VOLUME 25 · NUMBER 18 · JUNE 20 2007

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Intratumoral Balance of Regulatory and Cytotoxic T Cells Is Associated With Prognosis of Hepatocellular Carcinoma After Resection

Qiang Gao, Shuang-Jian Qiu, Jia Fan, Jian Zhou, Xiao-Ying Wang, Yong-Sheng Xiao, Yang Xu, Yi-Wei Li, and Zhao-You Tang

From the Liver Cancer Institute, Zhong Shan Hospital and Shanghai Medical School, Fudan University, Key Laboratory for Carcinogenesis & Cancer Invasion, the Chinese Ministry of Education, Shanghai, People's Republic of China.

Submitted October 15, 2006; accepted February 1, 2007.

Supported by grants from the National Natural Science Foundation of China (Grant No. 30200268) and the Foundation of China National "211" Project for Higher Education.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Address reprint requests to Jia Fan, MD, PhD, Liver Cancer Institute, Fudan University, 136 Yi Xue Yuan Rd, Shanghai 200032, People's Republic of China; e-mail: jiafan99@yahoo.com.

0732-183X/07/2518-2586/$20.00

DOI: 10.1200/JCO.2006.09.4565

ABSTRACT

Purpose
To investigate the prognostic value of tumor-infiltrating lymphocytes (TILs), especially regulatory T cells (Tregs), in hepatocellular carcinoma (HCC) patients after resection.

Patients and Methods
$CD3^+$, $CD4^+$, $CD8^+$, Foxp3-positive, and granzyme B-positive TILs were assessed by immunohistochemistry in tissue microarrays containing HCC from 302 patients. Prognostic effects of low- or high-density TIL subsets were evaluated by Cox regression and Kaplan-Meier analysis using median values as cutoff.

Results
$CD3^+$, $CD4^+$, $CD8^+$ TILs were associated with neither overall survival (OS) nor disease-free survival (DFS). The presence of low intratumoral Tregs in combination with high intratumoral activated $CD8^+$ cytotoxic cells (CTLs), a balance toward CTLs, was an independent prognostic factor for both improved DFS ($P = .001$) and OS ($P < .0001$). Five-year OS and DFS rates were only 24.1% and 19.8% for the group with intratumoral high Tregs and low activated CTLs, compared with 64.0% and 59.4% for the group with intratumoral low Tregs and high activated CTLs, respectively. Either intratumoral Tregs alone ($P = .001$) or intratumoral activated CTLs ($P = .001$) alone is also an independent predictor for OS. In addition, high Tregs density was associated with both absence of tumor encapsulation ($P = .032$) and presence of tumor vascular invasion ($P = .031$).

Conclusion
Tregs are associated with HCC invasiveness, and intratumoral balance of regulatory and cytotoxic T cells is a promising independent predictor for recurrence and survival in HCC. A combination of depletion of Tregs and concomitant stimulation of effector T cells may be an effective immunotherapy to reduce recurrence and prolong survival after surgery.

J Clin Oncol 25:2586-2593. © 2007 by American Society of Clinical Oncology

INTRODUCTION

The overall survival of patients with hepatocellular carcinoma (HCC) remains poor despite improved diagnostic and treatment strategies, among which resection is one of the first priorities.[1] A high postoperative recurrence rate is a major obstacle, and thus many biomarkers for prediction and intervention, mainly HCC metastasis-related chromosomes/genes/proteins, have been tried.[2] Recently, it was reported that biologic behaviors of HCC are associated with a unique immune response signature of the liver microenvironment,[3] and outcome is governed predominantly by immune responses at the primary tumor site,[4] which suggest that precise evaluation of local immune responses could be useful for predicting prognosis.[5]

Tumor-infiltrating lymphocytes (TILs) are considered manifestations of host immune reactions against cancers. Patients with a prominent lymphocyte infiltrate, especially T lymphocytes, who underwent resection for HCC have reduced recurrence and better survival.[6] However, tumor progression, which often also is seen in the presence of substantial lymphocytic infiltration, suggests that T cells are not capable of mounting effective immune responses to control tumor growth.[7] Evidence has accumulated that TILs are functionally defective, incompletely activated, and include regulatory subtypes, which varied with the types of cancers.[8-11] Therefore, if distinctions were made with respect to lymphocyte types, location, functional state, and their interactions, a more profound impact on prognosis was observed compared with only the overall degree of lymphoid infiltration.[4]

$CD4^+CD25^+$ regulatory T cells (Tregs) are believed to dampen T-cell immunity and to be the main obstacle tempering immunotherapy.[12] Foxp3, a critical regulator of the development and function of Tregs, fulfills the criteria of a Treg-specific marker.[5,13,14] It was reported that high Foxp3 expression is associated not only with dismal prognosis in ovarian cancer but also represents an independent predictor for overall survival (OS) and progression-free survival.[5,15] However, discrepancies remain among different cancer types. No prognostic influence of tumor-infiltrating Tregs was found in anal squamous cell carcinoma[14]; in head and neck squamous cell carcinoma, Tregs correlated positively with locoregional control.[16] Specifically, the balance between cytotoxic and regulatory T cells is believed to be of greater significance. The ratio of $CD8^+$ CTLs and Tregs in ovarian cancer,[5] as well as the concurrent low-Tregs density and high-CTLs density in Hodgkin's lymphoma,[17] is reported to be more valuable than the single TILs subtypes.

However, the prognostic role of TILs, especially Tregs, in patients with HCC who underwent resection is less clear. In this study, the significance of various subtypes of TILs (including regulatory, cytotoxic, and their balance) and their relation to established markers of HCC prognosis such as vascular invasion[18] were examined. We suggest that Tregs might have a role in the promotion of HCC invasiveness, and the intratumoral balance of Tregs and CTLs is a promising independent predictor for recurrence and survival.

PATIENTS AND METHODS

Patients and Specimens

Archival specimens were obtained from 302 patients at Zhong Shan Hospital (Shanghai, People's Republic of China) between 1997 and 1999 after informed consent. All of the patients without distant metastasis or any prior anticancer treatment, underwent curative resection for HCC, defined as complete macroscopic removal of the tumor.[19] Paraffin blocks were selected only on the basis of the availability of suitable formalin-fixed, paraffin-embedded tissue and complete clinicopathologic and follow-up data for the patients. The clinical typing of tumors was determined according to the TNM classification system of International Union Against Cancer (edition 6). The histologic grade of tumor differentiation was assigned by the Edmondson grading system. Liver function was assessed by Child-Pugh score system. Clinicopathologic characteristics are summarized in Appendix Table A1 (online only). Ethical approval was obtained from Zhong Shan Hospital research ethics committee.

Follow-Up and Postoperative Treatment

After surgery, patients with a high risk of recurrence, such as vascular invasion and spreading nodules, were treated with prophylactic transcatheter arterial chemoembolization (doxorubicin, cisplatin, fluorouracil, and iodized oil; one to three courses). Follow-up was completed March 15, 2006. The median follow-up was 58 months (range, 2 to 109 months). All patients were monitored prospectively by serum alpha-fetoprotein (AFP), abdomen ultrasonography, and chest x-ray every 1 to 6 months according to the postoperative time. For patients with test results suggestive of recurrence, computed tomography and/or magnetic resonance imaging were used to verify whether recurrence had occurred. A diagnosis of recurrence was based on typical imaging appearance in computed tomography and/or magnetic resonance imaging scan and an elevated AFP level. The treatment modality after relapse varied among individuals, and nine patients with recurrent disease and poor liver function received no treatment. Patients died as a result of recurrence, metastasis, or complicated liver cirrhosis. OS was defined as the interval between surgery and death or between surgery and the last observation for surviving patients. The data were censored at the last follow-up for living patients.

Tissue Microarray and Immunohistochemistry

Tissue microarrays were constructed as described previously.[4,14,17,20] All samples from HCC patients were reviewed histologically by hematoxylin and eosin staining, and representative areas with small round lymphocyte infiltrate were premarked in the paraffin blocks, away from necrotic and hemorrhagic materials. Duplicates of 1-mm-diameter cylinders from two different areas, tumor center and nearest noncancerous margin (designated as intratumor and peritumor, respectively; a total of four punches) were included in each case, along with different controls, to ensure reproducibility and homogenous staining of the slides (Shanghai Biochip Co Ltd, Shanghai, People's Republic of China). Thus, four different tissue microarray blocks were constructed, each containing 312 cylinders. Sections 4 μm thick were placed on slides coated with 3-aminopropyltriethoxysilane.

The mouse monoclonal antibodies used were antihuman CD3 (DAKO, Carpinteria, CA), CD4, CD8, granzyme B (Novocastra, Newcastle, United Kingdom), and Foxp3 (Biolegend, San Diego, CA). Immunohistochemistry of paraffin sections was carried out using a two-step protocol (Novolink Polymer Detection System, Novocastra) according to the manufacturer's instructions and as described previously.[21] Briefly, paraffin sections were first deparaffinized and then hydrated. After microwave antigen retrieval, as required, endogenous peroxidase activity was blocked with incubation of the slides in 0.3% H_2O_2, and nonspecific binding sites were blocked with Protein Block (RE7102; Novocastra). After serial incubation with primary antibodies, Post Primary Block (RE7111; Novocastra), and secondary antibody (Novolink Polymer RE7112), the sections were developed in diaminobenzidine solution under a microscope and counterstained with hematoxylin. Negative control slides omitting the primary antibodies were included in all assays.

Evaluation of Immunohistochemical Variables

The number of TILs was counted using a computerized image analysis system composed of a Hitachi HV-C20A CCD camera (Hitachi, Tokyo, Japan), installed on a Leica DMLA light microscope (Leica Microsystems, Wetzlar, Germany) and attached to a personal computer. Under 400× magnification, there exists at least 12 independent and intact computerized microscopic fields for the duplicates of each patient sample. Five independent microscopic fields (400×), representing the densest lymphocytic infiltrates, were selected for each patient sample to ensure representativeness and homogeneity. The results were expressed as the mean (± SE) number cells for one computerized 400× microscopic field (0.0768 mm^2/field). Granzyme B, constitutively expressed by natural-killer cells, is only expressed on activated $CD8^+$ CTLs.[17,22] Granzyme B–positive cells with a sparsely granulated pattern were evaluated as activated CTLs, and densely positive natural-killer cells were excluded.[23,24]

The evaluation of TILs was performed without knowledge of the clinicopathologic data by two independent investigators. Variations in the enumeration, within a range of 5%, were re-evaluated and a consensus decision was made.

Statistic Analysis

Statistical analyses were performed with SPSS 12.0 software (SPSS, Chicago, IL). Cumulative survival time was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. For all immunohistochemical markers, the cutoff for definition of subgroups was the median value.

A secondary analysis was performed to assess the relationship among lymphocytic variables and clinicopathologic characteristics. For the comparison of individual variables, χ^2 tests, Fisher's exact tests, and Spearman ρ coefficients tests were carried out as appropriate. Two-tailed $P < .05$ was judged to be significant.

RESULTS

Immunohistochemical Characteristics

By hematoxylin and eosin staining, immune cell infiltration was observed to be relatively homogenous within a tumor excluding necrotic, hemorrhagic, and fibrotic components. Lymphocytes

infiltrated HCC tissue in a diffuse manner or in lymphoid aggregates, with more abundant cells in peritumoral areas, and the ratio of $CD4^+$ and $CD8^+$ cells varied substantially among samples. The numbers of most TIL subtypes correlated with each other (range of correlation coefficients, 0.21 to 0.76; $P = 0.49$ to $< .0001$ for significant correlations; Appendix Table A2, online only). The duplicate of spots for each tumor showed a good level of homogeneity for stained cell density. Granzyme B–positive lymphocytes display a granular cytoplasmic staining pattern, which reflects the granular localization of granzyme B. Foxp3-positive cells exhibited distinct nuclear staining, reflecting transcription function of Foxp3 (Fig 1). Representative images (Appendix Figs A1 and A2, online only) and statistics of immunohistochemical variables (Table 1) are shown.

Prognostic Factors

The OS and disease-free survival (DFS) rates were 63.4% and 54.5% at 3 years, 49.1% and 46.1% at 5 years, and 39.9% and 39.0% at 7 years, respectively, for the whole study population.

On univariate analysis, age, sex, hepatitis history, cirrhosis, AFP level, ALT level, Child-Pugh score, and tumor differentiation showed no prognostic significance for OS and DFS. For DFS, both TNM stage and vascular invasion were predictors. In addition to TNM stage and vascular invasion, tumor size, number, and encapsulation were also associated with OS.

Table 1. Descriptive Statistics of Immunohistochemical Variables

Variable*	Mean	SE	Median	Range
$CD3^+$ TILs				
Intratumor	73.63	4.25	45.20	4.00-374.00
Peritumor	160.83	7.04	127.20	12.00-481.00
$CD4^+$ TILs				
Intratumor	32.92	1.96	20.20	0.80-166.40
Peritumor	76.35	3.02	61.60	6.00-221.20
$CD8^+$ TILs				
Intratumor	27.82	1.71	17.74	1.40-223.40
Peritumor	92.86	3.25	76.00	12.60-306.00
Gr B–positive TILs				
Intratumor	7.99	0.61	4.80	0-94.20
Peritumor	13.29	1.11	9.60	0-73.00
Foxp3-positive TILs				
Intratumor	5.16	0.49	2.24	0-77.40
Peritumor	7.95	0.64	6.00	0-56.20

Abbreviations: TILs, tumor-infiltrating lymphocytes; Gr B, granzyme B.
*Number of TILs per field (×400).

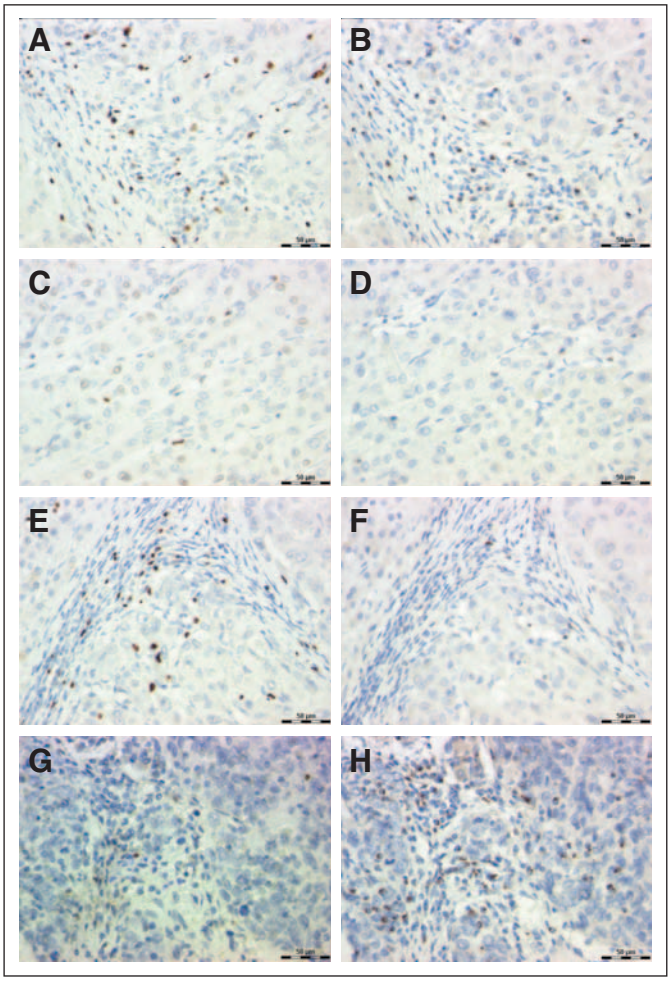

Fig 1. Tumor-infiltrating Foxp3-positive and granzyme B–positive cells. Consecutive sections were used for immunohistochemical study on (A, C, E, and G) Foxp3-positive and (B, D, F, and H) granzyme B–positive cells: (A and B) both high; (C and D) both low; (E and F) high Foxp3-positive and low granzyme B–positive cells; (G and H) low Foxp3-positive and high granzyme B–positive cells. Positive lymphocytes were stained brown (400× magnification).

No lymphocyte types infiltrating the peritumoral area showed any prognostic significance (Table 2). Intratumoral $CD3^+$, $CD4^+$, and $CD8^+$ TILs were not associated with either OS or DFS (Table 2; Appendix Fig A3, online only). Activated CTLs (granzyme B positive), which were reported to be prognostic in various cancer types,[14,25,26] were only associated with improved OS ($P = .026$) and had no influence on DFS ($P = .129$; Figs 2C and 2D; Table 2). Tregs (Foxp3 positive) were found to be prognostic for both OS ($P = .006$) and DFS ($P = .015$; Figs 2A and 2B; Table 2). Patients with low intratumoral Tregs had longer OS (median, 70 months) and DFS (median, 69 months) than did those with high intratumoral Tregs (median, 51 and 34 months, respectively). Although the numbers of most TIL subtypes correlated with each other, only these two types showed prognostic value.

The combined influence of low versus high number of intratumoral Tregs and activated CTLs was also evaluated. Using the median number as the cutoff, patients were classified into four groups: I, low Tregs and high activated CTLs (n = 50); II, low Tregs and low activated CTLs (n = 105); III, high Tregs and high activated CTLs (n = 100); IV, high Tregs and low activated CTLs (n = 47; Fig 1). Significant differences in both OS ($P < .0001$) and DFS ($P = .003$) were found among the four groups (Figs 2E and 2F; Table 2). In terms of median survival, group I had longer OS (86 months) and DFS (105 months) than did group IV (31 and 23 months, respectively). Five-year OS and DFS rates were only 24.1% and 19.8% for group IV, compared with 64.0% and 59.4% for group I, respectively. In groups II and III, the influence of intratumoral Tregs, low or high, on prognosis was probably counteracted by simultaneously low or high intratumoral activated CTLs, and vice versa. Therefore, irrespective of the absolute number of intratumoral Tregs and activated CTLs, the two groups had similar data for survival (hazard ratio = 1.05; 95% CI, 0.74 to 1.51; $P = .77$) and recurrence (hazard ratio = 1.11; 95% CI, 0.77 to 1.62; $P = .57$).

Clinicopathologic features showing significance by univariate analysis were adopted as covariates when multivariate Cox

Table 2. Univariate Analyses of Factors Associated With Survival and Recurrence

Variable	OS			DFS		
	Hazard Ratio	95% CI	*P*	Hazard Ratio	95% CI	*P*
Age, years (≤ 51 *v* > 51)	0.86	0.65 to 1.15	.313	1.02	0.75 to 1.38	.917
Sex (female *v* male)	0.92	0.61 to 1.38	.688	1.12	0.71 to 1.78	.626
Hepatitis history (no *v* yes)	1.24	0.80 to 1.90	.334	1.13	0.73 to 1.76	.581
AFP, ng/mL (≤ 20 *v* > 20)	1.18	0.89 to 1.58	.257	1.14	0.84 to 1.56	.394
Liver cirrhosis (no *v* yes)	1.15	0.76 to 1.75	.496	1.18	0.76 to 1.84	.455
ALT, U/L (≤ 40 *v* > 40)	1.26	0.93 to 1.70	.133	1.08	0.78 to 1.50	.635
Child-Pugh score (A *v* B)	1.91	0.61 to 5.98	.267	1.53	0.49 to 4.80	.466
Tumor differentiation (I-II *v* III-IV)	1.17	0.85 to 1.61	.320	1.22	0.87 to 1.71	.245
Tumor size, cm (≤ 5 *v* > 5)	1.64	1.23 to 2.19	.001	1.25	0.92 to 1.70	.159
Tumor number (single *v* multiple)	1.61	1.17 to 2.21	.003	1.21	0.84 to 1.73	.316
Tumor encapsulation (complete *v* none)	1.65	1.24 to 2.21	.001	1.14	0.83 to 1.56	.430
Vascular invasion (no *v* yes)	2.57	1.83 to 3.60	< .0001	1.98	1.35 to 2.92	.001
TNM stage (I *v* II + III)	2.00	1.50 to 2.67	< .0001	1.53	1.12 to 2.09	.008
Peritumoral TILs (low *v* high)						
$CD3^+$	0.88	0.53 to 1.35	.476	0.92	0.55 to 1.54	.743
$CD4^+$	0.84	0.53 to 1.35	.475	0.79	0.47 to 1.33	.382
$CD8^+$	0.68	0.42 to 1.71	.100	0.77	0.46 to 1.30	.329
Gr B positive	1.31	0.82 to 2.80	.260	0.92	0.55 to 1.55	.747
Foxp3 positive	1.10	0.69 to 1.75	.639	1.27	0.76 to 2.14	.363
Intratumoral TILs (low *v* high)						
$CD3^+$	0.84	0.63 to 1.12	.229	0.84	0.62 to 1.14	.255
$CD4^+$	0.82	0.62 to 1.10	.182	0.89	0.65 to 1.20	.435
$CD8^+$	0.84	0.63 to 1.12	.240	0.79	0.58 to 1.07	.133
Gr B positive	0.72	0.54 to 0.96	.026	0.79	0.58 to 1.07	.129
Foxp3 positive	1.50	1.12 to 2.00	.006	1.47	1.08 to 2.00	.015
Combination of Foxp3 positive and Gr B positive						
Overall	NA		< .0001	NA		.003
I *v* II	1.33	0.83 to 2.11	.237	1.48	0.90 to 2.42	.124
I *v* III	1.40	0.88 to 2.23	.162	1.64	1.00 to 2.68	.050
I *v* IV	3.25	1.97 to 5.36	< .0001	2.80	1.60 to 4.88	< .0001
I *v* II + III + IV	1.59	1.04 to 2.42	.031	1.70	1.09 to 2.66	.020

NOTE. Univariate analysis, Cox proportional hazards regression model.
Abbreviations: OS, overall survival; DFS, disease-free survival; AFP, alpha-fetoprotein; TILs, tumor-infiltrating lymphocytes; Gr B, granzyme B; NA, not applicable.

proportional hazards analyses were performed. Vascular invasion, an established prognostic predictor for HCC,[18,19] remained associated with both DFS and OS. However, TNM stage was not an independent prognostic factor, which may result from patients' heterogeneous distribution in TNM stage (I, n = 185; II, n = 52; III, n = 65), underlying cirrhosis,[1] and different treatment approaches (Tables 2 and 3; Appendix Table A3, online only) The presence of low intratumoral Tregs in combination with high intratumoral activated CTLs, with a balance toward CTLs, was an independent prognostic factor for both improved DFS ($P = .001$) and OS ($P < .0001$) (Figs 2E and 2F; Table 3). In another multivariate analysis, either intratumoral Tregs alone ($P = .001$) or intratumoral activated CTLs alone ($P = .001$) was an independent prognostic factor for OS, and patients with low intratumoral Tregs ($P = .051$) had a propensity of reduced recurrence (Appendix Table A3).

Correlation of Immunohistochemical Variables With Clinicopathologic Features

Activated CTLs did not correlate with any clinicopathologic features. Tregs were found to be associated with both tumor encapsulation and vascular invasion. HCC without complete encapsulation and/or with vascular invasion were prone to have more infiltrating Tregs (Table 4). $CD4^+$ cells correlated positively with high AFP level ($P = .011$) and poor tumor differentiation ($P = .008$). Increasing size of the primary tumor inversely correlated with the presence of $CD8^+$ cells ($P = .038$; Appendix Table A4, online only).

Postoperative Treatment and Prognosis

The percentage of patients who received prophylactic treatment was higher in the high Tregs and low activated CTLs group than in others (IV, 70%; III, 64%; II, 52%; I, 50%; $P = .071$), but their dismal outcome remained the worst. Appropriate adjuvant therapies were administrated after relapse. No significant differences in treatment approaches, prophylactic or postrecurrence, were seen among different subgroups. (Table 4 and Appendix Table A4).

DISCUSSION

Tregs have a crucial role in impeding immune surveillance against cancer and hampering the development of effective antitumor immunity.[12] It has been reported that the frequencies of Tregs are increased

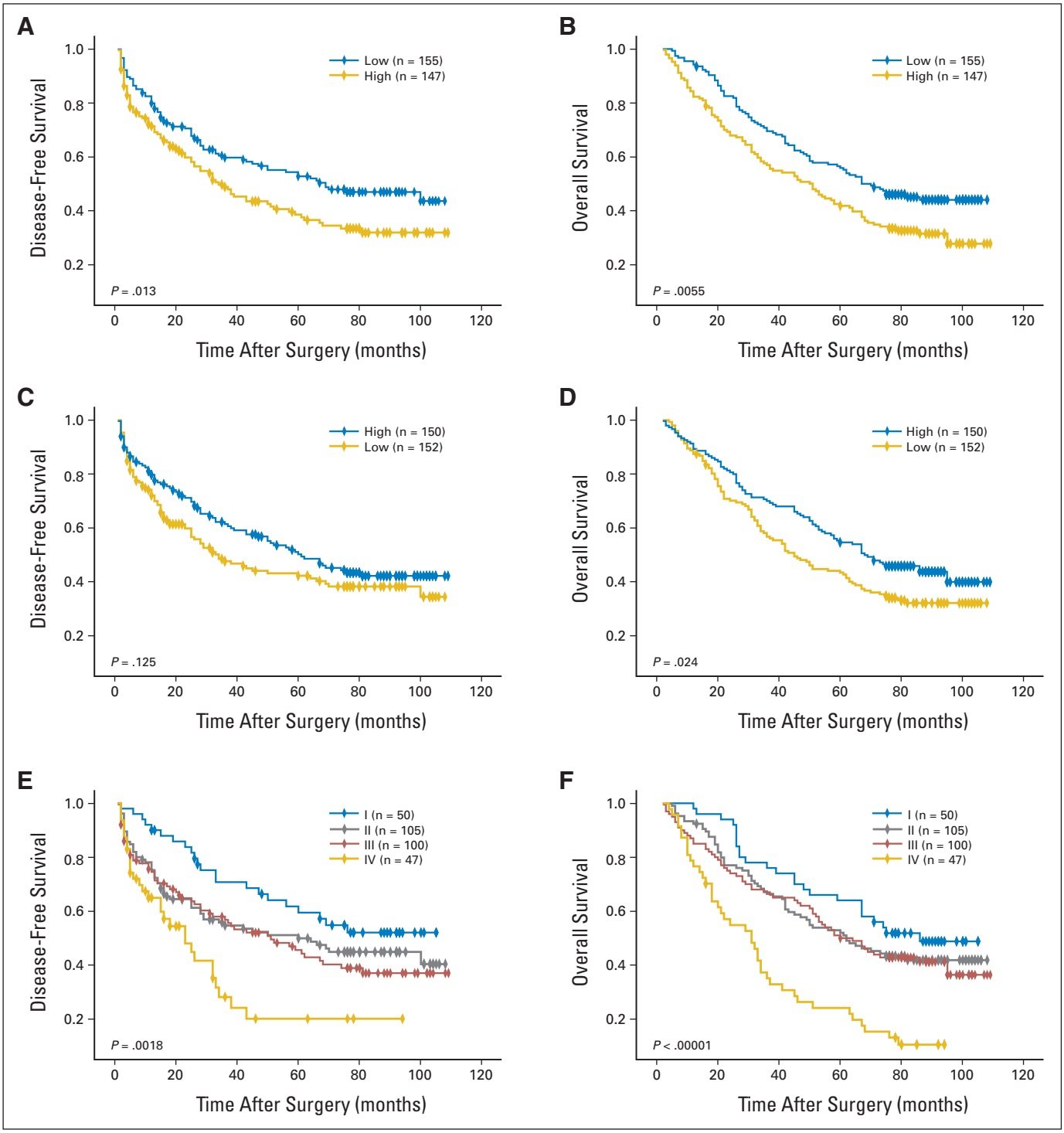

Fig 2. Kaplan-Meier analysis of disease-free survival and overall survival for (A and B) tumor-infiltrating regulatory T cells (Tregs), (C and D) activated CD8+ cytotoxic cells (CTLs), and (E and F) their balance. Low Tregs with concomitant high activated CTLs were associated with both prolonged survival and reduced recurrence.

in both peripheral blood and the tumor microenvironment in HCC, and Tregs from HCC can suppress proliferation and perforin expression of autologous T cells in vitro.[11,27,28] After removal of the tumors, elevated levels of circulating Tregs subsided. Similar to other cancer types, TILs in HCC are also functionally defective and incompletely activated, in which Tregs are suggested to have an important role.[11] However, these studies include no prognostic information on Tregs, and additional study failed to provide any association between Tregs infiltration and recurrence in HCC patients who underwent transplantation.[29] In addition, Yang et al[28] recently reported that an inverse correlation exists between Tregs and $CD8^+$ CTLs in the liver of HCC patients. In contrast, our data indicated a positive correlation between

Table 3. Multivariate Analyses of Factors Associated With Survival and Recurrence

Survival*	Median Survival		Hazard Ratio	95% CI	*P*
OS					
Tumor size, cm (≤ 5 *v* > 5)	73	38	1.28	0.93 to 1.75	.128
Tumor encapsulation (complete *v* none)	69	36	1.33	0.98 to 1.81	.065
Tumor number (single *v* multiple)	67	43	1.41	0.81 to 2.46	.219
Vascular invasion (no *v* yes)	67	21	1.92	1.09 to 3.38	.023
TNM stage (I *v* II + III)	75	37	0.97	0.52 to 1.81	.935
Combination of Foxp3 positive and Gr B positive					
Overall			NA		< .0001
I *v* II	86	62	1.28	0.80 to 2.05	.301
I *v* III	86	60	1.27	0.80 to 2.04	.309
I *v* IV	86	31	2.65	1.59 to 4.43	< .0001
DFS					
Vascular invasion (no *v* yes)	60	14	1.86	1.26 to 2.74	.002
TNM stage (I *v* II + III)	66	32	1.20	0.81 to 1.78	.355
Combination of Foxp3 positive and Gr B positive					
Overall			NA		.008
I *v* II	105	60	1.47	0.89 to 2.41	.130
I *v* III	105	51	1.58	0.96 to 2.59	.070
I *v* IV	105	23	2.61	1.49 to 4.58	.001

NOTE. Multivariate analysis, Cox proportional hazards regression model.
Abbreviations: OS, overall survival; DFS, disease-free survival; Gr B, granzyme B; NA, not applicable.
*Variables were adopted for their prognostic significance by univariate analysis.

Tregs infiltration and $CD8^+$ CTLs infiltration. Several aspects may contribute to this discrepancy. The first could be different sample sizes (25 *v* 302). Second, we used Foxp3-positive cells, a more specific marker than $CD4^+CD25^+$ cells, to assess tumor-infiltrating Tregs. Third, peritumoral regions contained part of the tumor in the study by Yang et al,[28] whereas the tumor center and noncancerous margin were investigated separately in our study. We found that lymphocytes in the noncancerous margin showed no influence on prognosis, which is different from their significant counterparts in tumor center. These differences attest to the value of TILs precise location. More importantly, even though the presence of intratumoral Tregs alone is an independent predictor for survival in HCC, our study demonstrates a relation of the balance of intratumoral Tregs and activated CTLs to both survival and recurrence. In patients with undesirable outcome, the balance is tipped in favor of Tregs (high Tregs and low activated CTLs), whereas in patients with relatively desirable outcome, the balance is tipped toward effector T cells (low Tregs and high activated CTLs). To our knowledge, this is the first report demonstrating prognostic values of tumor-infiltrating regulatory and cytotoxic T cells in human HCC.

These results are also in line with a series of recent reports. It is documented in mice sarcoma models that the ratio of regulatory to effector T cells, and not simply the presence or absence of Tregs, critically determined the in vivo growth behavior of the tumor.[30] Manipulating an increase in the intratumoral ratio of effector to regulatory T cells in poorly immunogenic mice melanoma can overcome Treg-mediated suppression and tip the balance toward tumor rejection.[31] Therefore, a combination of depletion or attenuation of Tregs and concomitant stimulation of tumor-specific effector T cells, systemically or locally in tumors, may be a feasible immunotherapy for HCC; such as combination has been proved to be effective in some other cancer types.[32,33] Notably, even though a larger percentage of patients received prophylactic treatment in the high Tregs and low activated CTLs group than in other groups (IV, 70%; III, 64%; II, 52%; I, 50%; $P = .071$), their dismal outcome remained unchanged. These may indicate that prognosis is governed mainly by local immune responses.[4] Thus, the development of strategies to tip the balance toward CTLs (encouraging the production of CTLs and depleting Tregs simultaneously) are urgently recommended.

Although tumor-infiltrating $CD8^+$ T lymphocytes are believed to be front fighters against cancers, they have no prognostic role in many cancer types,[14,23] as proved in this study. Two major points are suggested to be responsible for the compromised tumor-specific killing by $CD8^+$ CTLs: the dysfunction of cytolytic machineries and an increase in inhibitory molecules, in which both tumor and host play a part.[9,10] However, accumulating evidence has indicated that the activation state of CTLs, instead of just the existence of CTLs, were of great prognostic significance.[14,25,26] Our data showed that activated CTLs, unlike the total number of $CD8^+$ CTLs, were positively correlated with survival, authenticating the importance of the functional state of TILs. Nevertheless, in patients with high levels of activated CTLs infiltration, prognosis was not indiscriminately favorable but was dichotomized by Tregs infiltration, indicating the significance of the modulating effector function in Tregs of primed $CD8^+$ T cells and the key role of their balance. Although the widely used method of evaluating the distribution of TILs immunohistochemically was used, caution should be warranted in interpreting these results; the presence of specific T-cell subsets could also be influenced by other components in the tumor microenvironment. A prospective analysis on intratumoral expression profiles of groups of the immune-related genes using quantitative real-time reverse transcriptase polymerase chain reaction is being conducted to determine the local immune response in HCC more precisely.

We also found that high-density Tregs correlated positively with vascular invasion and correlated negatively with tumor encapsulation. Tumor encapsulation and vascular invasion are two

Table 4. Correlation Between Intratumoral Granzyme B–Positive and Foxp3-Positive TILs and Clinicopathologic Characteristics

Characteristic	Granzyme B Positive			Foxp3 Positive		
	Low	High	*P*	Low	High	*P*
No. of patients	152	150		155	147	
No. with postrecurrence treatment	80	73		70	83	
Age, years						
≤ 51	83	70	.168	77	76	.725
> 51	69	80		78	71	
Sex						
Male	125	135	.051	133	127	.883
Female	27	15		22	20	
Hepatitis history						
Yes	126	130	.362	128	128	.277
No	26	20		27	19	
Preoperative AFP, ng/mL						
≤ 20	70	62	.408	71	61	.450
> 20	82	88		84	86	
Liver cirrhosis						
Yes	123	131	.128	126	128	.169
No	29	19		29	19	
ALT (U/L)						
≤ 40	106	96	.290	111	91	.073
> 40	46	54		44	56	
Child-Pugh score*						
A	150	148	.999	152	146	.623
B	2	2		3	1	
Tumor size (cm)						
≤ 5	78	74	.730	86	66	.066
> 5	74	76		69	81	
Tumor encapsulation						
None	64	59	.624	101	78	.032
Complete	88	91		54	69	
Tumor number						
Single	118	114	.737	124	108	.179
Multiple	34	36		31	39	
Vascular invasion						
Yes	30	25	.489	21	34	.031
No	122	125		134	113	
TNM stage						
I	93	92	.979	103	82	.057
II/III	59	58		52	65	
Tumor differentiation						
I-II	113	102	.224	117	98	.091
III-IV	39	48		38	49	
Prophylactic TACE						
Yes	88	89	.800	67	58	.506
No	64	61		88	89	
Postrecurrence treatment†						
Re-resection	7	8	.234	3	12	.166
TACE	51	38		44	45	
Immunotherapy	4	7		4	7	
Others*	16	13		16	13	
No	2	7		3	6	

Abbreviations: TILs, tumor-infiltrating lymphocytes; AFP, alpha-fetoprotein; TACE, transcatheter arterial chemoembolization.
*Patients received radiofrequency ablation or percutaneous ethanol injection. Two of six in ethanol injection group, 11 of 23 in ablation group, six of 11 in immunotherapy group, and nine of 15 in re-resection group also received TACE treatment and are not included in the TACE group in this analysis.
†Fisher's exact tests; χ^2 tests for all the other analysis.

relatively putative clinicopathologic markers of HCC invasiveness,[29,34] which were both found to be prognostic in this study. These results sustain the hypothesis that Tregs could modify HCC cells in ways that potentiate their invasiveness.

In conclusion, our results demonstrate that intratumoral Tregs are associated with HCC invasiveness and that the concurrence of intratumoral low Tregs with high activated CTLs, with a balance toward CTLs, is associated with improved survival and reduced recurrence in HCC after resection. These results may provide a novel independent predictor for prognosis, and suggest that the combination of depletion of Tregs and concomitant stimulation of effector T cells may be an effective strategy for HCC to reduce recurrence and prolong survival.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Qiang Gao, Shuang-Jian Qiu, Jia Fan, Jian Zhou, Xiao-Ying Wang, Yong-Sheng Xiao

Financial support: Shuang-Jian Qiu, Jia Fan, Zhao-You Tang
Administrative support: Shuang-Jian Qiu, Jia Fan, Jian Zhou, Zhao-You Tang
Provision of study materials or patients: Shuang-Jian Qiu, Jia Fan, Jian Zhou, Xiao-Ying Wang, Yong-Sheng Xiao, Yang Xu
Collection and assembly of data: Qiang Gao, Xiao-Ying Wang, Yong-Sheng Xiao, Yang Xu, Yi-Wei Li
Data analysis and interpretation: Qiang Gao, Shuang-Jian Qiu, Jia Fan, Jian Zhou, Xiao-Ying Wang, Yong-Sheng Xiao, Yang Xu, Yi-Wei Li
Manuscript writing: Qiang Gao, Shuang-Jian Qiu, Jia Fan
Final approval of manuscript: Qiang Gao, Shuang-Jian Qiu, Jia Fan

REFERENCES

1. Llovet JM: Updated treatment approach to hepatocellular carcinoma. J Gastroenterol 40:225-235, 2005
2. Tang ZY, Ye SL, Liu YK, et al: A decade's studies on metastasis of hepatocellular carcinoma. J Cancer Res Clin Oncol 130:187-196, 2004
3. Budhu A, Forgues M, Ye QH, et al: Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell 10:99-111, 2006
4. Galon J, Costes A, Sanchez-Cabo F, et al: Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313:1960-1964, 2006
5. Sato E, Olson SH, Ahn J, et al: Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 102:18538-18543, 2005
6. Wada Y, Nakashima O, Kutami R, et al: Clinicopathological study on hepatocellular carcinoma with lymphocytic infiltration. Hepatology 27:407-414, 1998
7. Harlin H, Kuna TV, Peterson AC, et al: Tumor progression despite massive influx of activated CD8(+) T cells in a patient with malignant melanoma ascites. Cancer Immunol Immunother 55:1185-1197, 2006
8. Parmiani G: Tumor-infiltrating T cells: Friend or foe of neoplastic cells? N Engl J Med 353:2640-2641, 2005
9. Chiou SH, Sheu BC, Chang WC, et al: Current concepts of tumor-infiltrating lymphocytes in human malignancies. J Reprod Immunol 67:35-50, 2005
10. Yu P, Fu YX: Tumor-infiltrating T lymphocytes: Friends or foes? Lab Invest 86:231-245, 2006
11. Unitt E, Rushbrook SM, Marshall A, et al: Compromised lymphocytes infiltrate hepatocellular carcinoma: The role of T-regulatory cells. Hepatology 41:722-730, 2005
12. Zou W: Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 6:295-307, 2006
13. Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4:330-336, 2003
14. Grabenbauer GG, Lahmer G, Distel L, et al: Tumor-infiltrating cytotoxic T cells but not regulatory T cells predict outcome in anal squamous cell carcinoma. Clin Cancer Res 12:3355-3360, 2006 (11 pt 1)
15. Curiel TJ, Coukos G, Zou L, et al: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10:942-949, 2004
16. Badoual C, Hans S, Rodriguez J, et al: Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clin Cancer Res 12:465-472, 2006
17. Alvaro T, Lejeune M, Salvado MT, et al: Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res 11:1467-1473, 2005
18. Shah SA, Greig PD, Gallinger S, et al: Factors associated with early recurrence after resection for hepatocellular carcinoma and outcomes. J Am Coll Surg 202:275-283, 2006
19. Poon RT, Ng IO, Lau C, et al: Tumor microvessel density as a predictor of recurrence after resection of hepatocellular carcinoma: A prospective study. J Clin Oncol 20:1775-1785, 2002
20. Lee AM, Clear AJ, Calaminici M, et al: Number of CD4+ cells and location of Forkhead Box Protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. J Clin Oncol 24:5052-5059, 2006
21. Cai XY, Gao Q, Qiu SJ, et al: Dendritic cell infiltration and prognosis of human hepatocellular carcinoma. J Cancer Res Clin Oncol 132:293-301, 2006
22. Bleackley RC: A molecular view of cytotoxic T lymphocyte induced killing. Biochem Cell Biol 83:747-751, 2005
23. Nakano O, Sato M, Naito Y, et al: Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: Clinicopathologic demonstration of antitumor immunity. Cancer Res 61:5132-5136, 2001
24. Ahmed AF, Ohtani H, Nio M, et al: CD8+ T cells infiltrating into bile ducts in biliary atresia do not appear to function as cytotoxic T cells: A clinicopathological analysis. J Pathol 193:383-389, 2001
25. van Beek J, zur Hausen A, Snel SN, et al: Morphological evidence of an activated cytotoxic T-cell infiltrate in EBV-positive gastric carcinoma preventing lymph node metastases. Am J Surg Pathol 30:59-65, 2006
26. Schumacher K, Haensch W, Roefzaad C, et al: Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res 61:3932-3936, 2001
27. Ormandy LA, Hillemann T, Wedemeyer H, et al: Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res 65:2457-2464, 2005
28. Yang XH, Yamagiwa S, Ichida T, et al: Increase of CD4+ CD25+ regulatory T-cells in the liver of patients with hepatocellular carcinoma. J Hepatol 45:254-262, 2006
29. Unitt E, Marshall A, Gelson W, et al: Tumour lymphocytic infiltrate and recurrence of hepatocellular carcinoma following liver transplantation. J Hepatol 45:246-253, 2006
30. Bui JD, Uppaluri R, Hsieh CS, et al: Comparative analysis of regulatory and effector T cells in progressively growing versus rejecting tumors of similar origins. Cancer Res 66:7301-7309, 2006
31. Quezada SA, Peggs KS, Curran MA, et al: CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. J Clin Invest 116:1935-1945, 2006
32. Yu P, Lee Y, Liu W, et al: Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. J Exp Med 201:779-791, 2005
33. Dannull J, Su Z, Rizzieri D, et al: Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 115:3623-3633, 2005
34. Lockwood DS, Yeadon TM, Clouston AD, et al: Tumor progression in hepatocellular carcinoma: Relationship with tumor stroma and parenchymal disease. J Gastroenterol Hepatol 18:666-672, 2003

Acknowledgment

We thank Yuan Ji, MD, PhD, and Hai-Ying Zeng for their expertise in immunohistochemistry, and Shanghai Biochip Co Ltd for help in tissue microarray construction.

Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).