JGH Journal of Gastroenterology and Hepatology
JGHF
doi:10.1111/jgh.15475

HEPATOLOGY

Programmed cell death-ligand 1 expression in hepatocellular carcinoma and its correlation with clinicopathological characteristics

Haibo Mou,*[1] Qiu-an Yang,[†1] Lanfang Yu,* Ting Wang,[‡] Kui Liu,[§] Rong Shen,[¶] Xuedong Pan,** Yi Dai,** Qing Wan,** Fangling Zhou,** Lili Qian,** Donglin Chen,** Thomas Yau,[††] Xiaowei Dong,[‡‡] Xuemei Wang[§§] and Shuang Wang[¶¶,***]

Departments of *Medical Oncology, [‡]Hepatobiliary and Pancreatic Surgery, Shulan (Hangzhou) Hospital Affiliated to Zhejiang Shuren University Shulan International Medical College, Hangzhou, Zhejiang, [†]Cancer Center, Qilu Hospital of Shandong University, [¶]Department of Chemotherapy, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, [§]Department of Hepatobiliary Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, **Department of Clinical Pathology, OrigiMed, Minhang, [‡‡]Medical Laboratory, OrigiMed, Jiading, Shanghai, [††]Department of Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, [§§]Department of Pathology, China-Japan Union Hospital of Jilin University, Changchun, [¶¶]Department of Pathology, Nanfang Hospital, Southern Medical University, and ***Department of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China

Key words
E–S grade, Hepatocellular carcinoma, Immunohistochemistry, PD-L1, *TP53*, Tumor mutational burden.

Accepted for publication 22 February 2021.

Correspondence
Shuang Wang, Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China.
Email: shuangw@126.com

Xuemei Wang, Department of Pathology, China-Japan Union Hospital of Jilin University, Changchun 130033, China.
Email: wangxuemei@jlu.edu.cn

Declaration of conflict of interest:
All authors approved the submitted version of the manuscript, and the manuscript is not under consideration elsewhere. X. P., Y. D., Q. W., F. Z., L. Q., D. C., and X. D. are employees of OrigiMed. The other authors have no conflicts of interest to declare.
Author contribution: H. M., Q. Y., L. Y., T. W., K. L., R. S., and X. P. analyzed and interpreted the patient data. Y. D., Q. W., L. Q., F. Z., D. C., X. D., and S. W. performed the histological examination. H. M., Q. Y., X. P., T. Y., X. W., and S. W. are the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Abstract

Background and Aim: Programmed cell death-ligand 1 (PD-L1) immunohistochemistry score has been approved as the predictive biomarker for anti-PD1/PD-L1 therapy in several advanced malignancies. Although its predictive role remained inconclusive in hepatocellular carcinoma, ongoing study of anti-PD1/PD-L1 therapy showed promising results. However, less is known about the PD-L1 immunohistochemistry score and factors correlated with it in hepatocellular carcinoma. We investigated PD-L1 immunohistochemistry scores in a large cohort of hepatocellular carcinoma, as well as its correlation with various clinical and genomic factors.
Methods: Immunohistochemistry was performed to detect the expression of PD-L1 protein in 315 hepatocellular carcinoma tissues. All slides were independently reviewed by three senior pathologists. Next-generation YS panel (450 genes) sequencing was performed on 309 patients.
Results: Higher PD-L1 expression as measured by combined positive score (CPS) was associated with increased Edmondson–Steiner grade (grade III *vs* II, $P = 0.041$) and *TP53* mutations ($P = 0.021$). PD-L1 CPS had no correlation with tumor mutational burden (Spearman's correlation coefficient 0.067). PD-L1 CPS was not significantly associated with hepatitis B virus infection.
Conclusions: Our data indicated that patients with higher Edmondson–Steiner grade (grade III) had significantly higher PD-L1 CPS than patients with lower Edmondson–Steiner grade (grade II). Patients with *TP53* mutations had significantly higher PD-L1 expression.

Informed consent: All selected cases were informed, and a written informed consent of the patient was received according to the protocols and procedures approved by the institutional review board.

Financial support: Not available.
[†]These authors contributed equally to this work.

Introduction

Liver cancer causes over 750 000 deaths annually worldwide, and its incidence rate is still on the rise.[1,2] Hepatocellular carcinoma (HCC) accounts for most liver cancers, and it is frequently caused by chronic infections of hepatitis B (HBV) or hepatitis C viruses that led to inflammation and cirrhosis of liver. Most HCC patients are diagnosed at an advanced stage, for which not many systemic

therapies are available. A few multi-targeted tyrosine kinase inhibitors[3–7] and a monoclonal antibody against VEGFR2[8] were approved by the US Food and Drug Administration in recent years in the first-line or second-line settings for advanced HCC. However, the benefit in median overall survival was usually only a few months.

In recent years, immunotherapy has emerged as a promising cancer treatment due to the success of immune checkpoint inhibitors in many types of cancer. Two anti-programmed cell death-1 (PD-1) receptor checkpoint inhibitors, nivolumab and pembrolizumab, have demonstrated durable antitumor activity and long-term overall survival benefit in melanoma, non-small-cell lung cancer, and other malignancies.[9,10] In advanced HCC, both drugs were approved by the US Food and Drug Administration as second-line therapies based on promising single-arm phase II trial results achieving about 20% objective response rates.[7,11] Yet recently, the phase III trial of pembrolizumab as second-line therapy for advanced HCC (KEYNOTE-240) failed to show statistically significant improvement in progression-free survival and overall survival[12]; the phase III trial of nivolumab as first-line treatment for unresectable HCC evaluated against sorafenib (CheckMate-459) also failed to meet overall survival endpoint.[13] These results cast doubts on the clinical benefits that anti-PD-1 agents can bring to advanced HCC treatment and highlight the importance of identifying effective biomarkers predictive of response for patient selection.

Programmed cell death-ligand 1 (PD-L1) expression and tumor mutational burden (TMB) are the best studied biomarkers for checkpoint inhibitor response. Many studies have confirmed the association of elevated PD-L1 expression and higher TMB with clinical benefit to checkpoint inhibitors across different cancer types, and several immunohistochemistry (IHC) staining assays for PD-L1 have been developed as companion diagnostics.[14,15] HCC studies based on small cohorts have reported controversial results on the association between PD-L1 expression and response to anti-PD-1 therapy.[11,16] This is further complicated by the fact that there is considerable inter-assay variation of IHC results due to the use of different antibodies for detection, in particular for HCC.[17] Studies of large cohorts in the West have reported that less than 20% of HCC tumors were PD-L1 positive, and few HCC tumors had high TMB.[18,19] However, the etiology of HCC had considerable difference between the West[20,21] and Asian (the majority of HCC patients in China was HBV infected), and few studies have investigated the landscape of PD-L1 positivity and TMB in HCC patients.

In this study, we report the distribution of PD-L1 IHC scores assayed by Abcam 28-8, in a retrospective cohort of 315 HCC patients. We also explore the relationship between PD-L1 positivity with TMB and other clinical, pathological, and molecular variables in this cohort.

Materials and methods

Patients. Our cohort was a hospital-based retrospective cohort; 315 formalin-fixed, paraffin-embedded (FFPE) tumor tissue and matched normal blood were collected from patients with a diagnosis of primary HCC from different hospitals in China (detailed description in Table S1), all selected patients were informed, and a written informed consent of the patient was received. Immunohistochemistry analysis of PD-L1 protein expression was performed in all cases. DNA from 309 HCC patients was extracted, and next-generation sequencing (NGS) was performed.

Programmed cell death-ligand 1 immunohistochemistry staining. We performed IHC staining of FFPE tissue sections for PD-L1 protein using anti-PD-L1 antibodies clone 28-8 (Cat#ab205921, Abcam, Cambridge, UK) on the Dako Autostainer Link 48. Briefly, all slides were baked at 60 °C, deparaffinized in xylene, and rehydrated with graded ethanols to distilled water. Then antigen retrieval was performed using Dako's universal heat-induced epitope retrieval antigen reagent for 4 min at 99 °C in a pressure cooker. Nonspecific binding was blocked with the Dako EnVision FLEX peroxidase-blocking reagent for 10 min at room temperature. Dilutions 1:300 of the primary antibodies for 28-8 were used for antigen detection. All other staining was performed primarily with Dako series reagents (Cat#K8002; Dako, Carpinteria, CA, United States). According to the key guideline of PD-L1 testing, tonsil tissue had been selected for positive control tissue in each staining run. The PD-L1 expression in tonsil tissue should show strong staining in portions of the crypt epithelium and weak to moderate staining of the follicular macrophages in the germinal centers. PD-L1 expression of the endothelium, fibroblasts, and surface epithelium should be negative. In addition, tonsil stained with negative reagent shows no staining in the crypt epithelium and the follicular macrophages in the germinal centers, which makes sure of the specificity of staining. Meanwhile, the specimen of HCC samples stained with negative reagent shows the absence of cell membrane staining of viable tumor cells, lymphocyte, and macrophage, which excludes the nonspecific background staining of the sample.

Evaluation of programmed cell death-ligand 1 immunohistochemistry staining and histopathological grade. The IHC-stained tissue sections were independently reviewed and scored by three pathologists from different institutions using tumor proportion score (TPS) and combined positive score (CPS).[22] All the tissue sections were stained by hematoxylin and eosin, followed by histopathological grading according to Edmondson–Steiner (E–S) grade.[23] E–S grade was extracted from clinical notes (n = 315).

Panel sequencing. Genomic profiling was performed in a College of American Pathologists and Clinical Laboratory Improvement Amendments-certified laboratory in OrigiMed (Shanghai, China). At least 50 ng of malignant tissue DNA was extracted from tumor sample using a DNA Extraction Kit (QIAamp DNA FFPE Tissue Kit) according to the manufacturer's protocols, and DNA from paired blood samples was also extracted using QIAsymphony DSP DNA Kit. NGS was performed on hybridization-captured libraries of 450 clinically relevant cancer genes (cancer sequencing YS panel, CSYS[24]) in a College of American Pathologists-certified laboratory, to detect all classes of somatic genomic alterations including substitutions, short and long indels, copy number alterations, and gene rearrangements. TMB was calculated from the sequencing results for each patient

according to Cao *et al*.[24] Patients with more than 10 muts/Mb are definite as high TMB.

Data analysis. All statistical analyses were performed by R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria). For each sample, the median TPS or CPS from three pathologists was used as the consensus score. PD-L1 scores were binarized using cutoffs according to Shi *et al*.,[25] and consistency among pathologists was measured by Fleiss' kappa statistic (for binarized data) and intraclass correlation coefficient (for numerical values).

Kruskal–Wallis test was used to test the difference of PD-L1 CPS among all E–S grades, and Wilcoxon rank-sum test was then performed to test the difference of PD-L1 CPS between pairs of E–S grades. Spearman's correlation coefficient was used to evaluate the correlation between PD-L1 expression, and age of first diagnosis and α-fetoprotein. The function "spearman.test" in the R library "pspearman" was used to perform Spearman's rank correlation analysis, and $P < 0.05$ was considered statistically significant. Wilcoxon rank-sum test was used to test the difference of PD-L1 expression between male/female, primary/metastatic/relapse, and cirrhosis status. Kruskal–Wallis test was used to test the difference of PD-L1 CPS among stages. Wilcoxon rank-sum test was used to test the association of mutations with PD-L1 expression, and Benjamini–Hochberg adjusted $P < 0.05$ was considered as statistically significant.

Results

Demographics of our hepatocellular carcinoma cohort. Our cohort included 315 HCC patients with demographic and clinical information (Table 1). The median age of first diagnosis was 55 years (range, 16–83), and 87.6% (276/315) of the patients were male. Clinical information was available for most patients, and the proportions in the succeeding text were estimated based on available information only. Among 315 patients, 304 patients had virus infection test result record. Review of the clinical information revealed that 82.5% (260/315) of the patients were infected by HBV; 28.3% (89/315) had liver cirrhosis; and 73.3% (231/315) were of E–S grade II or III, including 41.9% (132/315) grade II patients and 31.4% (99/315) grade III patients. According to the eighth edition of the TNM staging system, 34.0% (107/315) of the patients were of stage III. Stage I, stage II, and stage IV each accounted for approximately one-fifth of the patients. From 315 of patients, 334 tissues were obtained; 89.2% (298 334) were samples from the primary site. Metastatic or relapse samples accounted for 5.7% (19/334) and 4.5% (15/334), respectively; 80.8% (270/334) of the samples were resected, and the remaining 19.2% (64/334) were from biopsy.

Table 1 Demographics of patients in the HCC cohort

Categories	Number
Number of patients	315
Median age of first diagnosis (years)	55 (range, 16–83)
Method	
PD-L1 staining	315
Next-generation sequencing	309
PD-L1 staining	
TPS < 1%	215 (68.3%, 215/315)
TPS ≥ 1%	37 (11.7%, 37/315)
Uncertain (TPS)	63 (20%, 63/315)
CPS < 1	256 (81.3%, 256/315)
CPS ≥ 1	59 (18.7%, 59/315)
Gender	
Male	276 (87.6%, 276/315)
Female	39 (12.4%, 39/315)
Histological subtype	
Hepatocellular carcinoma	315 (100%)
Viral infection (hospital reported)	
HBV	260 (82.5%, 260/315)
Not infected	44 (14.0%, 44/315)
Unknown	11 (3.5%, 11/315)
Liver cirrhosis	
Yes	89 (28.3%, 89/315)
No	218 (69.2%, 218/315)
Unknown	8 (2.5%, 8/315)
Edmonson–Steiner grade	
I	15 (4.8%, 15/315)
II	132 (41.9%, 132/315)
III	99 (31.4%, 99/315)
IV	4 (1.3%, 4/315)
I–II	9 (2.9%, 9/315)
II–III	56 (17.8%, 56/315)
Stage	
I	51 (16.2%, 51/315)
II	56 (17.8%, 56/315)
III	107 (34.0%, 107/315)
IV	47 (14.9%, 47/315)
III–IV	4 (1.3%, 4/315)
Unknown	50 (15.9%, 50/315)
Primary site or not (334 tissues from 315 patients)	
Primary site	298 (89.2%, 298/334)
Metastatic site	19 (5.7%, 19/334)
Relapse site	15 (4.5%, 15/334)
Unknown	2 (0.6%, 2/334)
Resection or biopsy (334 tissues from 315 patients)	
Resection	270 (80.8%, 270/334)
Biopsy	64 (19.2%, 64/334)

CPS, combined positive score; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PD-L1, programmed cell death-ligand 1; TPS, tumor proportion score.

Distribution of programmed cell death-ligand 1 combined positive score and tumor proportion score. Three senior pathologists reviewed 334 slides from 315 patients (six patients had three slides from different tumor sections assayed, and seven patients had two slides from different tumor sections assayed; the slide with the highest score was chosen for subsequent analyses). Overall, the three pathologists had modest agreement on TPS and CPS (Fleiss' kappa coefficient for binarized TPS/CPS between 0.63 and 0.93; Table 2). For each slide, the median of the three pathologists' scores was calculated and used as the consensus score. Distribution of PD-L1 TPS and CPS in this cohort was shown in Figure 1. Nearly 20% of HCC patients were

Table 2 Concordance among the three pathologists measured by Fleiss' kappa (for binarized data) or ICC (for specific numbers such as 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90)

Cutoffs	Kappa/ICC (95% CI)
CPS (binarized at 1)	0.75 (0.69, 0.81)
CPS (binarized at 10)	0.86 (0.77, 0.94)
CPS (binarized at 20)	0.80 (0.65, 0.91)
TPS (binarized at 1%)	0.63 (0.53, 0.74)
TPS (binarized at 10%)	0.67 (0.45, 0.82)
TPS (binarized at 25%)	0.80 (0.45, 1.0)
TPS (binarized at 50%)	0.80 (0.50, 1.0)
CPS (specific numbers)	0.93 (0.92, 0.94)
TPS (specific numbers)	0.86 (0.83, 0.88)

CI, confidence interval; CPS, combined positive score; ICC, intraclass correlation coefficient; TPS, tumor proportion score.

PD-L1 CPS positive (CPS ≥ 1), and more than 10% of HCC patients were PD-L1 TPS positive (TPS ≥ 1%).

Programmed cell death-ligand 1 combined positive score was not significantly associated with hepatitis B virus infection status. In 315 patients, HBV infection status of 260 patients was positive, while that of 44 patients was negative, according to clinical notes. The remaining 11 patients were unknown. No significant difference in PD-L1 CPS was observed between the HBV-infected group and the group without HBV infection (P = 0.15, Wilcoxon rank-sum test; supplementary plot in the Supporting Information).

Patients with Edmondson–Steiner grade III had significantly higher programmed cell death-ligand 1 combined positive score than Edmondson–Steiner grade II patients. Three hundred fifteen patients performed PD-L1 IHC examination, while E–S grade I–II (n = 9), E–S grade II–III (n = 56), and E–S grade IV (n = 4) patients were not included to explore the correlation of PD-L1 expression as measured by CPS and E–S grades. Hence, in the following investigation, 246 patients with specific E–S grades were included. E–S grade III patients had significantly higher PD-L1 CPS than E–S grade II patients (Benjamini–Hochberg corrected P = 0.041, Wilcoxon rank-sum test), and E–S grade III patients also had significantly higher PD-L1 CPS than E–S grade I patients (Benjamini–Hochberg corrected P = 0.11, Wilcoxon rank-sum test) (Fig. 2).

Association of programmed cell death-ligand 1 combined positive score with tumor mutational burden and gene mutations. Numerous studies have shown that TMB and PD-L1 expression are associated with response to immune checkpoint blockade in lung cancer and that these two biomarkers are independent predictors that only correlate weakly.[26,27] We explored the correlation between TMB and the PD-L1 expression in this cohort to determine if the same trend can be observed in HCC. In 315 patients, 309 had both PD-L1 expression examination and NGS data. TMB was calculated based on NGS result. Therefore, 309 patients were included in the following analysis. Across all individuals in this cohort, TMB and PD-L1 CPS had no correlation (Fig. 3, n = 309, Spearman's correlation coefficient 0.067, P = 0.23). Notably, a few samples with low TMB had strongly positive PD-L1 expression with high PD-L1 CPS scores. These results confirmed previous reports that PD-L1 expression and TMB are independent biomarkers.[19]

We then set out to explore the associations between PD-L1 positivity and individual gene mutations for the genes tested in our panel. As shown in Figure 4, commonly mutated genes (mutated in more than 10% patients) in our cohort included *TP53* (58.6%, 181/309), *TERT* (46.0%, 142/309), *CTNNB1* (20.3%, 63/309), *SPTA1* (15.2%, 47/309), *AXIN1* (14.6%, 45/309), and *LRP1B* (13.9%, 43/309). In 309 patients with NGS, 142 patients harbored *TERT* mutation, while 112 (78.9%, 112/142) of them were *TERT* promoter point mutations, the majority of which were c.-124C>T (90.2%; 101/112). Among these six commonly mutated

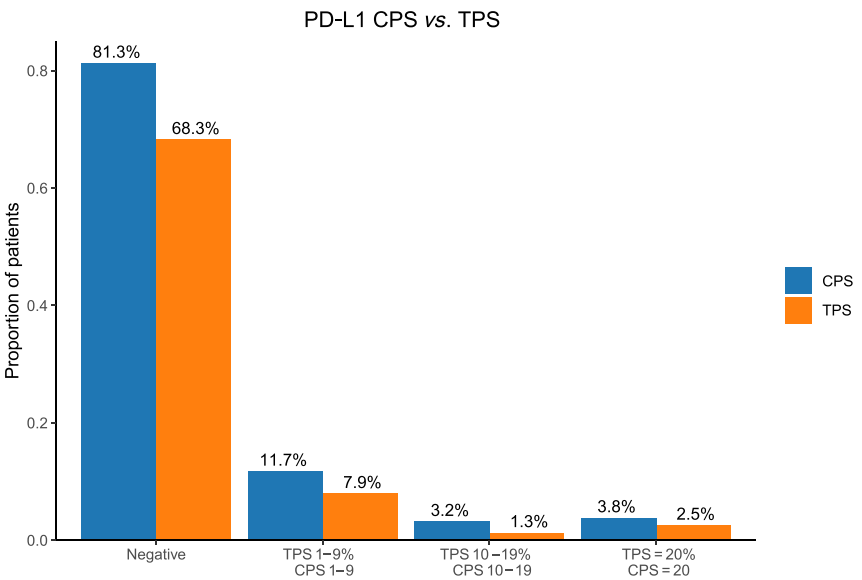

Figure 1 Distribution of programmed cell death-ligand 1 (PD-L1) tumor proportion score (TPS) and combined positive score (CPS) (n = 315). Blue bar represents CPS, and orange bar represents TPS.

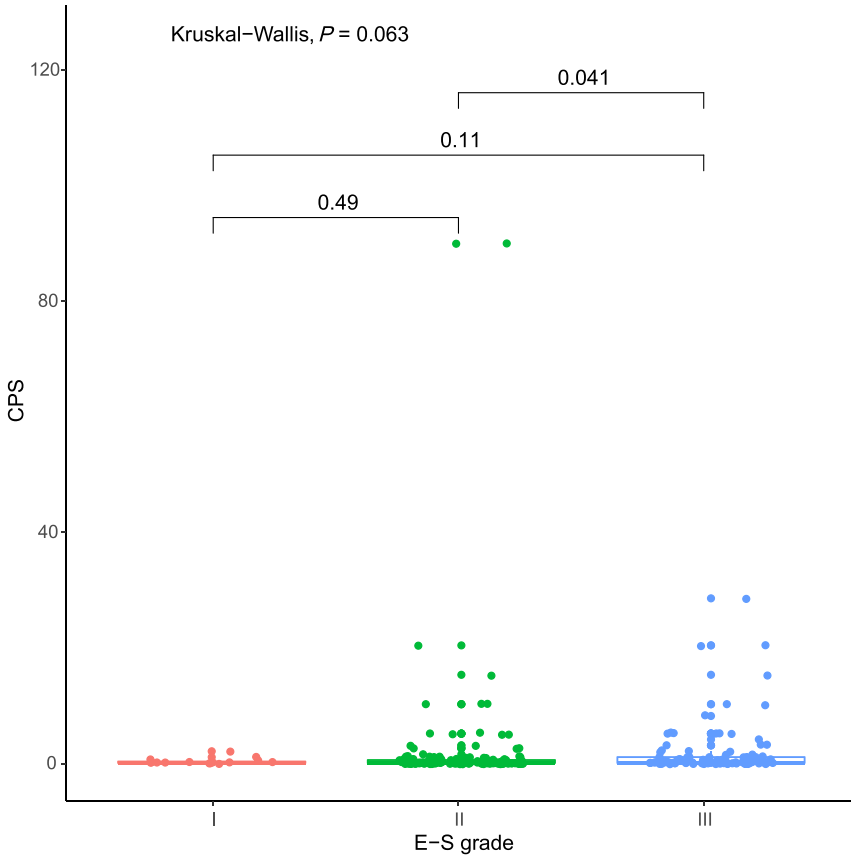

Figure 2 Distribution of programmed cell death-ligand 1 (PD-L1) combined positive score (CPS) by Edmondson–Steiner (E–S) grade. PD-L1 CPS was significantly different across E–S grades (P = 0.063, Kruskal–Wallis test). E–S grade III patients had significantly higher PD-L1 CPS than E–S grade II patients (P = 0.041, Benjamini–Hochberg corrected Wilcoxon rank-sum test). There were 246 of 315 patients with samples assayed by PD-L1 antibody and with E–S grade information. E–S grade I–II (n = 9), E–S grade II–III (n = 56), and E–S grade IV patients (n = 4) were excluded.

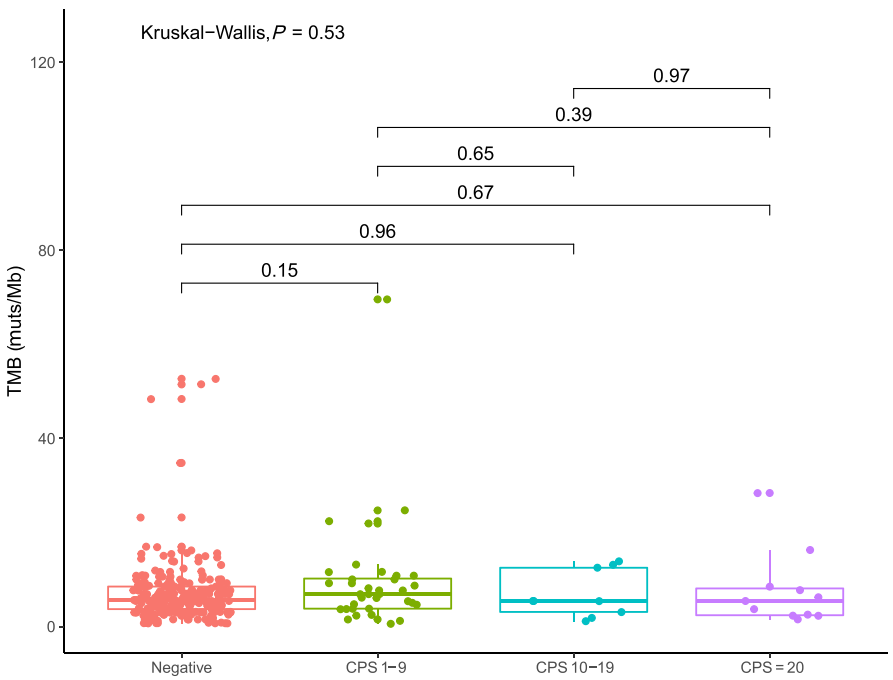

Figure 3 Distribution of tumor mutational burden (TMB) by programmed cell death-ligand 1 combined positive score (CPS). Spearman's correlation was 0.067, and the P value was 0.23; 309 patients with programmed cell death-ligand 1 expression examination and next-generation sequencing data were included in this analysis.

genes, *TP53* mutation was significantly associated with higher PD-L1 expression (Benjamini–Hochberg adjusted $P = 0.021$, Wilcoxon rank-sum test).

The mutation frequencies of homologous recombination deficiency genes were investigated. Major genes of DNA homologous recombination repair were *BRCA2*, *BRCA1*, *PALB2*, *CDK12*, *RAD51*, *CHEK2*, and *ATM*. Most of them were mutated at low frequency (< 5%) in our cohort but *ATM* (5.8%), and none of these genes were significantly correlated with PD-L1 expression.

The mutation frequencies of genes of SWI/SNF complex were investigated. *ARID1A* was mutated in 12.9% of patients, *ARID2* was mutated in 7.4% of patients, *ARID1B* was mutated in 3.5% of patients, and *SMARCA4* was mutated in 3.9% of patients. None of these genes were significantly correlated with PD-L1 expression.

Association of programmed cell death-ligand 1 combined positive score with other variables. The correlation of PD-L1 CPS with other variables, such as the age of first diagnosis, gender, cirrhosis status, stage, α-fetoprotein levels, or the site of origin (primary/metastatic/relapsed) of the samples, were observed. Only cirrhosis status was significantly associated with PD-L1 CPS (Benjamini–Hochberg corrected $P = 0.013$, Wilcoxon rank-sum test).

Discussion

TP53 mutation and higher E–S grade were both significantly associated with higher PD-L1 expression. We observed that both *TP53* mutation and higher E–S grade (grade III) were significantly associated with higher PD-L1 expression. Thus, *TP53* mutation status and E–S grade might be of interest in future studies.

One PD-L1 positive case in our cohort was treated with anti-PD-1 therapy and experienced clinical benefit. The case was a 38-year-old man who had chronic HBV infection for more than 10 years. The PD-L1 CPS was 25. Then the patient was treated with sorafenib and 200-mg pembrolizumab. As shown in Figure 5, computed tomography scan 1 month after the start of the treatment revealed reduced number of nodules in the lung and reduced size of the tumor in both the lung and the liver, suggesting that the treatment was effective. The case had a TMB of 4.8 muts/Mb. The case also harbored a *TP53* mutation. We note that this is just an anecdotal case, and larger-scale prospective studies are needed to further understand if anti-PD-1 treatment can result in clinical benefit in PD-L1-positive HCC patients.

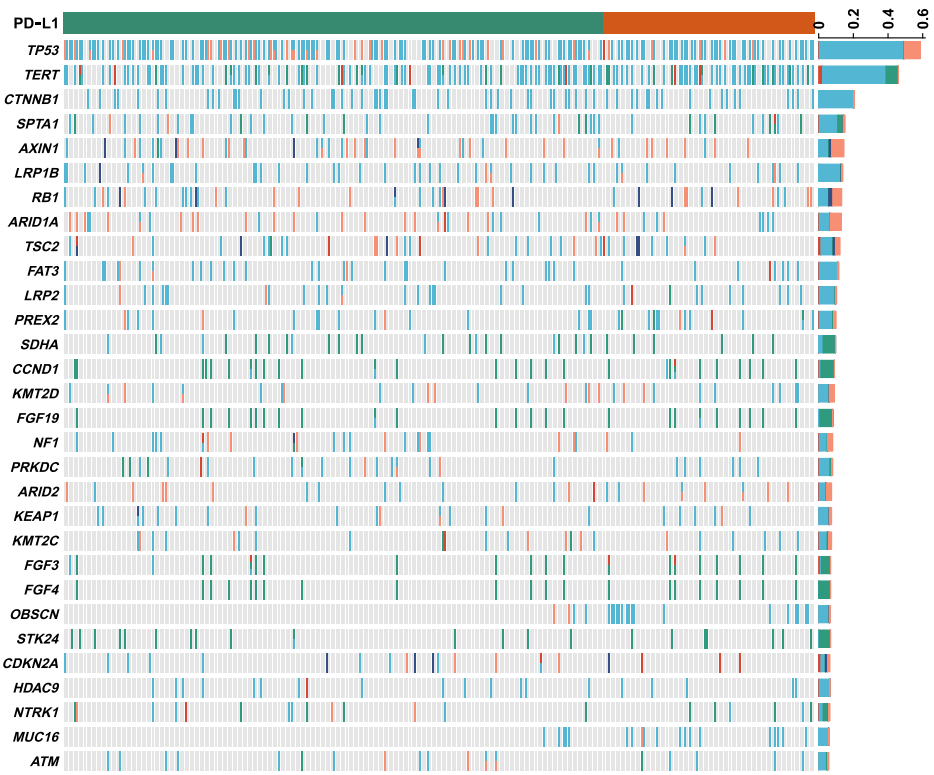

Figure 4 Somatic mutation profiles of programmed cell death-ligand 1 (PD-L1) assayed samples ($n = 309$). Samples with combined positive score ≥ 1 were marked as PD-L1 positive, and samples with combined positive score < 1 were marked as PD-L1 negative. Genes (rows) were ordered by mutation frequency (from high to low). Patients (columns) were first ordered by PD-L1 positivity and then by *TP53* mutation status; 309 patients with PD-L1 expression examination and next-generation sequencing data were included in this analysis. Alternations: , fusion/rearrangement; , substitution/indel; , gene amplification; , gene homozygous deletion; , truncation. PD-L1: , negative; , positive.

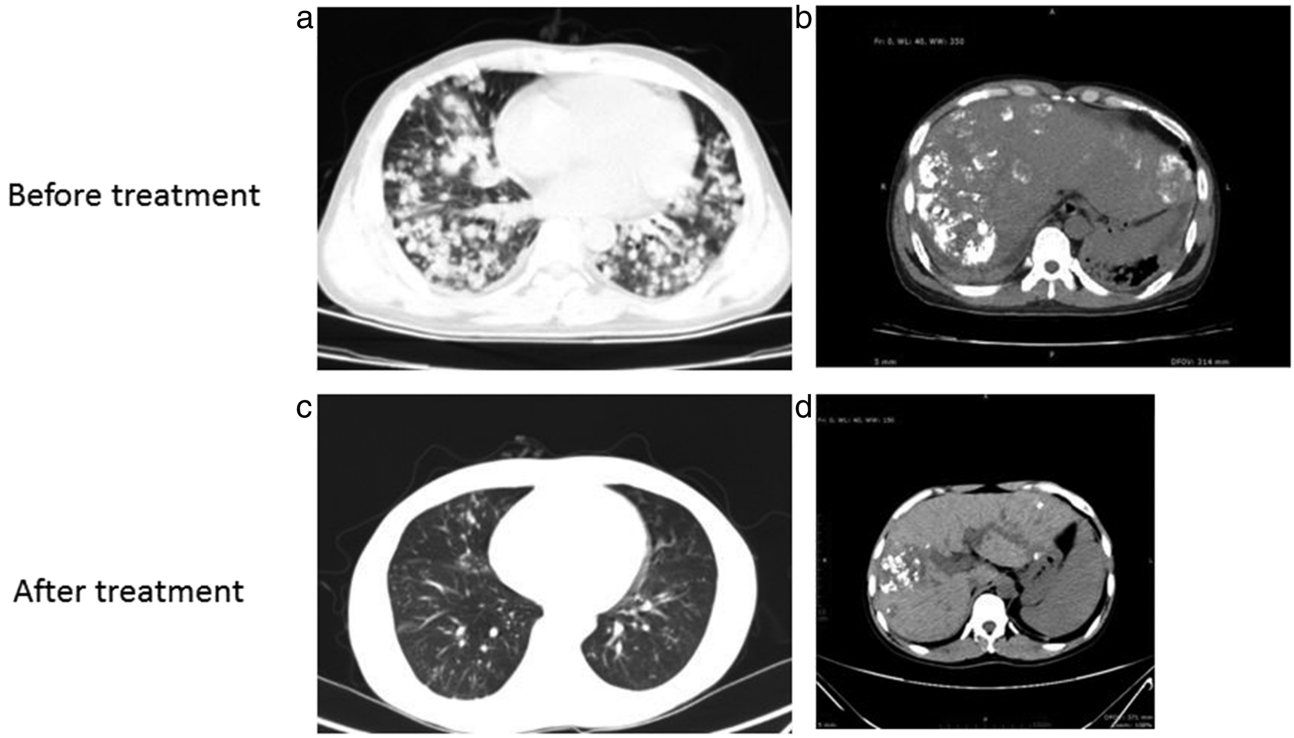

Figure 5 Computed tomography (CT) scan before and after treatment: (a) chest CT before treatment, (b) abdomen CT before treatment, (c) chest CT after treatment, and (d) abdomen CT after treatment.

TP53 mutations and E–S grade were significantly associated. A contingency table of *TP53* mutation status by E–S grade I/II against III/IV was constructed (Table S2). *TP53* mutations were significantly associated with E–S grade III/IV (P = 0.0006, Fisher's exact test). This observation supported the proposal of Calderaro *et al*. that *TP53* mutation was the molecular signature of poorly differentiated HCC.[28]

CTNNB1-mutated patients had lower E–S grade, supporting the hypothesis that *CTNNB1* was one of the molecular signatures of well-differentiated HCC. Calderaro *et al*. proposed that *CTNNB1* was one of the molecular signatures of well-differentiated HCC.[28] We then explored the association between *CTNNB1* mutations with E–S grade. A contingency table of *CTNNB1* mutation status by E–S grade I/II against III/IV was constructed (Table S3). *CTNNB1* mutations were significantly associated with E–S grade I/II (P = 4.804e − 06, Fisher's exact test).

Wnt/*CTNNB1*-mutated patients had lower PD-L1 expression. *CTNNB1*-mutated HCC patients had lower PD-L1 expression compared with *CTNNB1* wild-type patients (Benjamini–Hochberg adjusted P = 0.019). *AXIN1*-mutated HCC patients also had lower PD-L1 expression compared with *AXIN1* wild-type patients, although the difference was not significant (Benjamini–Hochberg adjusted P = 0.46). These observations were expected, as recently it was reported that Wnt/*CTNNB1*-mutated HCC was correlated with resistance to immunotherapies.[29] And it was generally expected that resistance to immunotherapies may be associated with lower PD-L1 expression.

Hepatitis B virus infection may not be associated with PD-L1 expression. It was reported that active HBV carriers had increased PD-L1 expression in myeloid dendritic cells.[30] However, in our cohort, PD-L1 expression was not significantly different between the HBV-positive and HBV-negative groups (P = 0.15, Wilcoxon rank-sum test; HBV status reported by hospital). Further investigation may be needed because of the low PD-L1-positive proportion in this cohort.

Preliminary results showed that no significant difference was observed between PD-L1 28-8 Abcam assay and PD-L1 22C3 Dako assay. The PD-L1 antibody used in our study was PD-L1 28-8 Abcam antibody. To compare its performance with the more commonly used PD-L1 22C3 Dako antibody, we performed PD-L1 22C3 Dako assay on 50 slides, the consecutive sections of which had already been stained by the PD-L1 28-8 assay (Figure S1). The difference between these two assays was not significant (P = 0.14 for TPS and P = 0.068 for CPS, Wilcoxon signed-rank test). Experimental details of the PD-L1 22C3 assay are available in the Supporting Information.

One major limitation of this study is that the cohort studied is a retrospective cohort consisting of patients from many hospitals in China. As a result, the characteristics of the tumors studied in this cohort are prone to selection bias. Moreover, a small number of the patients in this cohort received more than one PD-L1 IHC tests, and the use of the highest scores in subsequent analyses could introduce additional bias. Larger-scale prospective study is needed to further validate the conclusions from this work.

In conclusion, for HCC patients, higher E–S grade and *TP53* mutation significantly correlated with higher PD-L1 expression, while TMB or HBV infection was not significantly correlated with PD-L1 expression. These observations indicated interesting relationship between clinicopathological variables with PD-L1

expression in HCC. It might be helpful to incorporate E–S grade, *TP53* mutation status, and TMB in combination with PD-L1 expression in future studies.

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

References

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 2018; **68**: 394–424.

2 Cronin KA, Lake AJ, Scott S *et al*. Annual report to the nation on the status of cancer, part I: national cancer statistics. *Cancer* 2018; **124**: 2785–800.

3 Cheng A-L, Kang Y-K, Chen Z *et al*. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol.* 2009; **10**: 25–34.

4 Llovet JM, Ricci S, Mazzaferro V *et al*. Sorafenib in advanced hepatocellular carcinoma. *N. Engl. J. Med.* 2008; **359**: 378–90.

5 Abou-Alfa GK, Meyer T, Cheng A-L *et al*. Cabozantinib in patients with advanced and progressing hepatocellular carcinoma. *N. Engl. J. Med.* 2018; **379**: 54–63.

6 Bruix J, Qin S, Merle P *et al*. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet (London, England)* 2017; **389**: 56–66.

7 Kudo M, Finn RS, Qin S *et al*. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet (London, England)* 2018; **391**: 1163–73.

8 Zhu AX, Kang Y-K, Yen C-J *et al*. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* 2019; **20**: 282–96.

9 Hamid O, Robert C, Daud A *et al*. Five-year survival outcomes for patients with advanced melanoma treated with pembrolizumab in KEYNOTE-001. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* 2019; **30**: 582–8.

10 Leighl NB, Hellmann MD, Hui R *et al*. Pembrolizumab in patients with advanced non-small-cell lung cancer (KEYNOTE-001): 3-year results from an open-label, phase 1 study. *Lancet Respir. Med.* 2019; **7**: 347–57.

11 El-Khoueiry AB, Sangro B, Yau T *et al*. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet (London, England)* 2017; **389**: 2492–502.

12 Finn RS, Ryoo B-Y, Merle P *et al*. Results of KEYNOTE-240: phase 3 study of pembrolizumab (Pembro) vs best supportive care (BSC) for second line therapy in advanced hepatocellular carcinoma (HCC). *J. Clin. Oncol.* 2019; **37**: 4004. Available from. https://doi.org/10.1200/JCO.2019.37.15_suppl.4004

13 Squibb B-M. Bristol-Myers Squibb announces results from CheckMate -459 study evaluating Opdivo (nivolumab) as a first-line treatment for patients with unresectable hepatocellular carcinoma. 2019. https://news.bms.com/press-release/bmy/bristol-myers-squibb-announces-results-checkmate-459-study-evaluating-opdivo-nivol

14 Patel SP, Kurzrock R. PD-L1 expression as a predictive biomarker in cancer immunotherapy. *Mol. Cancer Ther.* 2015; **14**: 847–56.

15 Samstein RM, Lee C-H, Shoushtari AN *et al*. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. *Nat. Genet.* 2019; **51**: 202–6.

16 Zhu AX, Finn RS, Edeline J *et al*. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol.* 2018; **19**: 940–52.

17 Pinato DJ, Mauri FA, Spina P *et al*. Clinical implications of heterogeneity in PD-L1 immunohistochemical detection in hepatocellular carcinoma: the Blueprint-HCC study. *Br. J. Cancer* 2019; **120**: 1033–6.

18 Chalmers ZR, Connelly CF, Fabrizio D *et al*. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med.* 2017; **9**: 34.

19 Yarchoan M, Albacker LA, Hopkins AC *et al*. PD-L1 expression and tumor mutational burden are independent biomarkers in most cancers. *JCI insight* 2019; **4**.

20 Carrat F, Fontaine H, Dorival C *et al*. Clinical outcomes in patients with chronic hepatitis C after direct-acting antiviral treatment: a prospective cohort study. *Lancet (London, England)* 2019; **393**: 1453–64.

21 Adhoute X, Penaranda G, Raoul J-L *et al*. Hepatocellular carcinoma macroscopic gross appearance on imaging: predictor of outcome after transarterial chemoembolization in a real-life multicenter French cohort. *Eur. J. Gastroenterol. Hepatol.* 2019; **31**: 1414–23.

22 Kulangara K, Zhang N, Corigliano E *et al*. Clinical utility of the combined positive score for programmed death ligand-1 expression and the approval of pembrolizumab for treatment of gastric cancer. *Arch. Pathol. Lab. Med.* 2019; **143**: 330–7.

23 Zhou L, Rui J-A, Zhou W-X, Wang S-B, Chen S-G, Qu Q. Edmondson-Steiner grade: a crucial predictor of recurrence and survival in hepatocellular carcinoma without microvascular invasio. *Pathol. Res. Pract.* 2017; **213**: 824–30.

24 Cao J, Chen L, Li H *et al*. An accurate and comprehensive clinical sequencing assay for cancer targeted and immunotherapies. *Oncologist* 2019: e1294.

25 Shi L, Zhang S-J, Chen J *et al*. A comparability study of immunohistochemical assays for PD-L1 expression in hepatocellular carcinoma. *Mod. Pathol. Off. J. United States Can. Acad. Pathol. Inc.* 2019; **32**: 1646–56.

26 Alborelli I, Leonards K, Rothschild SI *et al*. Tumor mutational burden assessed by targeted NGS predicts clinical benefit from immune checkpoint inhibitors in non-small cell lung cancer. *J. Pathol.* 2019; **250**: 19–29.

27 Mok TSK, Wu Y-L, Kudaba I *et al*. Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-042): a randomised, open-label, controlled, phase 3 trial. *Lancet* 2019; **393**: 1819–30 Available from. https://doi.org/10.1016/S0140-6736(18)32409-7

28 Calderaro J, Ziol M, Paradis V, Zucman-Rossi J. Molecular and histological correlations in liver cancer. *J. Hepatol.* 2019.

29 Pinyol R, Sia D, Llovet JM. Immune exclusion-Wnt/CTNNB1 class predicts resistance to immunotherapies in HCC. *Clin. Cancer Res.* 2019; **25**: 2021–3.

30 Chen L, Zhang Z, Chen W *et al*. B7-H1 up-regulation on myeloid dendritic cells significantly suppresses T cell immune function in patients with chronic hepatitis B. *J. Immunol.* 2007; **178**: 6634–41.

Supporting information

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

Figure S1. Distribution of PD-L1 CPS between in HBV positive group (n = 260) and HBV negative group(n = 44). Blue plot represents HBV positive group, and Orange plot represents HBV negative group.

Table S1. Sample size per hospital.
Table S2. Contingency table of *TP53* mutation status across E-S grades.
Table S3. Contingency table of *CTNNB1* mutation status across E-S grades.