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 2 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 (ROC) curve. The activation, function and chemotaxis of intratumoral regulatory T (Treg) cells 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, while the proportion of IL-17+ cell and the number of trypase+ 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. Further, we found Treg cells 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 Treg cells among CD4+ T cells plays a central role.
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 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 (TILs) 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 (eg, mast cells, macrophage) and adaptive immune cells (eg. regulatory T [Treg] cells, T-helper 17 [Th17] cells). Tumor infiltration by Treg cells is often associated with a poor prognosis (9-12), while the present of cytotoxic T cells or Th17 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 two independent cohorts to investigate the relationship between the infiltration extent of 8 immune cell markers (CD3, CD4, CD8, CD56, CD68, FoxP3, IL-17, and tryptase), and the clinical outcome of HCC patients. Our data show that the proportion of FoxP3⁺ cells among CD4⁺ T cells is of the most importance in predicting recurrence and survival for HCC patients after curative resection, especially when it is combined with TNM stage and other immune parameters.
Materials and Methods

Patients

245 HCC samples were obtained from patients underwent curative resection from 2004 to 2011 in the first affiliated hospital, Zhejiang University School of Medicine, ZUSC (n=132), the Zhejiang Cancer Hospital, ZCH (n=82), and the second affiliated hospital, Wenzhou Medical College, WMC (n=31). Data from ZUSC patients were used as a training cohort to derive the survival prediction model, while ZCH plus WMC patients were used as an independent validation cohort (Figure 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 and flow cytometry. The study protocol was approved by Ethics Committee of Zhejiang University School of Medicine. 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 1.

Immunohistochemical staining and evaluation

Immunohistochemistry was done and evaluated as previously described (9,16). Sections were incubated with monoclonal antibodies against CD3, CD4, CD8, CD56, CD68 (Novocastra, Newcastle, UK), FoxP3, trypase (Abcam, Cambridge, UK) and IL-17, CXCL16 (R&D System, Minneapolis, MN). Ten different high-power fields (×400), representing the densest lymphocytic infiltrates, were selected for each sample, and counted by two investigators without knowledge of the clinicopathologic
data (9). Variations in counts exceeding 5% were re-counted 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 previous described (17). Briefly, the tissue was cut into small pieces and incubated in an enzyme mixture containing 0.05% collagenase IV (Invitrogen, Carlsbad, CA) and 0.001% DNase I (Sigma-Aldrich, St. Louis, MO) for 1h. 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, and CTLA-4, ICOS, granzyme B (BD PharMingen, San Diego, CA), CXCR6, and S1P1 ((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, three- or four-color flow cytometry was performed using LSR II flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).

Real-time PCR

RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) and
synthesized for cDNA using QuantiTech Reverse Transcription kit (Qiagen). Real-time PCR was conducted in SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) 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 GAPDH as endogenous controls: $2^{-\Delta\Delta CT}$. The primers were summarized in supplementary Table 2.

**Chemotaxis assay**

CD4$^+$CD25$^+$ (Treg) and CD4$^+$CD25$^-$ (Tconv) T cells were separated from TILs using CD4$^+$CD25$^+$ regulatory T cell isolation kit (purity, >95%; Miltenyi Biotec, Bergisch Gladbach, Germany). Then, chemotaxis assays were performed as previously described (18). Briefly, $5 \times 10^5$ CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells in a volume of 200μl were added to the upper wells (insert pore size, 5μm; Millipore, Billerica, MA). 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 900μl. After 4h at 37°C, cells migrating to the lower chamber were enumerated using a hemocytometer. Assays were performed 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 overall survival (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’s 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 performed with SPSS 15.0 software (SPSS, Chicago, IL).

The “minimum \( P \) value” approach, calculated by X-tile software (Yale University, New Haven, CT) (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: \( \leq 50=0, >5=1 \); Sex: male=0, female=1; AFP (21,22), ng/mL: \( \leq 400=0, >400=1 \); HBsAg: negative=0, positive=1; ALT, U/L: \( \leq 40=0, >40=1 \); Child-Pugh score: A=0, B and C=1; Tumor size (9,21,23,24), cm: \( \leq 5=0, >5=1 \); 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; 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 (Figure 2A) from one patient and statistics of immunohistochemical variables (supplementary Table 3) are shown.

In univariate analysis, we found the proportion of FoxP3+ cells among CD4+ T cells and the number of trypase+ cells was significantly associated with both DFS and OS (Table 1). Patients with tumors containing a low (versus high) proportion of FoxP3+ cells had better patient outcome (5-year DFS, 70% vs. 34%; 5-year OS, 83% vs. 41%; Figure 2B; Table 2). Contrary to FoxP3, the number of trypase+ 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 two hospitals (n = 113) confirmed the data obtained in the training cohort (Figure 2C, supplementary Table 4). The correlation of the three significant immunohistochemical parameters with clinicopathologic features was analyzed using Spearman’s coefficients tests. Neither the proportion of FoxP3+ cells nor that of IL-17+ cells correlated with any...
clinicopathologic features. The number of trypase+ cells was found to be negatively associated with the stage of tumor and vascular invasion, but positively with the number of tumor (All $P < 0.05$, supplementary Table 5). However, the correlation was rather weak.

**Establishment and validation of the predictive models**

We then performed 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 trypase+ 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, $\text{Cox}(R) = 1.334 \times \text{FoxP3+}/\text{CD4+} - 0.997 \times \text{IL-17+}/\text{CD3+} + 0.917 \times \text{TNM stage}$; that of being survival, $\text{Cox}(S) = 1.395 \times \text{FoxP3+}/\text{CD4+} - 0.874 \times \text{Trypase+} + 0.875 \times \text{TNM stage}$. The predictive performance of the two Cox models was evaluated using ROC analysis. The AUC of Cox(R) and Cox(S) was 0.825 (95% CI, 0.749 to 0.885; sensitivity = 67.3%, specificity = 86.3%) and 0.837 (95% CI, 0.762 to 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 Figure 1). 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 Figure 1).

**Phenotypes of tumor-infiltrating Treg cells**

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

Prevalence of 16 biomarkers on Treg cells were assessed using flow cytometry in tumors with a high proportion of FoxP3+ 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 S1P1) (25). Two activation and memory biomarkers (CD69 and HLA-DR), and especially, all biomarkers of suppressive and effector function were significantly more expressed in FoxP3Hi patients (All $P<0.01$, Figure 3A). Hierarchical clustering showed these five biomarkers clustered together (Figure 3A). S1P1, a receptor for lipid mediator sphingosine 1-phosphate (S1P), serves as a unique receptor system to negatively
regulate the function of Treg cells (26). The prevalence of S1P1 was markedly lower in FoxP3\textsuperscript{Hi} patients than in FoxP3\textsuperscript{Lo} patients ($P<0.001$, Figure 3A). These data collectively suggest that Treg cells in FoxP3\textsuperscript{Hi} patients are in a status of relatively active and functionally superior to their counterparts in FoxP3\textsuperscript{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 (Figure 3A).

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

Further, we qualified the expression of a panel of cytokine and chemokine genes related to Treg cells in the same tumor tissue using quantitative real-time PCR. The expression levels of IL-10 and IL35 (formed by Epstein-Barr virus-induced gene 3 [Ebi3] and p35), and IL-2 were significantly higher in FoxP3\textsuperscript{Hi} patients than in FoxP3\textsuperscript{Lo} patients (All $P<0.01$; Figure 3B). Among investigated chemokine genes, only the expression of CCL20 and CXCL16 was significantly increased in tumors with a high proportion of FoxP3\textsuperscript{+} cells (All $P<0.001$, Figure 3B). Also, these six genes clustered together in hierarchical clustering analysis. In contrast, the expression levels of the other cytokines (eg, IL-6, TGF-\(\beta\), and IL-9) and chemokines (eg, CCL1, CCL17, CXCL12) did not obviously influence FoxP3\textsuperscript{+} cell function and tumor infiltration, respectively.

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

Our study demonstrated that (1) the infiltration of Treg cells, Th17 cells (IL-17+), mast cells (trypase+) at tumor site is correlated with the clinical outcome in two independent cohorts of HCC patients, but other immune cells, such as cytotoxic T cells (CD8+), macrophages (CD68+) and NK cells (CD56+), not; (2) the proportion of intratumoral Treg cells had the highest predictive accuracy, while combination of immune factors and pathological staging showed the predominance for predicting tumor recurrence and patient survival; (3) increased aggregation and enhanced immunosuppressive function of regulatory T cells in tumor microenvironment are responsible for their critical role in prognostic prediction.

Tumor environment contains innate immune cells (e.g., mast cells, macrophages, and natural killer 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) (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 and -promoting effects (28). For example, Treg cells, 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 Treg cells is indicative of poor prognosis. Here, our data showed that the proportion of Treg cells provided an indicator of clinical
outcome beyond that predicted by their number: first, the proportion but not number of Treg cells 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 (AUC_{recurrence} = 0.603 vs. 0.551, AUC_{survival} = 0.628 vs. 0.533), though the difference is not significant. Similar to Treg cells, Th17 cells also exert both tumor-suppressive and –promoting effects. The beneficial impact of Th17 cells within the tumor microenvironment is well established for HCC, CRC and others. One important mechanism is that Th17 cells provide important help to boost cytotoxic immunity via production of IL-17A (29,30). In contrast, there are several previous reports that Th17 cells in solid tumor are involved in tumor promotion or progression (31,32). Mast cells are also important contributors to some tumors (33), while confer a better prognosis in other tumors (34). Taken together, our data showed that Treg cells are negative predictor, whereas Th17 cells and mast cells are positive ones.

In colorectal cancer, the immune reaction was found to be more powerful in predicting prognosis than the histopathologic criteria (including TNM staging) (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 two independent cohorts. According to Cox analysis, we found 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 immunological 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 Treg cells exert immunosuppressive function by two mechanisms (25): contact-dependent suppression, where CTLA4 and granzyme A are key molecules; cytokine-mediated suppression, where IL-10, IL-35, and TGF-β are key molecules. Our data showed that the expression of the majority of these molecules were markedly up-regulated in tumors with a high proportion of Treg cells. These results suggested that enhanced function of Treg cells was also responsible for poorer prognosis of HCC patients 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 Treg cells (39). Treg cells are attracted mostly through CCL22-CCR4 and CCL19-CCR7 (40,41). In HCC patients, our previous study showed selective recruitment of peripheral Treg cells into tumor tissue via signaling CCL20-CCR6 (18), which is also critical for Th17 infiltration (41). Here, we further found the pathway CXCL16-CXCR6 might also mediate the migration of peripheral Treg cells into tumor microenvironment. To a great extent, this pathway may be responsible for high Treg cells and low Th17 cells in the same tumor microenvironment.

In summary, our study reveals that the proportion of Treg cells in tumor microenvironment is the most important immune predictor of tumor recurrence and survival in patients with HCC. Improved understanding of the function and
chemotactic modes of Treg cells in tumorigenesis will help in the rational design of new therapeutic approaches for patients with HCC.
References


Figure Legends

Figure 1. Study design for establishment of Cox models with independent prognostic factors derived from the training cohort (ZUSM, n=132) and validated in an independent cohort of patients from ZCH (n=82) and WMC (n=31). HCC, hepatocellular carcinoma; IHC, immunohistochemical staining; ROC, receiver operating characteristics.

Figure 2. (A) Representative images for total T cells (CD3+), Helper T cells (CD4+), cytotoxic T cells (CD8+), nature killer cells (CD56+), macrophage (CD68+), Treg cells (FoxP3+), Th17 cells (IL-17+), and mast cells (trypase+) infiltration in hepatocellular carcinoma tissue from the same patient. Immunostained cells (brown) and tumor cells (blue). Magnification, ×100; inserted box, ×400. (B, C) Kaplan-Meier curves illustrate the duration of disease-free survival and overall survival according to the proportion of FoxP3+ among CD4+ T cells in the training cohort (B) and in the validation cohort (C).

Figure 3. (A, B) Unsupervised hierarchical clustering of 16 biomarkers (A) and 23 gene expression (B) related to Treg cells from different patient groups (n=15 for each group). Biomarker prevalences on Treg cells were assessed using flow cytometry after isolation of TILs from tumor tissue, while gene expression levels were investigated using real-time PCR. The expressions of biomarker or gene are plotted from the minial level of expression (blue) to the maximal level (red). The marker type is shown using a color bar on the right side of the figure: (A) activation and memory biomarkers are represented in green, homing and origin biomarkers in blue, function
biomarkers in red; (B) cytokine genes are represented in blue, chemokines in red. The Mann-Whitney test was used to compare the expression levels of biomarker or gene between patient groups. †, $P<0.05$.

**Figure 4.** (A) Representative pictures showing the expression of CXCL16 in patients with high (left panel) or low (right panel) proportion of FoxP3 in tumors. Bar=50$\mu$m. Magnification, $\times200$. (B) Correlation between the number of FoxP3$^+$ cells and CXCL16 expression levels as measured by immunohistochemical staining in corresponding tissues (Spearman’s r). (C) Treg cells (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.
Figure 1

Training cohort

Resected HCC samples
The first affiliated hospital, Zhejiang University School of Medicine (n = 132)

IHC

Number or proportion of immune cells

Kaplan-Meier

Significant immune cell markers

Cox regression

Establish Models with independent prognostic parameters

ROC analyses

Performance of parameter

Validation cohort

Resected HCC samples
Zhejiang Cancer hospital (n = 82), The second hospital of Wenzhou Medical College (n = 31)

IHC

Number or proportion of immune cells

Validation on independent cohort of HCC patients

Model validation

Performance of parameter
Figure 3

A

Proteins

- CCR4
- CXCR6
- CCR6
- CD25
- S1P₁
- CD103
- CD11a
- HLA-DR
- CTLA4
- ICOS
- GZMB
- CD69
- CD127
- CD45RO
- CCR7
- CD62L

P value*

- 0.071
- 0.561
- 0.950
- 0.340
- 0.000
- 0.178
- 0.309
- 0.000
- 0.000
- 0.002
- 0.009
- 0.011
- 0.648
- 0.207
- 0.663
- 0.065

FoxP3⁺/CD4⁺

Low

High

B

Genes

- IL9
- IL6
- IL10
- IL2
- ebi3
- P35
- CCL20
- CXCL16
- CXCL13
- CCL27
- CCL21
- CCL28
- CCL19
- CCL9
- CXCL12
- CXCL11
- CCL3
- CCL2
- CCL4
- CCL1
- CCL17
- CCL18
- CCL22

P value*

- 0.272
- 0.373
- 0.000
- 0.002
- 0.001
- 0.001
- 0.001
- 0.000
- 0.000
- 0.000
- 0.000
- 0.007
- 0.443
- 0.443
- 0.468
- 0.547
- 0.547
- 0.663
- 0.576
- 0.004

FoxP3⁺/CD4⁺

Low

High
Table 1. Univariate analysis of DFS and OS among patients with UICC-TNM stage I, II, or III hepatocellular carcinoma (training cohort) according to clinicopathologic or immune parameters.

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<th>Parameter</th>
<th>DFS</th>
<th>OS</th>
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<td>HR</td>
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<td><strong>Clinicopathologic parameters</strong></td>
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<td>ALT level, U/L (&lt;40/&gt;40)</td>
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<td>Liver cirrhosis (Yes/No)</td>
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<td>Tumor size, cm (&lt;5/&gt;5)</td>
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<tr>
<td>(Yes/No)</td>
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<td>Vascular invasion (no/yes)</td>
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<td>Tumor differentiation (I-II/III)</td>
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<td><strong>Immune parameters (low/high)†</strong></td>
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<tr>
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<tr>
<td>No. of CD4+ cells</td>
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<td>No. of CD56+ cells</td>
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<td>0.1-1.9</td>
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<td>No. of CD68+ cells</td>
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<td>0.4-1.3</td>
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<td>No. of FoxP3+ cells</td>
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<td>No. of IL-17+ cells</td>
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Cancer Research.
Proportion of FoxP3+ cells among CD4+ cells
2.4  1.3-4.6  0.007‡  3.9  1.7-8.8  0.001‡

Proportion of CD8+ cells among CD3+ cells
0.8  0.5-1.5  0.825  1.2  0.6-2.2  0.611

Proportion of IL-17+ cells among CD3+ cells
0.5  0.3-0.9  0.015‡  0.6  0.3-1.1  0.082

NOTE: Univariate analysis, Cox proportional regression.

Abbreviations: DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; HBsAg, Hepatitis B surface antigen; AFP, α-fetoprotein; ALT, alanine aminotransferase.

*Log-rank P value.
†All parameters were divided into two groups with the “minimus P value” approach.
‡significant.
Table 2. Multivariate analysis of DFS and OS among patients with UICC-TNM stage I, II, or III hepatocellular carcinoma according to clinicopathologic or immune parameter

<table>
<thead>
<tr>
<th>Parameters*</th>
<th>Training cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cox score</td>
<td>5-year Survival</td>
</tr>
<tr>
<td>DFS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFP, ng/mL (≤ 400/&gt;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>
<tr>
<td>OS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFP, ng/mL (≤ 400/&gt;400)</td>
<td>NA</td>
<td>65%</td>
</tr>
<tr>
<td>Tumor size, cm (≤ 5/&gt;5)</td>
<td>NA</td>
<td>70%</td>
</tr>
<tr>
<td>Vascular invasion (no/yes)</td>
<td>NA</td>
<td>65%</td>
</tr>
<tr>
<td>TNM stage (I+II/III)</td>
<td>0.875</td>
<td>74%</td>
</tr>
<tr>
<td>Parameter</td>
<td>HR (CI)</td>
<td>DFS (%)</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>Trypase^+ (low/high)</td>
<td>-0.874</td>
<td>48%</td>
</tr>
<tr>
<td>FoxP3^+/CD4^+ (low/high)</td>
<td>1.395</td>
<td>83%</td>
</tr>
</tbody>
</table>

NOTE: Multivariate analysis, Cox proportional regression.

Abbreviations: DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; AFP, α-fetoprotein; NA, not adopted.

*parameters were adopted for their prognostic significance by univariate analysis of training cohort.

†significant
<table>
<thead>
<tr>
<th>Variable</th>
<th>Training cohort</th>
<th>Validation cohort</th>
<th></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;0.001*</td>
<td>0.559</td>
</tr>
<tr>
<td>FoxP3+/CD4⁺</td>
<td>0.603</td>
<td>0.515-0.687</td>
<td>&lt;0.001*</td>
<td>0.607</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.701</td>
<td>0.616-0.778</td>
<td>0.016*</td>
<td>0.632</td>
</tr>
<tr>
<td>Cox(R)</td>
<td>0.825</td>
<td>0.749-0.885</td>
<td></td>
<td>0.788</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>0.003†</td>
<td>0.683</td>
</tr>
<tr>
<td>FoxP3+/CD4⁺</td>
<td>0.643</td>
<td>0.555-0.725</td>
<td>&lt;0.001†</td>
<td>0.621</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.673</td>
<td>0.586-0.752</td>
<td>&lt;0.001†</td>
<td>0.616</td>
</tr>
<tr>
<td>Cox(S)</td>
<td>0.837</td>
<td>0.762-0.895</td>
<td></td>
<td>0.815</td>
</tr>
</tbody>
</table>

*compared with AUC of Cox(R).
†compared with AUC of Cox(S).

AUC, area under curve; CI, confidence interval; DFS, disease-free survival; OS, overall survival.
Prediction of recurrence and survival in hepatocellular carcinoma based on two Cox models mainly determined by FoxP3+ regulatory T cells


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