Prediction of Recurrence and Survival in Hepatocellular Carcinoma Based on Two Cox Models Mainly Determined by FoxP3⁺ Regulatory T Cells

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Abstract
Hepatocellular carcinoma (HCC) is an aggressive disease with poor prognosis and limited methods to predict patient survival. Immune cells infiltrating tumors is known to impact clinical outcome. Here, we investigated the prognostic significance of immune infiltration within the tumor microenvironment in 245 specimens from two independent cohorts by immunohistochemical analyses. A Cox regression model was constructed using a training cohort and validated in an independent cohort. The diagnostic accuracy was evaluated by receiver operating characteristic curve. The activation, function, and chemotaxis of intratumoral regulatory T cells (Treg) were analyzed using flow cytometry, quantitative PCR, and chemotaxis assay. We identified that the proportion of FoxP3⁺ cells within tumors is negatively associated with patient prognosis, whereas the proportion of interleukin (IL)-17⁺ cell and the number of trypsin⁺ cells are positive predictor. The two Cox models, composed of independent predictors in multivariate analysis, provided a high diagnostic accuracy of prognosis for patients with HCC. The proportion of FoxP3⁺ cells showed the most significant predictive power, with the highest Cox score in the two models. Furthermore, we found Tregs from tumor with high FoxP3⁺ proportion were more active and powerful than the counterparts from tumor with low FoxP3⁺ proportion. In conclusion, two Cox models are established that have considerable clinical value in predicting tumor recurrence and survival of patients with HCC, respectively. In the both models, the proportion of Tregs among CD4⁺ T cells plays a central role. Cancer Prev Res; 6(6); 594–602. ©2013 AACR.

Introduction
Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of death from cancer worldwide (1). Despite improved diagnostic and treatment strategies, the overall survival (OS) of patients with HCC remains poor due to a high recurrence rate (2, 3). In the past decades, many biomarkers, mainly from tumor cells, have been extensively studied (4). Genetic and molecular criteria have been proposed to identify patients at high risk for recurrence (5), but none of these have been sufficiently informative for inclusion in clinical practice. Therefore, the identification of patients with a high-risk of disease recurrence remains a major clinical issue.

It is now recognized that cancer progression is regulated by both cancer cell-intrinsic and microenvironmental factors (6). Among the latter, the nature and localization of tumor-infiltrating lymphocytes (TIL) play a central role. In the past, TILs were considered as one of the manifestations of host immune reaction against cancers. Patients with a prominent lymphocyte infiltration have improved prognosis (7, 8). Nowadays, it has been clear that TILs are heterogeneous and contain various immune cell subsets, including innate cells (e.g., mast cells, macrophage, etc.) and adaptive immune cells [e.g., regulatory T cells (Treg), T-helper 17 (T₁₇) cells, etc.]. Tumor infiltration by Tregs is often associated with a poor prognosis (9–12), whereas the presence of CTLs or T₁₇ cells correlates with a reduced risk of relapse in several cancers (13–15).

However, those studies were limited by one or more of the following factors: failure to value or comprehensively analyze the predictive power of immune infiltration on prognosis, no validation in an independent cohort, and lack of functional orientation of immune cells.

We herein present a study of 245 patients from 2 independent cohorts to investigate the relationship between the...
infiltration extent of 8 immune cell markers (CD3, CD4, CD8, CD56, CD68, FoxP3, IL-17, and trypase) and the clinical outcome of patients with HCC. Our data show that the proportion of FoxP3^+ cells among CD4^+ T cells is of the most importance in predicting recurrence and survival for patients with HCC after curative resection, especially when it is combined with tumor–node–metastasis (TNM) stage and other immune parameters.

Materials and Methods

Patients

Two hundred and forty-five HCC samples were obtained from patients who underwent curative resection from 2004 to 2011 in the First Affiliated Hospital, Zhejiang University School of Medicine (ZUIUC; Hangzhou, China; n = 132), the Zhejiang Cancer Hospital (ZCH; Hangzhou, China; n = 82), and the Second Affiliated Hospital, Wenzhou Medical College (WMC; Wenzhou, China; n = 31). Data from ZUIUC patients were used as a training cohort to derive the survival prediction model, whereas ZCH plus WMC patients were used as an independent validation cohort (Fig. 1). A total of 30 fresh HCC samples (n = 15 for each group: tumors with high or low proportion of FoxP3^+ cells) were randomly obtained from the training cohort for real-time PCR (qRT-PCR) and flow cytometry. The study protocol was approved by Ethics Committee of ZUIUC. Informed written consent was obtained from patients according to the Declaration of Helsinki.

Clinical and demographic characteristics of the training and validation cohorts are summarized in Supplementary Table S1.

Immunohistochemical staining and evaluation

Immunohistochemistry was conducted and evaluated as previously described (9, 16). Sections were incubated with monoclonal antibodies (mAb) against CD3, CD4, CD8, CD56, CD68 (Novocastra), FoxP3, trypase (Abcam), and interleukin (IL)-17, CXCL16 (R&D System). Ten different high-power fields (×400), representing the densest lymphocytic infiltrates, were selected for each sample, and counted by 2 investigators without knowledge of the clinicopathologic data (9). Variations in counts exceeding 5% were recounted and a consensus decision was made. The proportion of FoxP3^+ cells among CD4^+ TILs and that of CD8^+ or IL-17^+ cells among CD3^+ cells were calculated using the mean number of total fields and the averages were compared.

Isolation of TILs

TILs were isolated as previously described (17). Briefly, the tissue was cut into small pieces and incubated in an enzyme mixture containing 0.05% collagenase IV (Invitrogen) and 0.001% DNase I (Sigma-Aldrich) for 1 hour. Dissociated tissues were then ground through a 70-μm strainer, and mononuclear cells were obtained by density gradient separation using Ficoll-Hypaque (Sigma-Aldrich).

Flow cytometry

TILs were stained with fluorochrome-conjugated mAbs against human CD3, CD4, CD25, CD45RO, CD69, HLA-DR, CCR4, CCR6, CCR7, CD11a, CD62L, CD103, FoxP3, CTLA-4, ICOS, granzyme B (BD Pharmingen), CXCR6, and CXCR3 (R&D System). Then, TILs were stained with fluorochrome-conjugated mAbs (anti-human CD3, CD4, CD25, CD45RO, CD69, HLA-DR, CCR4, CCR6, CCR7, CD11a, CD62L, CD103, FoxP3, CTLA-4, ICOS, granzyme B (BD Pharmingen), CXCR6, and CXCR3 (R&D System). For intracellular staining, the cells were permeabilized and fixed using Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer’s instructions. After staining, 3- or 4-color flow cytometry was conducted using LSRII flow cytometer (Becton Dickinson), and data were analyzed using FlowJo software (Tree Star, Inc.).

Real-time PCR

RNA was extracted using RNeasy Mini Kit (Qiagen) and synthesized for cDNA using QuantiTech Reverse Transcription kit (Qiagen). qRT-PCR was conducted in SYBR Green PCR Master Mix (Applied Biosystems) using the ABI Prism 7500 Real-time PCR System (Applied Biosystems). Samples were run in triplicate and their relative expression was calculated in the following formula using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous controls: \(2^{-\Delta\Delta C_t}\). The primers were summarized in Supplementary Table S2.

Chemotaxis assay

CD4^+CD25^+ (Treg) and CD4^+CD25^- (Tconv) T cells were separated from TILs using CD4^+CD25^+ Treg isolation kit (purity, >95%; Miltenyi Biotech). Then, chemotaxis assays were conducted as previously described (18). Briefly, 5 × 10^5 CD4^+CD25^+ or CD4^+CD25^- T cells in a volume of 200 μL were added to the upper wells (insert pore size, 5 μm; Millipore). Human chemokine (CCL1, CCL2, CCL3, CCL4, CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, CCL28, CXCL9, CXCL12, CXCL13, and CXCL16, 100 ng/mL of each; R&D System) alone or in combination were added to the lower chamber in a volume of 900 μL. After 4 hours at
37°C, cells migrating to the lower chamber were enumerated using a hemocytometer. Assays were conducted in triplicate.

Statistical analysis

The t test and the Mann–Whitney test were used to identify markers with a significantly different expression among patient groups. Kaplan–Meier curves were used to visualize differences between disease-free survival (DFS) and OS. The significance among patient groups was analyzed using the log-rank test. We used a multivariate Cox proportional hazards models to identify independent prognostic factors. Spearman coefficients tests were carried out to assess the correlation of lymphocytic variables with clinicopathologic characteristics. The predictive performance of each marker alone or in combination was assessed by receiver operating characteristic (ROC) curve analysis. All tests were two-sided, and a P < 0.05 was considered statistically significant. All statistical analyses were conducted with SPSS 15.0 software (SPSS).

The “minimum P value” approach, calculated by X-tile software (Yale University, New Haven, CT; ref. 19), was used to assess the cutoff for the best separation of immune markers referring to DFS outcome of patients (20). Parameters were transformed into numeric codes as follows: age, years: ≤50 = 0, >50 = 1; sex: male = 0, female = 1; α-fetoprotein (AFP; ref. 21, 22), ng/ml: ≤400 = 0, >400 = 1; hepatitis B surface antigen (HbsAg): negative = 0, positive = 1; alanine aminotransferase (ALT): UL/NL = 0, ALT > 40 = 1; Child–Pugh score: A = 0, B and C = 1; tumor size (9, 21, 23, 24), cm: ≤3 = 0, >3 = 1; tumor number: 1 = 0, >1 = 1; tumor differentiation: I and II = 0, III = 1; TNM stage: I and II = 0, III = 1; liver cirrhosis, tumor encapsulation, vascular invasion: no = 0, yes = 1; and immune markers: low = 1, high = 2.

Results

Identification and validation of immune markers predicting clinical outcomes of patients with HCC

We first evaluated the relationship between clinical outcome and the expression of 8 immune markers. The host reaction was investigated by determining immune marker number and/or proportion using immunohistochemical staining. Representative images (Fig. 2A) from one patient and statistics of immunohistochemical variables (Supplementary Table S3) are shown.

In univariate analysis, we found that the proportion of FoxP3+ cells among CD4+ T cells and the number of tryptase+ cells was significantly associated with both DFS and OS (Table 1). Patients with tumors containing a low (vs. high) proportion of FoxP3+ cells had better patient outcome (5-year DFS, 70% vs. 34%; 5-year OS, 83% vs. 41%; Fig. 2B; Table 2). Contrary to FoxP3, the number of tryptase+ cells was positively associated with clinical outcome (Table 1). In addition, the proportion of IL-17+ cells among CD3+ T cells was also positively related to prognosis, though the difference for OS was not significant (Table 1). Among the clinicopathologic parameters of this cohort, tumor size, TNM stage, and vascular invasion were significantly associated with survival (Table 1). An independent cohort from the other 2 hospitals (n = 113) confirmed the data obtained in the training cohort (Fig. 2C and Supplementary Table S4). The correlation of the 3 significant immunohistochemical parameters with clinicopathologic features was analyzed using Spearman coefficients tests. Neither the proportion of FoxP3+ cells nor that of IL-17+ cells correlated with any clinicopathologic features. The number of tryptase+ cells was found to be negatively associated with the stage of tumor and vascular invasion but positively associated with the number of tumor (All P < 0.05; Supplementary Table S5). However, the correlation was rather weak.

Establishment and validation of the predictive models

We then conducted Cox multivariate regression analysis by adding significant clinicopathologic and immune parameters revealed in univariate analysis into a model. The proportion of FoxP3+ cells and TNM stage were found to be significantly and independently associated with DFS and OS (FoxP3+ cell proportion: HR, 2.50 and 4.04, respectively; all P < 0.01; Table 2). In addition, the proportion of IL-17+ cells and the number of tryptase+ cells were positive independent predictor for DFS and OS, respectively. The independent cohort confirmed the results obtained from the first series (all P < 0.01; Table 2).

The relative importance of each parameter was assessed using its absolute Cox score (Table 2). Our data showed that the proportion of FoxP3+ cells was the most important parameter for both DFS and OS. Two Cox models were built combining the independent predictors: the predicted probability of being recurrent, Cox(R) = 1.334 × FoxP3+/CD4− – 0.997 × IL-17+ /CD3− + 0.917 × TNM stage; that of being survival, Cox(S) = 1.395 × FoxP3+/CD4− – 0.874 × Tryptase+ + 0.875 × TNM stage. The predictive performance of the 2 Cox models was evaluated using ROC analysis. The area under curve (AUC) of Cox(R) and Cox(S) was 0.825 [95% confidence interval (CI), 0.749–0.885; sensitivity = 67.3%; specificity = 86.3%] and 0.837 (95% CI, 0.762–0.895; sensitivity = 74.4%; specificity = 82.0%), respectively, significantly larger than that of immune or clinicopathologic parameter alone (P < 0.05; Table 3; Supplementary Fig. S1). This suggests better predictive performance of Cox(R) and Cox(S) than parameter alone. The diagnostic accuracy of the Cox models was then evaluated in the validation data. Similarly, the predicted AUC of the Cox(R) and Cox(S) was significantly larger than those of any parameter alone (P < 0.05; Table 3; Supplementary Fig. S1).

Phenotypes of tumor-infiltrating Tregs

According to our data above, we hypothesized that Treg (FoxP3+) cells are the major players in the tumor microenvironment. However, little information is available about the immune environment favoring the emergence and function of Tregs in liver cancer.

Prevalence of 16 biomarkers on Tregs were assessed using flow cytometry in tumors with a high proportion of FoxP3+ cells.
cells and compared with the levels in tumors with a low proportion of FoxP3\(^+\) cells (\(n = 15\) for each group). These biomarkers correlate with activation and memory (CD25, CD45RO, HLA-DR, CD127, and CD69), homing and origin (CCR4, CCR6, CCR7, CXCR6, CD11a, CD62L, and CD103), suppressive and effector function (granzyme B, ICOS, CTLA4, and S1P\(_1\); ref. 25). Two activation and memory biomarkers (CD69 and HLA-DR), and especially, all biomarkers of suppressive and effector function were significantly more expressed in FoxP3\(^{Hi}\) patients (all \(P < 0.01\); Fig. 3A). Hierarchical clustering showed these 5 biomarkers clustered together (Fig. 3A). S1P\(_1\), a receptor for lipid mediator sphingosine 1-phosphate (S1P), serves as a unique receptor system to negatively regulate the function of Tregs (26). The prevalence of S1P\(_1\) was markedly lower in FoxP3\(^{Hi}\) patients than in FoxP3\(^{Lo}\) patients (all \(P < 0.001\); Fig. 3A). These data collectively suggest that Tregs in FoxP3\(^{Hi}\) patients are in a status of relatively active and functionally superior to their counterparts in FoxP3\(^{Lo}\) patients. On the other hand, the expression levels of CD25, CD45RO, CD127, and all markers associated with homing and origin were not significantly different between patient groups (Fig. 3A).

**Treg-related cytokine and chemokine expression in tumor environment**

Furthermore, we qualified the expression of a panel of cytokine and chemokine genes related to Tregs in the same tumor tissue using quantitative RT-PCR (qRT-PCR). The expression levels of IL-10 and IL-35 [formed by Epstein–Barr virus–induced gene 3 (Ebi3) and p35] and IL-2 were significantly higher in FoxP3\(^{Hi}\) patients than in FoxP3\(^{Lo}\) patients (all \(P < 0.01\); Fig. 3B). Among investigated chemokine genes, only the expression of CCL20 and CXCL16 was significantly increased in tumors with a high proportion of FoxP3\(^+\) cells (all \(P < 0.001\); Fig. 3B). Also, these 6 genes clustered together in hierarchical clustering analysis. In contrast, the expression levels of the other cytokines (e.g., IL-6, TGF-\(\beta\), and IL-9) and chemokines (e.g., CCL1, CCL17, and CXCL12) did not
obviously influence FoxP3+ cell function and tumor infiltration, respectively.

Chemokines are critical for attracting immune cells (27). Our previous data have shown that CCL20 mediates the migration of Tregs into tumor microenvironment (18). Here, we further found the expression of CXCL16 correlated with the number of FoxP3+ cells ($P = 0.005$; $r = 0.668$; Fig. 4A and B), indicating that CXCL16, other than CCL20, are likely to attract Tregs into HCC tumor. In vitro chemotaxis assay showed significantly higher chemotactic responses of Tregs to CXCL16 and CCL20 ($P < 0.05$ for CXCL16 or CCL20 vs. other chemokines), and synergic effect between them ($P < 0.05$ for CXCL16 or CCL20 vs. CXCL16 plus CCL20; Fig. 4C).

Discussion

Our study showed that (i) the infiltration of Tregs, T$_\text{H17}$ cells (IL-17$^+$), or mast cells (tryptase$^+$) at tumor site is correlated with the clinical outcome in 2 independent cohorts of patients with HCC, but other immune cells, such as CTLs (CD8$^+$), macrophages (CD68$^+$), and natural killer (NK) cells (CD56$^+$), did not; (ii) the proportion of intratumoral Tregs had the highest predictive accuracy, whereas the combination of immune factors and pathologic staging showed the predominance for predicting tumor recurrence and patient survival; (iii) increased aggregation and enhanced immunosuppressive function of Tregs in tumor microenvironment are responsible for their critical role in prognostic prediction.

Tumor environment contains innate immune cells (e.g., mast cells, macrophages, and NK cells) and adaptive immune cells (T and B lymphocytes) in addition to the cancer cells and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes, and mesenchymal cells; ref. 28). These diverse cells communicate with each other to control and shape tumor growth. T lymphocytes are the most frequently found immune cells within the tumor microenvironment and can exert both tumor-suppressive

Table 1. Univariate analysis of DFS and OS among patients with Unio Internationale Contra Cancrum (UICC)-TNM stage I, II, or III HCC (training cohort) according to clinicopathologic or immune parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DFS HR (95% CI)</th>
<th>DFS P&lt;sup&gt;c&lt;/sup&gt;</th>
<th>OS HR (95% CI)</th>
<th>OS P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (&lt;50/50)</td>
<td>0.8 (0.5–1.4)</td>
<td>0.454</td>
<td>1.1 (0.6–2.1)</td>
<td>0.662</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>1.1 (0.4–2.8)</td>
<td>0.803</td>
<td>2.7 (0.6–11.1)</td>
<td>0.173</td>
</tr>
<tr>
<td>AFP, ng/mL (&lt;400/&lt;400)</td>
<td>2.3 (1.3–4.0)</td>
<td>0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9 (1.0–3.5)</td>
<td>0.031&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HBsAg, (negative/positive)</td>
<td>1.1 (0.5–2.3)</td>
<td>0.798</td>
<td>0.7 (0.3–1.4)</td>
<td>0.701</td>
</tr>
<tr>
<td>ALT level, U/L (&lt;40/&gt;40)</td>
<td>1.7 (1.9–3.1)</td>
<td>0.064</td>
<td>1.8 (0.9–3.4)</td>
<td>0.074</td>
</tr>
<tr>
<td>Liver cirrhosis (yes/no)</td>
<td>0.6 (0.3–1.5)</td>
<td>0.283</td>
<td>0.5 (0.2–1.0)</td>
<td>0.555</td>
</tr>
<tr>
<td>Child staging (A/B–C)</td>
<td>1.1 (0.6–1.9)</td>
<td>0.802</td>
<td>1.1 (0.6–2.2)</td>
<td>0.647</td>
</tr>
<tr>
<td>Tumor size, cm (&lt;5/&gt;5)</td>
<td>2.8 (1.6–4.8)</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 (1.8–5.9)</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tumor number (single/multiple)</td>
<td>0.6 (0.4–1.1)</td>
<td>0.115</td>
<td>0.6 (0.3–1.0)</td>
<td>0.059</td>
</tr>
<tr>
<td>TNM stage (I–II/III)</td>
<td>4.2 (2.4–7.5)</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1 (1.7–5.9)</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tumor encapsulation (yes/no)</td>
<td>1.4 (0.7–2.7)</td>
<td>0.315</td>
<td>0.7 (0.3–1.7)</td>
<td>0.465</td>
</tr>
<tr>
<td>Vascular invasion (no/yes)</td>
<td>3.0 (1.7–5.2)</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0 (1.1–3.6)</td>
<td>0.025&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tumor differentiation (I–II/III)</td>
<td>1.6 (0.9–2.8)</td>
<td>0.092</td>
<td>1.6 (0.9–2.9)</td>
<td>0.146</td>
</tr>
</tbody>
</table>

NOTE: Univariate analysis, Cox proportional regression.

<sup>a</sup>All parameters were divided into 2 groups with the "minimum P value" approach.

<sup>b</sup>Significant.

<sup>c</sup>Log-rank P value.
Table 2. Multivariate analysis of DFS and OS among patients with Union Internationale Contra Cancrum (UICC)-TNM stage I, II, or III HCC according to clinicopathologic or immune parameter

<table>
<thead>
<tr>
<th>Parametersa</th>
<th>Training cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cox score</td>
<td>5-y survival</td>
</tr>
<tr>
<td>DFS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFP, ng/mL (≤400/400)</td>
<td>NA</td>
<td>56%</td>
</tr>
<tr>
<td>Tumor size, cm (≤5/&gt;5)</td>
<td>NA</td>
<td>60%</td>
</tr>
<tr>
<td>Vascular invasion (no/yes)</td>
<td>NA</td>
<td>60%</td>
</tr>
<tr>
<td>TNM stage (I/II/III)</td>
<td>0.917</td>
<td>71%</td>
</tr>
<tr>
<td>Trypase− (low/high)</td>
<td>NA</td>
<td>37%</td>
</tr>
<tr>
<td>IL-17+/CD3+ (low/high)</td>
<td>−0.997</td>
<td>32%</td>
</tr>
<tr>
<td>FoxP3−/CD4+ (low/high)</td>
<td>1.334</td>
<td>70%</td>
</tr>
</tbody>
</table>

bParameters were adopted for their prognostic significance by univariate analysis of training cohort. 
Significant.

and-promoting effects (28). For example, Tregs, which are presumed to act mostly in a protumorigenic fashion through suppression of antitumor immune response, may also exert an antitumorigenic function under certain circumstances by suppressing tumor-promoting inflammation. In HCC, the higher number (9, 13) or proportion (12) of Tregs is indicative of poor prognosis. Here, our data showed that the proportion of Tregs provided an indicator of clinical outcome beyond the one predicted by their number: first, the proportion but not number of Tregs was independently associated with recurrence and survival in less-advanced HCC patients; second, the former had larger AUC than did the latter according to ROC analysis (AUCrecurrence = 0.603 vs. 0.551; AUCsurvival = 0.628 vs. 0.533), though the difference is not significant. Similar to Tregs, Th17 cells also exert both tumor-suppressive and-promoting effects. The beneficial impact of Th17 cells within the tumor microenvironment is well established.

Table 3. ROC analyses of training and validation cohort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Training cohort</th>
<th></th>
<th>Validation cohort</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>95% CI</td>
<td>P value</td>
<td>AUC</td>
</tr>
<tr>
<td>DFS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-17+/CD3+</td>
<td>0.560</td>
<td>0.471–0.646</td>
<td>&lt;0.001b</td>
<td>0.559</td>
</tr>
<tr>
<td>FoxP3−/CD4+</td>
<td>0.603</td>
<td>0.515–0.687</td>
<td>&lt;0.001a</td>
<td>0.607</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.701</td>
<td>0.616–0.778</td>
<td>0.016a</td>
<td>0.632</td>
</tr>
<tr>
<td>Fox(R)</td>
<td>0.825</td>
<td>0.749–0.885</td>
<td>0.788</td>
<td>0.701–0.860</td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypase−</td>
<td>0.666</td>
<td>0.579–0.746</td>
<td>0.003b</td>
<td>0.683</td>
</tr>
<tr>
<td>FoxP3−/CD4+</td>
<td>0.643</td>
<td>0.555–0.725</td>
<td>&lt;0.001b</td>
<td>0.621</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.673</td>
<td>0.586–0.752</td>
<td>&lt;0.001b</td>
<td>0.616</td>
</tr>
<tr>
<td>Fox(S)</td>
<td>0.837</td>
<td>0.762–0.895</td>
<td>0.815</td>
<td>0.731–0.882</td>
</tr>
</tbody>
</table>

aCompared with AUC of Cox(R).
bCompared with AUC of Cox(S).
for HCC, colorectal cancer (CRC), and others. One important mechanism is that T\textsubscript{H17} cells provide important help to boost cytotoxic immunity via production of IL-17A (29, 30). In contrast, there are several previous reports that T\textsubscript{H17} cells in solid tumor are involved in tumor promotion or progression (31, 32). Mast cells are also important contributors to some tumors (33), while they confer a better prognosis in other tumors (34). Taken together, our data showed that Tregs are negative predictor, whereas TH17 cells and mast cells are positive ones.

In CRC, the immune reaction was found to be more powerful in predicting prognosis than the histopathologic criteria (including TNM staging; ref. 35). We are not sure whether this is the same case in HCC, while our data showed the predominance of the combination of immune infiltration with TNM stage in 2 independent cohorts. According to Cox analysis, we found that the combination of immune infiltration with TNM stage had the highest predictive accuracy for recurrence and survival using ROC curve analysis. Therefore, some authors advocated that immunologic criteria should be of interest in clinical practice and added to tumor staging to improve the identification of high-risk patients (36). This approach should be extended to other cancers, particularly those where high numbers of infiltrating T cells have been associated with good prognosis (37).

It is now well established that Tregs exert immunosuppressive function by 2 mechanisms (25): contact-dependent suppression, where CTLA4 and granzyme A are key molecules; cytokine-mediated suppression, where IL-10, IL-35, and TGF-\beta are key molecules. Our data showed that the expression of the majority of these molecules was markedly upregulated in tumors with a high proportion of Tregs. These results suggested that enhanced function of Tregs was also responsible for poorer prognosis of patients with HCC to some extent.

Not only the suppressor potential but also appropriate localization, which are mediated mainly by chemokine and its receptor (38), determines the in vivo suppressive capacity of Tregs (39). Tregs are attracted mostly through CCL22-CCR4 and CCL19-CCR7 (40, 41). In patients with HCC, our previous study showed selective recruitment of peripheral Tregs into tumor tissue via signaling CCL20-CCR6 (18), which is also critical for T\textsubscript{H17} infiltration (41). Here, we further found the pathway CXCL16–CXCR6 might also mediate the migration of peripheral Tregs into tumor microenvironment. To a great extent, this pathway may be responsible for high Tregs and low T\textsubscript{H17} cells in the same tumor microenvironment.

In summary, our study reveals that the proportion of Tregs in tumor microenvironment is the most important immune predictor of tumor recurrence and survival in
Figure 4. A, representative pictures showing the expression of CXCL16 in patients with high (left) or low (right) proportion of FoxP3 in tumors. Bar, 50 μm. Magnification ×200. B, correlation between the number of FoxP3+ cells and CXCL16 expression levels as measured by immunohistochemical staining in corresponding tissues (Spearman r). IOD, integrated optical density. C, Tregs (CD4+CD25+) or effector T cells (CD4+CD25-) migrate in response to recombinant human chemokines alone or in combination (n=4 independent experiment for CCL20 and CXCL16; n=3 for the other chemokines). *, P<0.05 vs. control; **, P<0.05 vs. CCL20 and CXCL16.

patients with HCC. Improved understanding of the function and chemotatic modes of Tregs in tumorigenesis will help in the rational design of new therapeutic approaches for patients with HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Chen, H.-Y. Xie

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References


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