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ORIGINAL ARTICLE

An imbalance between stellate cells and γδT cells contributes to hepatocellular carcinoma aggressiveness and recurrence

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Abstract

Purpose The diagnostic potential of hepatic stellate cells (HSCs) and γδT cells for patients with hepatocellular carcinoma (HCC) and their synergistic contributions to the prognosis of these patients have not yet been investigated. The aim of this study was to elucidate the prognostic value of these cells in HCC.

Methods The prognostic significance of the ratio of HSCs to γδT cells (SGR) was assessed in a total of 339 HCC patients undergoing resection. The correlation between the circulating tumor cell (CTC) level and SGR in 71 HCC patients was determined using the CellSearch system. In vitro experiments were performed to validate the synergistic effects of HSCs and γδT cells on hepatoma cells.

Results Peritumoral SGR was closely associated with overall survival (OS) and recurrence-free survival (RFS) of HCC patients after resection. In the testing cohort, two nomograms incorporating the SGR were constructed for the prediction of OS and RFS. The predictive accuracy of the two nomograms was verified by the validation cohort. CTC levels were positively correlated with SGR ($r = 0.479$, $p < 0.001$). Among the patients with CTCs > 2/7.5 ml, those with a high SGR exhibited higher early recurrence rates than those with a low SGR. In vitro experiments revealed that the secretion of INF-γ, IL-17, and TNF-α from γδT cells was increased after culture with HSC-conditioned medium. In addition, γδT cells cultured with HSC-conditioned medium decreased the proliferative and invasive abilities of hepatoma cells.

Conclusions The peritumoral SGR is related to aggressive tumor behavior and has a powerful predictive value in HCC. Early recurrence in patients with a high peritumoral SGR might be associated with high CTC levels.

Keywords Hepatocellular carcinoma · γδT cell · Hepatic stellate cell · Circulating tumor cell · Prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and has a high mortality rate. Reciprocal communication between tumor cells and stromal cells in the tumor microenvironment is fundamental to the aggressiveness and metastasis of HCC [1]. Therefore, an in-depth understanding of the intercellular adhesive interactions could provide experimental evidence regarding the pathogenesis of HCC and thereby benefit the treatment and improve the prognosis of patients with HCC.

HCC is characterized by a tumor milieu in which the neoplastic transformation of hepatocytes might be influenced by interactions with stromal cells [2]. Among these stromal cells, γδT cells are a minor lymphocyte population (approximately 2–10% of the total T lymphocytes in the peripheral blood) that show a notable diversity of effector functions associated with inflammatory/immune responses, cellular stress, tissue damage responses, and tumor development [3, 4]. Upon activation in the periphery, γδT cells show strong cytotoxic effector activity by producing high levels of various proinflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, which are related to the immunosurveillance of malignancies [5]. These findings

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reveal the host response to malignancy in HCC and the antitumor immune function of γδT cells. In the context of liver cancer, although some studies [6] have firmly established the protective role of γδT cells during tumor development, the effector function of γδT cells is substantially impaired, and this effect is partially mediated by Treg cells [7]. These results show the functional transformation of γδT cells in response to various signaling stimuli, but the functional implications of communications among γδT cells, other stromal cells, and cancer cells in the HCC microenvironment have not yet been explicitly explored.

During HCC progression, activated hepatic stellate cells (HSCs) are critical stromal cells that accelerate hepatocarcinogenesis. In response to stimulation from injured hepatocytes, HSCs are activated and converted into highly proliferative myofibroblast-like cells that contribute to tumor aggressiveness and metastasis [8]. We previously revealed that activated HSCs produce high levels of IL-6 and TNF-α [9], which are important factors in the immune milieu that regulate the differentiation of Th17 cells [10]. Nevertheless, very little is known about the influence of HSCs on γδT cells in HCC.

In this study, we demonstrated that the peritumoral stellate cell-to-γδ T-cell ratio (SGR) is closely associated with the prognosis of HCC after resection. We also explored whether activated HSCs could influence the antitumor immunity of γδT cells in HCC, which could reflect the intrinsic relationships among various inflammatory/immune cells and cytokines involved in tumor progression.

Materials and methods

See details in supplementary material.

Patients and specimens

A total of 339 consecutive patients with pathologically confirmed HCC after surgical resection from January to December 2012 were enrolled. The inclusion criteria were as follows: (a) no prior anticancer therapy, (b) no other malignancies, (c) R0 curative resection, and (d) pathologically proven HCC. Patients were excluded if they had any infections or autoimmune diseases before the operation. All the patients were monitored postoperatively until July 2018 as described previously [11].

Immunohistochemical staining

Immunohistochemistry was performed according to the manufacturer's instructions (Invitrogen, Zymed Polymer Detection System) and as described previously [12].

Isolation of cells and CTC analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of ten HCC patients and ten healthy subjects by LymphoPrep™ (Axis-Shield) gradient centrifugation as described previously [10]. γδT cells were positively selected through flow cytometric analysis using PE-conjugated γδ TCR (11F2, BD Oncomark) and FITC-conjugated CD3 (SK7, BD Oncomark). Normally, 0.5–2.5 x 10^5 γδT cells/ml peripheral blood can be obtained and we collected 50–100 ml peripheral blood from each HCC patient or healthy subject, respectively. The HSC line LX-2 and the hepatoma cell lines HepG2 (ATCC HB-8065) and Hep3B (ATCC HB-8064) were cultured in DMEM at 37 °C as described in our previous studies. $EpCAM^+$ CTC analysis was performed using the CellSearch (Veridex, Raritan, NJ) method as described previously [13].

Enzyme-linked immunosorbent assay (ELISA)

The INF-γ, TNF-α, and IL-17 levels secreted by γδT cells were determined using ELISA kits (INF-γ, TNF-α, and IL-17, R&D Systems) according to the manufacturer's instructions.

Cell proliferation and invasion assay

The proliferative ability of hepatoma cells was detected using a cell counting kit (CCK-8, Dojindo Laboratories, Japan). For the transwell invasion assays, 1×10^5/well hepatoma cells were seeded on the upper chamber of a 24-well plate (8-μm pore size, Millipore, USA) with Matrigel (BD, USA). RPMI-1640, γδT cells cultured with RPMI-1640 (1×10^6/well) and γδT cells cultured with HSC-CM (1×10^6/well) were seeded in the lower chamber, and the plates were then incubated for 24 h. The hepatoma cells that adhered to the lower surface were determined by quantifying the numbers in five fields under a light microscope (200×).

Statistical analysis

SPSS 19.0 (SPSS, Inc., Chicago, IL) was employed for the statistical analyses, and differences were considered significant if $p < 0.05$. Multivariable analyses of the RFS and OS in the primary cohort were conducted to identify statistically significant ($p < 0.05$) variables, and two nomograms were then constructed based on these variables using R version 3.4.0 with the rms package (http://www.r-project.org/) as described previously [11].

Results

Baseline characteristics

The median ages of the patients in the testing and validation cohorts were 52 and 53 years, respectively, and these cohorts consisted of 190 and 100 male and 36 and 13 female, respectively. Most patients had a single tumor (testing: 198/226; validation: 99/113) with an early TNM stage (testing: 174/226; validation: 86/113), and the testing cohort had a higher incidence of microvascular invasion than the validation cohort (33.2% vs 17.5%). The mean OS of the 339 patients with HCC was 41.8 months (range, 1.0–75.0 months), and their 1-, 3-, and 5-year OS rates were 83.6%, 66.7%, and 44.1%, respectively. The mean RFS was 35.5 months (range, 1.0–75.0 months), and the 1-, 3-, and 5-year RFS rates were 64.2%, 50.7%, and 33.5%, respectively. The baseline characteristics of the 339 patients are shown in Table S1, and these parameters were broadly similar to the baseline clinicopathological characteristics of the two cohorts ($p > 0.05$).

Correlation of the ratio of stellate cells to γδT cells with clinicopathologic features in the two cohorts and survival analyses of the two cohorts

In the testing cohort, the counts of intratumoral and peritumoral stellate cells and γδT cells in the testing cohort were investigated to assess their association with clinicopathologic variables. In the liver tissues from HCC patients, the densities of intratumoral α-SMA- and peritumoral TCRG-positive cells were higher than those of peritumoral α-SMA- and intratumoral TCRG-positive cells, respectively (Fig. 1a–e, Table S2). The ratio of peritumoral α-SMA- to TCRG-positive cells (SGR, which represents the ratio of stellate cells to γδT cells, respectively) was associated with microvascular invasion ($p = 0.008$) and tumor size ($p = 0.005$). As shown in Table 1, the univariate analysis of our data revealed that the AST level ($p = 0.028$ and $p = 0.009$), tumor number ($p = 0.001$ and $p = 0.002$), tumor size (both $p < 0.001$), and microvascular invasion ($p = 0.002$ and $p < 0.001$) showed prognostic significance for both OS and RFS. Gamma-glutamyl transpeptidase (GGT, $p = 0.049$) and albumin (ALB, $p = 0.028$) exhibited predictive value for OS, and AFP ($p = 0.003$) could predict RFS. In addition, peritumoral α-SMA and TCRG were related to OS ($p = 0.007$ and $p = 0.040$) and RFS

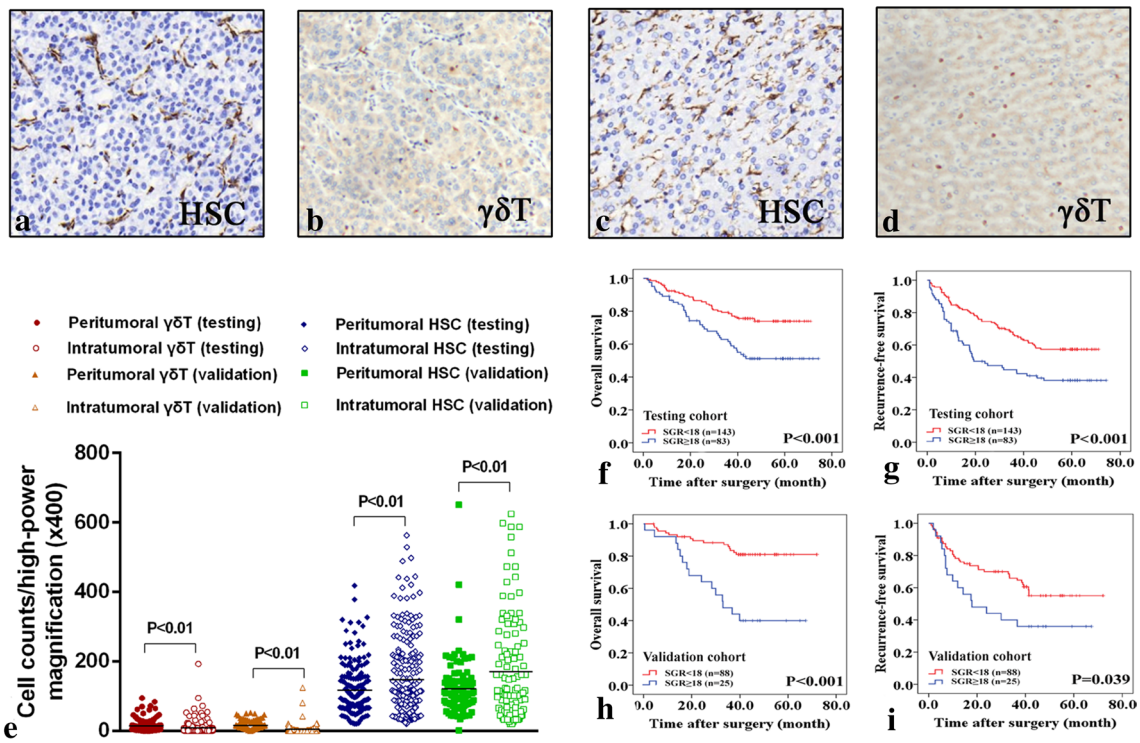

Fig. 1 Immunohistochemical staining of peritumoral (**a** and **b**) and intratumoral (**c** and **d**) HSCs and γδT cells showed that there were higher densities of intratumoral HSCs and peritumoral γδT cells than peritumoral HSCs and intratumoral γδT cells (**e**). Both in testing cohort (**f** and **g**) and in validation cohort (**h** and **i**), Kaplan–Meier analyses revealed that the ratio of HSCs to γδT cells (SGR) was associated with overall survival (**f** and **h**) and recurrence-free survival (**g** and **i**) of patients with hepatocellular carcinoma

Table 1 Prediction of survival and recurrence of HCC patients in the testing cohort ($n = 226$)

Factors	OS				RFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (years) (< 52 vs ≥ 52)		0.153		NA		0.040		0.052
Gender (Female vs male)		0.763		NA		0.493		NA
Cirrhosis (yes vs no)		0.486		NA		0.656		NA
ALT (U/L) (< 40 vs ≥ 40)		0.294		NA		0.197		NA
AST (U/L) (< 38 vs ≥ 38)	1.673 (1.051–2.664)	**0.028**		0.150	1.660 (1.130–2.439)	**0.009**		0.087
GGT (U/L) (< 54 vs ≥ 54)	1.582 (0.999–2.505)	**0.049**		0.694		0.210		NA
TB (μmol/L) (< 13.6 vs ≥ 13.6)		0.291		NA		0.287		NA
ALB (g/L) (< 44 vs ≥ 44)	0.599 (0.377–0.950)	**0.028**	0.625 (0.391–0.999)	**0.049**		0.242		NA
AFP (μg/L) (< 20 vs ≥ 20)		0.111		NA	1.820 (1.213–2.731)	**0.003**	1.607 (1.061–2.434)	**0.025**
HBsAg (negative vs positive)		0.927		NA		0.360		NA
Tumor number (single vs multiple)	2.471 (1.420–4.300)	**0.001**	2.194 (1.125–3.852)	**0.006**	2.124 (1.316–3.427)	**0.002**		0.058
Microvascular invasion (yes vs no)	2.339 (1.484–3.684)	**0.002**	1.638 (1.023–2.620)	**0.040**	2.609 (1.787–3.811)	**< 0.001**	1.992 (1.343–2.956)	**0.001**
Tumor encapsulation (yes vs no)		0.311		NA		0.051		NA
Tumor differentiation (I–II v III–IV)		0.119		NA		0.061		NA
Tumor size (cm, < 3.0 vs ≥ 3.0)	2.964(1.881–4.673)	**< 0.001**	2.674 (1.680–4.256)	**< 0.001**	2.425 (1.659–3.545)	**< 0.001**	2.018 (1.367–2.981)	**< 0.001**
SGR	2.204 (1.400–3.470)	**< 0.001**	2.324 (1.083–3.546)	**0.002**	2.392 (1.296–3.460)	**< 0.001**	1.980 (1.071–2.829)	**0.001**

Bold value (*p* value) indicates the significant difference ($p < 0.05$)

Univariate analysis: Kaplan–Meier method; multivariate analysis: Cox proportional hazards regression model

HCC hepatocellular carcinoma, *OS* overall survival, *RFS* recurrence-free survival, *CI* confidence interval, *HR* hazard ratio, *ALT* alanine aminotransferase, *AST* aspartate *aminotransferase*, *GGT* gamma-glutamyl transpeptidase, *TB* total bilirubin, *ALB* albumin, *AFP* alpha fetoprotein, *HBsAg* hepatitis B virus surface antigen, *SGR* stellate cell-to-γδT-cell ratio, *NA*: not adopted

($p < 0.001$ and $p = 0.009$, Fig S1a–d), and the peritumoral SGR could predict both OS and RFS (both $p < 0.001$, Fig. 1f–g, Table 1). In contrast, neither the intratumoral stellate cell counts nor the intratumoral γδ T-cell counts were associated with death or recurrence. The multivariate analysis showed that the tumor size (both $p < 0.001$), microvascular invasion ($p = 0.040$ and $p = 0.001$, respectively), and peritumoral SGR ($p = 0.002$ and $p = 0.001$, respectively) were all associated with death and elevated risks of recurrence. Both the ALB level ($p = 0.049$) and the tumor number ($p = 0.006$) were demonstrated to be related to OS, whereas AFP ($p = 0.025$) was found to be an independent predictor of RFS.

In the validation cohort, the peritumoral α-SMA and TCRG were also related to OS ($p = 0.008$ and $p < 0.001$) and RFS ($p = 0.009$ and $p = 0.029$, Fig S1e–h). The peritumoral SGR was associated with both microvascular invasion ($p = 0.015$) and tumor size ($p < 0.001$, Table S3). Similarly, a

high density of peritumoral SGR was significantly correlated with poor OS ($p < 0.001$) and RFS ($p = 0.039$, Fig. 1h–i).

Association between CTCs and SGR

CTCs isolated from HCC patients were used to further investigate their correlations with the peritumoral SGR (Fig. 2a). Scatter plot analyses showed that the peritumoral SGR was significantly positively correlated with the CTC level ($r = 0.479$; $p < 0.001$, Fig. 2b). As described previously [13], the patients with CTC levels > 2/7.5 ml were defined as the high subgroup. Specifically, the patients were divided into four groups: I, low CTC level and low SGR ($n = 12$); II, low CTC level but high SGR ($n = 14$); III, high CTC level but low SGR ($n = 11$); and IV, high levels of both markers ($n = 24$). There was 2-year follow-up (DOC S1), and we only found that a significant discrepancy in early recurrence was found ($p = 0.004$, Fig. 2c).

Development of nomograms for the testing cohort

The independent risk factors of OS (Fig. 3a) and RFS (Fig. 3b) identified in the multivariate analysis were incorporated into the nomograms. The C-indexes of the OS (0.769, 95% CI 0.713–0.825) and RFS (0.756, 95% CI 0.703–0.809) nomograms were higher than those of all other risk factors included in the two nomograms (Table S4). The calibration plot for the probability of the 1- and 5-year OS nomograms (Fig. 3c–d) showed optimal agreement between the probabilities and the actual observations in the testing cohort. Similarly, as demonstrated by ROC analyses, the OS nomogram also showed a greater AUC (0.792) than those found for the TNM staging system and other risk factors included in the nomograms (Fig. 3e). The RFS nomogram also showed good predictive power, as demonstrated by the calibration plot (Fig. 3f–g) and ROC analyses (AUC = 0.777, Fig. 3h).

Validation of the nomograms

ROS analysis of the validation cohort showed that the OS (AUC = 0.813, Fig S2a) and RFS (AUC = 0.787, Fig S2b) nomograms had a larger AUC than those found for the TNM staging system (AUC = 0.664 for OS and AUC = 0.619 for RFS) and all other risk factors ($p < 0.01$). Calibration curves of the nomograms showed good agreement between the predictions and the observed probabilities of the 1- and 5-year survival and recurrence of the patients (Fig S3).

Predictive performance of the SGR for recurrence and of the RFS nomogram for early recurrence

The predictive significance of the SGR for clinical subgroups within the set of 339 patients was further investigated. In the subgroups with a low risk of recurrence (Fig. 4a–e), the SGR could discriminate the patients with AFP < 400 ng/ml ($p = 0.008$), without microvascular invasion ($p = 0.003$), with a single tumor ($p < 0.001$), with a tumor size < 5 cm ($p = 0.028$), and with an early tumor stage (TNM I, $p = 0.001$). In each subgroup of HCC with these favorable clinicopathologic characteristics, there were several other adverse factors with significant difference between high and low SGR subgroups, which may determine the

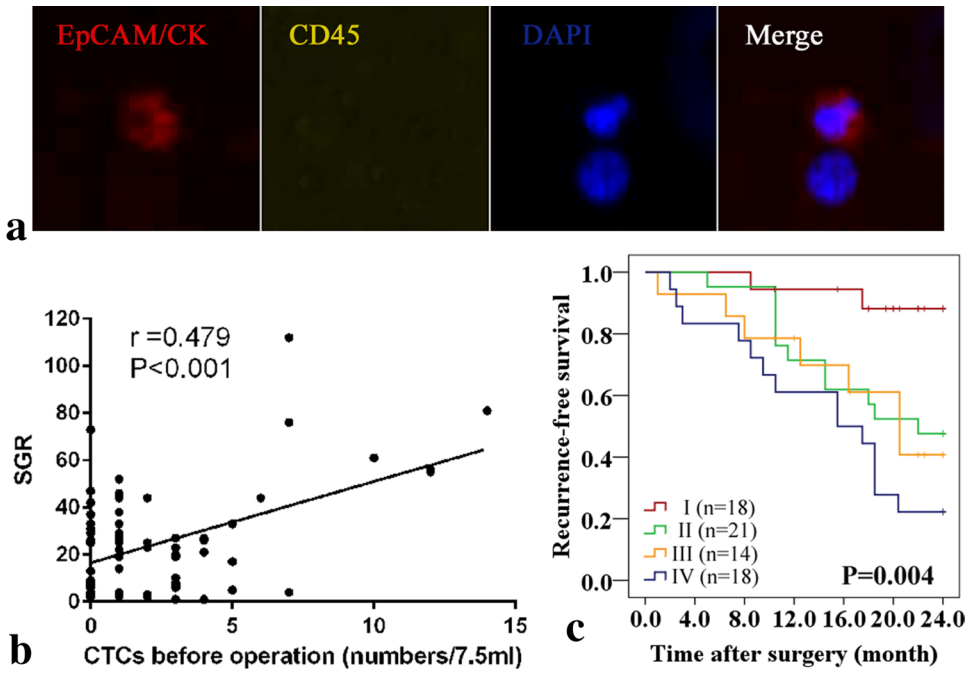

Fig. 2 EpCAM$^+$ circulating tumor cells (CTCs) were identified with the CellSearch system and defined as cells staining positively for cytokeratin (CK) and DAPI and negatively for CD45 (**a**). Peritumoral SGR had a significant positive correlation with CTC level (**b**). Patients with high peritumoral SGR and CTCs > 2/7.5 ml had higher early recurrence rates of hepatocellular carcinoma than other subgroups (**c**)

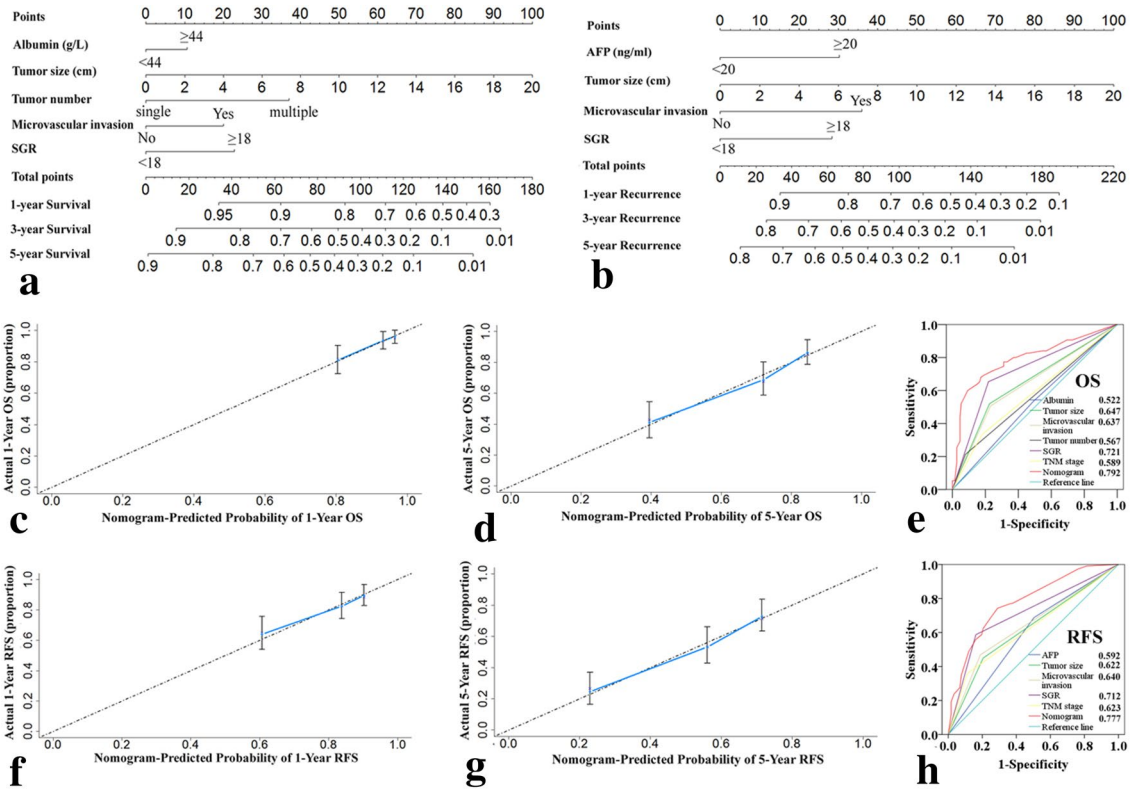

Fig. 3 Two nomograms of hepatocellular carcinoma patients after hepatectomy were constructed (**a** and **b**). Calibration curves showed good agreement between prediction and observation in the probability of 1- and 5-year overall survival (**c** and **d**) and recurrence-free survival (**f** and **g**) in the testing cohort. ROC curve analyses showed that the two nomograms had best predictive accuracy compared to other variables included in the nomograms and TNM stage (**e** and **h**) in the testing cohort

recurrence of these patients to a certain extent (Table S5). In the patients with early recurrence, high SGR was associated with a low OS ($p = 0.004$, Fig. 4f). The calibration plot for the probability of the 1- and 2-year RFS nomograms showed good agreement between the predictions and the observed probabilities of early recurrence (Fig. 4g–h).

Expression levels of INF-γ, IL-17, and TNF-α secreted by γδT cells

To investigate the effects of HSCs on γδT cells in the cancer milieu, we isolated human γδT cells from PBMCs (Fig. 5a). The level of INF-γ produced by γδT cells was decreased in HCC patients (36.12 ± 2.48 pg/ml) compared with healthy controls (44.47 ± 3.32 pg/ml, $p < 0.05$), whereas the levels of TNF-α (39.12 ± 3.82 pg/ml in HCC patients and 33.77 ± 3.94 pg/ml in healthy controls) and IF-17 (18.20 ± 1.77 pg/ml in HCC patients and 15.22 ± 2.01 pg/ml in healthy controls) secreted by γδT cells were increased in HCC patients compared with healthy controls (Fig. 5b–d, $p < 0.05$). After coculture with HSC-CM for 24 h, the γδT cells from both HCC patients and healthy controls secreted increased levels of INF-γ (63.28 ± 3.21 pg/ml and 47.54 ± 4.45 pg/ml, respectively), TNF-α (58.93 ± 4.23 pg/ml and 51.24 ± 3.21 pg/ml, respectively), and IF-17 (32.03 ± 3.29 pg/ml and 25.41 ± 1.90 pg/ml, respectively, Fig. 5b–d, $p < 0.01$).

In vitro validation of the effects of stellate cells and γδT cells on cancer cells

Compared with the blank, the proliferation of hepatoma cells (HepG2 and Hep3B) was inhibited by γδT cells, particularly after culture with HSC-CM (Fig. 5e–f). In control medium, more cancer cells could migrate through chambers. In contrast, γδT cells could decrease the aggressiveness of cancer cells ($p < 0.01$). Furthermore, the aggressiveness of both types of cancer cells was greatly reduced by γδT cells after incubation with HSC-CM ($p < 0.05$, Fig. 5g–i).

Discussion

In this study, consistent with our previous findings [14, 15], high counts of HSCs and low counts of γδT cells in peritumoral liver tissues exhibited specificity as a prognostic

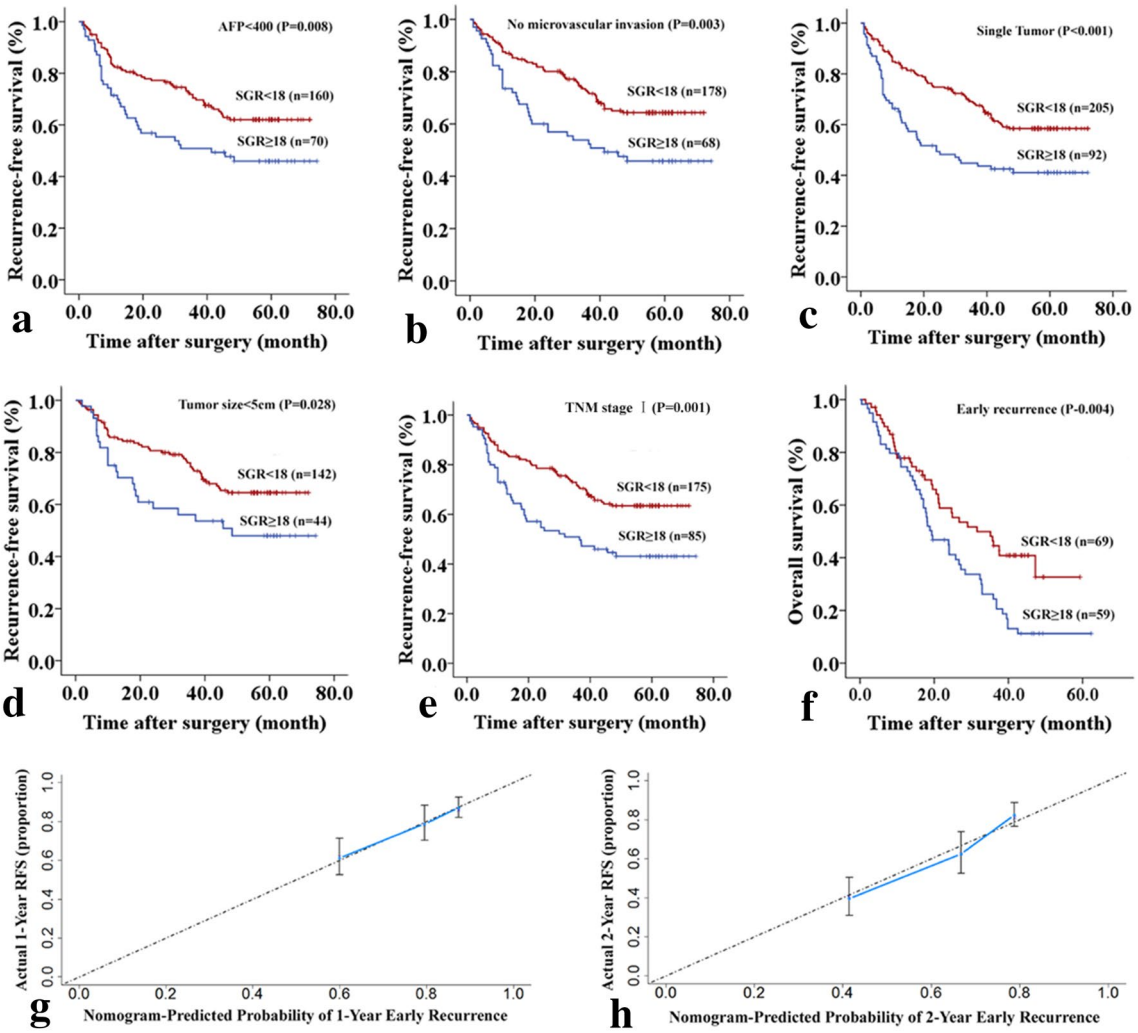

Fig. 4 In a total of 339 patients with hepatocellular carcinoma after resection, Kaplan–Meier analyses showed that the prognostic value of peritumoral SGR was significant in AFP < 400 ng/mL (**a**), no microvascular invasion (**b**), single tumor (**c**), tumor size < 5 cm (**d**), and early tumor stage (**e**), respectively. In the patients with early recurrence, high SGR had lower overall survival (**f**). The calibration plot for the probability of 1- and 2-year recurrence-free survival nomogram showed good agreement between prediction and observation in the probability of early recurrence (**g** and **h**)

marker for the dismal outcome of patients with HCC. Interestingly, we also found that the SGR is associated with poor prognosis of HCC patients after curative resection, which suggests that these cells exert distinct biological effects on tumor growth and that the balance between these cells might influence the tumor process. Furthermore, we developed novel nomograms comprising SGR, AFP, albumin, tumor number, tumor size, and microvascular invasion, and these provided an accurate prognosticator of the outcomes of HCC patients after surgery. Therefore, the balance between HSCs and γδT cells might serve as a critical homeostatic factor in the cancer milieu that interferes with tumor development.

We previously confirmed that HSCs could exert their biological functions to promote aggressive tumor cell behavior [9, 12, 14]. The present study provides new evidence, showing that HSCs are versatile mesenchymal cells that partially impact the direction of HCC development via crosstalk with γδT cells. First, although high expression of peritumorally activated HSCs was found to be associated with poor prognosis of HCC patients, their tumor-promoting ability might be impaired or protected by some antitumor immune cells, such as γδT cells. Here, patients with higher numbers of HSCs may have poor outcomes, and conversely, those who show substantial increases in the numbers of γδT cells might have relatively desirable prognoses. Second, SGR was positively related to CTCs, which were identified as predictive markers of tumor recurrence and metastases after surgery [16]. A high SRG in patients was closely associated with microvascular invasion and large tumors. Tumor cells enter the circulating blood to increase the CTC levels after

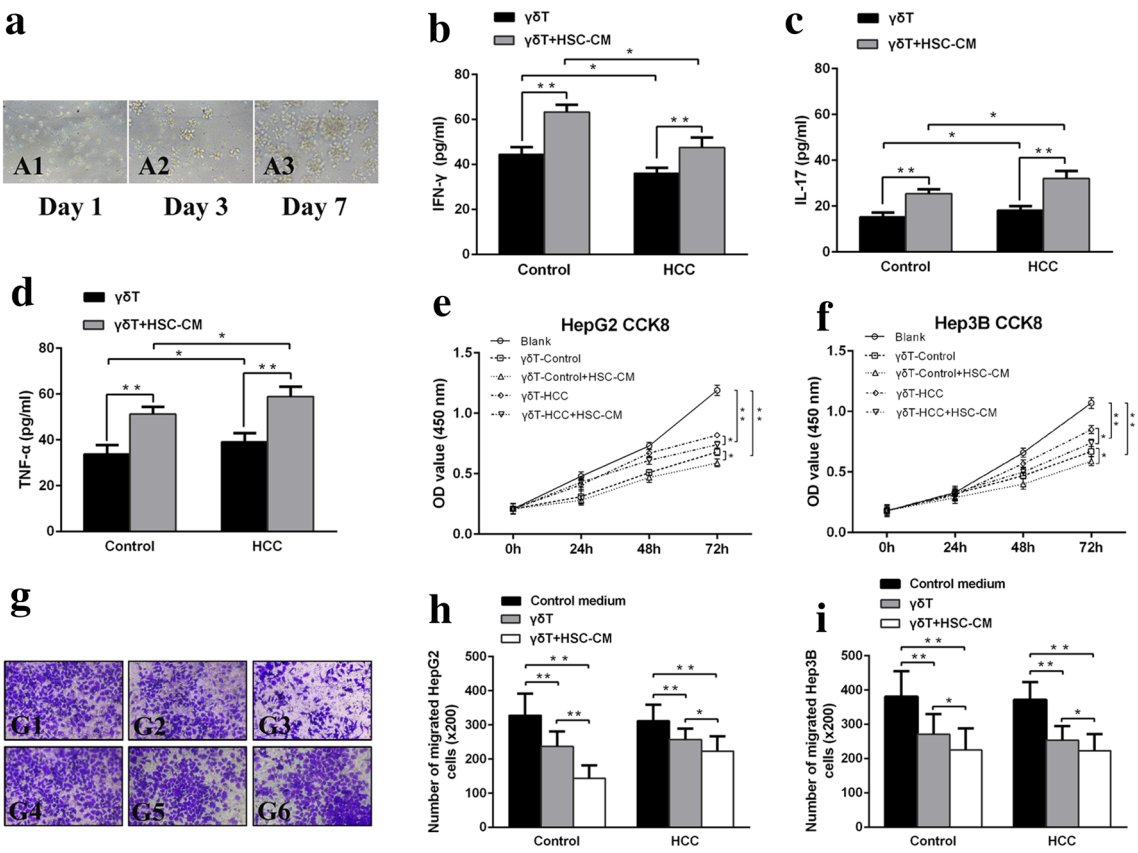

Fig. 5 Human γδT cells were isolated from peripheral blood mononuclear cells (**a**). The levels of INF-γ (**b**), TNF-α (**c**), and IF-17 (**d**) from γδT cells were detected by ELISA. The proliferative abilities of HepG2 (**e**) and Hep3B (**f**) were assessed by CCK8. In vitro invasion assay (**g** representative pictures of HepG2) showed that γδT cells cultured with or without HSCs conditioned medium inhibited the invasion of HepG2 (**h**) and Hep3B cells (**i**). $*p < 0.05$, $**p < 0.01$

aggressive tumor cells invade the microvasculature. On one hand, previous studies have revealed that tumor-activated HSCs can promote the dissemination of cancer cells through inflammation-associated mechanisms [9], and some proinflammatory cytokines (e.g., IL-6 and TNF-α) secreted by HSCs have potent tumor-promoting abilities [9]. On the other hand, the prevailing hypothesis indicates that γδT cells could engage in tumor immune surveillance and defense. We previously found that these abilities were substantially attenuated by Treg cells, which can directly suppress the cytotoxicity of peritumor-derived γδT cells and the secretion of IFN-γ from these cells [7, 15]. The inhibition of decreases in the number of γδT cells allows tumor cells to establish immune tolerance and subsequently increase the CTC level. This process might be one of the mechanisms through which both the SRG and CTCs are involved in the recurrence of HCC. Thus, we believe that this possible correlation of SGR and CTC levels is not random. Third, in vitro experiments support our clinical data showing the role of a high SGE in tumor promotion. We previously observed that HSCs are vital antigen-presenting cells and could produce cytokines to create a cytokine milieu [9, 10] that benefits the proliferation and expansion of γδT cells. In this study, some cytokines, such as IL-17, IFN-γ, and TNF-α, secreted from γδ T cells isolated from both healthy and HCC patients were increased after stimulation with CM from HSCs. Although the cytokines play protumor (IL-17 and TNF-α) and antitumor (IFN-γ) roles, our study revealed that γδT cells suppress the aggressiveness of cancer cells. Here, we propose that antitumor cytokines might play prevailing roles and contribute to the generation of a feedback response to the protumor stimulation of HSCs to maintain tumor microenvironment homeostasis. However, the data are not enough to support this hypothesis and further analysis is needed.

Here, we found that it was peritumoral SGR other than intratumoral SGR that had association with microvascular invasion and tumor size, and also, could predict OS and RFS. Consistent with our previous findings [9, 17], this difference may be involved in the roles of HSCs and γδT cells in hepatocarcinogenesis and the extremely heterogeneous inflammation microenvironment of HCC. On one hand, the peritumoral liver tissue may be indisputably the

principal target organ for the aggressiveness and recurrence of HCC. On the other hand, the different densities of peritumoral HSCs and γδT cells possibly stand for an inflammatory signal accelerating the formation of hepatic metastasis. Therefore, peritumoral inflammatory/immune cells such as HSCs and γδT cells provide us some important therapeutic targets in treating HCC, especially in immunotherapy, since the tumors tend to have a "stealth" behavior.

In clinical practice, it is challenging to establish a feasible tool for monitoring tumor relapse in HCC patients with factors of low recurrence risk, such as low AFP (< 400 ng/ml), no microvascular invasion, a single tumor, small tumors (< 5.0 cm), and early tumors [18–20]. The present study provides the first demonstration that the SGR retains its predictive power in the at-risk subgroups and might thus show more promise than conventional clinicopathological variables for providing valuable information for the prediction of tumor relapse. Moreover, early recurrence represents a true metastasis, but it is difficult to effectively monitor these patients. The SGR might help to predict the prognosis of HCC patients with early recurrence. However, considering most samples from early HCC tumors, in the current stage, early recurrence does not represent a significant problem, and the direct association between SGR and the dissemination of HCC cells was not investigated in this study; therefore, a conclusion about the proangiogenic role of SGR in early recurrence should be cautiously interpreted. Other types of HCC including more HCC patients with the advance stage are needed to further validate its prognostic significance in these subpopulations.

Based on the multivariate analysis, the two nomograms that included SGR, the preoperative AFP or albumin level, the tumor number, the tumor size, and microvascular invasion showed a better prognostic value for recurrence and overall survival than the TNM staging system and any indicator alone. We also found that γδT cells showed increased accumulation in peritumoral liver tissue than in the intratumoral area, and conversely, more HSCs infiltrated into the intratumoral tissue than into the peritumoral area. This difference might be associated with their distinct roles in hepatocarcinogenesis. In tumor tissue, γδT cells are involved in antitumor immunity and are sacrificed after fighting against tumor cells [7], and HSCs are converted into myofibroblasts in response to tumor stimuli and could be converted to malignant phenotypes that promote the carcinogenic process [14]. Our previous study suggested that the peritumoral liver tissue is indisputably the principal target organ, because it provides the optimal environment for accelerating the formation of hepatic metastasis and is related to the aggressiveness of the tumor [9, 14]. Therefore, the inclusion of the peritumoral SGR in the two nomograms might contribute to their significantly increased predictive accuracy.

In this study, the cohort size was relatively small, and the clinical data were collected from a single study center. Although we found that peritumoral SGR had a significant positive correlation with CTCs level, there was just 2-year follow-up of the cohort providing CTCs, and we only found that high CTCs levels could predict early recurrence, and could not completely conclude that the group with low CTCs and SGR favors for OS and RFS. Future in-depth studies are needed to explore the mechanisms regulating the balance between HSCs and γδT cells and their relationship with CTCs during the development of HCC. Because all patients were selected from surgical HCC patients, most of them had a single tumor and an early TNM stage. As a result, the present study is valid only for early HCC tumors.

To our knowledge, this study is the first to establish two nomograms that include SGR, and shows that these can be used as an objective and reliable model for predicting the prognosis of HCC patients following curative resection. We also confirmed that HCC patients with a high SGR may have increased CTC levels, which are associated with a high recurrence rate and aggressive behavior. Herein, the two models might be used as promising predictors for monitoring patients at risk of early postoperative relapse. A large-scale prospective validation study might determine the applicability of this model.

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Compliance with ethical standards

Conflict of interest Bao-Yong Zhou, Jun-Hua Gong, Xiao-Yan Cai, Jia-Xing Wang, Fang Luo, Ning Jiang, Jian-Ping Gong, Cheng-You Du, and Rui Liao declare that they have no competing interests.

Ethical approval This study protocol conformed to the Ethics Committee at the First Affiliated Hospital of Chongqing Medical University and the ethical guidelines of the 1975 Helsinki Declaration and was approved, and written informed consents were obtained from all patients.

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