RESEARCH ARTICLE

Low Counts of γδ T Cells in Peritumoral Liver Tissue are Related to More Frequent Recurrence in Patients with Hepatocellular Carcinoma after Curative Resection

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Abstract

Objectives: TCR- gamma-delta + T cells (γδ T cells) are non-conventional T lymphocytes that can recognize and eradicate tumor cells. Our previous studies showed that infiltration and function of γδ T cells were substantially attenuated in hepatocellular carcinoma (HCC). However, their prognostic value was not clarified. Methods: The association between γδ T cells and the clinical outcomes was determined by immunohistochemistry (IHC) in a HCC patient cohort (n = 342). Results:Immunohistochemistry showed decreased infiltration of γδ T cells in tumoral tissues compared with paired peritumoral tissues. The counts of γδ T cells in peritumoral tissues were negatively correlated with tumor size (*P* = 0.005). Survival analysis showed that the levels of peritumoral γδ T cells were related to both time to recurrence (TTR) and overall survival (OS) (*P* = 0.010 and *P* = 0.036, respectively) in univariate analysis, and related to TTR in multivariate analysis (*P* = 0.014, H.R. [95% CI] = 0.682 [0.502-0.927]). Furthermore, the level of peritumoral γδ T cells showed independent prognostic value for TTR in Barcelona Clinic Liver Cancer (BCLC) stage A patients (*P* = 0.038, H.R. [95% CI] = 0.727 [0.537-0.984]). However, tumoral γδ T cells did not show independent prognostic value for either TTR or OS in HCC patients. Conclusions: Low counts of γδ T cells in peritumoral liver tissue are related to a higher incidence of recurrence in HCC and can predict postoperative recurrence, especially in those with early-stage HCC.

Keywords: γδ T cells - hepatocellular carcinoma - prognosis - recurrence

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Introduction

The tumor microenvironment is a dynamic system includes cancer cells, stromal tissue (including immune cells, fibroblasts, myofibroblasts, vascular tissue and multiple cytokines), as well as the surrounding extracellular matrix (Dunn et al., 2002). It is now clear that the interplay between the immune cells and the tumor will define the final outcome of the immune response during the equilibrium phase in "cancer immunoediting" model, which includs the following three key events: elimination, equilibrium and escape (Dunn et al., 2002; Ikeda et al., 2002). As a key component of the tumor microenvironment, the immune cells infiltrated into the tumor and exerted a variety of effects, either impeding or favoring tumor progression, at least partly, if not totally, determined the prognosis of patients.

TCR-γδ+ T cells (γδ T cells) are a small subset of human T lymphocytes (1–10% of total T cells in peripheral blood) that express the γδ T cells receptors (Braza et al., 2010), a higher percentage of which was found in the skin, intestine, genitourinary, and especially in the liver (Norris et al., 1998; Gao et al., 2008). γδ T cells can discriminate the host and pathogens by reacting rapidly towards nonpeptide antigens following infection and thereby activating the innate immune cells and facilitating the adaptive immune responses of αβ T cells. Recent date showed that these cells not only served as guards in the innate immune system but also acted as a bridge between innate and adaptive immune responses by performing multiple functions (Bonneville et al., 2010). γδ T cells also played a prominent role in innate defenses against tumor formation by directly recognizing molecules expressed on cancer cells without antigen processing and presentation (Braza et al., 2011).

Liver, largely accepted as an immune organ, is one of the richest source of γδ T cells. γδ T cells account for 15% to 25% of total liver T cells both in normal mouse and human (Gao et al., 2012). The percentage of γδ T cells in the liver was significantly increased in the tumor-bearing mice, which implied the prominent role of these cells in tumor surveillance (Born et al., 2006). Our previous study found that the infiltration of γδ T cells in tumoral tissues was significantly reduced compared to paired peritumoral

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tissues, accompanied by impaired effector function (Yi et al., 2013a). Patients with advanced HCC had even lower infiltration of γδ T cells in tumors compared with patients with early-stage HCC, which potentially indicated their possibility of prognostic value in HCC. Not surprisingly, we found the relative count of γδ T cells in tumors was associated with tumor recurrence by flowcytometry analysis. Then we further examined the prognosis value of the absolute counts of γδ T cells in HCC patients by tissue microarray and immunohistochemical staining in a cohort of 240 patients. Unfortunately, the result was meaningless either in tumor or peritumor tissues. This unconsistency had prompted our speculation on the related factors such as patient selection, the methods of antigen retrieval, number of cases and so on. Re-examining their prognostic value in HCC is urgently needed.

In the present study, we evaluated the infiltration of γδ T cells in the other larger HCC cohort of 342 patients by IHC staining with pressure cooking for antigen retrieval. Then we investigated the relationship between the absolute counts of γδ T cells in HCC specimens and the clinical outcome of the patient cohort. We found that less counts of γδ T cells in peritumoral liver tissue, but not in tumoral tissue, were related to higher incidence of recurrence in hepatocellular carcinoma patients after curative resection.

Materials and Methods

Patients and tissue specimens

Archived tissues were obtained for a cohort of 342 patients who received curative resection of HCC at the Liver Cancer Institute of Fudan University (Shanghai, China) between 2007 and 2008 after informed consent and approval by the Research Ethics Committee of Zhongshan Hospital (Fudan University, China). The inclusion and exclusion criteria are listed as follow: (a) confirmed pathologic diagnosis of HCC, (b) without preoperative anticancer treatment or sign of distant metastasis, (c) with integrated clinicopathological characteristics and postoperative follow-up data as described previously (Yi et al., 2013b; Wu et al., 2012). The Barcelona Clinic Liver Cancer (BCLC) staging system were applied for clinical staging (Llovet et al., 2008). Tumor differentiation was graded by the Edmond-son-Steiner grading system (Guo et al., 2001). Time to recurrence (TTR) and overall survival (OS) time were defined as the interval from primary surgical resection to the first recurrence or death, respectively.

Tissue immunohistochemical staining and evaluation

Tissue microarray (TMA) was constructed as described in our previous study (Gao et al., 2009). Triplicate of 1 mm core from two different areas, tumor center and non-tumor tissues (over 1 cm away from tumor margin), was obtained for each case to ensure reproducibility of the staining.

A standard two-step protocol was applied with primary antibodies: mouse anti-human TCRG (1:200; ThermoScientific, Rochester, USA). MaxvisionTM2 HRP-Polymer anti-Mouse/Rabbit IHC Kit (Maixin-Bio Corp., Fuzhou, China) was used as second antibody. Firstly, slides were deparaffinized in xylene and hydrated through a graded alcohol series, antigen retrieval was carried out in a pressure cooker (using a standard household model) with 0.1M EDTA (pH 8) for 9 minutes. Secondly, sections were incubated with primary antibodies at 4°C overnight after being placed in blocking solution to inhibit endogenous peroxidase activity and nonspecific background staining. Thirdly, sections were incubated with second antibody for 20 minutes at room temperature and developed with DAB for 3 minutes. Blank controls were treated identically except that the primary antibodies were omitted.

The positive γδ T cells were observed with the use of a computerized image system composed of camera connected to OLYMPUS U-CAMD3 microscope. Under high-power magnification (×200), the number of positive γδ T cells in each 1-mm-diameter cylinder was totally counted by two experienced pathologists who were blinded to the clinicopathologic data of the patients and expressed as mean value of the triplicates (cells/spot).

Statistical analysis

Statistical Package of the Social Sciences (SPSS) 17.0 statistical package was employed. χ^2 test , paired t test and independent samples t test were done as appropriate. Univariate analyses were done using the Kaplan-Meier method and compared by the long-rank test. Cox multivariate analysis was used to adjust for potentially confounding variables and to determine the independent prognostic factors. The "minimum *P* value" approach (Galon et al., 2006; Li et al., 2011) was used to get optimal cut-off value for the best separation between groups of patients related to TTR or OS. Significance was accepted when $P < 0.05$.

Results

Characteristics of the patient cohort

The clinicopathological characteristics of the patients are shown in Supplementary Table S1, of which the

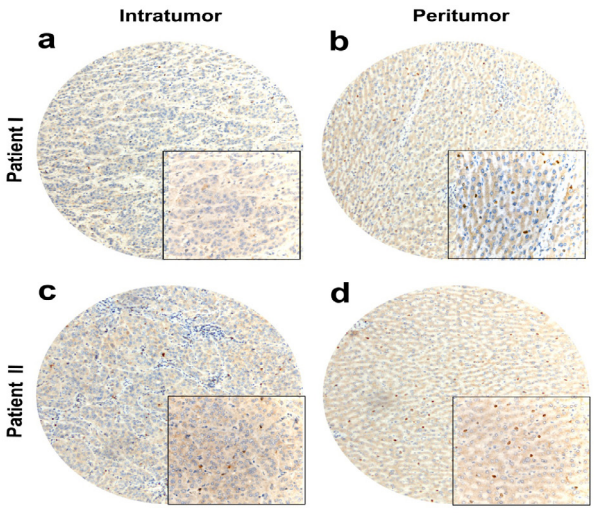

Figure 1. Representative Immunohistochemical Staining of γδ T Cells. Representative staining of γδ T cells in tumoral tissues (a and c) and paired peritumoral tissues (b and d), two cases were shown (magnification100× & 400×)

Table 1. Correlation of Clinicopathologic Characteristics with γδT Expression

Characteristics	iγδT			pγδT		
	low	high	*P* value	low	high	*P* value
Gender						
Male	207	83	0.698	151	139	0.121
Female	36	16		21	31	
Age (Years)						
≤52	124	45	0.265	87	81	0.587
>52	119	54		85	89	
HBV infection						
Yes	217	88	0.817	150	155	0.238
No	27	10		22	15	
Liver cirrhosis						
Yes	213	91	0.279	155	149	0.468
No	30	8		17	21	
ALT (U/L)						
>75	25	16	0.279	17	22	0.374
≤75	218	83		155	148	
AFP (ng/ml)						
>20	154	56	0.334	102	108	0.422
≤20	89	43		70	62	
γ-GT (U/L)						
>54	133	46	0.118	96	83	0.196
≤54	110	53		76	87	
Tumor size (cm)						
>5	89	24	0.022	69	44	0.005
≤5	154	75		103	126	
Tumor number						
≥2	29	15	0.381	17	27	0.098
1	214	84		155	143	
Tumor thrombi						
Yes	69	24	0.495	50	43	0.433
No	174	75		122	127	
Encapsulation						
No	117	42	0.326	72	87	0.084
Yes	126	57		100	83	
Differentiation						
III&IV	60	27	0.549	47	40	0.420
I&II	183	72		125	130	
BCLC stage						
A	166	67	0.816	115	118	0.613
B/C	77	32		57	52	

χ^2 tests for all the analysis; AFP, alpha-fetoprotein; γ-GT, γ-glutamyl transferase; OS, overall survival; TTR, time to recurrence; BCLC, Barcelona Clinic Liver Cancer; iγδT, intratumoral γδ T cells; pγδT, peritumoral γδ T cells

median age was 53 years. There were about 89.18% of patients (305 cases) with hard evidences of hepatitis B virus (HBV) infection among the cohort. The median follow-up was 42.9 months (range, 0.43-61.83 months; SD, 18.8 months). At the last follow-up (March 31st, 2012), 158 patients had recurrence. 125 patients died of recurrence. The 1-, 3-, and 5-year cumulative recurrence and survival rates (in brackets) were 33 % (81%), 50 % (65%), and 59 % (60%), respectively.

Expression of γδ T cells in tumoral and peritumoral tissues

We found that the positive cells, which were defined as brown-stained cell, principally scattered in the tumoral and peritumoral parenchyma (Figure 1a-d), Compared with paired peritumoral tissues, tumoral tissues had significantly less infiltration of positive γδ T cells (mean, 7.5 vs. 15.0, *P*<0.001; Figure 2). When stratified by HBV infection status, there was no difference between the count of infiltrating positive γδ T cells with and without HBV infection in tumoral or peritumoral tissues (mean, 7.79 vs 5.38, *P*=0.152; mean, 15.14 vs 13.41, *P*=0.634).

Table 3. Survival Analysis of γδT Count in HCC Patients when Stratified by Clinicopathologic Factors

Variables		iγδT -TTR *P* value	iγδT -OS *P* value	pγδT-TTR *P* value	pγδT-OS *P* value
BCLC stage					
A	233	0.351	0.003	0.003	0.043
B/C	109	0.855	0.811	0.808	0.451
HBV infection					
No	37	0.142	0.101	0.123	0.098
Yes	305	0.945	0.087	0.021	0.094
AFP					
≤20	132	0.746	0.511	0.122	0.109
>20	210	0.430	0.054	0.026	0.100
γ-GT (U/L)					
≤54	163	0.549	0.325	0.577	0.614
>54	179	0.837	0.111	0.007	0.048
tumor size					
≤5	229	0.723	0.514	0.443	0.372
>5	113	0.444	0.097	0.013	0.410
Tumor number					
1	298	0.240	0.005	0.004	0.006
≥2	44	0.158	0.352	0.88	0.472
Tumor thrombi					
No	249	0.882	0.048	0.015	0.100
Yes	93	0.195	0.488	0.376	0.269
Tumor capsule					
Yes	183	0.930	0.077	0.310	0.164
No	159	0.573	0.292	0.004	0.086
Differentiation					
I-II	255	0.286	0.022	0.107	0.201
III-IV	87	0.489	0.675	0.029	0.075

χ^2 tests for all the analysis. BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein; γ-GT, γ-glutamyl transferase; OS, overall survival; TTR, time to recurrence; iγδT, intratumoral γδ T cells; pγδT, peritumoral γδ T cells

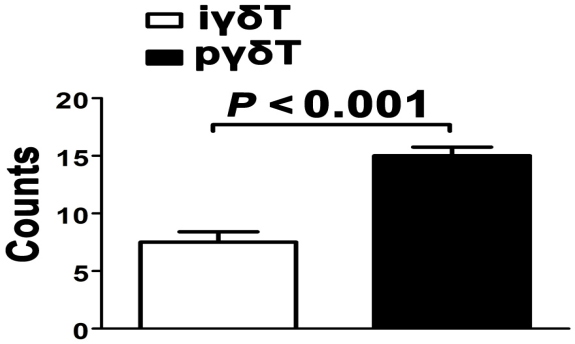

Figure 2. The Counts of γδ T Cells in Tumoral Tissues Compared with Paired Peritumoral Tissues. All the positive cells in 1 mm cylinder was counted. (mean ± SEM, 7.5 ± 0.9108 vs. 15.0 ± 0.7657, P < 0.001). (iγδT, intratumoral γδ T cells; pγδT, peritumoral γδ T cells)

Correlations of γδ T cells and clinicopathological characteristics of HCC

The clinicopathological factors of HCC were analyzed

Table 2. Univariate and Multivariate Analyses of γδT Associated with Recurrence and Survival

Variables	TTR			OS		
	Univariate	Multivariate		Univarate	Multivariate	
	P value	H.R. (95% CI)	*P* value	*P* value	H.R. (95% CI)	*P* value
BCLC stage A						
γ-GT, U/L (≤54 vs >54)	<0.001	1.531 (1.117-2.096)	0.007	0.003	1.498 (1.029-2.180)	0.041
AFP, ng/ml (≤20 vs >20)	0.079	ND	NA	0.038	1.446 (0.983-2.128)	0.056
Tumor size, cm (≤5 vs >5)	<0.001	1.632 (1.188-2.240)	0.003	<0.001	2.823 (1.962-4.062)	<0.001
Tumor number (single vs multiple)	0.703	ND	NA	0.075	ND	NA
Tumor capsule (yes vs no)	0.398	ND	NA	0.822	ND	NA
Differentiation (I&II vs III&IV)	0.670	ND	NA	0.640	ND	NA
iγδT (6)	0.351	ND	NA	0.003	0.725 (0.475-1.106)	0.125
pγδT (11)	0.003	0.727 (0.537-0.984)	0.038	0.043	0.805 (0.564-1.150)	0.231
TMA assays						
Age, year (≤52 vs >52)	0.196	ND	NA	0.668	ND	NA
Gender (female vs male)	0.982	ND	NA	0.752	ND	NA
HBV infection (no vs yes)	0.811	ND	NA	0.225	ND	NA
Liver cirrhosis (no vs yes)	0.303	ND	NA	0.277	ND	NA
ALT, U/L (≤75 vs >75)	0.392	ND	NA	0.288	ND	NA
γ-GT, U/L (≤54 vs >54)	<0.001	1.521 (1.107-2.090)	0.009	<0.001	1.480(1.014-2.160)	0.039
AFP, ng/ml (≤20 vs >20)	0.018	1.377 (0.998-1.899)	0.048	0.009	1.465 (0.997-2.152)	0.047
Tumor size, cm (≤5 vs >5)	<0.001	1.613 (1.167-2.230)	0.004	<0.001	2.837 (1.968-4.088)	<0.001
Tumor number (single vs multiple)	<0.001	1.664 (1.103-2.551)	0.021	0.081	ND	NA
Tumor capsule (yes vs no)	0.147	ND	NA	0.210	ND	NA
Differentiation (I&II vs III&IV)	0.147	ND	NA	0.147	ND	NA
Tumor thrombi (no vs yes)	<0.001	1.515 (1.090-2.104)	0.016	<0.001	1.621 (1.123-2.339)	0.012
BCLC stage (A vs B/C)	<0.001	ND	NA	<0.001	ND	NA
iγδT (6)	0.599	1.020 (0.732-1.423)	0.906	0.038	0.744 (0.487-1.135)	0.159
pγδT (11)	0.010	0.682 (0.502-0.927)	0.014	0.036	0.822 (0.575-1.174)	0.279

Note: Univariate analysis was calculated by the Kaplan–Meier method (the long-rank test). Multivariate analysis was done using the Cox multivariate proportional hazards regression model in a stepwise manner (backward, likelihood ratio). AFP, a-fetoprotein; 95% CI, 95% confidence interval; γ-GT, γ-glutamyl transferase; HR, hazard ratio; NA, not adopted; ND, no data; OS, overall survival; TTR, time to recurrence; BCLC, Barcelona Clinic Liver Cancer. iγδT, intratumoral γδT; pγδT, peritumoral γδT

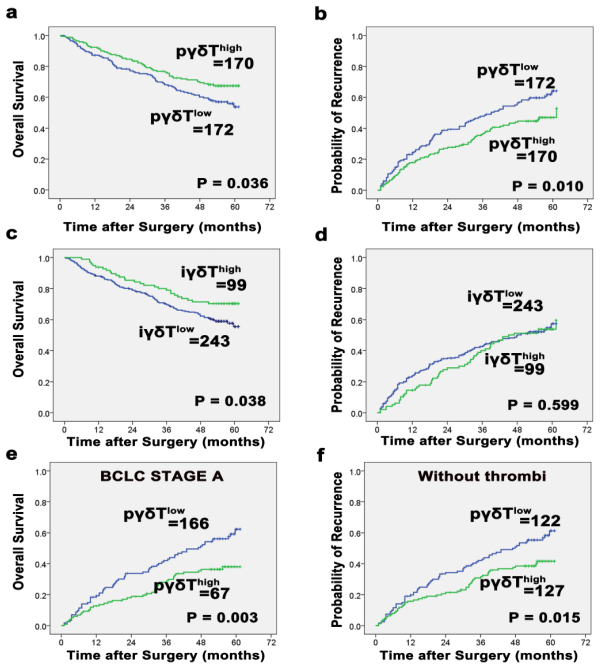

Figure 3. Kaplan-Meier Analysis of OS and TTR for the Expression Levels of γδ T Cells. Univariate analyses of the relationship between γδ T cells and OS or TTR (peritumoral: a and b, respectively; intratumoral: c and d, respectively). Survival analysis of the association between peritumoral γδ T cells and TTR when classified by BCLC stage A (e) and without thrombi (f) (iγδT, intratumoral γδ T cells; pγδT, peritumoral γδ T cells)

related to the levels of the intratumoral and peritumoral γδ T cells. As shown in Table 1, the number of γδ T cells was negatively correlated with tumor size grading both in tumoral and peritumoral tissues.

Prognostic significance of γδ T cells for HCC

By using the "minimum *P* value" approach, scoring value of 6 and 11 are the best cut-off value for intratumoral and peritumoral number of γδ T cells, respectively. On univariate analysis, the counts of peritumoral γδ T cells were related to both OS and TTR (Table 2, Figure 3a, b). We further performed multivariate Cox proportional hazard regression analyses, which enrolled clinicopathologic factors showed significant in univariate analysis, to reveal the independent prognostic role of γδ T cells. As expected, the levels of peritumoral T cells showed favorable independent prognostic value for TTR, but not for OS. Additionally, the counts of tumoral γδ T cells were related to OS in univariate analysis (Figure 3c), but did not show independent prognostic value in HCC patients when evaluated by multivariate analysis.

Moreover, when stratified by clinicopathological factors, the level of peritumoral γδ T cells showed favorable prognostic role for TTR in patients with early-stage HCC (BCLC A) or patients without thrombi by univariate analysis ($P = 0.003$ and $P = 0.015$, respectively; Table 3, Figure 3e, f). In addition, similar results showed in the patients with one of other seven clinicopathological

factors such as with HBV infection (Table 3). The prognostic power with early-stage HCC was further strengthened by multivariate analysis ($P = 0.038$; Table 2).

Discussion

It had been reported that immune status of tumor microenvironment in situ was an important element in the prognostic classification of HCC (El-Serag and Rudolph, 2007). Our previous reports supported the relations between various immune cells (including regulatory T cells (Gao et al., 2007), memory T cells (Gao et al., 2012), dendritic cells (Cai et al., 2006), neutrophils (Li et al., 2011), mast cells (Ju et al., 2009), and activated hepatic stellate cells (Liao et al., 2013) in tumoral or peritumoral tissues with different survival outcomes. Our previous study also showed that the percentage of γδ T cells in HCC was reduced in tumor compared with peritumoral tissues and advanced tumor had even lower infiltration of γδ T cells, indicating the importance of γδ T cells in the antitumor immunity (Yi et al., 2013a). In the current study, we observed that the number of γδ T cells was negatively correlated with tumor size grading both in tumoral and peritumoral tissues. More importantly, less counts of γδ T cells in peritumoral tissues were related to higher incidence of postoperative recurrence in HCC patients.

Our present data displayed that the counts of γδ T cells were much lower in tumoral tissues than that of peritumoral tissues in HCC (Figure 2), consistent with previous study conducted by using IHC and flow cytometry. This result was also in accordance with the low levels of intratumoral γδ T cells in other solid tumors, such as renal cancer (Kowalczyk et al., 1996; Inman et al., 2008). In the tumoral tissues, we found that the number of γδ T cells was positively associated with OS when patients were defined as following subgroups: patients with early-stage (BCLC stage A) HCC, or with single tumor, or with well differentiated tumor, or without thrombi, which indicated that the important antitumor role of γδ T cells in early-stage HCC. Unfortunately, both the infiltration and the cytotoxicity function of γδ T cells were attenuated in advance tumor, which may lead to the underestimation of the prognostic power of γδ T cells in the whole HCC population. Furthermore, the impact of intratumoral γδ T cells was removed by surgery.

The peritumoral tissue, which contained a significant amount of leukocyte infiltration, was long assumed to represent the host response to the malignancy and had the significance of prognostic value in HCC (Ju et al., 2009; Kuang et al., 2009; Kuang et al., 2010; Liao et al., 2013). In current study, the most important new conclusion was that less counts of γδ T cells in peritumoral liver tissue were related to a higher incidence of recurrence in HCC patients after curative resection. This result was not consistent with our previous finding in the cohort of 240 HCC patients. This discrepancy can be explained as follow: firstly, it was likely that the clinicopathologic features with enormous implications for prognosis of HCC was much different between these two study cohorts, six of which had prognostic value in HCC. Secondly, the current patient cohort contained more cases with early-stage or small tumor, mainly due to the availability of earlier diagnosis and treatment.

It has been reported that hepatitis B virus (HBV) is responsible for 80% to 89% of HCCs in developing countries (Kew, 2012). In our cohort, we found that about 89.18% of patients had hard evidences with HBV infection. Recently, Wu et al. (2013) has reported that decreased Vδ2 γδ T cells are associated with liver damage in patients with chronic hepatitis B. However, we found the majority of γδ T cells were Vδ1 T cells in normal liver tissue and cirrhotic peritumor tissue in previous study (Yi et al., 2013a), which may explain that the number of total γδ T cells had no difference between two groups (with or without HBV infection) in this study. Furthermore, the level of peritumoral γδ T cells showed favorable prognostic role for TTR in patients with HBV infection in stratified analysis.

Therefore, we proposed that the peritumoral γδ T cells may represent the host response to the malignancy in HCC and the level of γδ T cells in peritumoral tissues be connected with the abilities of host antitumor immunity and the possibility of recurrence after hepatectomy, especially in early-stage HCC. Our data also demonstrated that some items such as the presence of vascular invasion were prognostic predictors after liver resection, just as reported by Ikai et al. among intermediate to advanced stages of HCC (Ikai et al., 2004). Although the prognosis of early stage HCC is far from homogenous and existing classification systems have shown limitations on precise prediction of patients' outcome, in consideration of the lack of precise indicator for the early-stage HCC, the absolute counts of γδ T cells in peritumoral tissues will distinguish the possibility of recurrence and guide further treatment after hepatectomy. The current study also provides further evidences to support that the peritumoral immune environment is important in understanding the mechanism of intrahepatic metastasis of HCC. As the front line of defense to prevent tumor growth, the peritumoral tissues, which were endowed with abundant γδ T cells, may play a role in defending metastatic tumor cells. Accordingly, our colleagues had demonstrated that higher level of interleukin (IL)-2 and IL-15, which were important for γδ T cells homeostasis, was significantly associated with a decreased incidence of intrahepatic tumour recurrence and a prolonged overall survival (Caccamo et al., 2005; Zhou et al., 2010).

In conclusion, on the whole, the present study indicated that γδ T cells in peritumoral liver tissues can predict the postoperative recurrence free survival time of patients with HCC, and also highlighted the important role of the residual liver in recurrence and metastasis. Increasing the function of γδ T cells may become a new strategy for anti-tumor immunotherapy for HCC.

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