The distribution of immune cells within combined hepatocellular carcinoma and cholangiocarcinoma predicts clinical outcome

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Abstract

Background: This study aimed to investigate the clinical relevance of the immune microenvironment in patients with combined hepatocellular carcinoma and cholangiocarcinoma (cHCC-ICC).

Patients and Methods: The density of tumor-infiltrating CD3⁺, CD8⁺, CD163⁺, and Foxp3⁺ immune cells, as well as Programmed cell death 1, Programmed cell death-ligand 1, and Tumor necrosis factor receptor superfamily member 4, was measured in the peritumor liver, tumor invasive margin, and intratumor subregions of 56 cHCC-ICC by immunohistochemistry. The immune index was established to stratify patients. Prognostic significance of immune cell subsets and immune indices was evaluated.

Results: The distribution of immune cells was highly heterogeneous among different subregions of cHCC-ICC. As compared with the hepatocellular carcinoma (HCC) component, the lower density of CD8⁺ T cells and higher intensity of Foxp3⁺ and immune checkpoints in the intrahepatic cholangiocarcinoma (ICC) component may indicate a stronger immune evasive ability of ICC. Based on clustering classification or a combination of random forest and lasso-cox, two models of immune indices were established and both were identified as independent prognostic factors for cHCC-ICC patients. The selected immune variables in the immune prognostic

Abbreviations: cHCC-ICC, combined hepatocellular carcinoma and cholangiocarcinoma; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; ICC, intrahepatic cholangiocarcinoma; IDH1, isocitrate dehydrogenase-1; IM, invasion margin; OS, overall survival; OX40, Tumor necrosis factor receptor superfamily member 4; PD1, Programmed cell death 1; PD-L1, Programmed cell death-ligand 1; TNM, tumor-node-metastases.
models derived from both HCC and ICC subregions, indicating that the prognosis of cHCC-ICC patients was a complex interaction of both components.

Conclusions: The immune contexture was heterogeneous among different subregions of cHCC-ICC patients and contributed differently to patient prognosis. Immune score based on the densities of immune cells might serve as a promising prognostic predictor for cHCC-ICC patients.

KEYWORDS
liver cancer, programmed cell death 1 receptor, T lymphocytes, tumor microenvironment

1 | BACKGROUND

Liver cancer is the fourth leading cause of cancer-related deaths, with more than 85,000 new cases annually worldwide. Combined hepatocellular carcinoma and cholangiocarcinoma (cHCC-ICC), a rare type of primary liver cancer, accounts for 1-14.2% of all primary liver malignancies. The survival of cHCC-ICC is significantly worse than hepatocellular carcinoma (HCC) and more similar to intrahepatic cholangiocarcinoma (ICC). Due to the relatively low incidence of cHCC-ICC, the molecular pathogenesis and the clinical behavior of these tumors remain ill-defined. To date, clinical guidelines do not propose a specific treatment recommendation for cHCC-ICC patients. Hepatectomy remains the only curative treatment that amenable for early-stage patients, albeit modest benefits and high recurrence rate. For those cHCC-ICC patients in the advanced stage, standard systemic therapies are still not available. Hence, new treatment strategies are urgently needed for cHCC-ICC patients.

Recent data have demonstrated significant benefits of immunotherapy in various solid tumors, including nonsmall cell lung cancer, genitor-urinary cancer, HCC, and ICC. However, there is still no such ongoing clinical trials for cHCC-ICC patients. The basic principle of immunotherapy is the modulation of tumor-immune interactions. Several studies have reported the epigenetic, genetic, and transcriptomic signatures of cHCC-ICC patients, but the understanding of the immune microenvironment in cHCC-ICC is still lacking. Based on the density and distribution of CD3+ and CD8+ T cells, the “hot and cold” classification for the tumor was postulated, which could predict clinical outcomes of patients with various cancers and “hot” indicated potential sensitivity to immunotherapy. It is rational to speculate that a comprehensive analysis of the type, density, and spatial distribution of immune components within the local microenvironment may provide important clues for developing immunotherapy for cHCC-ICC patients.

In this study, we carried out a preliminary quantitative and qualitative assessment of immune contexture in cHCC-ICC patients. Immunohistochemical characterization of CD3 (Pan-T cells), CD8 (T-killer cells), Foxp3 (Regulatory T cells [Tregs]), and CD163 (macrophages), as well as immune checkpoints Programmed cell death 1 (PD-1), Programmed cell death-ligand 1 (PD-L1), and Tumor necrosis factor receptor superfamily member 4 (OX40), was conducted in a consecutive cohort of 56 cHCC-ICC patients. We showed a tumor subregion-specific infiltration of immune cells that contributed differently to patient prognosis. As compared with HCC subregions, lack of CD8+ T cells, enriched Tregs, and a higher level of immunoinhibitory checkpoints in ICC subregions may indicate a stronger immune evasive ability of ICC.

2 | PATIENTS AND METHODS

2.1 | Patient selection

This retrospective analysis included 56 consecutive cHCC-ICC patients who underwent curative resection of their primary tumors between 2011 and 2015 at Zhongshan Hospital. The criteria for the enrolled patients were listed in Additional file 1. Tumors were assigned a pathological tumor-node-metastases (TNM) stage according to the American Joint Committee on Cancer (AJCC) 8th edition. According to the Allen and Lisa criteria, cHCC-ICC was divided into three types: separated tumor, combined type, and mixed type (Additional file 2). This study was approved by the Institutional Review Board (B2017-060) and was performed following the Declaration of Helsinki.

Postoperative surveillance and treatment modality was performed as we previously described. The definition of overall survival (OS) was the span from the first resection to death or censored for living patients.
2.2 | Immunohistochemistry

Immunohistochemistry was conducted as described previously.18 Detailed procedures were depicted in Supplementary Method. Details of antibodies used were presented in Table S1.

2.3 | Microanatomical annotation

To evaluate the spatial heterogeneity of immune components, tumor sections were microanatomically divided into intratumor, invasion margin (IM), and peritumor liver. The tumor IM was defined as the region within 500 μm on each side of the border between the tumor and normal liver tissue19 (Figure 1A). For separated and combined subtypes of cHCC-ICC, the intratumor area was divided into HCC and ICC components, whereas the IM was divided into HCC IM and ICC IM. For mixed subtype, the HCC and ICC components were defined as areas where HCC and ICC cells accounting for 90% of all the tumor cells, respectively. Details of the annotation strategy were presented in Figure S2.

2.4 | Quantification of CD3, CD8, Foxp3, and PD1 positive immune cells

The density of positive cells was evaluated as described previously.19 In brief, three microscopic fields (magnification, ×200) of each hot area were selected and captured. Positively stained cells were counted by using the IHC toolbox.20,21 Then, the density of positive cells was calculated by averaging.

2.5 | Quantification of the expression of PD-L1 and OX40

For the immune checkpoints, a digital image system was used to evaluate the signals as described previously.22 Detailed information was listed in Additional file 1.

2.6 | Clustering analysis and development of the immune score

Unsupervised clustering analysis of the densities of immune cells and immune checkpoints was performed using Euclidean distance. Random forest for survival analysis using the “randomForestSRC” package was used to select the most important prognostic factors. Then, the lasso-cox using the “glmnent” package was implemented to establish an immune score. All analyses were carried out in R (R foundation for statistical computing, Vienna, Austria; URL: http://www.R-project.org; 2016). Then, C-index was used to evaluate the prognostic significance of the two categories and compared via the Delong test.

2.7 | Statistical analysis

The results were presented as mean ± standard deviation (SD) or median (range). The Fisher’s exact test, Chi-squared test, or Mann-Whitney U test was used as appropriate. Paired Wilcoxon signed-rank tests was used to analysis the difference in the distribution of immune cells, whereas Spearman’s rank correlation analysis was performed to analysis the correlations between the immune cells. The survival curve was depicted by using Kaplan-Meier and compared via the log-rank test. The Cox hazard regression model was carried out for univariate and multivariate analyses. A two-tailed P < .05 was considered statistical significance. SPSS 22.0 software (Chicago, IL, USA) and Graphpad Prism 7 software (La Jolla, CA, USA) were used to conduct the statistical analyses.

3 | RESULTS

3.1 | Baseline characteristics of patients

The characteristics of the study cohort are listed in Table 1. Among the 56 cHCC-ICC cases, one was defined as separated type, 24 were combined type, and 31 were mixed type (Figure S1). The number of patients at TNM stages Ia, Ib, II, and IV were 34, 10, 10, and 2, respectively. The 1-, 3-, and 5-year postoperative survival rates were 86%, 67%, and 57%, respectively. In our study, the 5-year survival rate was better than previously reported ones,15 possibly due to that most patients were at the early stage and all patients received R0 resection. Besides, only two patients were found to have lymph node metastases.

3.2 | Density and distribution of immune cells and checkpoints in cHCC-ICC

Tumor slides were divided into five subregions, including the HCC component, ICC component, HCC-IM, ICC-IM, and the peritumor liver. Positive staining of Hep-Par1 and GPC3 was defined as the HCC component, whereas the positivity of CK7 and CK19 was defined as the ICC component16,23,24 (Figure S1). The representative staining of immune variables (including CD3, CD8, CD163, Foxp3, PD1, PD-L1, and OX40) using consecutive sections is presented in Figure 1B. Accordingly, the intensity of immune cells and checkpoints were evaluated at different microanatomical subregions (Figures 1C and 1D).
FIGURE 1 Representative staining pictures and spatial distribution of immune variables in combined hepatocellular carcinoma and cholangiocarcinoma (cHCC-ICC). A. Tumor micro-annotation and the definition of the tumor invasive margin (magnification, ×4 and ×100). B. The representative images of indicated immune variables in cHCC-ICC. Positive cells were stained brown (magnification, ×100). C. Statistics depicting the spatial distribution of infiltrating immune cells (*P < .05; **P < .01; ***P < .001). D. Statistics depicting the spatial distribution of the immune checkpoints

CD3⁺ T cells were predominately enriched in the peritumor liver (672.50/mm²), followed by HCC-IM (458.50/mm²), ICC-IM (456.50/mm²), and HCC component (239/mm²), with the least in ICC component (143.50/mm²). The distribution of CD8⁺ T cells was similar to CD3⁺ T cells, which was abundant in the peritumor liver (123/mm²), followed by HCC-IM (111/mm²), ICC-IM (112/mm²), and HCC component (103/mm²), and generally low in ICC component (48/mm²). Also, CD163⁺ macrophages were enriched in the peritumor liver (95/mm²); moderate in HCC-IM (73/mm²)
TABLE 1  Clinicopathological features of combined hepatocellular carcinoma and cholangiocarcinoma (cHCC-ICC) patients (n = 56)

| Variables                        | Values                   |
|----------------------------------|--------------------------|
| Age, median (range)              | 56 (29-74)               |
| Gender                           |                          |
| Male                             | 45                       |
| Female                           | 11                       |
| Liver cirrhosis                  |                          |
| Absent                           | 13                       |
| Present                          | 43                       |
| Max tumor size, cm               |                          |
| Median (range)                   | 3.5 (0.5-8.0)            |
| Tumor number                     |                          |
| Single                           | 46                       |
| Multiple                         | 10                       |
| Microvascular invasion           |                          |
| Absent                           | 44                       |
| Present                          | 12                       |
| Lymph node metastases            |                          |
| Absent                           | 54                       |
| Present                          | 2                        |
| Macrovascular invasion           |                          |
| Absent                           | 54                       |
| Present                          | 2                        |
| CA19-9, ng/mL                    |                          |
| Median (range)                   | 25.6 (3.2-254.5)         |
| AFP, ng/mL                       |                          |
| Median (range)                   | 46.7 (0.7-60 500)        |
| Pathological type                |                          |
| Separated                        | 1                        |
| Combined                         | 24                       |
| Mixed                            | 31                       |
| TNM stage                        |                          |
| Ia                               | 34                       |
| Ib + II + IV                     | 22                       |

Abbreviations: TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; CA19-9, antigen carbohydrate 19-9.

and ICC-IM (57/mm²); and very low in HCC component (28/mm²) and ICC component (20/mm²). In contrast, the density of Foxp3+ Tregs in ICC component was the highest (10/mm²), followed by HCC-IM, ICC-IM (10/mm²/9/mm²), HCC component (7.5/mm²), and the peritumor liver (4/mm²). The density of PD-1+ cells was high in HCC-IM (33/mm²), intermediate in ICC-IM (29/mm²) and the peritumor liver (28/mm²), and very low in ICC component (10/mm²) (Figure 1C; Table 2). These results indicated that ICC component was specifically enriched with Tregs and sparsely infiltrated with CD8+ T cells, as compared with other microanatomical subregions.

Then, the expression patterns of OX40 and PD-L1 were analyzed (Figure 1D; Table 2). We found that the positive rate of OX40 was similar between each tumor subregion and their corresponding IM (HCC: 42.9% vs HCC-IM: 48.2%, \( P = .285 \); ICC: 35.7% vs ICC-IM: 39.3%, \( P = .789 \)), but lower in the peritumor liver (14.3%). Of importance, the positive rate of OX40 in HCC component was slightly higher than that in ICC component (HCC: 42.9% vs ICC: 35.7%, \( P = .033 \)). For PD-L1, the positive rate was higher in IM, with comparable levels between HCC-IM and ICC-IM (HCC-IM: 50.00% vs ICC-IM: 42.86%, \( P = .345 \)). In contrast, the positivity of PD-L1 in ICC component was significantly higher than that in HCC component (ICC: 39.29% vs HCC: 28.57%, \( P = .002 \)). Taken together, all these data showed obvious spatial heterogeneity of immune contexture in the cHCC-ICC microenvironment. The lower density of CD8+ T cells and higher intensity of immune checkpoints in the ICC component and ICC invasive margin may indicate a stronger immune evasive ability of ICC.

### 3.3 The prognostic values of immune cells and checkpoints

Due to their heterogeneous distribution, the prognostic values of immune variables in different subregions were analyzed individually. Patients were stratified into high and low groups based on the optimal cutoff of each immunostaining variable determined by cutoff finder.25

For T cells, patients with high density of CD3+ or CD8+ T cells were associated with better survival, including HCC component, ICC-IM, or peritumor liver. For CD163+ macrophages or Foxp3+ Tregs, patients with high density in HCC or ICC components were associated with worse survival. Likewise, patients with high PD1+ immune cells in HCC component, ICC-IM, or ICC-IM were associated with worse survival. No prognostic significances were observed for OX-40 expression across all subregions, whereas patients with positive PD-L1 in HCC or ICC components were associated with worse survival (Table S2).

Then, multivariate analyses identified that CD3 in HCC component (hazard ratio [HR] = 0.299; 95% confidence interval [CI], 0.111-0.807; \( P = .017 \)) and ICC-IM (HR = 0.344; 95% CI, 0.134-0.886; \( P = .027 \)), CD8 in HCC component (HR = 0.234; 95% CI, 0.092-0.592; \( P = .002 \)), ICC-IM (HR = 0.375; 95% CI, 0.142-0.992; \( P = .048 \)), peritumor liver (HR = 0.254; 95% CI, 0.092-0.705; \( P = .009 \), Foxp3 in ICC component (HR = 3.426; 95% CI, 1.328-8.841; \( P = .011 \)), PD-L1 in HCC-IM (HR = 0.239; 95% CI, 0.085-0.672; \( P = .007 \)), PD-L1 in HCC (HR = 3.132; 95% CI, 1.258-7.796; \( P = .014 \)), and ICC components (HR = 3.844;
TABLE 2 Descriptive statistics of immunohistochemical variables

| Subregions            | CD3 (cell/mm²) | Median | Range     | CD8 (cell/mm²) | Median | Range     | CD163 (cell/mm²) | Median | Range     | Foxp3 (cell/mm²) | Median | Range     | PD1⁺ cells (cell/mm²) | Median | Range     |
|-----------------------|----------------|--------|-----------|----------------|--------|-----------|------------------|--------|-----------|------------------|--------|-----------|----------------------|--------|-----------|
| HCC component         | 239            | 40-1011| 103       | 13-662         | 28     | 9-210     | 7.5              | 3-116 | 19        | 0-159            |        |           |                     |        |           |
| ICC component         | 143.5          | 26-573 | 48        | 5-281          | 20     | 3-155     | 10.0             | 1-100 | 10        | 0-158            |        |           |                     |        |           |
| HCC-IM                | 458.5          | 70-1627| 111       | 26-419         | 73     | 14-429    | 10.0             | 1-154 | 33        | 0-131            |        |           |                     |        |           |
| ICC-IM                | 456.5          | 59-1314| 112       | 33-382         | 57     | 12-294    | 9                | 1-71  | 29        | 0-152            |        |           |                     |        |           |
| Peritumor liver       | 672.5          | 157-2072| 123      | 8-493          | 95     | 19-434    | 4                | 1-39  | 28        | 0-176            |        |           |                     |        |           |

P-values

- HCC vs ICC <.001 <.001 <.001 .665 .011
- HCC vs HCC-IM <.0001 .454 <.001 .254 .005
- ICC vs ICC-IM <.001 <.001 .025 .828 .000
- HCC-IM vs peritumor .076 .107 .004 <.001 .655
- ICC-IM vs peritumor <.001 .367 <.001 <.001 .458
- HCC-IM vs ICC IM .925 .791 .002 .203 .808
- HCC vs peritumor <.001 <.001 <.001 <.001 <.001
- ICC vs peritumor <.001 <.001 <.001 <.001 <.001

Note. Wilcoxon signed-rank test.

TABLE 3 Multivariable cox proportional hazards models for overall survival

| Variables            | Multivariable analysis | HR   | 95%CI    | P-value |
|----------------------|------------------------|------|---------|---------|
| HCC CD3 (high vs low)| 0.299                  | 0.111-0.807 | .017   |
| ICC-IM CD3 (high vs low)| 0.344              | 0.134-0.886 | .027   |
| Peritumor CD3 (high vs low)| –                | –    | –       | .664    |
| HCC CD8 (high vs low)| 0.234                  | 0.092-0.592 | .002   |
| HCC-IM CD8 (high vs low)| –                  | –    | –       | .089    |
| ICC-IM CD8 (high vs low)| 0.375              | 0.142-0.992 | .048   |
| Peritumor CD8 (high vs low)| 0.254              | 0.092-0.705 | .009   |
| HCC CD163 (high vs low)| –                   | –    | –       | .545    |
| ICC CD163 (high vs low)| –                   | –    | –       | .381    |
| HCC-IM CD163 (high vs low)| –              | –    | –       | .093    |
| HCC Foxp3 (high vs low)| –                   | –    | –       | .218    |
| ICC Foxp3 (high vs low)| 3.426                 | 1.328-8.841 | .011   |
| HCC PD1 (high vs low)| –                     | –    | –       | .161    |
| HCC-IM PD1 (high vs low)| 0.239              | 0.085-0.672 | .007   |
| ICC-IM PD1 (high vs low)| –                  | –    | –       | .065    |
| HCC PD-L1 (positive vs negative)| 3.132        | 1.258-7.796 | .014   |
| ICC PD-L1 (positive vs negative)| 3.844        | 1.459-10.414 | .008   |
| Cluster (2 vs 1)     | 4.191                  | 1.005-18.253 | .023   |
| Immune score (high vs low)| 29.266            | 8.157-105.00 | <.001  |

Abbreviation: IM, invasive margin.

95% CI, 1.419-10.414; $P = .008$ were independent prognostic factors for OS in chHCC-ICC patients (Table 3). The survival analysis indicated that the prognostic significance of immune variables varied among different tumor subregions. Thus, we assumed that a panel of immune variables, including immune cell density and spatial distribution, should be identified and integrated to stratify patient prognosis.

3.4 | Patient classification based on unsupervised clustering of immune variables

The correlations between immune variables were analyzed (Figure 2A). The densities of most tumor-infiltrating immune subsets significantly and positively correlated with each other (range of correlation coefficients, 0.237-0.686; $P = .007$ to <.001 for significant correlations).

Several recent studies have demonstrated that tumors could be divided into different subtypes according to the density and distribution of immune cells. Herein, unsupervised clustering based on the immune density identified two subgroups with significantly different survival, where cluster 1 had a significantly better OS than cluster 2 ($P = .023$) (Figure 2B). Multivariate analysis further identified this clustering as an independent prognostic factor for chHCC-ICC patients ($HR = 4.191$; 95% CI, 1.005-18.253; $P = .023$; Table 3). Patients in cluster 2 were associated with larger tumor size ($P = .029$), advanced TNM stage ($P = .002$), and higher hepatitis B virus (HBV) infection rate ($P = .021$) (Additional file 6). Notably, patients in cluster 1 were more abundant in CD3⁺ and CD8⁺ T cells among all subregions (Figure 2C), whereas no significant differences were observed for the densities of CD163⁺ macrophages and Foxp3⁺ Tregs.
FIGURE 2  The correlation between the immune cells and the cluster based on the density of immune cells. A, The correlation between the immune cells among different subareas (hepatocellular carcinoma [HCC] [H]; intrahepatic cholangiocarcinoma [ICC] [I]; HCC invasive margin [H.IM]; ICC invasive margin [I.IM]; peritumor [P]). B, The clustering classification based on the density of the immune variables. The green bar means cluster 1 (n = 18), whereas the red bar means the cluster 2 (n = 38). Kaplan-Meier curve of overall survival indicated that patients in cluster 1 (n = 18) correlated with better outcomes (P = .023). C, Patients in cluster 1 were abundant in CD3+ and CD8+ T cells in HCC, ICC, HCC invasive margin, ICC invasive margin, and peritumor areas. *P < .05; **P < .01; ***P < .001
FIGURE 3 The representative images of the cold and hot tumors. A, A representative case of a hot tumor with abundant CD3+ and CD8+ immune cells (magnification, ×200). B, A representative case of a cold tumor with sparse CD3+ and CD8+ immune cells (magnification, ×200).

Interestingly, this clustering was consistent with the classical concept of immune “hot and cold.”23,28,29 The typical cases of hot and cold tumors were presented in Figure 3.

3.5 Establishment and prognostic significance of an immune index based on lasso-cox

Although the immune clustering could predict the postoperative survival of cHCC-ICC, it is unable to predict for linear measurement of risk. Thus, we utilized the random forest to select factors that contributed most to patient prognosis. Accordingly, the expression of PD-L1 in HCC, the densities of CD3+ T cells and CD163+ macrophages in HCC-IM, the densities of CD8+ T cells in the peritumor liver, HCC-IM, and ICC-IM, and the density of Foxp3+ Tregs in the HCC components were selected out (Figure 4A). Then, lasso-cox was used to establish the immune index based on the selected immune variables (Figure 4B), using the following formula: $-2.7640938 -0.9704039*PD-L1^{HCC} -1.3569411*Foxp3^{HCC} -1.67459*CD163^{HCC-IM} + 0.6143227*CD8^{peritumor} + 0.4881430*CD8^{HCC-IM} + 1.5869789*CD3^{ICC-IM}$. Notably, patients with a low immune score had a significantly worse survival than those with a high immune score ($P < .001$; Figure 4C). The selected immune variables in this score derived from both HCC and ICC subregions, indicating that the prognosis of cHCC-ICC patients was a complex interaction of both components. Multivariate analysis further identified this immune score as an independent prognostic factor for cHCC-ICC patients (HR = 0.034; 95% CI, 0.010-0.123; $P < .001$; Table 3). Furthermore, the immune score had significantly higher C-index than the clustering classification (0.850 vs 0.672; $P < .001$), indicating the superiority of the immune score for prognostic stratification of cHCC-ICC patients.

Furthermore, we found that patients with a high immune score were positively associated with smaller tumor diameter ($P = .003$), early TNM stage ($P < .001$), and lower HBV infection rate ($P = .037$) Table S3. Although it has been reported that the immune microenvironment varied with the pathological subtypes of cHCC-ICC,16 no correlation was found between cHCC-ICC subtypes and the immune index ($P = .612$). All these results indicated that patients with a
FIGURE 4 The establishment and the prognostic significance of the immune score. A, The results of the random forest for selecting variables related to survival. Seven parameters (the red ones) were selected out by the Random forest. B, Six parameters were selected out by the lasso-cox to establish the immune score. C, The Kaplan-Meier curve of overall survival showed that patients with high immune score were associated with better overall survival ($P < .001$)
low immune score had more aggressive tumors, authenticating that immune surveillance played a critical role in the clinical outcome of cHCC-ICC patients, irrespective of its pathological subtypes.

4 | DISCUSSION

Growing evidence has suggested that the type, density, and location of immune cells within the local milieu may strongly influence tumor evolution and patient prognosis. 29 In this study, we carried out a preliminary investigation on the spatial distribution and composition of the immune contexture in cHCC-ICC patients, finding that the density of each type of immune cell varied among different tumor subregions and contributed differently to patient prognosis.

We found that the densities of CD3+ and CD8+ T cells in the HCC component were higher than those in the ICC component, whereas the density of Tregs in ICC component was higher than that in HCC component. These findings may indicate that ICC was more likely to be immune suppressed than HCC, possibly imprinting the different genetic profiles of HCC and ICC. Recently, several studies have reported the genetic profiling of HCC 30,31 and ICC, 32 finding distinct gene mutation patterns between the two types of liver cancer. For instance, isocitrate dehydrogenase-1 (IDH1) mutation has been reported as a frequent mutation in ICC, occurring in 10-20% of patients, whereas this mutation was rarely found in HCC. Recently, Gary and colleagues found that IDH1 mutations could suppress STAT1 signaling and CD8+ T cell accumulation to promote the immune evasion ability of gliomas. 33 This may partly explain why the immune microenvironment of ICC was more likely to be immune suppressed than that of HCC.

As is well known, tumor immune microenvironment is spatially heterogeneous, especially between tumor core and invasive margin. Previous studies in HCC have reported that B cells, T cells, and monocytes were enriched in the invasive margin and correlated with patient prognosis. 19,35,36 In colorectal cancer, the heterogeneous density and location of T cell subsets in the tumor microenvironment have also been identified as a superior prognostic factor to the traditional TNM stage. 37 Consistent with these previous studies, distinct infiltration of immune subsets within each subregion was observed and contributed differentially to the prognosis of cHCC-ICC patients, suggesting that the subregion-specific immune enrichment was a promising prognostic factor for cHCC-ICC patients. Besides, it has been demonstrated that immune cell density may affect the response to immunotherapy, 38 as exemplified by the findings that PD1+ cell was a potential biomarker for anti-PD-1-immunotherapy in head and neck cancer. 39 HCC, 40 and ICC. 41 Of note, we found that the distribution of PD1+ cells was highly heterogeneous among HCC and ICC subregions, which may result in different responses to PD1 subregions. This assumption was consistent with a recent report that showed heterogeneous immune microenvironment among liver, lung, and peritoneum metastases from the same colorectal cancer patients, leading to either sensitivity or resistance to immunotherapy of each metastasis. 42

In our study, the immune cluster based on the density of immune cells indicated that patients were able to be divided into two subgroups with the different distribution of immune cells. The cluster 1 was abundant in CD3+ and CD8+ T cells across all tumor subregions, whereas the cluster 2 was relatively in shortage of both T-cell subsets across all subregions. This distribution fitted the classical “hot and cold” category. 28,43,44 Meanwhile, an immune score was established by the lasso-cox method that selected immune variables from both HCC and ICC subregions, indicating that the prognosis of cHCC-ICC patients was a complex interaction of both components. However, this immune score was derived from a mathematic algorithm, and its biological significance still needs further investigation. Further prospective analysis with a larger cohort of cHCC-ICC patients and evaluating more immune subsets such as B cells and dendritic cells are needed for an in-depth understanding of the clinical relevance of heterogeneous immune microenvironment in this fetal malignancy.

5 | CONCLUSION

In conclusion, our results demonstrated the spatial heterogeneity of immune microenvironment in cHCC-ICC, possibly due to the distinct genetic background of the two types of liver cancer. Prognostic immune indices were established based on the density of immune cells in HCC and ICC components, indicating that the prognosis of cHCC-ICC patients was a complex interaction of both cancer components.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

QG, JYS, and BHZ were associated with conception and design of the study. BHZ, JQM, and LYT performed the experiments. QG, JYS, BHZ, and JQM drafted the manuscript. BHZ, LQD, GHS, JMP, YML, and SXY drafted
and interpreted the data. XYW, JZ, JF, XMZ, JYS, and QG reviewed and edited the manuscript. All authors approved the manuscript.

**DATA AVAILABILITY**

Data are available upon reasonable request. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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