Hepatocellular Carcinoma–Circulating Tumor Cells Expressing PD-L1 Are Prognostic and Potentially Associated With Response to Checkpoint Inhibitors

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Hepatocellular carcinoma (HCC) is a leading cause of mortality. Checkpoint inhibitors of programmed cell death protein-1 (PD-1) and programmed death-ligand 1 (PD-L1) have shown great efficacy, but lack biomarkers that predict response. Circulating tumor cells (CTCs) have promise as a liquid-biopsy biomarker; however, data on HCC CTCs expressing PD-L1 have not been reported. We sought to detect PD-L1-expressing HCC-CTCs and investigated their role as a prognostic and predictive biomarker. Using an antibody-based platform, CTCs were enumerated/phenotyped from a prospective cohort of 87 patients with HCC (49 early-stage, 22 locally advanced, and 16 metastatic), 7 patients with cirrhosis, and 8 healthy controls. Immunocytochemistry identified total HCC CTCs (4′,6-diamidino-2-phenylindole–positive [DAPI+]/cytokeratin-positive [CK+]/clusters of differentiation 45–negative [CD45–]) and a subpopulation expressing PD-L1 (DAPI+/CK+/PD-L1+/CD45–). PD-L1+ CTCs were identified in 4 of 49 (8.2%) early-stage patients, but 12 of 22 (54.5%) locally advanced and 15 of 16 (93.8%) metastatic patients, accurately discriminating early from locally advanced/metastatic HCC (sensitivity = 71.1%, specificity = 91.8%, area under the receiver operating characteristic curve = 0.807; $P < 0.001$). Compared to patients without PD-L1+ CTCs, patients with PD-L1+ CTCs had significantly inferior overall survival (OS) (median OS = 14.0 months vs. not reached, hazard ratio [HR] = 4.0, $P = 0.001$). PD-L1+ CTCs remained an independent predictor of OS (HR = 3.22, $P = 0.010$) even after controlling for Model for End-Stage Liver Disease score (HR = 1.14, $P < 0.001$), alpha-fetoprotein (HR = 1.55, $P < 0.001$), and overall stage/tumor burden (beyond University of California, San Francisco, HR = 7.19, $P < 0.001$). In the subset of 10 patients with HCC receiving PD-1 blockade, all 5 responders demonstrated PD-L1+ CTCs at baseline, compared with only 1 of 5 nonresponders, all of whom progressed within 4 months of starting treatment. *Conclusion:* We report a CTC assay for the phenotypic profiling of HCC CTCs expressing PD-L1. PD-L1+ CTCs are predominantly found in advanced-stage HCC, and independently prognosticate OS after controlling for Model for End-Stage Liver Disease, alpha-fetoprotein, and tumor stage. In patients with HCC receiving anti-PD-1 therapy, there was a strong association with the presence of PD-L1+ CTCs and favorable treatment response. Prospective validation in a larger cohort will better define the utility of PD-L1+ CTCs as a prognostic and predictive biomarker in HCC. (*Hepatology Communications* 2020;4:1527-1540).

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer mortality worldwide and remains an important global health burden, with an estimated one million deaths attributable to HCC by 2030.[1] In the United States, the age-adjusted incidence of HCC has increased

Abbreviations: AFP, alpha-fetoprotein; ASGPR1, asialoglycoprotein receptor 1; AUROC, area under the receiver operator characteristic curve; BCLC, Barcelona Clinic Liver Cancer; CD45, clusters of differentiation 45; CI, confidence interval; CK, cytokeratin; CTC, circulating tumor cell; DAPI, 4′,6-diamidino-2-phenylindole; EMT, epithelial-to-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; GPC-3, glypican-3; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; ICC, immunocytochemistry; IQR, interquartile range; LT, liver transplantation; MELD, Model for End-Stage Liver Disease; MC, Milan criteria; NASH, nonalcoholic steatohepatitis; OS, overall survival; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PD-L1, programmed-death ligand 1; TACE, transarterial chemoembolization; UCSF, University of California, San Francisco.

dramatically over the last 2 decades,[2] and it remains one of the only malignancies with an increasing mortality rate.[3] Unfortunately, the current clinicopathologic staging systems and serum biomarkers poorly discriminate outcome for early-stage patients undergoing potentially curative surgical resection and liver transplantation (LT), in whom postoperative recurrence remains a significant challenge.[4,5] Additionally, for most patients presenting with advanced-stage, incurable HCC, predictors of response to systemic therapy remain unavailable. Until 2017, the multikinase inhibitor sorafenib has been the only systemic treatment option for patients with advanced disease, conferring a modest increase in median overall survival from 7 to 10 months, with an overall response rate of 2%.[6] However, there has been renewed optimism for the treatment of advanced HCC over the past several years, with numerous effective systemic agents approved for both front-line therapy[7] and second-line therapy,[8-13] underscoring the importance of identifying both prognostic and predictive biomarkers in HCC.

One class of these drugs, the checkpoint inhibitors, has garnered particular attention. The programed cell death protein 1 (PD-1) and programed death-ligand 1 (PD-L1) axis is an inhibitory immune checkpoint widely implicated in the suppression of antitumor immunity. Tumor overexpression of PD-L1 enables evasion of immune-mediated tumor surveillance by inducing T-cell anergy or apoptosis. Immune checkpoint blockades targeting PD-1 or PD-L1 restore antitumor immunity and have demonstrated durable efficacy in several malignancies.[14] Although a wide range of PD-L1 tumor expression has been reported in HCC, studies have consistently demonstrated PD-L1 tumor expression to be associated with more aggressive pathologic features and worse prognosis.[15-17] The PD-1 checkpoint inhibitor, nivolumab, was the first in this class of drugs to receive accelerated approval as a second-line treatment for patients with HCC failing sorafenib on the basis of the phase 2 CheckMate 040 trial.[10] In this study, the objective response rate was 18%, but tumor PD-L1 status assessed by immunohistochemistry was not predictive of response to

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nivolumab. Pembrolizumab, another monoclonal antibody against PD-1, also demonstrated a 17% objective response rate in advanced-stage patients progressing on sorafenib in the phase 2 KEYNOTE-224 trial,[12] but again, tumor PD-L1 expression was not significantly associated with response. Subsequent large phase 3 randomized controlled trials have confirmed the efficacy of both nivolumab[18] and pembrolizumab[11] in prolonging survival in the subset of patients who respond; however, a predictive biomarker has not been established. These findings highlight the urgent need to identify predictive biomarkers in HCC to identify patients likely to benefit from anti-PD-1/PD-L1 therapy.

Circulating tumors cells (CTCs) are intravasated tumor cells released into the bloodstream from primary tumor sites, and are implicated in the development of metastases.[19] CTCs are easily accessible through a simple blood draw, amenable to serial sampling, and recognized as a potential alternative to invasive, technically challenging tissue biopsies.[20] Although the prognostic significance of CTCs is established in several malignancies,[21,22] data for their utility in HCC remain limited.[23] More recently, subset characterizations of CTCs expressing various phenotypic profiles such as epithelial-to-mesenchymal transition markers[24,25] and stem cell markers[26] have been shown to have significant prognostic utility. Furthermore, characterization of CTCs expressing PD-L1 have recently been reported in breast cancer,[27] and shown to be associated with progression-free survival in patients receiving immunotherapy in both melanoma[28] and non-small cell lung cancer.[29]

To our knowledge, characterization of HCC CTCs expressing PD-L1 has not been previously reported. In this study, we sought to evaluate the feasibility of isolating and detecting CTCs expressing PD-L1 in a prospective cohort of patients with HCC, and subsequently evaluate their clinical significance. Using the microfluidic, antibody-based NanoVelcro Chip,[30] which has been validated in several malignancies[21,30,31] including HCC,[25] we herein report the characterization of an HCC-CTC subpopulation expressing PD-L1. We aimed to evaluate (1) the association of PD-L1-expressing CTCs with HCC tumor stage, (2) the prognostic impact of PD-L1+ CTCs on survival, and (3) the predictive ability of PD-L1+ CTCs, to potentially identify patients who may benefit from anti-PD-1 therapy.

Patients and Methods

STUDY DESIGN

After written informed consent was obtained (University of California, Los Angeles Internal Review Board No. 14-001932), peripheral venous blood was collected from healthy individuals, patients with liver cirrhosis, and patients with a pathologic or radiographic (Liver Imaging Reporting and Data System 5) diagnosis of HCC. Individuals with no history of underlying liver disease or chronic medical conditions were enrolled as healthy controls. Patients who met the following criteria were enrolled as the cirrhotic cohort: (1) histology or findings characteristic of cirrhosis in cross-sectional imaging studies and (2) at least 6 months of follow-up after blood collection to confirm the absence of HCC on screening ultrasonography, or a negative contrast-enhanced multiphasic computed tomography or magnetic resonance imaging at study enrollment. Participants were excluded if they had concomitant neoplasms or history of extrahepatic malignancy within the last 5 years.

Demographic, clinical, and pathologic data were collected and maintained in a prospective manner. At the time of blood draw, the tumor burden of patients with HCC was assessed radiographically based on the University of California, San Francisco (UCSF) transplant criteria. By definition, patients with early-stage HCC were within UCSF transplant criteria, patients with locally advanced HCC were beyond UCSF transplant criteria but without evidence of extrahepatic disease, and patients with metastatic HCC had evidence of distant metastases.[32] For patients with prior treatment, their stage at HCC diagnosis was also characterized and reported.

Disease progression was assessed using the revised Response Evaluation Criteria In Solid Tumors (RECIST, version 1.1).[33] In patients who underwent locoregional therapy, modified Response Evaluation Criteria In Solid Tumors (mRECIST) was used to assess for disease progression.[34] In the cohort of patients who received anti PD-1 therapy, RECIST version 1.1 was used to designate treatment response. Patients were considered as *responders* if they had a partial response or stable disease, and *nonresponders* if they had progressive disease or died within 6 months of initiating treatment from any cause.

BLOOD SAMPLE PROCESSING

Venous blood from participants were collected in acid-citrate dextrose-containing vacutainers (BD Bioscience, San Jose, CA) and processed within 24 hours. Peripheral blood mononuclear cells (PBMCs), including CTCs, were separated from venous blood by gradient centrifugation with the use of Ficoll-Paque solution (Sigma-Aldrich, St. Louis, MO) using the manufacturer's protocol.

CTC ENRICHMENT USING NANOVELCRO CHIP

PBMCs separated from 2.0 mL of venous blood were incubated with a HCC-specific, mulitmarker capture antibody cocktail (biotinylated anti–epithelial cell adhesion molecule [EpCAM] antibody [R&D Systems, Minneapolis, MN], biotinylated anti-ASGPR1 [LifeSpan BioSciences, Seattle, WA], and biotinylated anti-GPC3 [R&D Systems]). After washing off the leftover antibodies, the sample was then loaded into the NanoVelcro Chip,[30] which combines a streptavidin-coated nanostructure-embedded substrate and an overlaid polydimethylsiloxane microfluidic chaotic mixer to capture CTCs. For each sample, the whole process was run in duplicate. Thus, CTC counts were recorded per 4 mL of blood. The CTC capture relied on antigen-specific immobilization on the nanostructured interface using selected antibodies directed against HCC cell surface markers. The operating parameters were determined by the optimization experiments previously described and detailed in our studies.[25,35]

CTC IMMUNOSTAINING, IMAGING, AND ENUMERATION

The cells captured on the chip were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and stained using immunocytochemistry (ICC), allowing for both cytometric and immunofluorescent identification parameters. Mouse anti-PD-L1 (R&D Systems), rabbit anti-pan-cytokeratin (Life Technologies, Grand Island, NY, and Abcam, Cambridge, MA), rat anti–clusters of differentiation 45 (CD45; BD Biosciences and Abcam), Alexa Fluor 555-conjugated anti-mouse, Alexa Fluor 488-conjugated anti-rabbit (Life Technologies), and Alexa Fluor 647-conjugated anti-rat (Abcam) were used. PD-L1 antibody was validated using the HepG2 cell line known to express PD-L1 as the positive control and PBMCs from healthy donor as the negative control (Supporting Fig. S1). Chips were mounted in ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole [DAPI] (Life Technologies).

An automated scan of the NanoVelcro Chip was carried out (×10) for enumeration, and images of cells were acquired (×40) using an fluorescence microscope (Eclipse 90i; Nikon, Toyo, Japan) with NIS-Element imaging software (Nikon), which allowed visualization of the entire slide with full zoom capacity from the equivalent of a ×1 original magnification to ×400 original magnification. Scans were performed under DAPI (nucleus), fluorescein isothiocyanate (cytokeratin [CK]), tetramethyl rhodamine isothiocyanate (PD-L1), and Cy5 (CD45) channels. When analyzing the multichannel ICC image, white blood cells were defined as round/ovoid cells (DAPI+/CK–/PD-L1–/CD45+); and HCC CK+ CTCs were defined as round/ovoid cells (DAPI+/CK+/PD-L1–/CD45–) (Fig. 1A). PD-L1+ CTCs are the subpopulation of HCC CK+ CTCs defined as round/ovoid events (DAPI+/CK+/PD-L1+/CD45–) (Fig. 1B). PD-L1 positivity was defined as ×2 or higher background fluorescence intensity located in the target cell membrane. Any CD45 positivity of greater than ×2 background fluorescence intensity disqualified a cell as a CTC. HCC CTCs were enumerated by the same blinded researcher (S.H.), and CTC counts were represented as a total count per 4-mL venous blood. For patients with multiple blood draws, the PD-L1 expression status for patients undergoing systemic therapy was determined from the blood draw closest to the time of treatment initiation.

STATISTICAL ANALYSIS

Continuous variables were summarized as medians and interquartile ranges (IQRs) and compared using Wilcoxon rank sum test. Categorical variables were summarized as frequencies and percentages and compared using the chi-square test/Fisher exact test. CTC enumeration was reported per 4 mL of peripheral venous blood and compared among groups using the nonparametric Mann-Whitney U test. Diagnostic performance of CTCs was evaluated using receiver operating characteristic curves for determination of

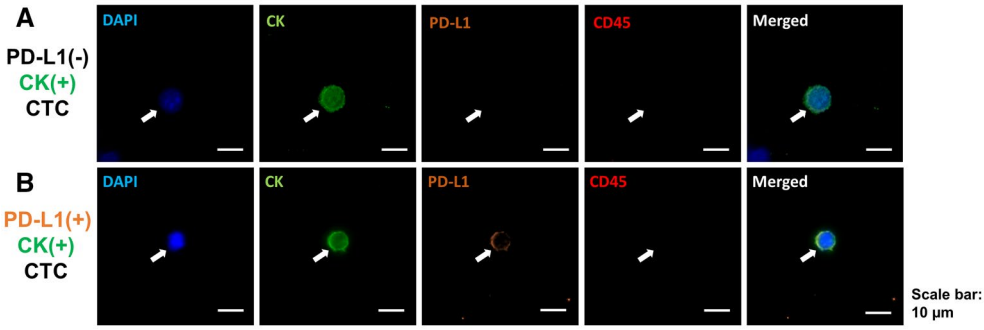

FIG. 1. Schematic depicting identification of CTCs through four-color ICC approach in conjunction with high-resolution fluorescent microscopy shown at ×40 magnification. (A) Representative images of a standard epithelial phenotype HCC CTC not expressing PD-L1 (PD-L1– CK+ CTC) stains positive for DAPI and CK but negative for PD-L1 and the universal leukocyte marker CD45. (B) The phenotypic subpopulation of PD-L1+ CTCs stain positive for DAPI, CK, and PD-L1 but negative for CD45.

the area under the receiver operating characteristic curve (AUROC) and sensitivity and specificity calculations. Optimal cutoff points for CTC enumeration were evaluated using Youden's J statistic.

Overall survival (OS) was calculated as the time from enrollment blood draw to death or last follow-up. Kaplan-Meier survival curves were compared using the log-rank test. A P-spline plot was used to identify the optimal CK+CTC cutoff on which to stratify survival. Cox proportional hazards regression model was used to determine univariate and multivariate hazard ratios (HRs). Alpha-fetoprotein (AFP) values were log-transformed for multivariate analysis. A two-tailed *P* value less than 0.05 was considered statistically significant.

All statistical analyses and calculations were performed with the assistance of GraphPad Prism 8.0a (GraphPad Software, La Jolla, CA), R software (R Foundation for Statistical Computing, Vienna, Austria), and SAS (SAS Institute, Cary, NC).

Results

PATIENT CHARACTERISTICS

Between April 2015 and November 2017, 102 participants were prospectively enrolled in the study. Eight participants were healthy controls, 7 participants had liver cirrhosis without HCC, and 87 participants had HCC (49 early-stage HCC, 22 locally advanced HCC, and 16 metastatic HCC) (Fig. 2). The clinicopathologic features of the HCC cohort are summarized in Table 1.

At the time of blood draw, the median age of patients with HCC was 63 (IQR 60-71), and 65 (74.7%) were male. Of the 77 (88.5%) patients with HCC with cirrhosis, 59 (76.6%), 10 (13.0%), and 8 (10.4%) were Child class A, B and C, respectively, with a median laboratory Model for End-Stage Liver Disease (MELD) score of 7 (IQR 7-10). Regarding the underlying etiologic risk factor for HCC, 52 (59.8%) patients had hepatitis C virus (HCV) cirrhosis, 17 (19.5%) had hepatitis B virus (HBV) cirrhosis, and 8 (9.2%) had nonalcoholic steatohepatitis (NASH) cirrhosis; the remaining 10 (11.5%) patients with HCC did not have underlying liver disease (noncirrhotic). Regarding Barcelona Clinic Liver Cancer (BCLC) staging, 22 (25.3%), 22 (25.3%), 36 (41.4%), and 7 (8.0%) patients were stage A, B, C, and D, respectively, with 49 patients (56.3%) within UCSF criteria and 38 (43.7%) patients beyond UCSF, 22 (25.3%) of whom had locally advanced HCC without metastases, and 16 (18.4%) with extrahepatic metastases. Fifty-four (62.1%), 14 (16.1%), 7 (8.0%), and 12 (13.8%) patients had one, two, three, and four or more lesions, respectively, with a median cumulative tumor diameter of 4.8 cm (IQR 3.0-7.8), and with 16 (18.4%) patients with vascular involvement. The median AFP at blood draw was 14 ng/mL (IQR 5.2-610), with a maximum predraw AFP of 38 (IQR 7-1148). Thirty-five of the 87 patients with HCC (40.2%) had treatment before blood draw and study enrollment, including LT (n = 3), hepatic resection (n = 6), transarterial

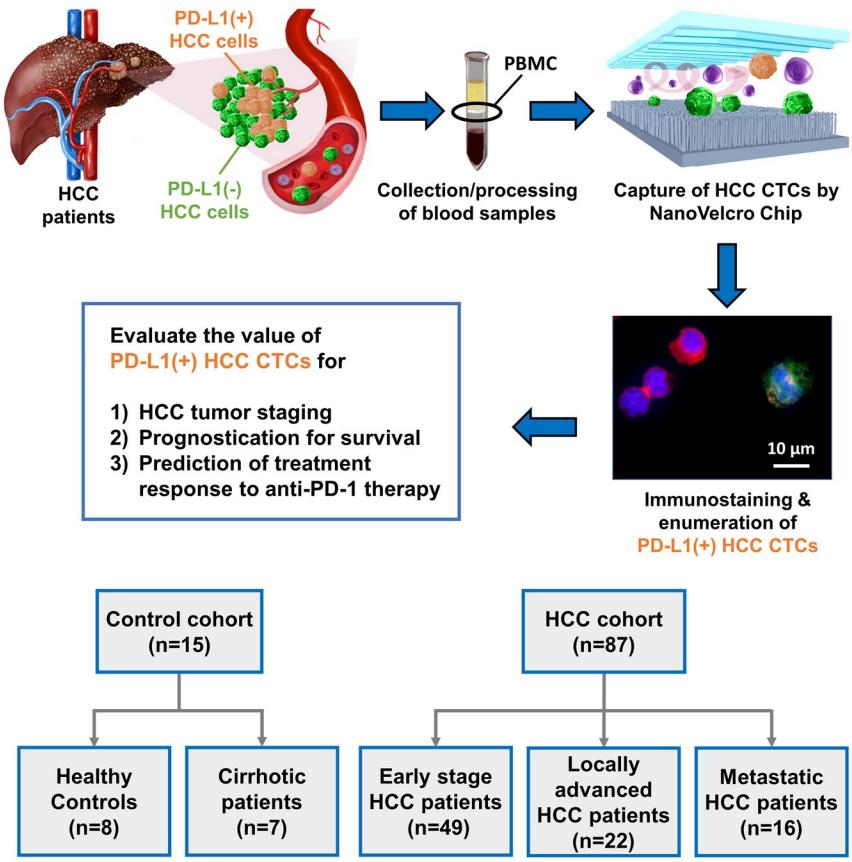

FIG. 2. Overall study design and overview of enrolled control patients and patients with HCC.

chemoembolization (TACE; n = 15), thermal ablation (n = 13), Y90 (n = 3), sorafenib (n = 14), checkpoint inhibitors (n = 5), or other systemic therapy (n = 1). For these 35 patients, stages at diagnosis were as follows: 22, 2, 10, and 0 had BCLC stage A, B, C, and D at diagnosis, and 3, 3, 26, and 3 had BCLC stage A, B, C, and D at the time of study enrollment, respectively. For these 35 patients, the median time from HCC diagnosis to enrollment for blood draw was 605 days (IQR 280-910 days). Twenty-eight (32.2%) patients died over the course of the study. The overall median follow-up time was 628 days.

HCC CTCs

The CK+ CTC enumeration data for healthy controls, patients with cirrhosis, and patients with HCC are presented in Fig. 3A,C. CK+ CTCs were detected in 84 of 87 (96.6%) patients with HCC (median = 6, IQR= 3-10, range = 0-27). The median CK+ CTC counts correlated with more advanced disease stage (early stage vs. locally advanced and metastatic, $P < 0.001$) and were lowest in patients with early-stage HCC (median = 4, IQR = 2-7, range = 0-17), increasing in patients with locally advanced HCC (median = 8, IQR = 4-11, range = 0-27), and highest in patients with metastatic HCC (median = 9.5, IQR = 8-13, range = 2-22). In the control cohorts, a single CK+ CTC was found in 2 (25%) of the 8 healthy controls, and a single CK+ CTC was found in 3 (43%) of the 7 patients with cirrhosis. HCC CTCs were also significantly associated with OS. Using a P-spline plot, an optimal CK+ CTC cutoff of 7 was identified (Supporting Fig. S2), as well as a highly discriminated OS (Fig. 4A), with HCC patients having more than 7 CK+ CTCs with significantly inferior survival (median OS = 29 months, 12-month Kaplan-Meier survival = 62%) compared to patients with seven or fewer CTCs (median OS not reached, 12-month Kaplan-Meier survival = 84%, $P = 0.006$).

TABLE 1. PRE-ENROLLMENT CLINICAL, RADIOLOGIC, AND TREATMENT CHARACTERISTICS OF HCC COHORT

Characteristics	n = 87
Clinical	
Age, median (IQR), years	63 (60-71)
Male, n (%)	65 (74.7)
Cirrhosis, n (%)	77 (88.5)
Child's class, n (% with cirrhosis)	
A	59 (76.6)
B	10 (13.0)
C	8 (10.4)
HCC etiology, n (%)	
HCV	52 (59.8)
HBV	17 (19.5)
NASH	8 (9.2)
Patients without cirrhosis	10 (11.5)
Physiologic MELD, median (IQR)	7 (7-10)
BCLC stage, n (%)	
A	22 (25.3)
B	22 (25.3)
C	36 (41.4)
D	7 (8.0)
AFP at draw, median (IQR)	14 (5.2-610)
Maximum predraw AFP, median (IQR)	38 (7-1,148)
Radiologic	
Transplant criteria	
Within UCSF criteria, n (%)	49 (56.3)
Outside UCSF/locally advanced, n (%)	22 (25.3)
Outside UCSF/metastatic, n (%)	16 (18.4)
Number of lesions, n (%)	
1	54 (62.1)
2	14 (6.1)
3	7 (8.0)
4+	12 (13.8)
Cumulative tumor diameter, median (IQR)	4.8 (3.0-7.8)
Vascular involvement	
Absent	71 (81.6)
Present	16 (18.4)
Pre-enrollment Treatment Characteristics	
Any predraw treatment, n (% of all HCC)	35 (40.2)
Type of treatment, n (% of treated patients)	
LT	3 (8.6)
Hepatic resection	6 (17.1)
TACE	15 (42.9)
Thermal ablation	13 (37.1)
Radioembolization (Y90)	3 (8.6)
Sorafenib	14 (40)
PD-1 checkpoint inhibitor	5 (14.3)
Other systemic therapy	1 (2.9)

PD-L1 EXPRESSION ON HCC CTCs

PD-L1+ CTC enumeration data for all groups are provided in Fig. 3B,C. PD-L1+ CTCs were detected in 31 of 87 (35.6%) patients with HCC. PD-L1+ CTCs were identified in 4 of 49 (8.2%) patients with early-stage HCC (median = 0, IQR = 0-0, range = 0-6), 12 of 22 (54.5%) locally advanced patients (median = 2, IQR = 0-2, range = 0-20), and 15 of 16 (93.8%) metastatic patients (median = 2, IQR = 2-4, range = 0-14). No PD-L1+ CTCs were identified in any healthy controls or patients with cirrhosis. The presence of PD-L1+ CTCs accurately discriminated patients with early-stage HCC from patients with locally advanced or metastatic HCC, with a sensitivity of 71.1%, specificity of 91.8%, and an AUROC of 0.807 (95% confidence interval [CI] = 0.707–0.907, $P < 0.001$).

The clinicopathologic characteristics of the HCC cohort stratified by CTC PD-L1 expression are given in Table 2. Compared to patients without PD-L1+ CTC, patients with PD-L1+ CTC were less likely to have cirrhosis (77.4% vs. 94.6%, $P = 0.030$), but more likely to have advanced-stage disease as assessed by BCLC (C, 67.7% vs. 26.8%; D, 12.9% vs. 5.4%; $P < 0.001$) and UCSF criteria (locally advanced, 38.7% vs. 17.9%; metastatic, 48.4% vs. 1.8%; $P < 0.001$); and had significantly greater number of lesions ($P = 0.004$), cumulative tumor diameter (7.3 cm vs. 3.7 cm, $P < 0.001$), vascular involvement (18.4% vs. 7.1%, $P < 0.001$), and pre-enrollment treatment (61.3% vs. 28.6%, $P = 0.006$), specifically related to receipt of sorafenib (52.6% vs. 25%, $P = 0.005$). There were no significant differences in age, gender, underlying liver disease etiology, MELD, or immediate or maximum pre-enrollment AFP.

PROGNOSTIC SIGNIFICANCE OF PD-L1 EXPRESSION ON HCC CTCs

Over the study period, 17 of 31 (54.8%) patients with PD-L1+ CTCs and 11 of 56 (19.6%) patients without PD-L1+ CTCs died. Patients with PD-L1+ CTCs had significantly worse OS compared to patients without PD-L1+ CTCs (median OS = 14.0 months vs. median OS not reached; HR = 4.0, 95% CI = 1.8-9.2, $P = 0.001$; Fig. 4B). Univariate and multivariate predictors of survival are

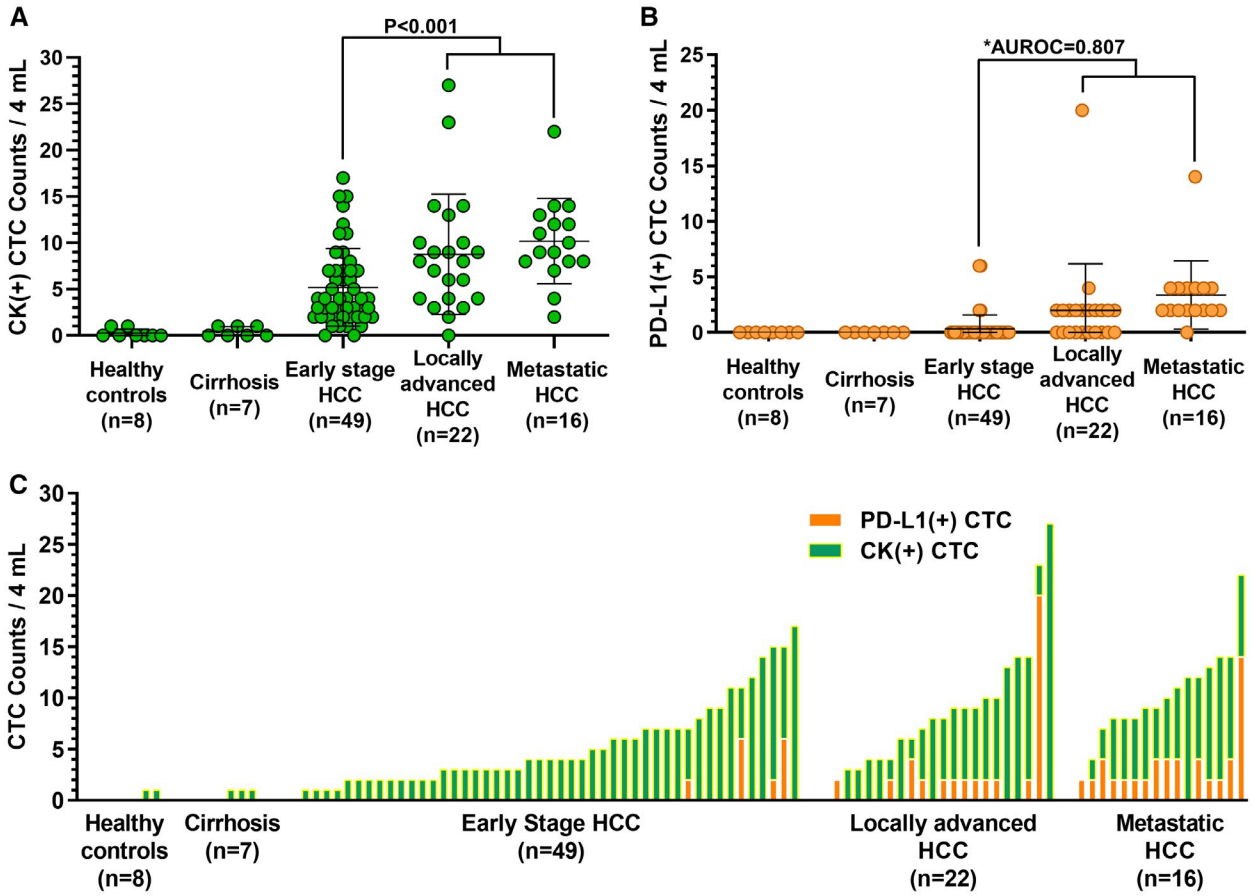

FIG. 3. CTC enumeration of both standard CK+ CTCs (A) and PD-L1+ CTCs (B) per 4 mL of venous blood shown in healthy controls, patients with cirrhosis, early-stage HCC, locally advanced HCC, and metastatic HCC. PD-L1+ CTCs were predominantly observed in patients with locally advanced and metastatic HCC, and accurately discriminated early stage from advanced/metastatic disease with an AUROC of 0.807. **P* < 0.001. (C) Comparative graph showing CTC enumeration per 4 mL of venous blood for both CK+ and PD-L1+ CTCs for healthy controls, patients with cirrhosis, and patients with HCC stratified by stage of disease.

given in Table 3. Univariate predictors of OS include Child class (B, HR = 2.58, *P* = 0.029; C, HR = 4.84, *P* < 0.001), Eastern Cooperative Oncology Group 2+ (HR = 9.07, *P* < 0.001), MELD (HR = 1.08 per unit, *P* < 0.001), and AFP (HR = 1.64 per log unit, *P* < 0.001), beyond Milan (HR = 4.90, *P* < 0.001) and UCSF (HR = 8.40, *P* < 0.001) criteria, radiological number of lesions, cumulative tumor diameter (HR = 8.17 per log unit, *P* < 0.001), vascular involvement (HR = 6.04, *P* < 0.001), and presence of PD-L1+ CTCs of two (HR = 3.45, *P* = 0.001) and four or more (HR = 4.74, *P* < 0.001). On multivariate Cox regression analysis, presence of four or more PD-L1+ CTCs remained an independent predictor of OS (HR = 3.22, 95% CI = 1.33-7.79, *P* = 0.010), even after controlling for MELD score (HR = 1.14 per unit increase, 95% CI = 1.08-1.19, *P* < 0.001), AFP (HR = 1.55 per log unit increase, 95% CI = 1.20-2.00, *P* < 0.001), and overall stage as assessed by UCSF criteria (outside UCSF HR = 7.19, 95% CI = 3.03-16.7, *P* < 0.001).

ASSOCIATION OF PD-L1+ CTCs WITH TREATMENT RESPONSE TO IMMUNOTHERAPY

In the subset of 10 patients receiving anti-PD-1 treatment, the association between PD-L1+ CTC status and treatment course is illustrated by a swimmer plot in Fig. 5. Nine patients received nivolumab, and 1 received pembroilzumab. Five patients responded to anti-PD-1 treatment (mean duration of treatment of

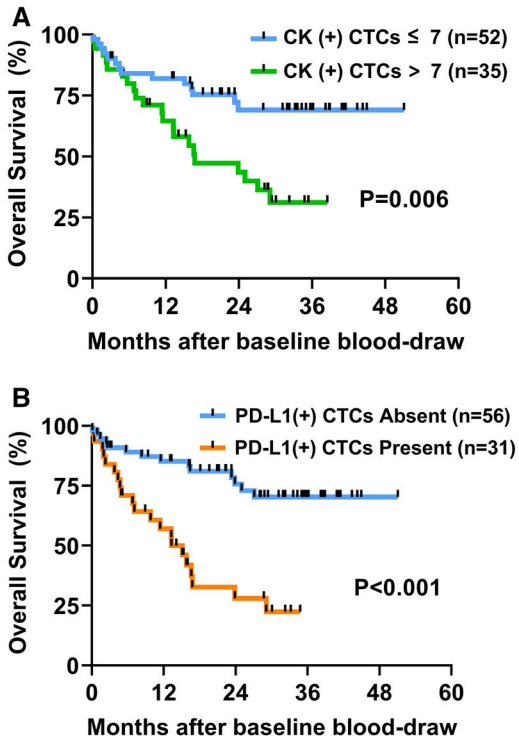

FIG. 4. Kaplan-Meier OS estimates comparing patients with HCC patients by CK+ CTCs (A) and PD-L1+ CTCs (B).

588 days), while the remaining 5 patients failed treatment (mean duration of treatment of 106 days). Six of the 10 patients receiving immunotherapy had PD-L1+ CTCs present at baseline, of whom 5 responded to treatment; the remaining 1 patient with PD-L1+ CTCs failed treatment. Conversely, all 4 patients who did not have PD-L1+ CTCs were nonresponders, with a median time to death of 10.4 months. All 5 treatment responders had PD-L1+ CTCs present at baseline; 1 patient (H193) was taken off treatment secondary to elevated LFTs. Of the 5 patients who failed to respond to treatment, only 1 (20%) had PD-L1+ CTCs. All nonresponders progressed within 4 months of starting treatment, with all but 1 (H222) dying by 15 months.

Discussion

The emergence of immunotherapy as a therapeutic option for patients with HCC appears poised to revolutionize the treatment algorithm for patients with all but the earliest stage of HCC. However, it is clear that the benefits of immunotherapy are not universal, and appear to be limited to the approximately 20% of patients with HCC who respond.[10-12,18] In other malignancies, expression of PD-L1 or PD-1 by the primary tumor,[36] tumor mutational burden,[37,38] T-cell infiltration of the tumor,[39] and microsatellite instability-high or presence of DNA mismatch repair gene defects[40] have all been associated with response to immunotherapy; unfortunately, expression of PD-L1 in HCC has not been associated with treatment response,[10,12] and a widely accepted predictive biomarker remains elusive. In this study, we evaluated the feasibility and potential significance of detecting PD-L1 expression on CTCs in patients with HCC. This study characterizes a phenotypic subpopulation of CTCs expressing PD-L1 in HCC, and evaluates the association of CTC PD-L1 expression with overall prognosis and treatment response to immunotherapy. Unequivocally, our data strongly support that PD-L1-expressing CTCs are prognostic, with further studies required to confirm their association with response to immunotherapy.

The presence of PD-L1+ CTCs was first reported in breast cancer,[27] with subsequent reports in lung, melanoma, colon, prostate, and bladder cancers.[28,41-44] These studies used various CTC enrichment and detection techniques, most commonly using antibodies to the cell surface marker EpCAM. For patients with HCC, only a minority of tumors express EpCAM (20%-35%), which limits the utility of EpCAM-dependent CTC platforms.[45] Additionally, recently identified important subpopulations of CTCs undergoing epithelial-to-mesenychmal transition (EMT) do not express EpCAM and cannot be isolated by EpCAM-dependent technology.[46] For this study, it was essential to use a technology capable of capturing CTCs with an EMT-like phenotype, as studies have demonstrated PD-L1 expression on CTCs undergoing EMT in both breast and bladder cancer.[41,43] We therefore used our previously described multimarker, microfluidic-based CTC capture platform, given its ability to identify the subpopulation of HCC CTCs undergoing EMT, as demonstrated by the presence of the EMT marker vimentin.[25]

One of the key findings of this study was the prognostic importance of PD-L1 expression in HCC CTCs. PD-L1 CTC expression was evaluated in 87 patients with HCC across all disease stages (early, locally advanced, and metastatic). While

TABLE 2. PRE-ENROLLMENT CLINICAL, RADIOLOGIC, AND TREATMENT CHARACTERISTICS STRATIFIED BY PD-L1 CTC STATUS

Characteristics	PD-L1 CTC Status		*P* Value
	Negative (n = 56)	Positive (n = 31)	
Clinical			
Age, median (IQR), years	63 (60.8-70.5)	64 (56.5-71.5)	0.703
Male, n (%)	41 (73.2)	24 (77.4)	0.799
Cirrhosis, n (%)	53 (94.6)	24 (77.4)	0.030
Child's class, n (% with cirrhosis)			0.751
A	34 (73.9)	25 (80.6)	
B	7 (15.2)	3 (9.7)	
C	5 (10.9)	3 (9.7)	
HCC etiology, n (%)			0.775
HCV	35 (62.5)	17 (54.8)	
HBV	11 (19.6)	6 (19.4)	
NASH	5 (8.9)	3 (9.7)	
No cirrhosis	5 (8.9)	5 (16.1)	
Physiologic MELD, median (IQR)	8.0 (7.0-11.2)	7.0 (6.0-9.0)	0.182
BCLC stage, n (%)			<0.001
A	20 (35.7)	2 (6.5)	
B	18 (32.1)	4 (12.9)	
C	15 (26.8)	21 (67.7)	
D	3 (5.4)	7 (12.9)	
AFP at draw, median (IQR)	13.7 (5.8-77.0)	94.7 (4.3-4,401.5)	0.265
Maximum predraw AFP, median (IQR)	25.9 (6.7-369)	124 (6-4,401)	0.301
Radiologic			
Transplant criteria			<0.001
Within UCSF criteria, n (%)	45 (80.4)	4 (12.9)	
Outside UCSF/locally advanced, n (%)	10 (17.9)	12 (38.7)	
Outside UCSF/metastatic, n (%)	1 (1.8)	15 (48.4)	
Number of lesions, n (%)			0.004
1	41 (73.2)	13 (41.9)	
2	9 (16.1)	5 (16.1)	
3	1 (1.8)	6 (19.4)	
4+	5 (8.9)	7 (22.6)	
Cumulative tumor diameter, median (IQR)	3.7 (2.5-5.3)	7.3 (4.9-10.9)	<0.001
Vascular involvement			<0.001
Absent	52 (92.9)	19 (81.6)	
Present	4 (7.1)	12 (18.4)	
Pre-enrollment Treatment Characteristics			
Any predraw treatment, n (% of all HCC)	16 (28.6)	19 (61.3)	0.006
Type of treatment, n (% of treated patients)			
LT	1 (6.3)	2 (10.5)	0.288
Hepatic resection	2 (12.5)	4 (21.1)	0.181
TACE	8 (50)	7 (36.8)	0.380
Thermal ablation	10 (62.5)	3 (15.8)	0.364
Radioembolization (Y90)	1 (6.25)	2 (10.5)	0.288
Sorafenib	4 (25)	10 (52.6)	0.005
PD-1 checkpoint inhibitor	2 (12.5)	3 (15.8)	0.343
Other systemic therapy	0 (0.0)	1 (5.3)	0.356

TABLE 3. UNIVARIATE AND MULTIVARIATE COX REGRESSION ANALYSIS OF OS

	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Clinical						
Age (per year increase)	1.00	0.97-1.04	0.992			
Gender						
Female	1.00	Ref	Ref			
Male	1.13	0.52-2.49	0.755			
Cirrhosis	1.02	0.36-2.89	0.968			
Child's class						
A	1.00	Ref	Ref			
B	2.58	1.11-6.02	0.029			
C	4.84	1.96-11.97	<0.001			
ECOG status						
0	1.00	Ref	Ref			
1	1.79	0.88-3.65	0.111			
2+	9.07	3.19-25.79	<0.001			
Physiologic MELD (per unit)	1.08	1.03-1.12	<0.001	1.14	1.08-1.19	<0.001
AFP (per log unit)	1.64	1.30-2.06	<0.001	1.55	1.20-2.00	<0.001
Radiologic						
Transplant criteria						
Within Milan criteria	1.00	Ref	Ref			
Outside Milan	4.90	2.12-11.1	<0.001			
Within UCSF	1.00	Ref	Ref	1.00	Ref	Ref
Outside UCSF	8.40	3.70-20.0	<0.001	7.19	3.03-16.7	<0.001
Number of lesions						
1	1.00	Ref	Ref			
2	1.80	0.69-4.68	0.230			
3	8.84	3.60-21.7	<0.001			
4+	8.19	3.19-21.1	<0.001			
Cumulative tumor diameter (per log unit)	8.17	2.40-27.6	<0.001			
Vascular involvement						
Absent	1.00	Ref	Ref			
Present	6.04	2.99-12.21	<0.001			
CTC Characteristics						
PDL1+ CTCs (n)						
0	1.00	Ref	Ref	1.00	Ref	Ref
2	3.45	1.62-7.31	0.001			
4+	4.74	2.00-11.26	<0.001	3.22	1.33-7.79	0.010

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

the standard epithelial phenotype CK+ CTCs were observed in most of the patients with HCC (84 of 87), the subpopulation of CTCs expressing PD-L1 were observed predominantly in patients with locally advanced or metastatic HCC, and accurately discriminated patients with more advanced disease from those with early-stage HCC (AUROC = 0.807; Fig. 3B). Furthermore, all but 1 of the 16 patients with HCC with metastatic disease were found to have PD-L1+ CTCs. Subsequently, when patients with HCC were stratified based on the presence or absence of PD-L1+ CTCs, patients with PD-L1+ CTCs demonstrated significantly inferior survival (Fig. 4B). Most importantly, the presence of PD-L1+ CTCs remained an independent predictor of survival even after controlling for tumor stage, AFP, and MELD score, supporting its utility as a prognostic biomarker that adds to what is clinically knowable about the patient. These

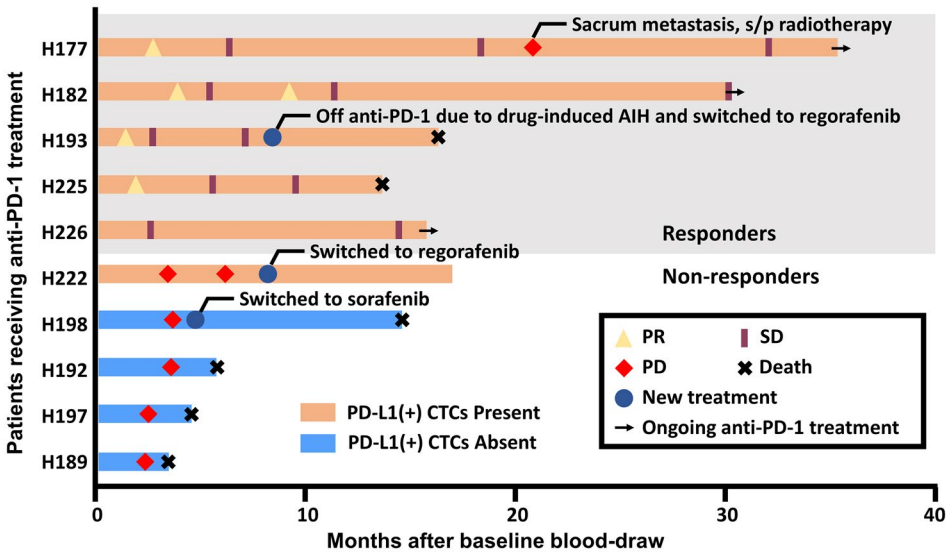

FIG. 5. Swimmer plot depicting patients with HCC receiving PD-1 inhibitors. Each patient is represented by an individual bar. The color of each bar indicates the presence (orange) or absence (blue) of PD-L1+ CTCs at baseline. The top 5 patients in the gray box were treatment responders, whereas the bottom 5 patients were nonresponders. All reported events were tracked from the start of treatment. Abbreviations: PD, progressive disease; PR, partial response; SD, stable disease.

findings are highly consistent with the known role of tumor PD-L1 expression, which contributes not only to immune evasion, but also initiating an EMT and cancer-stem cell phenotype, which has been linked to tumor invasiveness and metastases.[47]

Perhaps the most intriguing finding of our study was the observed association between PD-L1+ CTCs and response to anti-PD-1 therapy. In the subset of 10 patients receiving checkpoint inhibitors, all patients who responded had PD-L1+ CTCs, and all patients without PD-L1+ CTCs failed treatment (Fig. 5). Very few prior studies have examined the association of PD-L1+ CTC phenotype and response to immunotherapy, and none in HCC. Khattak et al. examined 40 patients with metastatic melanoma receiving pembrolizumab. Of the 25 patients with detectable CTCs, the 14 with PD-L1+ CTCs had significantly longer progression-free survival compared to patients with PD-L1− CTCs, and the presence of PD-L1+ CTCs was an independent predictive biomarker for progression-free survival.[28] In contrast, Nicolazzo et al. reported outcomes in 24 patients with non-small cell lung cancer receiving nivolumab. The presence of PD-L1+ CTCs at baseline and at 3 months was associated with poor patient outcome, although the high-frequency of PD-L1+ CTCs impaired the ability to draw definitive conclusions.[42] Although our findings in HCC are descriptive and certainly not powered to draw any definitive conclusions, if validated on a larger scale, would suggest that assays evaluating PD-L1 expression on CTC may potentially serve as a biomarker for predicting response to anti-PD-1 immunotherapy in patients with HCC.

Our study has several limitations. Although PD-L1 phenotyping was performed in a large number of patients with HCC, allowing us to establish its prognostic utility, only a small subset of 10 patients were receiving immunotherapy, limiting our power to draw definitive conclusions about the utility of PD-L1+ CTCs as a predictive biomarker for treatment response. Furthermore, only the baseline PD-L1+ CTC evaluation was correlated with treatment response, as very few of these patients had serial blood draws over the course of treatment. Such a dynamic assessment at numerous time points would have been essential to correlate clinical events (stable disease, partial response, or progression) with the CTC phenotype, allowing for a more robust interpretation of results. Finally, as patients receiving immunotherapy were all metastatic, there was no access to pathologic tissue to assess PD-L1 expression in the tumors and compare with their CTC PD-L1 expression. Nonetheless, the

large clinical studies to date that have included protocol biopsies have not confirmed a predictive role for immunohistochemical tumor testing for PD-L1 expression.[10,11] This fact, coupled with issues of sampling, tumor heterogeneity, and invasiveness of traditional biopsies, certainly makes the evaluation of CTCs an attractive alternative for dynamic, repeated, and real-time monitoring of treatment response.

In summary, we report a CTC assay for the evaluation of PD-L1 expression in HCC CTCs using a multimarker antibody-based CTC capture platform specific for HCC CTCs. This report describes the phenotyping of PD-L1+ CTCs in HCC. The presence of PD-L1+ CTCs were found predominantly in patients with HCC with locally advanced or metastatic disease, and independently prognosticated OS. Furthermore, despite relatively small numbers, there was a strong association of the presence of PD-L1+ CTCs with a favorable treatment response in the subset of patients with HCC receiving immunotherapy. Prospective validation of our findings in a larger cohort is necessary to better define the utility of enumerating and phenotyping PD-L1+ HCC CTCs as a prognostic and predictive biomarker in HCC.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1577/suppinfo.