical School.

Surgery and materials

The mice were anesthetised with inhaled isoflurane and subjected to sham operation or 70% partial hepatectomy (PHx) as previously described (n >3 for each group and time point) [15]. Then, the mice were euthanized at indicated time points after surgery. The 85% PHx surgical procedure was identical to 70% PHx but with the additional resection of the right lower and caudate lobes [16]. Carbamazepine (CBZ) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in a stock solution of 50 mg/ml dimethyl sulfoxide (DMSO). The mice were orally administered 250 mg/kg of CBZ or an equivalent volume of DMSO 2 h before surgery. The CBZ dosage was determined based on a previous *in vivo* study [14]. Temsirolimus was purchased from Sigma-Aldrich and dissolved in a stock solution of 20 mg/ml DMSO. The mice were injected intraperitoneally with 5 mg/kg of temsirolimus or an equivalent volume of DMSO 4 h before surgery. The temsirolimus dosage was determined based on a previous *in vivo* study reporting its inhibitory effects on mTOR [17].

Blood tests

To measure serum AST and ALT levels, blood was collected from the inferior vena cava of mice and centrifuged at 10,000*g* at room temperature for 15 min. Serum AST and ALT levels were measured by a standard method at the Oriental Kobo Life Science Laboratory (Nagahama, Japan).

Histological analyses

The dissected livers were fixed in formalin and embedded in paraffin. The sections were stained with haematoxylin and eosin (H&E). To assess hepatocyte proliferation, the sections were further processed for immunohistochemistry (IHC) with anti-Ki-67 antibody (Sigma-Aldrich) and anti-PCNA antibody (Cell Signaling Technology, Beverly MA). For IHC, antigen retrieval was performed by steaming for 20 min in 1× Target Retrieval Solution (pH 6.0) (DAKO, Glostrup, Denmark). The quenching of the endogenous peroxidase was accomplished with a 10-min incubation in 3% hydrogen peroxide in methanol. Sections were stained using the immunoperoxidase technique and counterstained with haematoxylin. We also stained liver sections for nuclear BrdU incorporation as previously described [18].

Western blot analysis

A piece of frozen liver tissue was lysed in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1× protease inhibitor cocktail [Nacalai Tesque, Kyoto Japan], 1× phosphatase inhibitor cocktail [Nacalai Tesque], phosphate-buffered saline, pH7.4). The homogenates were purified by centrifugation at 10,000*g* at 4 °C for 15 min. The protein concentrations were determined using a bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL). Equal amounts of protein extract were electrophoretically separated by SDS polyacrylamide gels and transferred onto a polyvinylidene fluoride membrane. For immunodetection, the following antibodies were used: anti-cyclinE1, anti-Akt, anti-phospho Akt (Thr 308), anti-phospho Akt (Ser 473), anti-mTOR, anti-phospho mTOR (Ser 2448), anti-S6K, anti-phospho S6K (Thr 389), anti-4EBP1, anti-phospho-4EBP1 (Thr 37/46), anti-ERK, and anti-phospho ERK (Thr 202/Tyr 204), anti-JNK, anti-phospho JNK (Thr 183/Tyr 185) (Cell Signaling Technology), anti-cyclinA (Santa Cruz Biotechnology Inc., Santa Cruz, CA), PCNA and β-actin (Sigma-Aldrich).

Real-time quantitative PCR

Total RNA isolated from liver tissues using an RNeasy Mini Kit (QIAGEN) was reverse transcribed and subjected to real-time reverse transcription PCR (RT-PCR) as previously described [18]. The mRNA expression levels of the specific genes were quantified using TaqMan Gene Expression Assays (Applied Biosystems) as follows: murine *ccna2* (assay ID:Mm00438063_m1), murine *ccne2* (assay ID: Mm00438077_m1), murine *hgf* (assay ID:Mm01135193_m1), murine *il6* (assay ID:Mm00446190_m1) and murine *actb* (assay ID:Mm00607939_s1). The transcript levels are presented as fold change relative to the controls.

Statistics

Data are expressed as mean ± SD. Statistical analyses between two groups were performed by an unpaired Student's *t* test unless otherwise indicated. Multiple comparisons were performed by a one-way ANOVA, and differences in the mean values among groups were examined by a Fischer *post hoc* correction. *p* values less than 0.05 were considered to be statistically significant.

Results

CBZ promotes liver regeneration after PHx

To test whether CBZ has any effect on liver regeneration, male C57BL6/J mice were orally administered CBZ or vehicle and underwent 70% PHx. The PHx procedure allows for a well-established liver regeneration model in which the liver recovers full volume after surgery. In the sham-operated mice, no difference was found in liver to body weight ratio at 48 h after drug administration between the CBZ-treated and vehicle-treated groups (Supplementary Fig. 1). In the hepatectomised mice, the ratio was significantly higher in the CBZ-treated group than in the vehicle-treated group (Supplementary Fig. 1). We then examined the liver to body weight ratio at several time points after surgery with or without one-time oral CBZ administration. After PHx, the liver to body weight ratio was rapidly recovered in the CBZ-treated mice and was significantly higher than in the vehicle-treated mice at 2, 3 and 5 days after PHx (Fig. 1A). The liver to body weight ratio reached similar levels by 14 days after surgery in both groups (Fig. 1A). These findings demonstrate that CBZ promoted liver regeneration after PHx in mice.

CBZ enhances hepatocyte proliferation after PHx

During liver regeneration, hepatocyte proliferation is critically important in compensating for the lost liver mass and liver function recovery. To determine whether CBZ affects hepatocyte proliferation in the hepatectomised mice, hepatocyte DNA synthesis was assessed by immunohistochemical staining of liver sections with Ki-67 and BrdU—two principal markers of DNA replication. We first confirmed that there was no difference in liver injury after PHx in the CBZ- or vehicle-treated mice, by evaluation of serum AST and ALT levels (Fig. 1B and C). H&E staining also revealed that there was no inflammatory cell infiltration or necrosis in the livers of either group (Fig. 1D). The number of Ki-67 positive cells increased to a peak at 48 h after PHx in both groups (Fig. 1E and F), but the peak value was significantly higher in the CBZ-treated livers (Fig. 1E and F). Similarly, the number of BrdU-positive nuclei was also significantly higher in CBZ-treated mice than in vehicle-treated mice at 36 h after PHx (Fig. 1G and H). Western blotting indicated higher protein expression levels for proliferating nuclear antigen (PCNA), another well-known marker of DNA replication, in CBZ-treated livers at 48 h after PHx (Fig. 1I). These findings indicate that CBZ increased the number of proliferative hepatocytes after PHx in mice. We also observed the similar hepato-proliferative effect and amelioration of liver regeneration in hepatectomized mice even after repeated CBZ administration for 3 consecutive days (Supplementary Fig. 2A and B), which is a more clinically relevant regimen since CBZ requires multiple administrations to reach steady state levels [19]. To determine whether this favourable effect of CBZ is only observed in a resected liver, CBZ-treated mice were administered a single injection of carbon tetrachloride (CCl_4), which causes acute liver injury, and followed compensative liver regeneration [20]. CBZ treatment did not affect the liver damage but enhanced hepatocyte proliferation (Supplementary Fig. 3A–C) suggesting that the hepato-proliferative effect of CBZ may not be limited to the hepatectomised liver.

We then examined the gene expression of several cyclins, accelerators of cell cycle progression, which are important for hepatocyte proliferation in regenerating livers [21]. A real-time RT-PCR analysis revealed that the mRNA levels of *ccne2* and *ccna2* were significantly higher in CBZ-treated mice than in

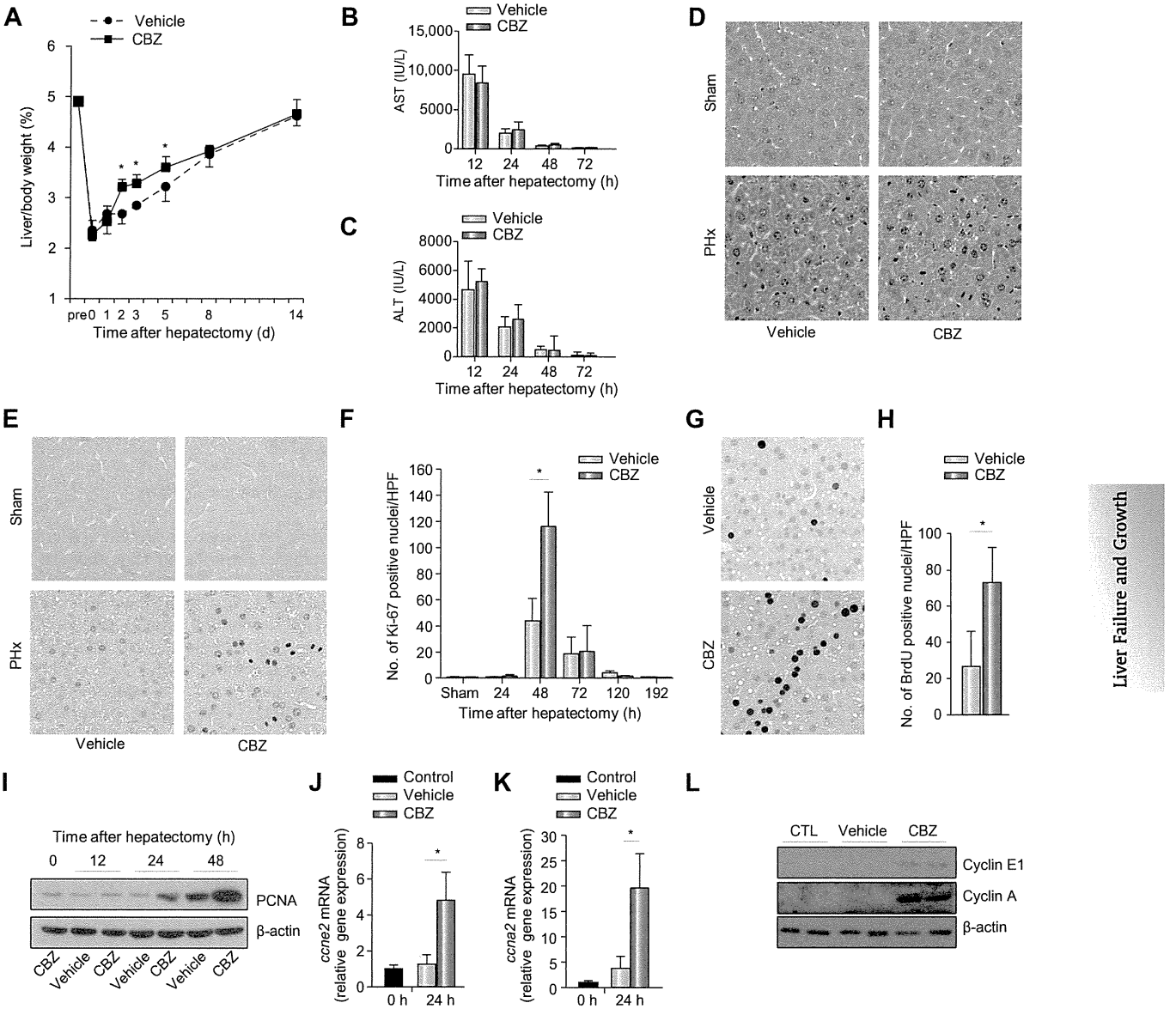

Fig. 1. CBZ promotes liver/body weight ratio recovery and enhances hepatocyte proliferation after 70% partial hepatectomy. Mice were administered 250 mg/kg of CBZ or DMSO orally and subjected to 70% partial hepatectomy 2 h later (3 mice per group). CBZ, PHx, and CTL stand for carbamazepine, 70% partial hepatectomy and control, respectively. (A) Changes in liver/body weight ratio over time in mice receiving PHx with vehicle or CBZ, $^{*}p$ <0.05 *vs.* vehicle. (B and C) Serum AST (B) and ALT (C) levels in vehicle- or CBZ-treated mice. (D) Liver sections at 48 h after PHx or sham operation were stained with H&E; original magnification, 400×. (E) Liver sections after surgery were evaluated for hepatocyte proliferation with anti-Ki-67 staining; original magnification, 400×. (F) The number of Ki-67 positive nuclei/high-power field (HPF) at 48 h after surgery in sham-operated mice and at indicated time in hepatectomised mice with vehicle or CBZ treatment. Six fields of view (FOVs) were counted in liver sections of individual mice, $^{*}p$ <0.05. (G) Liver sections at 36 h after PHx were stained with BrdU; original magnification, 400×. (H) The number of BrdU positive nuclei/HPF at 36 h after PHx in vehicle-treated and CBZ-treated mice. Six FOVs were counted in liver sections of individual mice, $^{*}p$ <0.05. (I) Expression of PCNA protein in liver tissue from vehicle- or CBZ-treated mice after PHx was assessed by Western blot analysis. (J and K) *ccne2* (J) and *ccna2* (K) mRNA levels in the liver were determined by real time RT-PCR at 24 h after PHx, $^{*}p$ <0.05. (L) Protein expression of cyclin E1 and cyclin A in liver tissue was assessed by Western blot analysis at 24 h after PHx.

vehicle-treated mice at 24 h after PHx (Fig. 1J and K). Evaluation by Western blot also demonstrated that protein levels of cyclin E1 and cyclin A were increased in CBZ-treated mice (Fig. 1L). Collectively, these results suggest that CBZ upregulated the cyclin levels in remnant hepatocytes, leading to an increase in the number of hepatocytes entering the cell cycle after PHx.

CBZ strongly activates the Akt-mTOR pathway after PHx

Mood stabilisers, including CBZ, have been reported to modulate the Akt and MAPK pathways [10–13], both of which are also involved in initiating the cell cycle progression of remaining liver cells upon liver resection [22–25]. Thus, we examined the effect

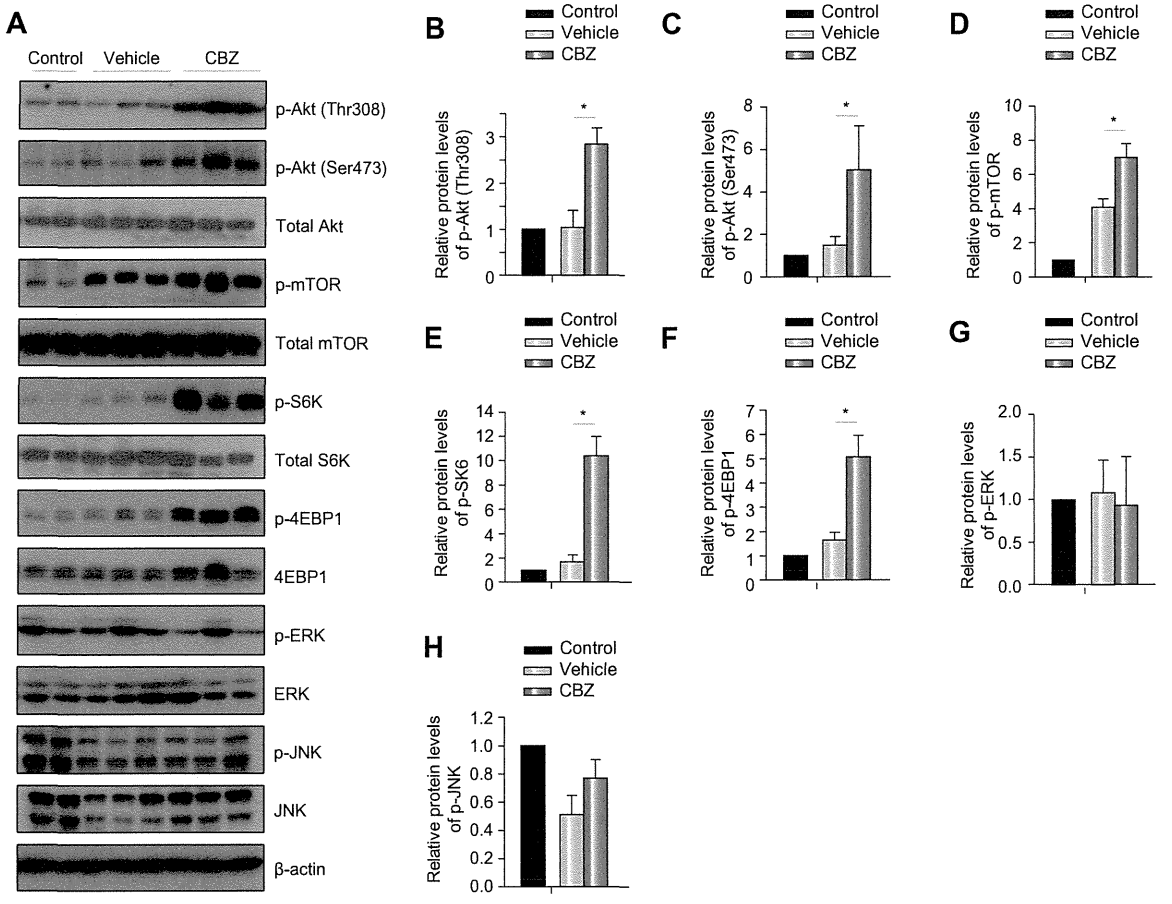

Fig. 2. CBZ strongly activates Akt-mTOR signalling. Mice were administered 250 mg/kg of CBZ or DMSO orally and subjected to 70% partial hepatectomy 2 h later (4 mice per group). (A) The phosphorylation status of Akt, mTOR, S6K, 4EBP1, ERK and JNK was assessed by Western blot analysis at 12 h after PHx. (B–H) Relative expression levels of phosphorylated proteins were calculated as the optical densities of their blots normalized to the β-actin blots; p-Akt (Thr308) (B), p-Akt (Ser473) (C), p-mTOR (D), p-S6 K (E), p-4EBP1 (F), p-ERK (G) and p-JNK (H). CBZ, carbamazepine; *p <0.05.

of CBZ on the activation of these two pathways in the livers of hepatectomised mice. PHx induced phosphorylation of Akt (Thr308, Ser473) and activated its downstream effectors, mTOR, S6K, and 4EBP1, at 12 h after surgery. All of these signalling molecules were enhanced by CBZ treatment (Fig. 2A–F). By contrast, the phosphorylation of ERK was not different between the CBZ-treated and vehicle-treated mice (Fig. 2A and G). We also evaluated the activation of the c-jun N-terminal kinase (JNK) pathway, which is closely related to liver regeneration [26], and found no difference between the two groups (Fig. 2A and H).

Activation of the mTOR pathway is responsible for enhanced hepatocyte proliferation in hepatectomised mice following CBZ treatment

To investigate whether the strong activation of Akt-mTOR pathway was ascribable to the hepato-proliferative effect of CBZ after PHx, we blocked mTOR signalling by the use of the mTOR inhibitor temsirolimus. Temsirolimus administration blocked the enhancement of mTOR pathway activation in the CBZ-treated hepatectomised livers to a level similar to the vehicle-treated hepatectomised liver (Fig. 3A), while phosphorylation of Akt, an upstream signalling molecule of mTOR, was upregulated in both mice likely due to a compensative response (Fig. 3A). Under these conditions, temsirolimus abrogated the upregulation of *ccne2* and *ccna2* mRNA expression and PCNA protein expression in the CBZ-treated hepatectomised mice (Fig. 3B–E), suggesting that the hepato-proliferative effect of CBZ is attributable to the enhanced activation of the mTOR pathway. In addition, mTOR inhibition also prevented CBZ-induced acceleration of liver mass recovery 48 h after PHx (Fig. 3F). Altogether, these findings indicate that, following PHx surgery, CBZ treatment potentiated the activation of the mTOR pathway, which enhanced hepatocyte proliferation and promoted liver regeneration.

CBZ improves the survival rate of mice that undergo 85% massive hepatectomy

Finally, we evaluated the therapeutic significance of CBZ in regeneration of the resected liver using a severe 85% massive hepatectomy model. This PHx model typically presents extremely high mortality (82%) within 2 days after surgery [27]. Consistent

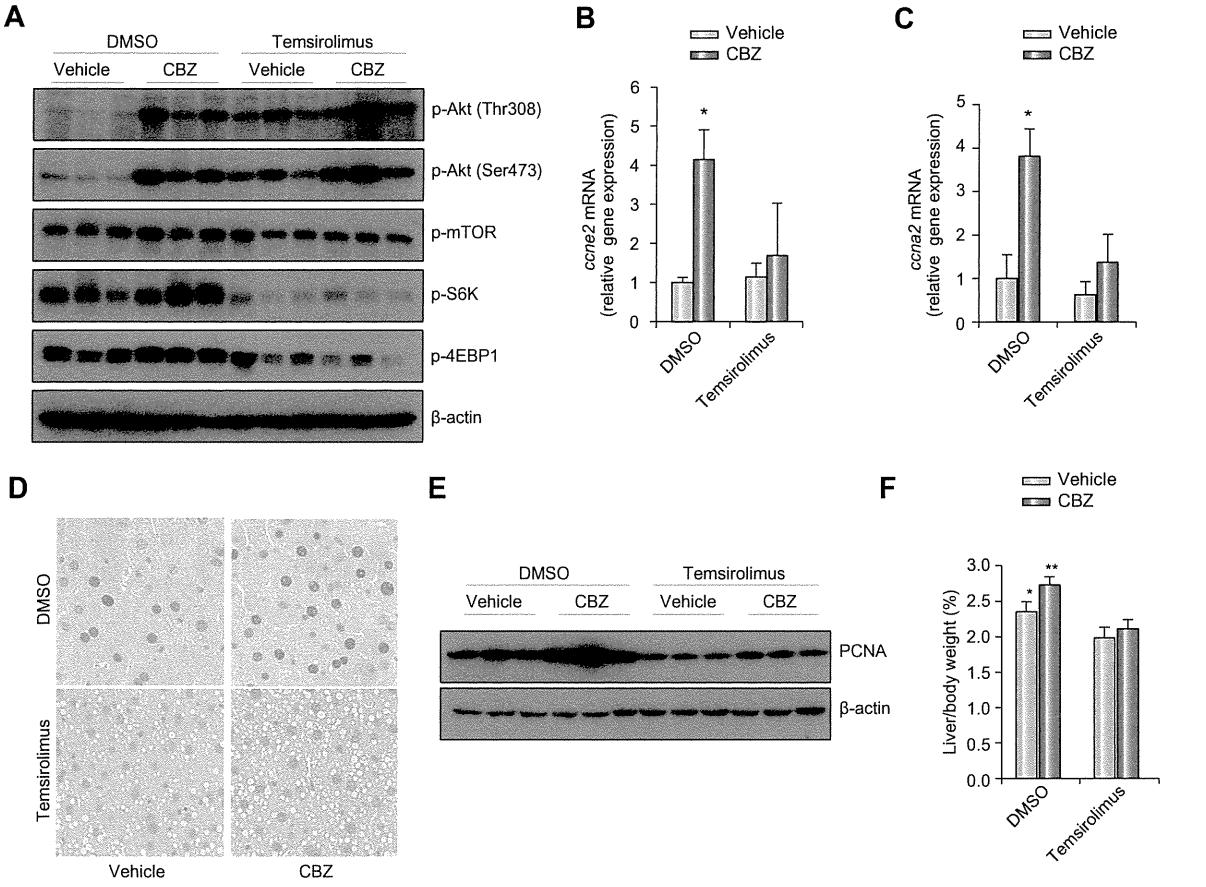

Fig. 3. mTOR inhibitor abrogates the hepato-proliferative effect of CBZ in hepatectomised mice. Mice were injected with temsirolimus or DMSO 4 h before PHx and orally administered 250 mg/kg of CBZ or DMSO 2 h before PHx. Then, mice were subjected to 70% partial hepatectomy and euthanized at indicated time points. (A) The phosphorylation status of Akt, mTOR, S6K, and 4EBP1 at 12 h after PHx was assessed by Western blot analysis. (B and C) Real-time RT-PCR analysis of *ccne2* (B) and *ccna2* (C) mRNA expression at 24 h after PHx, $^{*}p$ <0.05 *vs.* all. (D and E) The expression of PCNA proteins at 48 h after PHx was assessed by (D) immunohistochemistry and (E) Western blot analysis. (F) Liver/body weight ratio at 48 h after PHx in indicated groups. CBZ, carbamazepine; 3 mice per group. Statistical analyses were performed by one-way ANOVA. $^{*}p$ <0.05 *vs.* temsirolimus-vehicle group; $^{**}p$ <0.05 *vs.* all.

with the effect of CBZ observed in the 70% PHx model, CBZ did not affect liver injury but enhanced hepatocyte proliferation in the liver after the 85% PHx (Fig. 4A and B). Consequently, while only 4 of the 25 vehicle-treated mice survived for 7 days after 85% PHx, 11 of 25 CBZ-treated mice were alive at 7 days. The CBZ-treated mice survival rate was significantly higher than that of vehicle-treated mice (44% *vs.* 16%, p <0.05) (Fig. 4C).

Discussion

Liver regeneration after surgical resection or injury is a complex phenomenon primarily dependent on hepatocyte proliferation. In the present study, we identified a new aspect of CBZ, increasing hepatocyte proliferation after partial resection of the liver in mice. We also clarified the involvement of the mTOR signalling pathway in this hepato-proliferative effect. mTOR and its downstream effectors S6K and 4EBP1, all of which were intensively upregulated by CBZ treatment, have been shown to stimulate cell cycle progression via modulation of the expression of several cyclins, such as cyclin E and cyclin A [28]. In fact, in our hepatectomised mice, CBZ enhanced upregulation of their mRNA levels, which were dependent on mTOR activation. These findings suggest that mTOR activation may produce a profound effect on cell cycle progression via upregulating cyclin expression in CBZ-treated remnant livers. In this study, we also found that CBZ enhanced Akt phosphorylation following PHx, which might be an event that is upstream of mTOR activation. As mood stabilising drugs have been described to trigger activation of PI-3K and subsequent phosphorylation of Akt in neuronal cells by generating lipid second messengers (i.e., PI-3,4,5-P3 or PI-3,4-P2) [10,13], such a mechanism might be relevant to CBZ-mediated Akt activation in resected livers. Further studies are necessary to elucidate the exact mechanism by which CBZ activates the mTOR signalling pathway.

Given that CBZ has complicated pharmacokinetic properties, a variety of mechanisms other than those involving the mTOR pathway could be related to the enhanced liver regeneration in

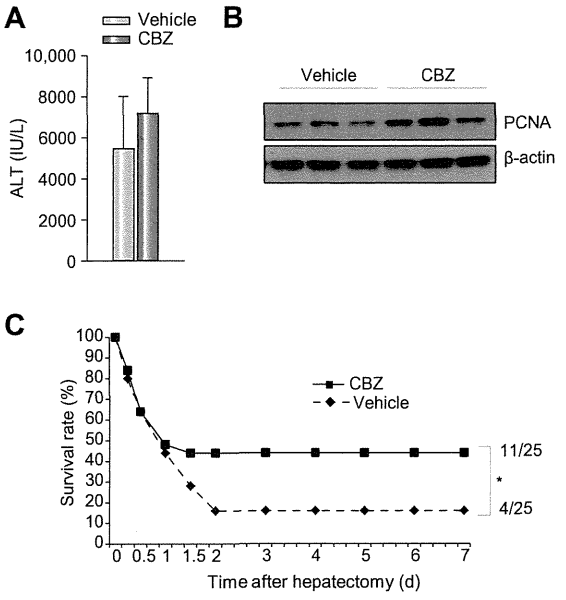

Fig. 4. CBZ improves survival of mice that undergo 85% massive hepatectomy. Mice were orally administered 250 mg/kg of CBZ or DMSO and subjected to 85% partial hepatectomy 2 h later. (A and B) Mice were euthanized 24 h after PHx (4 mice per group). (A) Serum ALT levels. (B) Expression of PCNA protein in liver tissue from vehicle- or CBZ-treated mice was assessed by Western blot analysis. (C) The survival rate was assessed at 7 days after surgery (25 mice per group). Statistical analysis was performed using Chi-square test. CBZ, carbamazepine; *p <0.05.

CBZ-treated mice. To further investigate underlying mechanisms, we performed microarray analysis of the mouse liver tissues collected after CBZ administration. Pathway analysis of microarray data revealed activation of PXR/RXR and FXR/RXR pathways (data not shown), both of which have been reported to be involved in liver regeneration [29–31]. These pathways might be also involved in the hepato-proliferative effect of CBZ.

Following PHx, both hepatocytes and non-parenchymal cells (NPCs) are activated and integrate multiple signals originating from immune, hormonal, and metabolic networks to induce hepatocyte proliferation [24]. In particular, after PHx, hepatic stellate cells and Kupffer cells produce hepatocyte growth factor (HGF) and IL-6, respectively, both of which contribute to liver regeneration partially through modulating the intrahepatic signalling pathways focused on in this study [3,32]. Therefore, we investigated the involvement of NPCs in the CBZ-induced hepato-proliferative effect in hepatectomised mice. Neither *HGF* nor *IL6* gene expression levels were different between the CBZ-treated livers and vehicle-treated livers following PHx (Supplementary Fig. 4A and B). By contrast, in the *in vitro* study, primary hepatocytes presented sustained phosphorylation of Akt (Ser473) with transient and moderate activation of mTOR in response to the administration of CBZ (Supplementary Fig. 5). These findings support the idea that CBZ may directly activate intracellular signalling pathways in hepatocytes contributing to enhanced liver regeneration. Meanwhile, in this *in vitro* setting, primary hepatocytes did not show a proliferative response to CBZ administration (Supplementary Fig. 6). This may be because hepatocytes require additional priming stimulus to start proliferation *in vitro*, same as our *in vivo* finding that CBZ administration did not start liver regeneration in the sham-operated mice (Fig. 1E and F, and Supplementary Fig. 1). We cannot exclude the possibility that CBZ does not primarily target hepatocytes, but affects other cell types in the liver to promote liver regeneration. Actual targets of CBZ in the liver will be determined in future studies.

In rodents, 70% hepatectomy is well tolerated, but beyond 70%, resection is accompanied by higher mortality due to acute liver failure despite the inherent ability of the liver to recover to full size. This suggests that insufficient functional compensation of the remnant liver fails to maintain homeostasis in the animal [16,27]. In clinical settings, extended liver resection is reportedly associated with severe hepatic dysfunction, leading to a significant increase in postoperative mortality [33,34]. In this context, the promotion of the recovery of impaired liver function is critically important for any therapeutic drug potentially used to aid in liver regeneration. In the present study, CBZ treatment significantly improved the survival rate of the mice that underwent lethal 85% massive hepatectomy. This result elucidates the therapeutic potential of CBZ to prevent postoperative liver failure after major hepatectomy or living donor liver transplantation with extended criteria.

When considering the therapeutic application of this study, it is important to apply clinically relevant doses of CBZ to obtain relevant physiological serum levels of CBZ (4-12 μg/ml) [35]. In the present study, 2 h after oral administration of 250 mg/kg of CBZ, its serum level reached 22.9 μg/ml (Supplementary Fig. 7A) and was relatively higher than the therapeutic range in humans. It is known that repeated administration of CBZ shortens its half-life, and therefore consecutive administration is required to acquire steady state levels [19]. Thus, we evaluated CBZ serum levels after repeated administration at 250 mg/kg for 3 consecutive days. This administration method acquires physiological levels of CBZ (4.8 μg/ml) (Supplementary Fig. 7B), and importantly, the favorable effect on liver regeneration was retained in the subsequently performed 70% PHx (Supplementary Fig. 2A and B). This result may support the potential therapeutic use of CBZ. We also studied the influence of hepatectomy on serum levels of CBZ because it reduces the total amount of metabolizing cells in the liver. Serum levels of CBZ were not different between the hepatectomized mice and the sham operated mice 3 h after the surgery (Supplementary Fig. 7C), suggesting that CBZ treatment may be applicable after liver resection.

In conclusion, we demonstrated that CBZ promoted hepatocyte proliferation via the mTOR signalling pathway, resulting in early liver regeneration in mice. We also demonstrated the therapeutic implications of this drug in an 85% massive hepatectomy model. Despite a large number of basic studies searching for novel therapeutic agents to enhance liver regeneration, few options are currently available for clinical use [6,7]. Our study suggests the possibility that CBZ may enhance liver regeneration in a clinical setting, leading to a reduction in postoperative liver failure and improving survival.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.07.018.

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