Prognostic value of immunoscore to identify mortality outcomes in adults with HBV-related primary hepatocellular carcinoma

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Abstract
This study aimed to determine if the immunoscore (IS) staging system would be a potential prognostic factor in hepatitis B virus-related hepatocellular carcinoma (HBV-HCC) in China.

IS was performed in a consecutive cohort of HBV-HCC patients (n = 92). CD3+, CD8+, and CD45RO+ T cells were quantified by immunohistochemical analyses. The patients were stratified into 5 IS groups: I0, I1, I2, I3, I4 for every 2 cell phenotypes (IS1 (CD8/CD45RO), IS2 (CD3/CD8), and IS3 (CD3/CD45RO), respectively. ImagePro Plus software was used in the calculation of the paraffin-embedded tumor sections.

The staining of CD3+, CD8+, and CD45RO+ cells in the HBV-HCC tissue demonstrated that there were higher density and larger area of lymphocytes in the invasive margins (IM) region than in the center (CT). Univariate analysis showed that preoperative TNM staging (P = .01), serum gamma-glutamyl transpeptidase (GGT) level (P = .03), vascular invasion (P = .00), and density of CD3+T (CT) (P = .01) were correlated significantly with disease-free survival (DFS); serum alpha-fetoprotein (AFP) level (P = .02), tumor size (P = .00), serum cholinesterase (CHE) (P = .04), and GGT level (P = .01), density of CD3+T (CT) (P = .00), CD8+T (CT) (P = .00), CD45RO+T (CT) (P = .00), and CD45RO+T (IM) (P = .02) were correlated with overall survival (OS). Multivariate analysis showed that TNM staging was not an independent prognostic factor of DFS and OS. Our results showed ISs did not have a significantly correlation with DFS (P = .35, .19, and .07, respectively), but it was correlated significantly with OS (P = .00, .00, and .00, respectively). There were statistical differences among the OS of every ISs subgroup except I0 and I1 by the Cox regressions analysis.

The IS staging was closely related to the outcome of patients. It can compensate the TNM tumor classification system in predicting the prognosis of HBV-HCC patients.

Abbreviations: AFP = alpha-fetoprotein, CHE = cholinesterase, CRC = colorectal cancer, CT = center, DFS = disease-free survival, GGT = gamma-glutamyl transpeptidase, GSH = glutathione, HBV = hepatitis B virus, HBV-HCC = hepatitis B virus-related hepatocellular carcinoma, HCC = hepatocellular carcinoma, Hi = high, IM = invasive margins, IS = immunoscore, Lo = low, OS = overall survival, TIL = tumor infiltrating lymphocyte, TNM = tumor-node-metastasis.

Keywords: HBV-related primary hepatocellular carcinoma, immunoscore, prognostic marker

1. Introduction
Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. The occurrence incidence of HCC is relatively high in China.[1] Chronic hepatitis B virus (HBV) infection is the primary risk factor for HCC in China.[2] Conventional prognosis assessments of HCC are based on histopathological evaluation of the primary tumor tissue obtained during operation. The tumor-node-metastasis (TNM) staging system by the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) summarizes the data of tumor burden (T). The presence of cancer cells in regional lymph nodes (N) and evidence for metastases (M) have been verified to be valuable in the outcome estimation of patients in a variety of tumors.[3–5] However, it provides limited information in the estimation of the postoperative outcome of HCC patients. Some patients with comparable histological tumor stages had clinical outcomes that varied significantly.[6] Increasing evidence supports that the host immune system significantly influences cancer development and clinical outcomes. The evaluation of systemic and local immunological biomarkers could offer useful prognostic information and be helpful for clinical decision making.[7–9]

Many researchers have found that immune contexture of the primary tumor is an essential prognostic factor.[10] Recently, large cohorts of various tumors have demonstrated that the number, type, and location of tumor-infiltrating lymphocytes (TILs) are essential for the prediction of clinical outcome.[11–16] The establishment of immunoscore (IS) is clinical translation of these observations, primarily based on the enumeration of

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2 lymphocyte populations (CD3/CD45RO, CD3/CD8, CD8/ CD45RO), both in the center (CT) and the invasive margins (IM) of tumors, as a clinically useful prognostic marker. This scoring system ranging from I0 (low densities of both cell types in both regions) to I4 (high densities of both cell types in both regions). Pages et al investigated the prognostic value of this scoring system in patients of early CRC (colorectal cancer), 5 different IS were associated with great differences in disease-free survival (DFS) and overall survival (OS) ($P < .0001$). Five years after diagnosis, only 4.8% of patients with high densities of CD8 and CD45RO cells had tumor recurrence, and 86.2% survived. In contrast, the tumor recurred in 75% of patients with low densities of these cell populations and only 27.5% survived. Since then it has been extensively used in a variety of tumor types, including colon, rectal, melanoma, and breast cancers. However, the predictive role of IS in patients with hepatitis B virus-related hepatocellular carcinoma (HBV-HCC) who underwent resection remains unknown. This study on 92 HBV-HCC patients with TNM staging I to IV demonstrated the importance of the localized IS in predicting survival in patients with HBV-HCC.

### 2. Materials and method

#### 2.1. Patients and materials

Patients who underwent curative resection with pathologically confirmed HBV-HCC between January 2006 and December 2010 in Beijing Youan Hospital, Capital Medical University, were retrospectively identified; relevant clinical and laboratory data were collected from their medical records. We excluded patients who met the following criteria: (1) patients with hepatitis B surface antigen negative; (2) patients who had coinfection with hepatitis C virus or history of alcoholism; (3) patients who had received radiotherapy, chemotherapy, gene therapy and/or molecular targeted therapy before or after surgery. Paraffin-embedded tumor samples were obtained from the pathology department following informed consent from all enrolled patients. A total of 92 cases were included in this analysis. Demographic, clinical information related to tumor stage/type, liver disease severity, clinical biochemistries and IS score are illustrated in Table 1. The average age (range) of participants was 46.7(22–77) years old. The protocol was approved by the Ethical Board of the Institutional Review Board of the Beijing Yuan Hospital, Capital Medical University. All procedures performed in study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments.

#### 2.2. Immunohistochemistry

The Paraffin-embedded tumor sections were dewaxed in xylene and rehydrated with distilled water. Following incubation with antibodies against human CD3 (clone LN 10, ZSGB-BIO Inc., Beijing, China), human CD8 (clone SP 16, ZSGB-BIO Inc., Beijing, China), and human CD45RO (clone UCHL1, ZSGB-BIO, Beijing, China), the adjacent sections were stained with Polink-1 HRP DAB Detection System (PV 6000, GBI Inc., USA). All section stained slides were examined by the reviewers who have no knowledge of any clinical data. Under $\times 400$ magnification, 3 representative views of every CT and IM were chosen from each tumor section as previously described (Fig. 1).

### Table 1

| Parameter                        | No. of pts (%) | DFS P | OS P |
|----------------------------------|----------------|-------|-------|
| Gender, male                     |                |       |       |
| Age                              |                |       |       |
| $<40$                            | 12 (13.04)     | 0.69  | 0.24  |
| $\geq40$                         | 80 (86.96)     |       |       |
| Number, single/ multiple         |                |       |       |
| $\leq5$                          | 78 (84.78)     | 0.91  | 0.36  |
| vascular invasion, Y/N           |                |       |       |
| $<5$                             | 19 (20.65)     | 0.06  | 0.52  |
| UICC, TNM, stage                 |                |       |       |
| $<4$                             | 9 (9.89)       | 0.48  | 0.45  |
| pathoholical differentiation     |                |       |       |
| Poorely differentiated           | 46 (50.00)     |       |       |
| Moderately differentiated        | 34 (36.96)     |       |       |
| Well-differentiated              | 12 (13.04)     |       |       |
| Cirrhosis                        | 73 (79.35)     |       |       |
| Child-Pugh score                 |                |       |       |
| $<6$                             | 91 (98.91)     |       |       |
| $\geq6$                          | 1 (1.09)       |       |       |
| Tumor size                       |                |       |       |
| $\leq5$                          | 48 (52.17)     | 0.23  | 0.00  |
| $>5$                             | 44 (47.83)     |       |       |
| AFP                              |                |       |       |
| $<10$ ng/mL                      | 28 (30.43)     |       |       |
| $<10$ ng/mL                      | 64 (69.57)     |       |       |
| CHE                              |                |       |       |
| $>4000$ µ/mL                     | 82 (89.13)     | 0.03  | 0.11  |
| $>4000$ µ/mL                     | 10 (10.87)     |       |       |
| GGT                              |                |       |       |
| $<50$ µ/mL                       | 43 (46.74)     |       |       |
| $>50$ µ/mL                       | 49 (53.26)     |       |       |
| CD3 (IM)                         | 45 (49.10)     | 0.91  |       |
| CD3 (CT)                         | 0.01           | 0.01  |
| CD8 (IM)                         | 0.60           | 0.06  |
| CD8 (CT)                         | 0.15           | 0.00  |
| CD45RO (IM)                      | 0.74           | 0.02  |
| CD45RO (CT)                      | 0.19           | 0.00  |
| IS1 (CD3/CD45RO)                 | 0.35           | 0.00  |
| IS2 (CD3/CD8)                    | 0.19           | 0.00  |
| IS3 (CD3/CD45RO)                 | 0.07           | 0.00  |

AFP = serum alpha-fetoprotein, CHE = serum cholinesterase, CT = center, DFS = disease-free survival, GGT = gamma-glutamyl transpeptidase, IM = invasive margins, IS = Immunoscore, OS = overall survival, pts = patients, UICC-TNM, International Union Against Cancer (UICC) tumor-node-metastasis (TNM) staging system.

** Significant.

#### 2.3. Immunoscore calculation

Precise quantification was performed to 3 markers (CD3+, CD8+, and CD45RO+) in 2 regions (CT and IM). The positive cells were quantified using ImagePro Plus software (Media Cybernetics), and the average score of the 3 different viewers was taken for analysis. Using the cutoff value that yielded from the minimum $P$ value for overall survival, the densities of CD3+, CD8+, and CD45RO+ cells in each tumor region (CT and IM) allowed the stratification of patients into 2 groups of high (Hi) or low (Lo) (“Hi” means above the cutoff value; “Lo” means under the cutoff value) (Table 2). Analysis the combination of every 2 types of immune cells in 2 regions, (IS1 = CD8+/CD45RO+, IS2 = CD3+/CD8+, and IS3 = CD3+/CD45RO+), the patients were divided into group 10–4. For instance, I 0 (I0) was defined as low densities of
both cell types in both regions (LoLoLoLo) and I1 (I1) was high density in 1 region (HiLoLoLo), I2(I2) means high densities in 2 region (HiHiLoLo), and so on.[12,16]

2.4. Statistical analysis

Stata software (version 13.1, Stata Corp.) was used for all statistical analyses. Survival analysis was used to illustrate the survival curves and to obtain the estimators of the median and survival rates for OS and DFS. The correlation between the densities of immune cells and survival time were using Cox regression analysis. Significant differences between groups were determined using unpaired 2-tailed t tests unless otherwise specified; P <.05 was considered significantly different.

3. Results

3.1. Disease free survival and overall survival rates

The 1, 3, and 5-year overall DFS rates of the 92 cases HBV-HCC patients after surgery were 68.2%, 52.4%, and 44.7% (Fig. 2A) and the OS were 91.1%, 81.3%, and 75.1%, respectively (Fig. 2B).

| Cell type | Mean (cell number) | Standard deviation | Rang (cell number) | Cut-off value (cell number) | Lo (%) | Hi (%) |
|-----------|--------------------|--------------------|--------------------|-----------------------------|--------|--------|
| CD3 (IM)  | 143                | 97.79              | 7                  | 418                         | 69     | 80     |
| CD3 (CT)  | 54                 | 51.31              | 2                  | 289                         | 55     | 60     |
| CD8 (IM)  | 84                 | 45.94              | 12                 | 221                         | 37     | 44     |
| CD8 (CT)  | 38                 | 37.98              | 3                  | 228                         | 29     | 37     |
| CD45RO (IM) | 196              | 103.00             | 8                  | 586                         | 272    | 32     |
| CD45RO (CT) | 85                | 59.87              | 6                  | 278                         | 45     | 50     |

CT = center, HI = high, IM = invasive margins, Lo = low.

* The average number of the 3 fields for each section.
3.2. The distribution of CD3+, CD8+, and CD45RO+ T lymphocytes in the tumor

The staining of CD3+, CD8+, and CD45RO+ cells in the HBV-HCC tissue demonstrated that the lymphocyte densities differed significantly between the CT and IM regions, with a higher density and larger area of lymphocytes in the IM region. The average number of CD3+ (IM), CD8+ (IM), or CD45RO+ (IM) T cells was significantly higher than those of CD3+ (CT), CD8+ (CT), or CD45RO+ (CT) T cells (P < .05).

Based on the methodology described by Galon et al.,

patients were divided into 2 groups according to the minimum P-value cutoffs for CD3+, CD8+ or CD45RO+ densities. The cut off values were 69, 55, 37, 23, 272, and 45 for CD3+ (IM), CD3+ (CT), CD8+ (IM), CD8+ (CT), CD45RO+ (IM), and CD45RO+ (CT), respectively in each tumor region (Table 2). When every 2 cell phenotypes were combined to predict clinical outcomes, 13.04%, 22.83%, and 11.96% of the patients presented with a high infiltration of CD8+/CD45RO+, CD3+/CD8+, and CD3+/CD45RO+ cells in CT and IM regions of the tumor (HiHiHiHi) respectively, whereas 7.61%, 8.70%, and 15.22% had a low infiltration of these cells in both tumor regions (LoLoLoLo), respectively (Table 3). Strikingly, 79.35%, 68.47%, and 72.82% of the patients presented with discrepancies between the densities of the immune markers, respectively.

3.3. Prognostic value of immunoscore in predicting DFS and OS

The univariate analysis showed that preoperative TNM staging, GGT level, vascular invasion, and density of CD3+T (CT) were correlated significantly with DFS, AFP level, tumor size, serum CHE and GGT level, density of CD3+T (CT), CD8+T (CT), CD45RO+T (CT), and CD45RO+T (IM) were correlated significantly with OS.

According to the IS system raised by Angell and Galon, we stratified the patients into 5 groups: (I0) 0Hi, (I1) 1Hi, (I2) 2Hi, (I3) 3Hi, (I4) 4Hi for every 2 cell phenotypes (I1/CD8/CD45RO, I2/CD3/CD8, and I3/CD3/CD45RO), respectively. DFS and OS were illustrated by Kaplan–Meier curves (Fig. 3). We found the OS (Fig. 3C, E, G) and DFS (Fig. 3D, F, H) of patients were gradually prolonged if correspondent IS increased: Patients in I4 group with the longest survival. ISs did not have a significantly correlation with DFS (P = .35, .19, and .07, respectively), but it was correlated significantly with OS (P = .00, .00, and .00, respectively) (Table 1). Cox regressions showed that there were statistical differences among the OS of every IS subgroup except I0 and I1 in IS1/CD8/CD45RO, IS2 (CD3/CD8) (Table 3).

4. Discussion

HCC is a major public health problem in the world. China has a high incidence of HCC with chronic HBV infection be the primary risk factor. Simple and effective prognostic markers are needed to predict the survival, which helps to avoid improper treatment in HCC patients.

AFP has been used as a serum marker for the detection of HCC. Since 1968. The AFP elevation in HCC has been shown to correlate with poor tumor differentiation, tumor burden, and early recurrence after tumor resection, and unfavorable prognosis. But several studies have evaluated the sensitivity and specificity of utilizing AFP with ranges of 21% to 64% and 82% to 93%, respectively. AFP sensitivity is lower with small HCC lesions. One major disadvantage is that AFP levels can be falsely raised in patients who have active hepatitis.

Although not showing significance in multivariate analysis, preoperative elevated GGT level was correlation with decreased OS and DFS in univariate analysis. GGT is a crucial enzyme of glutathione (GSH) metabolism, and it is related to biotransformation, nucleic acid metabolism, and tumorigenesis, GGT has been widely used as a marker enzyme for several cancers. Recently, serum GGT has been identified as a useful risk predictor in addition to traditional risk factors for cancer because it is a marker of oxidative stress. Elevated serum GGT has been associated with a worse prognosis in many cancers, including HCC, endometrial cancer, esophageal squamous cell carcinoma.

The traditional staging system assumed that tumor progression mainly is a cell-autonomous process, focusing only on cancer cells, paying no attention to the host immune response, this apparently limited predictive accuracy. Some patients with comparable histological tumor stages had clinical outcomes that varied significantly. Histopathological analysis has revealed that tumors are often infiltrated by a variable degree of inflammatory and lymphocytic cells. They are organized in more or less dense infiltrates in the CT and the IM of tumor. The presence of immune cells may reflect a distinct underlying biology of the tumor, includes of innate immune activation, chemokines for T cell recruitment, immune effector molecules, and expression of immune regulatory factors.

The distribution of immune cells also varies between tumor types, suggesting that different immune cell populations may
have different roles in tumor control.\[24\] In our study, the staining of CD3+, CD8+, and CD45RO+ cells in the HBV-HCC tissue demonstrated that the lymphocyte densities differed significantly between the CT and IM regions, with a higher density and larger numbers of CD3+, CD8+, and CD45RO+ cells in the HBV-HCC tissue in patients with HCC. In addition, the number, type, and location of TILs are essential for the prediction of clinical outcome.\[11\]

In this study, the results demonstrated that the HCC patients with lower IS was significantly associated with poor prognosis, and patients with higher IS are significantly associated with longer OS. Although in univariate analysis the TNM stage was significantly correlated with DFS, but it was not significantly correlated with OS. When be further analyzed, TNM stage could differentiate patients with different prognoses (Table 3, Fig. 3A and B). As the result showed, the HBV-HCC patients with higher ISs are significantly correlated with DFS; densities of CD3+ T (CT), CD8+ T (CT), CD45RO+ T (CT), and CD45RO+ T (IM) were correlated with OS. These results further verified the views raised by Galon et al.\[10\] that immune contexture of the primary tumor is an essential prognostic factor for identifying DFS and OS in patients with HCC. In addition, the number, type, and location of TILs are essential for the prediction of clinical outcome.\[11–16\]

In order to more efficiently predict survival in patients with HCC, the indicators of CD3+, CD8+, or CD45RO+ T cells in CT or IM regions in tumors were incorporated into the scoring systems (IS), respectively.\[9,12,14,16,21\] Galon et al.\[17\] first demonstrated the predictive accuracy of IS staging system in colorectal cancer patients in 2006. Subsequent to these studies, other clinical data have further confirmed that the prognostic value of IS classification is superior to the AJCC/UICC TNM classification in various tumor types, including rectal cancer, melanoma, and breast cancers patients.\[9,13,15,16,20,21\] Up to now, the predictive role of IS system in HBV-HCC has never been reported.

In this study, the results demonstrated that the HCC patients with lower IS was significantly associated with poor prognosis, and patients with higher IS are significantly associated with longer OS. Although in univariate analysis the TNM stage was significantly correlated with DFS, but it was not significantly correlated with OS. When be further analyzed, TNM stage could differentiate patients with different prognoses (Table 3, Fig. 3A and B). As the result showed, the HBV-HCC patients with higher ISs are significantly associated with longer OS compared with lower IS. (Fig. 3D, F, H). This suggests that IS has an advantage on predicting the prognosis in HCC patients over the current TNM tumor classification, or can be a complementary classification method for TMN.

Unlike previous studies that showed IS was significantly associated with various cancer patients’ DFS and OS\[9,11–13,15,16,20,21\], this study showed that IS could only predict HBV-HCC patients’ survival, and the prediction abilities of IS staging with combination of CD3+/CD45RO+ was not so strong as the combination of CD3+/CD8+ or CD8+/CD45RO+. These differences may be due to a variety of reasons, including

### Table 3

Cox regression analysis of DFS and OS among patients according to TNM staging and IS staging.

| Parameter | No of pts (%) | DFS HR (95%CI) | P | OS HR (95%CI) | P |
|-----------|--------------|---------------|---|--------------|---|
| TNM stage |              |               |   |              |   |
| I0        | 28 (30.43)   | 1 (base)      |   | 1 (base)     |   |
| I1        | 36 (39.13)   | 1.86 0.40     | .70 | 0.93 0.30     | .90 |
| I2        | 26 (28.26)   | 1.68 0.78     | .34 | 1.50 0.45     | .51 |
| I3        | 2 (2.17)     | 6.74 1.42     | .41 | 5.24 0.61     | .91 |
| IS1 (CT/CD45RO) | | | | | |
| I0        | 7 (7.61)     | 1 (base)      |   | 1 (base)     |   |
| I1        | 15 (16.30)   | 1.73 0.36     | .25 | 0.42 0.11     | .21 |
| I2        | 18 (19.57)   | 1.63 0.36     | .53 | 0.21 0.05     | .06 |
| I3        | 40 (43.48)   | 1.15 0.27     | .36 | 0.10 0.02     | .42 |
| I4        | 12 (13.04)   | 0.58 0.11     | .11 | 0.06 0.01     | .55 |
| IS2 (CT/CD8) | | | | | |
| I0        | 8 (8.70)     | 1 (base)      |   | 1 (base)     |   |
| I1        | 8 (8.70)     | 2.21 0.40     | .13 | 0.78 0.19     | .73 |
| I2        | 33 (35.87)   | 2.09 0.49     | .32 | 0.25 0.07     | .09 |
| I3        | 22 (23.91)   | 1.08 0.23     | .32 | 0.19 0.04     | .86 |
| I4        | 21 (22.83)   | 0.91 0.19     | .11 | 0.05 0.01     | .47 |
| IS3 (CT/CD45RO) | | | | | |
| I0        | 14 (15.22)   | 1 (base)      |   | 1 (base)     |   |
| I1        | 17 (18.48)   | 1.76 0.60     | .30 | 0.60 0.19     | .86 |
| I2        | 24 (26.09)   | 0.77 0.25     | .21 | 0.29 0.08     | .60 |
| I3        | 20 (21.72)   | 1.12 0.39     | .20 | 0.11 0.02     | .57 |
| I4        | 11 (11.96)   | 0.28 0.05     | .14 | 0.00 0.00     | 1* |

- CI: confidence interval, DFS: disease-free survival, HR: hazard ratio, IS: immunoscore, OS: overall survival, pts: patients, TNM: International Union Against Cancer-TNM stage.
- \*: Significant <0.05.
- \*: In this staging, no patient dies during the following period.
different tumor type/size influencing the prognostic value of IS or that the sample size was not sufficiently large enough to effectively illustrate the association between IS and DFS or OS. A larger sample size in each tumor category may be needed to confirm the current findings.

In summary, this study confirmed that IS was a valuable marker in predicting HBV-HCC patients' survival. This method is easy to use, economical, and reproducible in clinical settings and thus provides a promising approach to assess DFS and OS in HBV-HCC.

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