CLEC1B Expression and PD-L1 Expression Predict Clinical Outcome in Hepatocellular Carcinoma with Tumor Hemorrhage

Kuan Hu*, Zhi-Ming Wang*, Juan-Ni Li†, Sai Zhang‡, Zhong-Fu Xiao§ and Yi-Ming Tao*

*Department of Hepatobiliary Surgery, Xiangya Hospital, Central South University, Changsha, Hunan, China; †Department of Pathology, Xiangya Hospital, Central South University, Changsha, Hunan, China; ‡Institute of Medical Sciences, Xiangya Hospital, Central South University, Changsha, Hunan, China; §Department of Anesthesiology, Xiangya Hospital, Central South University, Changsha, Hunan, China

Abstract
Spontaneous tumor hemorrhage (TH) is frequently observed in solid tumors including human hepatocellular carcinoma (HCC). TH implies fast-growing and worse tumor immunological microenvironment; however, the underlying mechanism remains largely unknown. CLEC1B is a signature gene highly associated with tumor progression. PD-L1 expression is a key biomarker predictive of immune checkpoint therapies, which showed astonishing effect on various types of tumor. We assume that, in HCC, TH may closely associate with the expression of these two molecules. In this study, 136 patients with HCC were enrolled. qRT-PCR showed that CLEC1B expression is significantly lower in HCC tumor tissue. Immunohistochemistry of HCC tissue microarrays demonstrated that PD-L1high and CLEC1Blow expressions were significantly correlated with TH and clinicopathological features indicating worse HCC progression. According to univariate/multivariate analysis, a combination of PD-L1high and CLEC1Blow expression was an independent prognostic factor indicating the poor outcome. The prognostic value of PD-L1high and CLEC1Blow was validated by Cox proportional-hazard analyses. Collectively, tumor with TH is closely associated with CLEC1Blow & PD-L1high expression, which may imply high response of PD-L1/PD-1 immune checkpoint therapies. CLEC1B may be a potential therapeutic target for PD-L1/PD-1 immunotherapy. PD-L1high and CLEC1Blow can be a valuable prognosis factor implying worse clinical outcomes.

Introduction
Hepatocellular carcinoma (HCC) is one of the most common tumors in the world [1]. Increasing evidence supports that tumor microenvironment, which has high heterogeneity among different individuals with HCC, plays a pivotal role in regulating HCC progression [2,3]. Tumor microenvironment in HCC is composed of growth factors or inflammatory cytokines, stromal cells, and extracellular matrix proteins [4]. Some characteristics such as hypoxia and tumor hemorrhage also belong to the category of the tumor microenvironment. Change of the tumor microenvironment can result in totally different aggressive type of HCC [5].

As an important factor of the tumor microenvironment, tumor hemorrhage (TH) is recognized to be involved in tumor growth and metastasis. It is known that during TH, red blood cells and platelets aggregate around tumor cells, facilitating the formation of cancer cell nests and playing a protective role from immune responses and shear stress. Activated platelets also secret some growth factors which could markedly promote angiogenesis and tumor growth [6]. So the presence of TH in HCC specimens may be one key malignant clinicopathological feature of HCC. Exploration of the molecular

Address all correspondence to: Yi-Ming Tao, 87 Xiang Ya Road, Changsha, Hunan, China 410008. E-mail: yimingtao@cusu.edu.cn

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contexts associated with these pathogenesis changes will help to identify novel targets for HCC metastasis treatment.

C-type lectin domain family 1 member B (CLEC1B) is a novel platelet-related molecule that we assumed is associated with TH in HCC. This molecule is secreted by the activated platelets around tumor and proved to have an inhibitory effect on platelet aggregation and tumor metastasis by binding to the surface of tumor cells in colon carcinoma [7]. Although, recently, CLEC1B has been reported to have a dramatic downregulation in the tumor of HCC [8], the role of CLEC1B in HCC remains mostly unclear.

These days immunological microenvironment has been extensively studied for its role in HCC progression. The immune checkpoint therapy based on immunological microenvironment showed a surprising curative effect against some types of cancers [9]. However, immune therapies applied to HCC have not shown very satisfying responses for all patients, suggesting that more underlying mechanism remains to be revealed [10]. Antibodies targeting programmed cell death protein 1 (PD-1)/programmed cell death 1 ligand 1 (PD-L1) are representative antitumor immunotherapies that have been successfully applied in some clinical trials for several cancers including HCC [11]. Therapeutic mechanism is that blocking the interaction between PD-1 and PD-L1 results in dramatic increase of anticancer immune response [12]. Since anti-PD-1/PD-L1 therapy only works on about 30% of patients, the biomarkers predicting this effect are urgently needed. Data from these clinical trials showed that PD-L1 immunohistochemical expression in the tumor can predict the effect of anti-PD-1/PD-L1 therapies in many tumor types. In addition, high expression of PD-L1 also worked as a prognostic biomarker indicating poor clinical outcomes in several studies [13]. So, a study about PD-L1 in HCC is of great value.

There is, to the best of our knowledge, no study about the correlation among PD-L1, CLEC1B expression, and TH in HCC. In this present study, we aim to investigate the expression of both PD-L1 and CLEC1B in a cohort of HCC patients and explore their potential correlation with clinicopathologic parameters, especially TH, as well as the clinical outcomes.

**Materials and Methods**

**Patient Populations and Specimens**

This study was approved by the Ethics Committee of Xiangya Hospital, China. Informed consent was obtained from each patient as required for research purposes. One hundred thirty-six patients with HCC were enrolled. HCCs were diagnosed according to the current World Health Organization criteria. All the patients were treatment-naive before surgery. Patient follow-up was terminated on 31 January 2017. The clinical outcomes of HCC patients are summarized in Table S1. The clinical and pathological characteristics from Table 1 were comprehensively recorded. All research protocols strictly complied with REMARK guidelines for reporting prognostic biomarkers in cancer [14]. Fresh paired specimens of HCC and adjacent nontumorous liver tissue were randomly collected from HCC patients undergoing hepatic resection between September 2011 and March 2012 in the Department of Hepatobiliary Surgery, Xiangya Hospital, China.

**Bioinformatics Analysis**

We use Tumor Immune Estimation Resource (TIMER) web server (https://cistrome.shinyapps.io/timer/), a comprehensive analytic web tool which reanalyzed data from The Cancer Genome Atlas, Table 1. Correlation among PD-L1 and CLEC2 Expression and Clinicopathological Characteristics in 136 HCC Patients

| Clinicopathologic Variable              | n   | PD-L1 Expression | CLEC2 | P Value | P Value |
|-----------------------------------------|-----|------------------|-------|---------|---------|
| Patient number                          | 136 | 26               | 110   | .819    | 40      |
| Age (years)                             |     |                  |       |         | 96      |
| ≤60                                     | 81  | 16               | 65    | .946    | 24      |
| >60                                     | 55  | 10               | 45    | 57      | 16      |
| Sex                                     |     |                  |       |         | 39      |
| Male                                    | 107 | 21               | 86    | .829    | 31      |
| Female                                  | 29  | 5                | 24    | 37      | 9       |
| HBsAg                                   |     |                  |       |         | 20      |
| Negative                                | 12  | 4                | 8     | .329    | 5       |
| Positive                                | 124 | 22               | 102   |         | 7       |
| AFP (ng/ml)                             |     |                  |       |         |         |
| ≤20                                     | 55  | 6                | 49    |         | 35      |
| >20                                     | 81  | 20               | 61    | .143    | 20      |
| CPC                                      |     |                  |       |         | 61      |
| A                                        | 109 | 21               | 90    | .149    | 29      |
| B                                        | 27  | 7                | 20    |         | 80      |
| Liver cirrhosis                         |     |                  |       |         |         |
| Absence                                 | 15  | 5                | 10    | .340    | 6       |
| Presence                                | 121 | 21               | 100   |         | 9       |
| Tumor encapsulation                     |     |                  |       |         |         |
| Absence                                 | 50  | 13               | 37    | .094    | 120     |
| Presence                                | 86  | 13               | 73    |         | 19      |
| Tumor size (cm)                         |     |                  |       |         | 31      |
| ≤5                                      | 45  | 4                | 41    | .21     | .033    |
| >5                                      | 91  | 22               | 69    |         | 19      |
| Tumor number                            |     |                  |       |         | 26      |
| Single                                  | 73  | 10               | 63    | .190    | .084    |
| Multiple                                | 63  | 16               | 47    |         | 18      |
| Satellite nodules                       |     |                  |       |         | 55      |
| Absent                                  | 54  | 5                | 49    | .006    | .018    |
| Present                                 | 82  | 21               | 61    |         | 23      |
| Vascular invasion                       |     |                  |       |         | 31      |
| Absent                                  | 52  | 4                | 48    | .009    | .008    |
| Present                                 | 84  | 22               | 62    |         | 22      |
| Tumor differentiation                   |     |                  |       |         | 30      |
| I-II                                    | 69  | 8                | 61    | .032    | .024    |
| III-IV                                  | 67  | 18               | 49    |         | 26      |
| Tumor hemorrhage                        |     |                  |       |         | 43      |
| Absent                                  | 60  | 4                | 56    | .002    | .001    |
| Present                                 | 76  | 22               | 54    |         | 26      |
| TNM stage                               |     |                  |       |         | 34      |
| I                                        | 43  | 4                | 39    | .078    | .048    |
| II-III                                  | 93  | 22               | 71    |         | 17      |

Abbreviations: CPC, Child-Pugh classification; HBsAg, hepatitis B surface antigen.
to detect the CLEC1B expression in various cancer types. “DiffExp module” was used to study the difference in mRNA expression of CLEC1B between tumor and adjacent normal tissue [15]. GEPIA web server (http://gepia.cancer-pku.cn/) was used to generate the Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) stratified by CLEC1B [16]. Data from 334 patients with HCC were enrolled. Group cutoff was set as “median.”

**Quantitative Real-Time RT-PCR (qRT-PCR)**

The procedures of RNA isolation, reverse transcription, and SYBR Green fluorescent-based qRT-PCR were performed as previously described [17]. Primer sequences of CLEC1B (accession number NM_016509.3) are as follows: forward: 5′-ATTCTGCTGATCCTGTGCGT-3′; reverse: 5′-TCCAGTTTGTGTCA CAGGGG-3′.

**Tissue Microarray (TMA) and Immunohistochemistry (IHC) Analysis**

TMAs of representative HCC tissues and adjacent normal tissues were assembled as recommended. IHC staining was performed on TMA. The following antibodies were used: anti–PD-L1 (rabbit monoclonal 2 μg/ml; Abcam, Cambridge, MA) and anti-CLEC1B (ab197349 1:50; Abcam, Cambridge, MA). IHC staining was performed as previously described [17]. All IHC results were reviewed by two independent pathologists in our hospital. PD-L1 expression was considered as high when the proportion of cells with membranous staining was >1% in all the neoplastic cells [18]. CLEC1B expression was assessed as high if a moderate or strong membranous staining was observed.

**Statistical Analysis**

Statistical analysis was performed using Prism software (v.7.01; GraphPad Prism Software, La Jolla, CA) and SPSS 24.0 software (SPSS, Chicago, IL). Quantitative values are presented as mean ± SD or median (range). Spearman’s correlation coefficient (r) was used to access the correlation between PD-L1 and CLEC1B expression. \( \chi^2 \) test was applied to categorical data. The recurrence-free survival (RFS) and OS were evaluated using the Kaplan-Meier method and the log-rank test. Prognostic factors of RFS and OS were analyzed by univariate and multivariate analyses. Cox proportional-hazards regression model was used to determine if CLEC1B expression combined with PD-L1 has prognostic value. \( P < .05 \) was considered to be statistically significant.

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**Figure 1.** CLEC1B mRNA level is significantly reduced in tumors of LIHC and associated with poor clinical outcome. (A) Differential expression of CLEC1B between tumor and adjacent normal tissues from various types of cancer. Data are extracted from TIMER web server. ***P < .001. (B) Kaplan-Meier analysis of OS and DFS stratified by CLEC1B. Data are generated from GEPIA web server. Abbreviation: LIHC, liver hepatocellular carcinoma.
Results

Patient Demographics

Patients were most males (107/136, 78.6%). There are 124 patients with HBV infection; 81 patients have a high preoperative serum alpha-fetoprotein (AFP) level (>20 ng/ml, 59.6%). Patients were classified as A (109, 80.1%) or B (27, 19.9%) according to Child Purge Classification. For the pathological features, the tumor mean size was 7.5 (2-17.4) cm. Multiple tumor number, tumor encapsulation, satellite nodules, vascular invasion, and liver cirrhosis were observed in 63 (46.3%), 86 (63.2%), 82 (60.2%), 84 (61.7%), and 121 (88.8%) patients, respectively. The distribution of tumor-node-metastasis (TNM) stage in patients is as follows I (43/136, 31.7%) and II to III (93/136, 68.3%). Seventy-six (55.9%) of HCC tumor samples were considered TH positive according to tumor with/without hemorrhage.

Association between CLEC1B and TH and Poor Survival

As Figure 1A showed, by using web server of TIMER, CLEC1B mRNA expression was proven to decrease dramatically in HCC when compared with adjacent normal liver tissues (P < .001). Survival analysis from GEPIA web server demonstrated that lower expression of CLEC1B indicates poorer survival rate, both for OS (P = .017) and DFS (P = .014) (Figure 1B). In order to confirm this, we tested CLEC mRNA expression in cohort by qRT-PCR. As expected, consistent result was obtained. The CLEC1B expression is significantly lower in HCC tumor tissues (P < .01). In addition, when 76 tumors of HCC are classified into subgroup by TH or not (Figure 2A), tumors with TH showed even less expression of CLEC1B (P < .001) (Figure 2B).

CLEC1B and PD-L1 Expression and Their Association with Clinicopathological Features

CLEC1B membranous staining was detected in 29.4% (40/136) of tumors (Table 1). PD-L1 was assessed to have membranous reactivity in 19.1% (26/136) of tumors (Table 1). Representative images of CLEC1B and PD-L1 IHC staining are shown in Figure 3. The correlation between these two molecules and clinicopathological
features is shown in Table 1. It is low expression of CLEC1B that was significantly correlated with markers of HCC progression, including tumor size ($P = .021$), satellite nodules ($P = .006$), vascular invasion ($P = .009$), and tumor differentiation ($P = .032$). As expected, TH was markedly related to low CLEC1B protein level ($P = .002$).

On the contrary, high PD-L1 expression was significantly correlated with high AFP levels ($P = .045$), tumor size ($P = .033$), satellite nodules ($P = .018$), vascular invasion ($P = .008$), and tumor differentiation ($P = .024$), all of which indicate worse HCC progression. Notably, high PD-L1 was also significantly associated with TH ($P = .001$), suggesting a high level of red blood cells/platelets infiltration and hypoxia.

### Prognostic Role of CLEC1B and PD-L1

Univariate analysis revealed the following features as prognostic factors related with RFS and OS: high AFP level, tumor diameter (>5 cm), multiple tumor number, vascular invasion, tumor differentiation, satellite nodules, TNM stage, TH, high PD-L1 expression, low CLEC1B expression, and combination of CLEC1Blow and PD-L1high expression (Table 2). Multivariate analysis further screened that only tumor diameter (hazard ratio [HR] = 1.413, $P = .023$; HR = 1.313, $P = .038$), satellite nodules (HR = 1.705, $P = .011$; HR = 1.724, $P = .014$), TNM stage (HR = 1.564, $P = .012$; HR = 1.760, $P = .01$), PD-L1 expression (HR = 2.152, $P = .001$; HR = 1.936, $P = .006$), CLEC1B expression (HR = 2.395, $P = .0004$; HR = 2.124, $P = .001$), and combination of CLEC1Blow and PD-L1high expression (HR = 4.827, $P < .0001$; HR = 3.944, $P < .0001$) behaved as independent predictors of RFS and OS (Table 2).

Next, survival analysis of OS and RFS was performed in the cohort to assess the predictive value of CLEC1B integrated with PD-L1. Three risk groups were stratified by CLEC1B and PD-L1 expression: group I (32/136, 23.5%), CLEC1Bhigh and PD-L1low; group II (83/136, 61.0%), PD-L1High and CLEC1BLow; and group III (21/136, 15.4%), CLEC1BLow and PD-L1High. Notably, the 1- and 3-year OS rates in group III were significantly lower than those in II and I groups (52.8% versus 73.4% and 23.5% versus 61.0%, respectively).
among these significant markers, the presence of TH was frequently observed by us in the postoperative tumor specimen of HCC (Table 1, Figure 2A). Emerging evidence supported the strong association between TH and tumor microenvironment [22,23]. Complicated microenvironment in tumor results in high heterogeneity of HCC, making the therapeutic effect totally varied. TH in HCC implies tumor microenvironment of red blood cells releasing and platelet aggregation, which could promote tumor growth, invasion, and metastasis partially through the activation of NF-kB pathway [24,25]. The inflamed tumor microenvironment is another cause of tumor vessel injury [26]. Tumor immune microenvironment also closely interacts with hemorrhage. For instance, peritumoral T-cell infiltrates in melanoma are shown to have an association with low tumor hemorrhage, both of which reflect lower risk of metastases [27]. This evidence, to some extent, is in accordance with our finding between TH and PD-L1. Our study is the first to show that TH in HCCs displayed high PD-L1 expression.

Taken together, we believe further exploration is worthy following the direction of TH and PD-L1. We hypothesized that CLEC1B may be another key molecule related to TH because growing evidence showed that: 1) CLEC1B makes an inhibitory contribution to platelet aggregation [28]; 2) CLEC1B is significantly downregulated in HCC [8]; and 3) CLEC1B is involved in metastasis of various cancer types [29]. However, in the field of HCC, we did not find any data exploring the correlation among clinicopathological features, CLEC1B, and clinical outcomes. In our study, analytical data returned from TIMER and GEPIA showed that decreased CLEC1B expression in HCC is associated with poor prognosis. This decreased CLEC1B expression in tumor was further confirmed by qRT-PCR in our cohort. Interestingly, opposite to high PD-L1 expression, it is low CLEC1B expression that associated with clinicopathological features indicating progressive HCC. Additionally, as expected, there is a prominent correlation between low CLEC1B expression and high risk of TH. This correlation was also supported by the qRT-PCR result, which showed the lowest CLEC1B mRNA expression in tumor with TH. Based on the above findings, we provided evidence that TH, the frequent clinicopathologic observation in tumors of HCC, may be a preliminary sign for screening patients with high PD-L1 expression; CLEC1B may be the biomarker reflecting TH.

PD-L1 together with CXCL12, an inflammation-related chemokine, has been reported to be an independent prognosis factor [30]. Coinciding with these studies, our research data were the first to validate the predictive role of CLEC1B and PD-L1 in the prognosis of HCC. Patients with the expression pattern of CLEC1Blow and PD-L1high had the worst survival outcome. This intriguing finding also indirectly reflected that HCC tumor with hemorrhage could be a clinical-pathologic feature indicating bad outcome.

In conclusion, this work is the first to reveal that HCC with hemorrhage is closely associated with CLEC1Blow and PD-L1high expression, therefore providing some estimation about whether to use PD-L1/PD-1 immune checkpoint therapies. CLEC1B may be a potential therapeutic target for PD-L1/PD-1 immunotherapy. CLEC1Blow and PD-L1high can be a valuable prognosis factor implying worse clinical outcomes.

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