

Immunological Microenvironment Predicts the Survival of the Patients with Hepatocellular Carcinoma Treated with Anti-PD-1 Antibody

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Keywords

Hepatocellular carcinoma · Wnt/ β -Catenin pathway · Immune checkpoint inhibitors · Survival · Immunological microenvironment

Abstract

Introduction: Although immune checkpoint inhibitors (ICIs) have been considered as promising agents for the treatment of advanced hepatocellular carcinoma (HCC), previous clinical trials revealed that the response to anti-programmed cell death protein 1 (anti-PD-1) monotherapy was as low as 20%. Identifying subgroups that respond well to ICIs is clinically important. Here, we studied the prognostic factors for anti-PD-1 antibody treatment based on the molecular and immunological features of HCC. **Methods:** Patients who were administered anti-PD1 antibody for advanced HCC at Kindai University Hospital were included. Clinicopathological backgrounds and antitumor responses were examined in 34 cases where tumor tissues before treatment were available. Transcriptome analysis was performed using 40 HCC samples obtained from surgical resection, and immune status was compared between 20 HCCs with activating mutations

in β -catenin and those without the mutations using transcriptome-based immunogram. **Results:** Univariate analysis showed that the disease control rate was significantly better in patients with α -fetoprotein < 400 ng/mL, negative for β -catenin/glutamate synthetase (GS) staining, high combined positive score (CPS) of programmed death-ligand 1 (PD-L1), and increased infiltration of CD8⁺ cells in tumor tissues. Among them, negative staining of β -catenin/GS, CPS of PD-L1 ≥ 1 , and high degree of CD8⁺ tumor-infiltrating lymphocytes (TILs) were significantly associated with longer survival in both progression-free survival (PFS) and overall survival (OS). The combination of these factors well stratified the survival of the patients on anti-PD-1 antibody in both PFS and OS ($p < 0.0001$ and $p = 0.0048$ for PFS and OS, respectively). In addition, the immunogram revealed that tumor-carrying mutations in β -catenin showed downregulation of immune-related genes, especially in those related to priming and activation by dendritic cells, interferon- γ response, inhibitory molecules, and regulatory T cells. **Discussion/Conclusion:** The combined score including Wnt/ β -catenin activation, CPS of PD-L1, and degree of CD8⁺ TILs in HCC is informative for predicting the response to ICI in HCC cases. Constitutive activation of β -catenin can induce an immune

cold phenotype with downregulation of immune-related genes, and immunohistochemistry-based evaluation is beneficial for identifying the subgroup that shows a good response to ICI.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and a leading cause of cancer-related mortality worldwide [1, 2]. Although there is recent progress in locoregional treatment of HCC, recurrence after curative treatment leads to tumor progression, where systemic chemotherapy is inevitable. Although several studies have reported the impact of molecular targeted agents (MTAs) on the survival of advanced HCC [3–7], the accumulation of genetic and epigenetic alterations in cancer cells may result in the resistance of HCC to MTAs, where escape from the inhibitory effect on tumor growth by MTAs will occur. From this point of view, intervention in the immune microenvironment should be a promising approach for patients with advanced-stage HCC.

In recent years, several clinical trials of immune checkpoint inhibitors (ICIs) have been performed in patients with HCC. A phase-I/II clinical trial (CheckMate 040) using the anti-programmed cell death protein 1 (anti-PD-1) antibody nivolumab revealed an objective response rate (ORR) of 20% and disease control rate (DCR) of 64% in patients with advanced tumors in the 1st-line setting [8]. Another phase-II trial using the anti-PD-1 antibody pembrolizumab (KEYNOTE-224) showed an ORR of 17% and DCR of 62% in patients who showed tumor progression on sorafenib [9]. However, the phase-III clinical trial failed to show positive results on survival using pembrolizumab in advanced HCC cases, although higher antitumor responses were observed in the groups treated with the anti-PD-1 antibody compared to the control groups [10, 11]. Collectively, almost 80% of the patients with HCC do not respond effectively to anti-PD-1 monotherapy, indicating that identification of a subgroup that would show a good response to ICIs is crucial for the management of HCC, especially in patients who fail to show antitumor response to MTAs.

On the other hand, recent reports suggest that Wnt/ β -catenin signaling may be involved in the exclusion of CD8⁺ cells in tumor tissues in melanoma cases [12]. Ruiz de Galarreta et al. [13] showed the role of β -catenin acti-

vation in immune escape and resistance to anti-PD-1 therapy in a mouse model of HCC. Harding et al. [14] also showed a negative impact of Wnt/ β -catenin activation on DCR and progression-free survival (PFS) with anti-PD-1 therapy. Therefore, constitutive activation of the Wnt/ β -catenin signaling, which is generally observed in around 30% of HCC by mutations of the genes involved in the Wnt/ β -catenin pathway [15], would be a promising marker for predicting immune resistance to anti-PD-1 therapy in HCC cases. However, clinical evidence for the role of Wnt/ β -catenin activation and subsequent acquisition of the “immune cold phenotype” that may affect the response to ICIs is still lacking in HCC cases. In this study, we focused on this clinical question and analyzed the status of Wnt/ β -catenin activation, and factors associated with antitumor immunity on ICIs, such as expression of immune checkpoint molecules and infiltration of CD8⁺ cells, in HCC tissues of patients who underwent anti-PD-1 monotherapy thereafter. In addition, to determine the role of the activating mutation of Wnt/ β -catenin signaling on immune phenotype, characteristics of the immunological microenvironment of HCCs were examined using the transcriptome data of HCC tissues in the context of activating mutations in the Wnt/ β -catenin pathway.

Materials and Methods

Patients and Follow-Up Schedule

Between August 2015 and September 2017, 45 patients with advanced stage of HCC were treated with anti-PD-1 antibody monotherapy under clinical trials and real-world clinical practice at Kindai University Hospital. Among them, HCC tissues before treatment were available for 35 patients; 34 were eligible for immunohistochemical evaluations. Details of the administration and follow-up schedule are shown in the see online suppl. Material and methods; see www.karger.com/doi/10.1159/000516899 for all online suppl. material. We analyzed the background of these 34 patients who received anti-PD-1 monotherapy and examined the prognosis, including DCR, ORR, PFS, and overall survival (OS).

We also collected data associated with tumor progression and systemic inflammation from blood examination, including α -fetoprotein (AFP), lymphocyte count, red blood cell distribution width (RDW), neutrophil-to-lymphocyte ratio (NLR), platelet-lymphocyte ratio (PLR), and lactate dehydrogenase (LDH) because these laboratory findings were reportedly associated with the tumor response to ICIs in other types of cancer [16–20]. Based on the previous reports, cut-off values of each blood chemical data for analyzing the efficacy of ICIs in tumor response were set as follows: baseline AFP ≥ 400 ng/mL, lymphocyte count $\geq 1,000/\mu\text{L}$, RDW $\geq 16\%$, NLR ≥ 5 , PLR ≥ 200 , and LDH \geq upper normal limit, respectively [17–20].

Evaluation of Effectiveness

Antitumor responses were evaluated by the attending physicians according to RECIST version 1.1 [21]. Complete response (CR), partial response (PR), or stable disease (SD) persisting for ≥ 4 months were considered as disease control (DC) based on the report regarding treatment using ICIs in patients with HCC [14]. Objective response was defined as PR or CR.

Immunohistochemistry

We reviewed formalin-fixed paraffin embedded (FFPE) tumor samples obtained from the HCC tissues before the treatment and confirmed the histology of HCC by at least 2 liver specialized pathologists. To determine the activation of the Wnt/ β -catenin pathway, we examined the expression of β -catenin and its downstream target, glutamine synthetase (GS), using immunohistochemistry (IHC). It is reported that nuclear staining of β -catenin is associated with the activating mutation of the catenin beta-1 (*CTNNB1*) gene [22–24]. Furthermore, strong diffuse expression of GS reportedly reflects constitutive activation of Wnt/ β -catenin signaling, that is attributed to the β -catenin mutation [25]. Therefore, the presence of nuclear staining of β -catenin in $\geq 5\%$ of tumor cells [26, 27], or diffuse strong staining of GS were considered as positive findings for activation in Wnt/ β -catenin signaling [27–29].

On the other hand, it is conceivable that infiltration of CD8⁺ lymphocytes in cancerous tissues and expression of programmed death-ligand 1 (PD-L1) are also associated with the response of tumors to anti-PD-1 therapy because blockade of the PD-1/PD-L1 axis should restore the antitumor immunity induced by CD8⁺ lymphocytes in tumors [30, 31]. For this purpose, we performed IHC for CD8 and PD-L1 using the same tissues examined for the expression of β -catenin and GS. We counted the number of PD-L1-positive cells in tumor and tumor-infiltrated cells to calculate the combined positive score (CPS) as reported previously, where CPS was determined as the number of PD-L1-positive cells (tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells $\times 100$ [9]; CPS of PD-L1 (PD-L1-CPS) as reported previously [9]. The number of tumor-infiltrating lymphocytes (TILs) expressing CD8 was also counted. Cells expressing β -catenin, GS, PD-L1, and CD8 were visually counted and quantified at 400-fold magnification. Based on the CPS shown in the results of phase-II clinical trial of pembrolizumab, where PD-L1-CPS was positively associated with antitumor response to ICIs, CPS ≥ 1 was defined as high PD-L1-CPS and CPS < 1 was defined as low PD-L1-CPS [9]. Regarding the expression of CD8, the mean degree of CD8⁺ TILs was 28.4 cells/HPF (95% CI: 16.3–40.5) in tumors; values above the mean (28.4 cells/HPF) were defined as high degree of CD8⁺ TILs [30, 32]. Details of the conditions of the IHCs are shown in the online suppl. materials and methods.

Mutational and Transcriptome Analyses of HCC Tissues

Previously, we analyzed the mutational status of 154 HCC samples using the Ion AmpliSeq™ Comprehensive Cancer Panel that covered somatic mutations across 409 cancer-associated genes (Illumina®, San Diego, CA, USA) [31]. To determine the effect of activation of the Wnt/ β -catenin pathway on the antitumor immune response of HCC tissues, we compared the gene expression that determined the immune status in tumors between those with the *CTNNB1* mutation and those without mutations using 40 fresh-frozen HCC samples, where 20 were from HCC with activating *CTNNB1* mutation and 20 were from those with wild-type

CTNNB1. For the extraction of RNA from FFPE samples, we used the AllPrep DNA/RNA FFPE kit (QIAGEN, Venlo, the Netherlands). For library preparation of transcriptome analysis using next-generation sequencing, the Ion AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used according to the manufacturer's protocol. Sequencing was performed using the Ion 550 Kit and the Ion S5™ XL System (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

For the comprehension of the immunological microenvironment of the tumor, we constructed the immunogram, a scoring system based on RNA-sequence data describing individual cancer-immunity interactions [33–35]. We performed single-sample gene set enrichment analysis (ssGSEA) of these 40 tumors with 10 gene sets, innate immunity: natural killer cells (axis 1), priming and activation: dendritic cells (DCs) (axis 2), T cells: CD8⁺ T-cell response (axis 3), interferon- γ (IFNG) response (axis 4), inhibitory molecules (axis 5), inhibitory cells (regulatory T cells, axis 6), inhibitory cells (myeloid-derived suppressor cells, axis 7), recognition of tumor cells, antigen processing and presentation (axis 8), proliferation (axis 9), and glycolysis (axis 10), as described previously [34]. We obtained the mean (M) and standard deviation (SD) of the ssGSEA scores of the 40 HCC patients for each gene set. The immunogram score (IGS) for each axis was calculated as $IGS = 3 + 1.5 \times (ssGSEA \text{ score} - M)/SD$ [34]. This formula was applied to all the axes of the immunogram in each patient. To depict immunograms on radar chart, the lower and upper limits of the IGS were set at 1 and 5, respectively.

Statistics

We used Pearson's χ^2 test or Fisher's exact test for the comparison of categorical variables and the Wilcoxon rank-sum test for continuous variables. Nominal logistic regression analysis was used for the multivariate analysis. For the survival analysis, the Kaplan-Meier survival curve was used, and the *p* value was calculated using the log-rank test. The details of the immunogram, shown using radar charts, have been reported previously [34]; hierarchical clustering analysis was performed using IGS and for the characterization of the transcriptome-based immune status of HCC with the *CTNNB1* mutation. All *p* values were 2-sided, and $p < 0.05$ was considered statistically significant. All statistical analyses were conducted using the JMP v.14 software (SAS Institute Inc., Cary, NC, USA).

Results

Backgrounds of the Patients

Clinical backgrounds of the patients are as follows: the average age was 67.1 years, 28 patients were male, and 6 were female. Nine patients were positive for hepatitis B surface antigen, and 13 were positive for hepatitis C antibody. Sixteen HCCs were well differentiated, and 18 were moderately to poorly differentiated tumors. Portal vein tumor thrombus and extrahepatic spread were observed in 10 and 14 patients, respectively. Fourteen patients had tumors with Barcelona Clinic Liver Cancer (BCLC) stage B and 20 patients with BCLC stage C. Regarding back-

Table 1. Backgrounds of the patients in this study

Clinicopathological backgrounds	Distribution
Age: median, distribution, years old	67.1, 40–80
Sex (male/female)	28/6
ECOG performance status (PS0/PS1)	33/1
HBV (yes/no)	9/25
HCV (yes/no)	13/21
Differentiation (well/moderately – poorly)	16/18
PVTT (yes/no)	10/24
EHS (yes/no)	14/20
BCLC stage (B/C)	14/20
Pretreatment of sorafenib (yes/no)	13/21
Serum AFP level: median, distribution, ng/mL	1,634.4, 3.0–92,360
Lymphocyte count: median, distribution, / μ L	1,073.5, 251–2,265
RDW: median, distribution, %	14.5, 11.9–21.7
NLR: median, distribution	2.6, 1.1–5.1
PLR: median, distribution	123.6, 51–287.1
LDH: median, distribution, U/L	265, 180–582

ECOG, Eastern Cooperative Oncology Group; HBV, hepatitis B virus; HCV, hepatitis C virus; PVTT, portal vein tumor thrombus; EHS, extrahepatic spread; BCLC stage, Barcelona Clinic Liver Cancer stage; AFP, α -fetoprotein; RDW, red blood cell distribution width; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LDH, lactate dehydrogenase.

ground liver function, all cases were Child-Pugh grade A. Among them, 13 had a history of pretreatment with sorafenib, and 21 had none (Table 1).

IHC of β -catenin, Glutamine Synthetase, PD-L1, and CD8 in HCC Tissues

We performed IHC for the expression of β -catenin, GS, PD-L1, and CD8 in HCC tissues before anti-PD-1 therapy (Fig. 1). Nine out of 34 (26.5%) were positive for nuclear staining for β -catenin, 11 out of 34 were positive for diffuse strong staining of GS; collectively, activation of the Wnt/ β -catenin pathway was considered to be positive in 14 of the 34 cases (41.2%). Similarly, cases with high-degree CD8⁺ TILs were observed in 9 out of 34 (26.5%); the number of patients with high PD-L1-CPS was 13 of 30 (43.3%) of the cases (Table 2).

Association between Clinicopathological Backgrounds and Response to Anti-PD-1 Therapy in HCC

Regarding the best response of the 34 patients who received anti-PD-1 monotherapy, CR, PR, SD, and PD were observed in 3, 2, 10, and 19 patients, respectively. Univariate analysis revealed that AFP \geq 400 ng/mL was nega-

tively associated with DC ($p = 0.0186$ by Pearson's χ^2 test; Table 2). In addition, based on the IHC analyses, we found that Wnt/ β -catenin activation was negatively, and high degree of CD8⁺ TILs and high PD-L1-CPS were positively associated with achievement of DC on anti-PD-1 antibody ($p = 0.0034$, for Wnt/ β -catenin activation, $p = 0.0252$ for CD8⁺ TILs and $p = 0.0352$ for PD-L1-CPS, respectively; Table 2). Subsequently, we performed multivariate analysis using serum AFP level, activation status of Wnt/ β -catenin, degree of CD8⁺ TILs, and PD-L1-CPS as covariables. Although no items were independently associated with achievement of DC, the status of Wnt/ β -catenin activation showed a borderline association ($p = 0.0509$ by nominal logistic regression analysis, Fig. 2) and could mostly affect the achievement of DC on anti-PD-1 antibody, where the negative for Wnt/ β -catenin activation showed an odds ratio (OR) of 6.93 (95% CI: 0.83–57.78), followed by that for high PD-L1-CPS (OR = 4.87, 95% CI: 0.70–33.94), AFP <400 ng/mL (OR = 3.86, 95% CI: 0.57–26.07), and high-degree CD8⁺ TILs (OR = 2.17, 95% CI: 0.26–18.35, Fig. 2). It is also reasonable to speculate that most benefit of immune therapy is derived from the patients with objective responses. Therefore, we analyzed association between objective response and clinicopathological findings although the number of the cases with objective response is small ($n = 5$). Univariate analysis revealed that RDW \geq 16% showed a borderline association with objective response ($p = 0.0476$ by Fisher's exact test, online suppl. Table 1).

As absence of Wnt/ β -catenin activation was associated with DC in univariate analysis and showed borderline association in multivariate analysis, we compared the background of the patients carrying HCCs with Wnt/ β -catenin activation and those without activation with respect to relevant clinical and prognostic parameters. Univariate analysis revealed that RDW \geq 16% was negatively associated with Wnt/ β -catenin activation and low grade of CD8⁺ TILs showed borderline association ($p = 0.0262$, and $p = 0.0504$ for RDW \geq 16% and grade of CD8⁺ TILs, respectively, by Fisher's exact test, online suppl. Table 2).

Survival of the Patients Stratified by the Expression of Wnt/ β -Catenin Activation, PD-L1 Expression, CD8⁺ Cell Infiltration on PD-1/PD-L1 Blockade

Next, we examined the survival of the patients classified based on IHC findings as shown in Figure 3 depicting the survival curve of HCC patients after the initiation of anti-PD-1 monotherapy. No deaths occurred before PD judgment. The median PFS and 95% CI were as follows: median PFS durations were 56.5 days (95% CI: 42–89) and

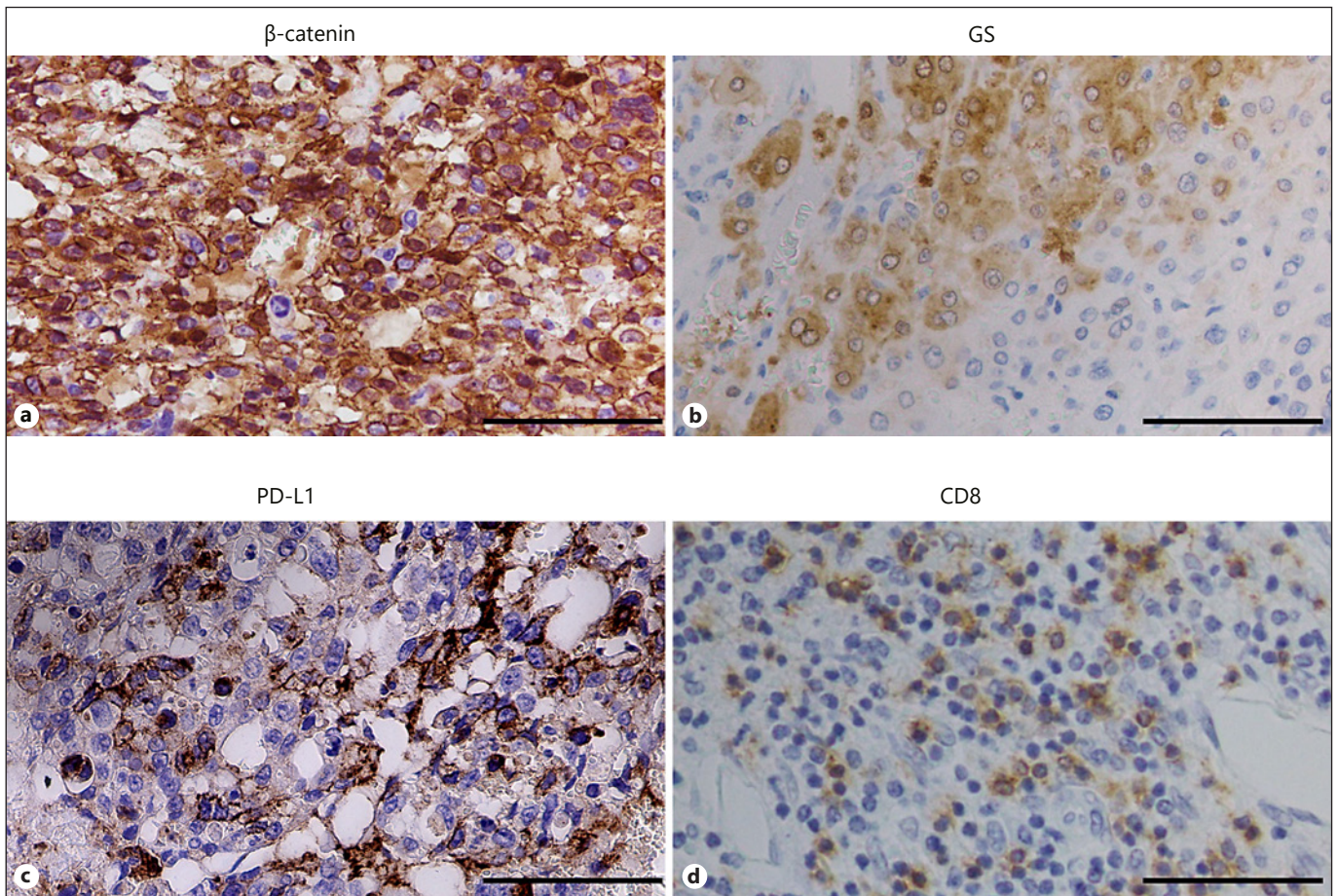


Fig. 1. Representative images of each immunostaining. Representative images of nuclear staining of β -catenin (a), strong diffuse expression of GS (b), expression of PD-L1 on cell membranes (c),

and infiltration of CD8⁺ lymphocytes in cancerous tissues (d) by IHC. Scale bar, 50 μ m. GS, glutamate synthetase; IHC, immunohistochemistry.

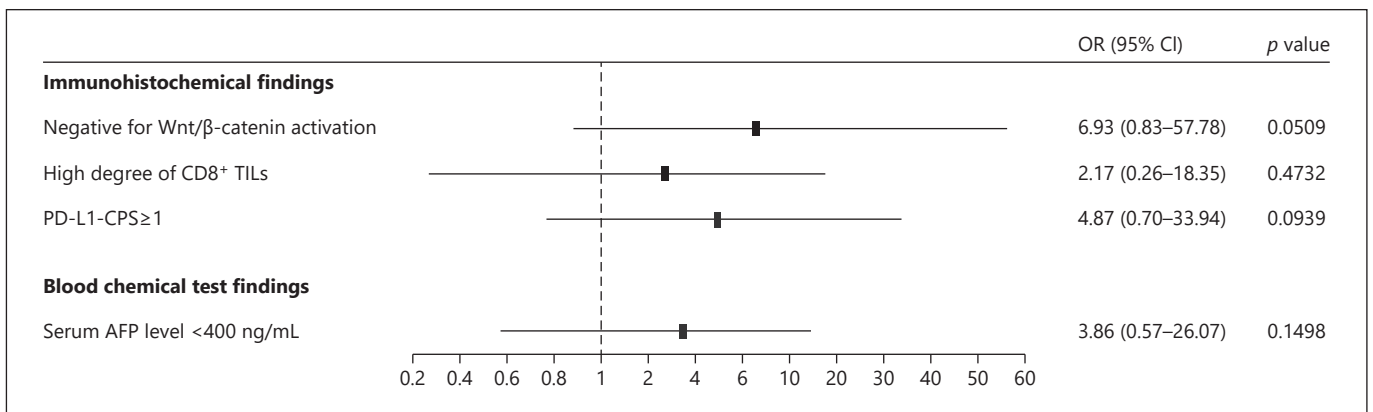


Fig. 2. Multivariate analysis and ORs for achievement of DC for Wnt/ β -catenin activation, PD-L1-CPS, CD8⁺TILs, and serum AFP level. Multivariate analysis in DC was performed for Wnt/ β -catenin activation, combined with degree of CD8⁺ TILs, positive score of PD-L1 (PD-L1-CPS), and serum AFP level. Although no

statistically significant difference was observed, the absence of Wnt/ β -catenin activation showed the highest OR = 6.93. DC, disease control; CPS, combined positive score; TIL, tumor-infiltrating lymphocyte; OR, odds ratio; AFP, α -fetoprotein; DC, disease control.

Table 2. Association between clinicopathological findings and achievement of DC on the treatment with anti-PD-1 antibody in HCC patients

Clinicopathological categories	DC		<i>p</i> value for univariate analysis ¹	<i>p</i> value for multivariate analysis ²
	with	without		
Clinical backgrounds				
Age (<65/≥65)	5/10	5/14	0.7176*	
Sex (male/female)	12/3	16/3	1.0000*	
ECOG performance status (PS0/PS1)	15/0	18/1	1.0000*	
HBV (yes/no)	4/11	5/14	1.0000*	
HCV (yes/no)	6/9	7/12	0.8508	
Degree of differentiation (well/moderately – poorly)	5/10	11/8	0.1542	
PVTT (yes/no)	4/11	6/13	1.0000*	
EHS (yes/no)	6/9	8/11	0.9014	
BCLC stage (B/C)	6/9	8/11	0.9014	
Pretreatment of sorafenib (yes/no)	8/7	5/14	0.1075	
Baseline blood chemical findings				
AFP (high [≥400 ng/mL]/low [<400 ng/mL])	5/10	14/5	0.0186	0.1498
Lymphocyte count (high [≥1,000/μL]/low [<1,000/μL])	8/7	11/8	0.7903	
RDW (high [≥16%]/low [<16%])	4/11	3/16	0.6722*	
NLR (high [≥5]/low [<5])	1/14	3/16	0.6128*	
PLR (high [≥200]/low [<200])	4/11	3/16	0.6722*	
LDH (high [≥upper normal limit]/low [<upper normal limit])	9/6	11/8	0.9014	
IHC				
Wnt/β-catenin activation (positive/negative)	2/13	12/7	0.0034	0.0509
Grade of CD8+ TILs (high/low)	7/8	2/17	0.0252*	0.4732
PD-L1-CPS (high [≥1]/low [<1])	8/4	5/13	0.0352	0.0939

Univariate analysis revealed that AFP ≥400 ng/mL was negatively associated with DC ($p = 0.0186$). In addition, based on the immunohistochemical analyses, Wnt/β-catenin activation, which was determined by the nuclear staining of the β-catenin and/or strong diffuse staining of the glutamine synthetase, was negatively, and high CPS of PD-L1 (PD-L1-CPS) and high grade of CD8+ TILs were positively associated with achievement of DC on anti-PD-1 antibody ($p = 0.0034$, for Wnt/β-catenin activation, $p = 0.0352$ for PD-L1-CPS, $p = 0.0252$ for CD8+ TILs, respectively). Although, no items revealed independently associated with achievement of DC in multivariate analysis, status of Wnt/β-catenin activation could mostly affect the achieving DC on anti-PD-1 antibody ($p = 0.0509$). ECOG, Eastern Cooperative Oncology Group; HBV, hepatitis B virus; HCV, hepatitis C virus; PVTT, portal vein tumor thrombus; EHS, extrahepatic spread; BCLC stage, Barcelona Clinic Liver Cancer stage; well, well differentiated; moderately poorly, moderately differentiated or poorly differentiated; AFP, α-fetoprotein; RDW, red blood cell distribution width; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LDH, lactate dehydrogenase; TILs, tumor-infiltrating lymphocyte; PD-L1-CPS, combined positive score of programmed cell death-ligand 1; HCC, hepatocellular carcinoma; DC, disease control; IHC, immunochemistry. ¹ p value by Pearson's χ^2 test or Fisher's exact test (The asterisk shows p value by Fisher's exact test). Bolds denote the p value <0.05. ² p value by logistic regression analysis.

231 days (95% CI: 115–547) in patients with positive for activation of the Wnt/β-catenin pathway and those with negative for activation, respectively ($p < 0.0001$ by log-rank test, Fig. 3a). Similarly, the median PFS was 297 days (95% CI: 89–514) and 58 days (95% CI: 54–115) in patients with PD-L1-CPS ≥1 and those with PD-L1-CPS <1 ($p = 0.0109$, Fig. 3b); median PFS durations were 246 days (95% CI: 84–1,064) and 89 days (95% CI: 56–164) in patients with a high degree of CD8+ TILs and those with a low degree of CD8+ TILs, respectively ($p = 0.0344$, Fig. 3c).

There was no significant difference in PFS among patients classified by serum AFP level. The median duration of PFS was 84 days (95% CI: 56–164) and 210 days (95% CI: 55–460) ($p = 0.2677$ by log-rank test, online suppl. Fig. 1a) in patients with AFP ≥400 ng/mL and those with AFP <400 ng/mL.

The median OS and 95% CI of each subgroup were as follows: median OS was 432 days (95% CI: 103–487) and 764 days (95% CI: 315–1,021) in patients with positive for activation of the Wnt/β-catenin pathway and those with

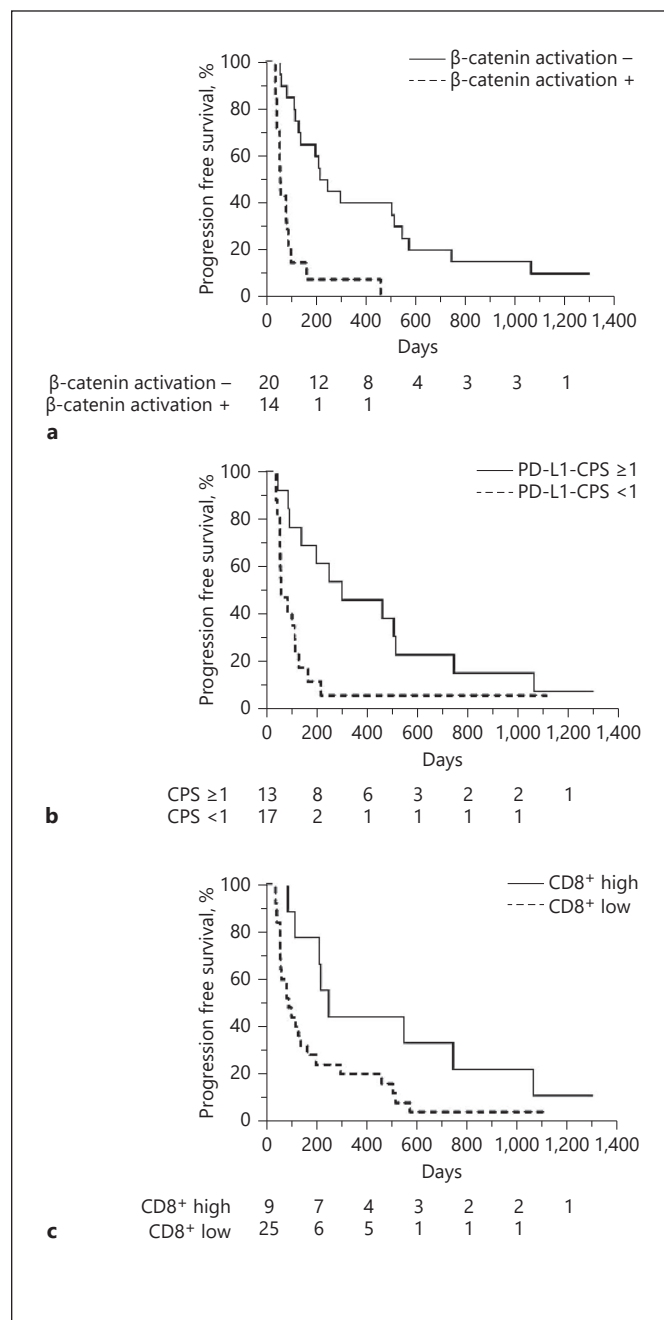
negative for activation, respectively ($p = 0.0003$ by log-rank test, Fig. 4a). Similarly, the median durations of OS and their 95% CI were 793 days (95% CI: 315–1,163) and 432 days (95% CI: 170–493) in patients with PD-L1-CPS ≥ 1 and those with PD-L1-CPS < 1 , respectively ($p = 0.0204$, Fig. 4b); median OS was 1,021 days (95% CI: 170–not determined) and 487 days (95% CI: 238–532) in patients with a high degree of CD8⁺ TILs and those with a low degree of CD8⁺ TILs ($p = 0.0089$, Fig. 4c), respectively. The median duration of OS was 430 days (95% CI: 187–532) and 668.5 days (95% CI: 466–1,163) in patients with AFP ≥ 400 ng/mL and those with AFP < 400 ng/mL ($p = 0.0289$ by log-rank test, online suppl. Fig. 1b).

Combined Immunohistochemical Findings for Wnt/ β -Catenin Activation, Expression of PD-L1, and Infiltration of CD8⁺ Cells Stratified by the Prognosis of Patients with HCC on anti-PD-1 Antibody

In this analysis, we performed the assignment for Wnt/ β -catenin activation, PD-L1-CPS, and infiltration of CD8⁺ cells through IHC and found that the absence of staining for β -catenin or GS represented the most valuable risk for favorable prognosis of HCC patients on anti-PD-1 therapy. However, high PD-L1-CPS and high degree of CD8⁺ TILs are also associated with a good tumor response to anti-PD-1 antibody. As IHC analyses sometimes yielded false-negative results because of the heterogeneity of staining in the tissue, and expression of PD-L1 and infiltration of CD8⁺ cells are also considered to be important for antitumor response to anti-PD-1 therapy, we assigned high PD-L1-CPS and high-degree CD8⁺

TILs, in addition to the negative staining of β -catenin or GS, as positive prognostic factors on ICLs, and studied the impact of accumulation of these 3 items on the prognosis of patients who received anti-PD-1 antibody. As shown in Figure 5, the median duration of PFS and 95% CI for patients without any positive prognostic factors, those with 1 positive prognostic factor, and those with 2 or more factors were 55 days (95% CI: 37–100), 89 days (95% CI: 42–460), and 271.5 days (95% CI: 136–547), respec-

Fig. 3. Kaplan-Meier curves of PFS on anti-PD-1 antibody in patients with HCC. PFS after the initiation of anti-PD-1 antibody administration was compared between HCC patients with positive and negative for activation of the Wnt/ β -catenin pathway (a), PD-L1-CPS ≥ 1 and PD-L1-CPS < 1 (b), and a high degree of CD8⁺ TILs and a low degree of CD8⁺ TILs (c). The median PFS and 95% CI were as follows: median durations of PFS were 56.5 days (95% CI: 42–89) and 231 days (95% CI: 115–547) in patients with positive for activation of the Wnt/ β -catenin pathway (β -catenin activation +) and those with negative for activation (β -catenin activation -), respectively ($p < 0.0001$ by log-rank test; panel a). Similarly, the median PFS was 297 days (95% CI: 89–514) and 58 days (95% CI: 54–115) in patients with a CPS of PD-L1 (PD-L1-CPS) ≥ 1 and those with PD-L1-CPS < 1 ($p = 0.0109$; panel b); median PFS durations were 246 days (95% CI: 84–1,064) and 89 days (95% CI: 56–164) in patients with a high degree of tumor-infiltrating CD8⁺ cells (CD8⁺ high) and those with a low degree of tumor-infiltrated CD8⁺ cells (CD8⁺ low), respectively ($p = 0.0344$; panel c). HCC, hepatocellular carcinoma; CPS, combined positive score; PFS, progression-free survival; TIL, tumor-infiltrating lymphocyte.



tively ($p < 0.0001$ by log-rank test, Fig. 5a). Similarly, the median duration of OS and 95% CI for patients without any positive prognostic factors, those with 1 positive prognostic factor, and those with 2 or more factors were 432 days (95% CI: 73–493), 479.5 days (95% CI: 187–547), and 859 days (95% CI: 225–1,163), respectively ($p = 0.0048$, Fig. 5b). This evidence suggests that comprehensive assessment of Wnt/ β -catenin activation and immune-related molecules could represent the immunological status that affects the response to anti-PD-1 therapy;

assessment of IHC could be a useful tool for selecting patients who may respond well to ICIs.

Evaluation of the Immunological Microenvironment Using Transcriptome-Based Immunogram in HCC with and without Activating Mutations in the Wnt/ β -Catenin Pathway

Constitutive activation of the Wnt/ β -catenin pathway in HCC is mostly due to mutations in the *CTNNB1* gene [15, 36, 37]. The immunograms represent the 10 kinds of immune status classified using the ssGSEA and were shown in radar chart (online suppl. Fig. 2a). Each immunogram for 20 HCCs with mutation in the *CTNNB1* gene and 20 HCCs without mutation in the *CTNNB1* gene was shown in online suppl. Fig. 2b, c, respectively. We compared the IGS of each axis of the immunogram between 20 HCCs with mutations in *CTNNB1* and 20 HCCs without mutations in *CTNNB1* (Table 3). Among the 10 axes that represent the corresponding molecular profiles shown in the immunogram, no significant differences were found in axis 1 (innate immunity, $p = 0.0884$ by Wilcoxon rank-sum test), axis 3 (T cells, $p = 0.0699$), axis 7 (inhibitory cells [myeloid-derived suppressor cells], $p = 0.0583$), axis 8 (recognition of tumor cells, $p = 0.1517$), axis 9 (proliferation, $p = 0.0989$), and axis 10 (glycolysis, $p = 0.3040$). However, we found significant differences in axis 2 (priming and activation, $p = 0.0020$), axis 4 (IFNG response, $p = 0.0011$), axis 5 (inhibitory molecules, $p = 0.0265$), and axis 6 (inhibitory cells [regulatory T cells], $p = 0.0029$) (Table 3). Subsequently, we performed mul-

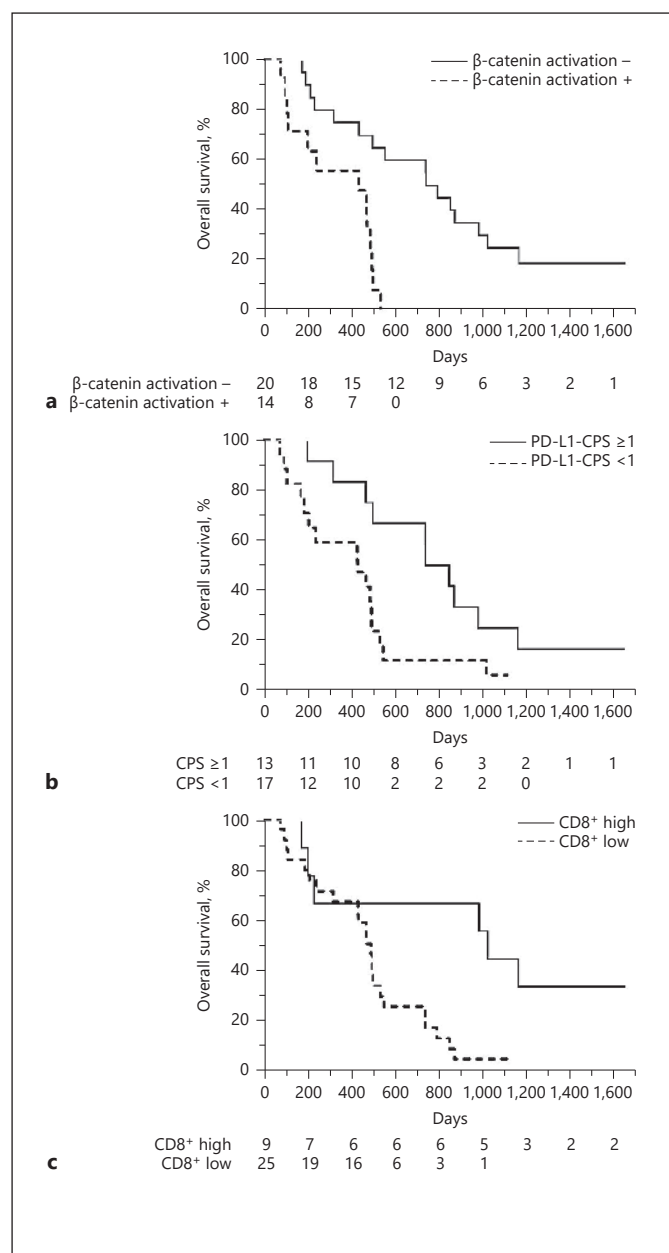


Fig. 4. Kaplan-Meier curves of OS on anti-PD-1 antibody in patients with HCC. The OS after the initiation of anti-PD-1 antibody was compared between HCC patients with positive and negative for activation of the Wnt/ β -catenin pathway (**a**), PD-L1-CPS ≥ 1 and PD-L1-CPS < 1 (**b**), and a high degree of CD8⁺ TILs and a low degree of CD8⁺ TILs (**c**). The median OS and their 95% CI of each subgroup were as follows: median OS, 432 days (95% CI: 103–487), and 764 days (95% CI: 315–1,021) in patients with positive for the Wnt/ β -catenin pathway (β -catenin activation +) and those with negative for activation of this pathway (β -catenin activation -), respectively ($p = 0.0003$ by log-rank test; panel **a**). The median durations of OS and their 95% CI were 793 days (95% CI: 315–1,163) and 432 days (95% CI: 170–493) in patients with a CPS of PD-L1 (PD-L1-CPS) ≥ 1 and those with PD-L1-CPS < 1 , respectively ($p = 0.0204$; panel **b**); median OS was 1,021 days (95% CI: 170 – not determined) and 487 days (95% CI: 238–532) in patients with a high degree of tumor-infiltrated CD8⁺ cells (CD8⁺ high) and those with a low degree of tumor-infiltrated CD8⁺ cells (CD8⁺ low) ($p = 0.0089$; panel **c**), respectively. HCC, hepatocellular carcinoma; CPS, combined positive score; OS, overall survival; CI, confidence interval; TIL, tumor-infiltrating lymphocyte.

tivariate analysis using the IGS of each axis that showed a difference in univariate analysis for the mutational status of the *CTNNB1* gene and found significant differences in axis 4 (IFNG response, $p = 0.0490$; Table 3).

We performed hierarchical clustering analysis using IGS of each axis corresponding to the stage of the immune response, and successfully detected 2 clusters (clus-

ter 1 and cluster 2) on a heatmap (online suppl. Fig. 3a); HCCs with *CTNNB1* mutation tended to be predominant in cluster 1 that showed lower expression of immune-related genes, which should be attributed to the lower infiltration of immune cells ($p = 0.0578$ by Pearson's χ^2 test, online suppl. Fig. 3b).

Discussion/Conclusion

Recent developments in systemic chemotherapy for HCC have improved the prognosis of patients with advanced HCC. For example, phase-III clinical trials for several MTAs, such as lenvatinib, sorafenib, regorafenib, ramucirumab, and cabozantinib have revealed the considerable effect of MTAs on survival [3–7]. However, acquired mutations in cancer cells induce resistance to TKIs because of the development of the escape pathway. On the other hand, ICIs are promising agents for the treatment of HCC, even in patients who show resistance to TKIs. It modulates the immune suppressive microenvironment, where mutations that induce resistance to ICI may rarely occur [38]. However, although phase-I/II clinical trial (CheckMate 040) and another phase-II clinical trial (KEYNOTE-224) of ICIs for HCC showed promising effects on survival, the phase-III clinical trial failed to show a superior effect on survival in patients who were treated with anti-PD-1 antibody compared to the control groups [8–10]. Based on the reports of the trials, around 20% of the patients showed tumor response to ICI monotherapies, suggesting that ICIs could be beneficial in a subset of patients with HCC, and currently, no effective biomarker has been established for predicting the effect of ICIs. It has also been reported that PD-L1 expression in tumors is associated with tumor response to this type of agent [20], indicating that the status of the tumor microenvironment is important to draw a benefit on ICI treatments. From this point of view, understanding the immunological microenvironment in HCC is required to develop biomarkers for ICIs in HCC treatment. In this study, we clearly showed that simple IHC-based analyses for evaluating immunological status could stratify the survival of patients with HCC on ICIs.

We examined the association between the data from the blood chemical test and tumor response to anti-PD-1 antibody. In our study, a significant association was observed between lower serum AFP levels and DC. Another report also showed that patients with serum AFP levels <400 ng/mL tended to show a longer survival than those with serum AFP level ≥ 400 ng/mL on anti-PD-1 therapy

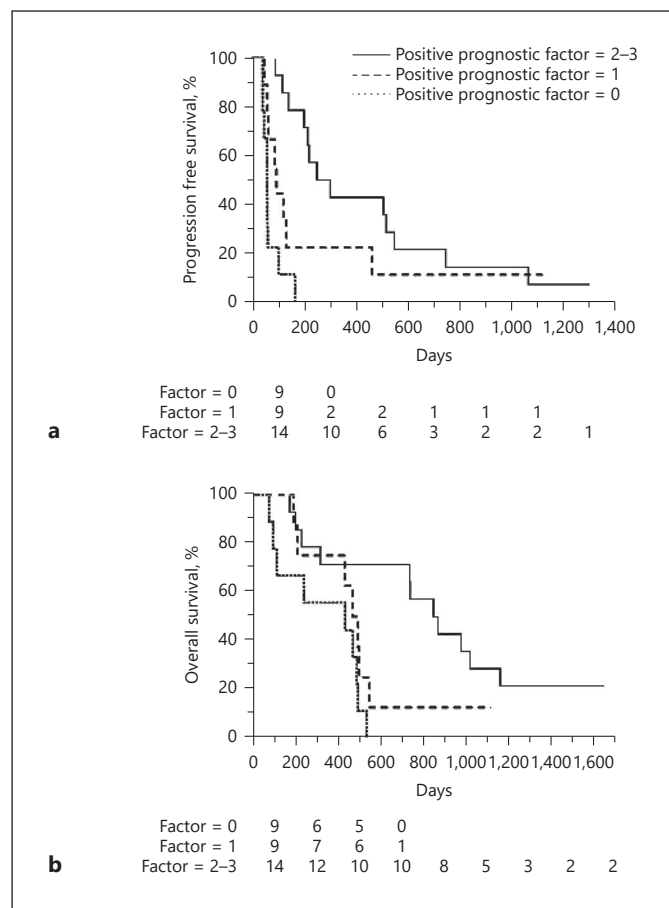


Fig. 5. Kaplan-Meier curves for survival classified based on the number of positive prognostic factors in patients with HCC. Kaplan-Meier curves for PFS and OS stratified by the members of positive prognostic factors are shown in (a, b), respectively. **a** The median duration of PFS and 95% CI for patients without any positive prognostic factors (positive prognostic factor = 0), those with one positive prognostic factor (positive prognostic factors = 1), and those with 2 or more factors (positive prognostic factors = 2–3) were 55 days (95% CI; 37–100), 89 days (95% CI; 42–460), and 271.5 days (95% CI; 136–547), respectively ($p < 0.0001$ by log-rank test). **b** The median duration of OS and 95% CI for patients without any positive prognostic factors, those with one positive prognostic factor, and those with 2 or more factors were 432 days (95% CI; 73–493), 479.5 days (95% CI; 187–547), and 859 days (95% CI; 225–1,163), respectively ($p = 0.0048$). HCC, hepatocellular carcinoma; PFS, progression-free survival; OS, overall survival.

Table 3. Presence or absence of the *CTNNB1* mutation and immune status defined by the immunogram

Immune status by immunogram	<i>CTNNB1</i> mutation	IGS median (distribution)	<i>p</i> value for univariate analysis ¹	<i>p</i> value for multivariate analysis ²
Innate immunity (axis 1)	With Without	2.0 (1.2–5.9) 3.5 (0.9–8.2)	0.0884	
Priming and activation (axis 2)	With Without	2.2 (0.1–4.8) 3.8 (1.4–7.0)	0.0020 ³	0.5271
T cells (axis 3)	With Without	2.4 (1.0–5.7) 3.3 (1.1–7.4)	0.0699	
Interferon- γ response (axis 4)	With Without	1.9 (0.7–4.4) 3.8 (1.5–6.2)	0.0011	0.0490
Inhibitory molecules (axis 5)	With Without	2.2 (–0.3–5.2) 3.7 (0.6–5.7)	0.0265	0.1173
Inhibitory cells (Tregs) (axis 6)	With Without	2.2 (1.1–4.3) 3.2 (1.3–8.4)	0.0029	0.8489
Inhibitory cells (MDSCs) (axis 7)	With Without	2.3 (0.1–5.1) 3.9 (0.6–6.3)	0.0583	
Recognition of tumor cells (axis 8)	With Without	2.5 (0.1–4.5) 3.2 (1.3–6.3)	0.1517	
Proliferation (axis 9)	With Without	3.1 (–1.9–4.5) 3.4 (1.2–5.4)	0.0989	
Glycolysis (axis 10)	With Without	2.8 (0.6–5.4) 3.0 (0.1–6.7)	0.3040	

HCC, hepatocellular carcinoma; IGS, immunogram score; MDSC, myeloid-derived suppressor cell; Tregs, regulatory T cells. The IGS was compared between HCCs with and without mutation of the *CTNNB1* gene in each axis of the immunological diagram. Twenty HCCs with and without *CTNNB1* mutation, respectively, were analyzed for immunogram. Among the 10 axes that represent the corresponding molecular profiles shown in the immunogram, significant differences were found in the IGS of axis 2, axis 4, axis 5, and axis 6 depending on presence or absence of the *CTNNB1* gene mutation. Multivariate analysis showed a significant difference for IGS in axis 4 ($p = 0.0490$). ¹*p* value by Wilcoxon rank-sum test. ²*p* value by logistic regression analysis. ³Bolds denote the *p* value <0.05.

[20]. In addition, other markers for systemic inflammation, such as elevated NLR and PLR, are also reportedly associated with poor prognosis in HCC [20]. Although we failed to show an association between NLR and PLR and tumor response, relatively small number of the analyzed cases may be attributed to the negative findings for the association between NLR, PLR, and tumor response. In contrast, lymphocyte count, RDW, and LDH have also been reported to be associated with a good response to ICI in lung cancer and melanoma cases [16–19]. In this study, we did not find any associations between these laboratory data and DCR in HCC cases although cases with high RDW were more frequent in patients who showed objective response (online suppl. Table 1). A larger cohort

is required to determine the role of markers related to systemic inflammation as a biomarker of ICIs in HCC cases.

On the other hand, we found significant association between negative staining related to Wnt/ β -catenin activation, high grade of CD8⁺ TILs, and high PD-L1-CPS and DC. However, as the benefits of ICI are presumed to be large in the case of objective response, we also analyzed the relationship between clinical parameters and an achievement of objective response; borderline association was observed only for high RDW. It is possible that the number of objective response cases in this cohort was small that might not be enough for statistical analyses. A previous report also evaluated the impact of Wnt/ β -catenin

activation on anti-PD-1 therapy using DCR because ICI can be expected to have a long-term effect even in SD cases [14]. Therefore, it is reasonable to focus on DCR for tumor response, which may well reflect the longer survival on ICIs; further study with larger cohort containing more cases with objective response is required.

Contrastingly, it has been suggested that immune hot status is critical for the successful treatment of cancer with ICI [39, 40]. In this context, currently, several studies showed the immune subclass of HCC based on the expression of cytokine, chemokine, immune checkpoint molecules, and infiltration of immune cells in the tumor [27, 41–45]. In addition, constitutive activation of the Wnt/ β -catenin pathway has been reported to be associated with the immune cold phenotype of cancer, including HCC [12, 13]. Harding et al. [14] reported that HCC patients with activating mutations of the Wnt/ β -catenin pathway in tumors showed shorter PFS than those without mutations on treatment with ICIs. Recently, atezolizumab plus bevacizumab combination therapy was revealed to show a superior effect on antitumor response rate compared to ICI monotherapy [8–10, 46]. From this point of view, it is intriguing to know the efficacy of combination of ICI with MTA on HCCs carrying Wnt/ β -catenin activation.

We also found that the absence of staining of the molecules in Wnt/ β -catenin signaling, high infiltration of CD8⁺ cells, and high CPS of PD-L1 were all significant contributors to the antitumor response on anti-PD-1 antibody. In addition, these findings were also associated with good prognosis. In this context, we hypothesized accumulation of these favorable factors might show an impact on the survival of the patients. As expected, the accumulation of these factors defined by IHC well stratifies the survival of HCC patients on anti-PD-1 antibody, suggesting that the immunological microenvironment should affect the survival of patients with HCC and IHC for assessing the activation of Wnt/ β -catenin signaling, degree of CD8⁺ TILs, and expression of PD-L1 is a simple and effective way to select patients who benefit from anti-PD-1 antibody.

Finally, among the ORs of Wnt/ β -catenin activation, the degree of CD8⁺ TIL and PD-L1-CPS on DCR that of Wnt/ β -catenin activation showed the highest value, suggesting the impact of role of Wnt/ β -catenin signaling on the effect of ICIs in HCC cases. To clarify the characteristics of HCC with activation of the Wnt/ β -catenin pathway, we compared the immune status of tumors between HCCs with activating mutations in the *CTNNB1* gene and those without mutation using transcriptome-based

immunogram, where 10 molecular profiles that represent the characteristics of each immune step can be visualized [34]. In the HCC with *CTNNB1* mutation, in addition to the IGS related to the priming and activation, IGS of the IFNG response, inhibitory molecules, and inhibitory cells were significantly lower in HCCs carrying the *CTNNB1* mutation than in those without mutations, where the IFNG response showed the most prominent difference. This evidence clearly supports the idea that activation of the Wnt/ β -catenin pathway is associated with an immune cold phenotype with fewer infiltrating immune cells in tumor. On the contrary, tumors without *CTNNB1* mutation may carry enough TILs, a subset of such HCC may have TILs showing an exhausted phenotype because the IGS of the inhibitory molecules (axis 5) and inhibitory cells (axis 6) is high in HCC without *CTNNB1* mutation compared to those with the mutation, as suggested previously [31, 45, 47, 48].

We found a considerable effect of anti-PD-1 antibody on HCC cases with a lack of β -catenin activation, suggesting that blockade of the PD-1/PD-L1 axis might, at least partially, overcome the presence of exhausted TILs with other immunosuppressive molecules. Although the classification of HCCs using the hierarchical clustering analysis based on the IGS showed borderline significance for the association between the transcriptome-based immune phenotype and *CTNNB1* mutation ($p = 0.0578$, online suppl. Fig. 3b), 2 axes that could be affected by the characteristics of cancer cells (axis 9 that represented proliferation and axis 10 showing glycolysis) were also involved in the immunogram, which might make the association between the transcriptome-based immune classification and *CTNNB1* mutation obscure. Indeed, classification with hierarchical clustering analysis using IGS from axis 1 to 8 represented a significant difference in this association (data not shown). In addition, the expression of IFNG, which induces the expression of PD-L1 [49], was enhanced in HCC without β -catenin mutation. This evidence clearly supports the idea that the Wnt/ β -catenin pathway plays an important role in determining the immunological microenvironment of HCC.

The limitation of this study includes the fact that only 34 cases were analyzed, and most of the tissue was obtained from liver biopsy specimens, where heterogeneity of staining in IHC was observed. It is possible that there was a false-negative result for immune staining of immune-related molecules because of its heterogeneity in expression. Indeed, although the immunogram suggests the relationship between the mutation of *CTNNB1* and immune cold phenotype, the IHC fails to show the sig-

nificant association between the activation of Wnt/ β -catenin pathway and low grade of CD8⁺ TILs and PD-L1-CPS, although the HCCs with Wnt/ β -catenin activation tend to carry low CD8⁺ TILs and PD-L1 expressions (online suppl. Table 2). Therefore, we believe that counting the total number of IHC findings, β -catenin/GS, CD8, and PD-L1 expression could minimize the false-negative results in terms of the presence of an immune cold phenotype. Another limitation of this study is only the limited molecules have been analyzed for evaluating immunological microenvironments of HCC. For example, several oncogenic signals are known to affect antitumor immunity [50]. Regarding HCC cases, a functional link between mutation of ARID2 and chemokine production related to tumor-associated macrophage, which led to noninflamed tumor, was reported [51]. Inactivation of Hippo pathway in HCC is also known to induce immune suppressive status through the induction of tumor-associated macrophage due to the expression of C-C motif chemokine 2 by yes-associated protein in mouse model [52]. Even in HCCs with enough TILs, a recent report showed that tumor related to nonalcoholic steatohepatitis (NASH) might be less responsive to ICIs, probably owing to the NASH-related aberrant T-cell activation that resulted in the impaired immune surveillance [53]. Because of the complexity of the antitumor immunity, data based on multi-omics analysis might be more informative for developing innovative biomarker of ICIs. Nevertheless, predicting the prognosis of HCC patients on ICIs by simple IHC evaluations is clinically significant because, currently, there are no biomarkers, and analysis using IHC is cost-effective compared to omics-based analysis. This result is considered beneficial for selecting the appropriate agents for personalized medicine in HCC treatment.

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Statement of Ethics

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. We used archived tissue samples for this study that were obtained with patient's written consent. This study was approved by the Ethics Committee of Kindai University Hospital (IRB approved number: #31–145).

Conflict of Interest Statement

Masatoshi Kudo reports honoraria and consulting or advisory fees from Bayer AG, Roche, and Eisai Co. Ltd.; honoraria from Bayer AG, Bristol Myers Squibb, EA Pharma Co., Ltd., Eisai Co. Ltd., and Merck Sharp & Dohme; and research funding from Bayer AG, Daiichi Sankyo Co., Ltd., Chugai Pharmaceutical Co., Ltd., Merck Sharp & Dohme, Otsuka Pharmaceutical Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Ono Pharmaceutical Co., and Taiho Pharmaceutical. M.K. is an Editor-in-Chief, and N.N. is an Editorial Board member of *Liver Cancer*.

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Author Contributions

Study concept and design: N.N. Acquisition of data: M.M., N.N., K.S., T.A., H.C., T.M., M.T., S.H., Y.M., H.L., K.U., and M.K. Analysis and interpretation of the data: M.M., N.N., K.S., K.N., Y.K., and K.K. Drafting of the manuscript: M.M. and N.N. Revision of the manuscript for intellectual content: all authors. Statistical analysis: M.M. and N.N. Study supervision: N.N. and M.K.

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