WNT/β-catenin Signaling and CD8+ Tumor-infiltrating Lymphocytes in Tremelimumab Plus Durvalumab for Advanced Hepatocellular Carcinoma

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Abstract. *Background/Aim: Tremelimumab plus durvalumab is an approved first-line therapy for advanced hepatocellular carcinoma (HCC). Previous studies identified WNT/β-catenin mutations or CD8+ tumor-infiltrating lymphocytes (TILs) as biomarkers that can predict responsiveness to immune checkpoint inhibitor therapy in HCC. However, biomarkers for effectiveness of tremelimumab plus durvalumab in HCC have not been reported. This study investigated whether evaluation of WNT/β-catenin signaling and CD8+ TILs by immunohistochemical staining of tumor biopsy tissues can predict the response to tremelimumab plus durvalumab in patients with HCC. Patients and Methods: Fifteen HCC patients who underwent tumor biopsies were classified into three groups based on WNT/β-catenin signal activation and CD8+ TIL infiltration. The clinical responses to treatment in the groups were evaluated. Results: Four patients had HCC with WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration, four patients had HCC with WNT/β-catenin signal activation and low-level CD8+ TIL infiltration, and seven patients had WNT/β-catenin signal activation and high-level CD8+ TIL infiltration or WNT/β-catenin signal inactivation and low-level CD8+ TIL infiltration. A better response rate was observed in the WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration group, and a worse response rate was observed in the WNT/β-catenin signal activation and low-level CD8+ TIL infiltration group. Conclusion: Although the present study involved a small number of patients, the findings suggest that the efficacy of tremelimumab plus durvalumab may be affected by WNT/β-catenin signaling and CD8+ TIL infiltration.*

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In 2020, hepatocellular carcinoma (HCC) accounted for 900,000 new cancer cases and 830,000 deaths worldwide, making it the sixth most common neoplasm and the third leading cause of cancer-related deaths worldwide (1, 2). Patients with advanced HCC have benefited from recent advances in systemic chemotherapy, including therapy with immune checkpoint inhibitors (ICIs) and molecular targeted agents (3-9).

Tremelimumab is a human monoclonal antibody that targets cytotoxic T-lymphocyte-associated protein-4 (CTLA-4). Tremelimumab inhibits CTLA-4 activity, thereby contributing to T-cell activation, priming the immune response to cancer, and fostering the death of cancer cells. As a monoclonal antibody, durvalumab binds to programmed death ligand (PD-L1) and blocks PD-L1 interactions with PD-1 and CD80, thus countering the tumor immune-evading tactics and releasing the inhibition of immune responses. Combination therapy with tremelimumab plus durvalumab, which targets CTLA-4 and PD-L1, showed superior outcomes in the HIMALAYA trial compared with sorafenib, displaying a median survival time of 16.4 months (8). Therefore, atezolizumab plus bevacizumab or tremelimumab plus durvalumab may be offered as first-line therapy for patients with advanced HCC, Child–Pugh class A liver disease, and Eastern Cooperative Oncology Group performance status 0-1 (10).

The prognosis of patients with advanced HCC may be improved by selecting the appropriate chemotherapy. Thus, it is

Table I. *Characteristics of the study patients.*

Characteristics	All (n=15)	Group A (n=4)	Group B (n=7)	Group C (n=4)	*p*-Value
Age, years	71 (65-79)	69 (65-79)	64.5 (55.5-70.5)	64.5 (55.5-70.5)	0.0478
Sex, n (male/female)	13/2	2/2	7/0	4/0	0.0433
MVI positive, n	5	2	2	1	0.7133
EHS positive, n	3	2	0	1	0.0834
Intrahepatic max tumor size, cm	4.5 (1.9-12.7)	1.9 (0.7-4.6)	7.7 (3.1-14.0)	3.5 (0,7-11.8)	0.1308
Numbers of tumors >5	8	2	4	2	0.9624
Etiology					0.2567
HBV	1	0	0	1	
HCV	7	3	3	1	
NBNC	7	1	4	2	
Child-Pugh score A/B	11/4	3/1	6/1	2/2	0.4466
Alb, g/dl	3.3 (2.9-3.7)	3.4 (2.9-3.6)	3.3 (2.8-3.7)	3.3 (3.1-3.9)	0.9145
T.Bil, g/dl	1.0 (0.6-1.6)	0.7 (0.5-1.4)	1.1 (1.0-1.8)	1.0 (0.4-1.6)	0.4946
BCLC stage					
B/C	8/7	0/4	6/1	2/2	0.0089
Tumor marker					
AFP, ng/ml	1,416.3 (7.0-68,870.0)	758.4 (78.9-107,276)	7,464.8 (8.0-68,870.0)	5.3 (2.1-118,445.0)	0.4154
PIVKA-II, mAU/ml	389.0 (107.0-24,090.0)	2,066.0 (109.0-202,594.5)	9,597.0 (389.0-36,921.0)	128.5 (17.0-305.3)	0.0899

Data are expressed as median (interquartile range) unless otherwise indicated. Group A: WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration. Group B: WNT/β-catenin signal activation and high-level CD8+ TIL infiltration or WNT/β-catenin signal inactivation and low-level CD8+ TIL infiltration. Group C: WNT/β-catenin signal activation and low-level CD8+ TIL infiltration. TIL: Tumor-infiltrating lymphocyte; HCV: hepatitis C; HBV: hepatitis B; NBNC: non-hepatitis B non-hepatitis C; MVI: macroscopic portal vein invasion; EHS: extrahepatic spread; BCLC: Barcelona Clinic Liver Cancer; AFP: α-fetoprotein; PIVKA-II: protein induced by vitamin K absence or antagonist-II.

critical to choose agents that are suitable for personalized HCC treatment. Consequently, it is essential to identify potential predictive biomarkers and understand the mechanisms of resistance or response to systemic chemotherapy regimens. Tremelimumab plus durvalumab has not been associated with any established biomarkers that can predict responsiveness in HCC patients.

HCC progression depends on the tumor immune microenvironment, which facilitates interactions between tumor cells and immune cells. Treatment for HCC has focused on the classification of tumors according to WNT/β-catenin mutations. Approximately 40% of HCC patients harbor WNT/β-catenin mutations that result in the immune microenvironment lacking immune cell infiltration, so-called 'immune-exclusion HCC' or 'non-inflamed cold HCC' (11, 12). Immune-exclusion HCC associated with WNT/β-catenin signal activation is resistant to ICI therapy (13-15). As well as reflecting the local immune response, CD8+ tumor-infiltrating lymphocytes (TILs) may also play an important role in controlling tumor progression (16-18). In the tumor immune microenvironment, CD8+ T cells can promote the accumulation of distinct endogenous CD8+ and CD4+ T cells that support antitumor activity (19-21). Positive correlations between CD8+ TIL levels in the tumor immune microenvironment and responses to ICI therapy have been noted in various types of cancer (22, 23). Patients with immune-exclusion HCC, who have decreased CD8+ TIL infiltration, are resistant to ICI therapy, based on a recent study (11). CD8+ TILs may also be correlated with the clinical response to atezolizumab plus bevacizumab (24, 25).

While WNT/β-catenin signal activation is known to cause immune exclusion in other cancer types, HCC with WNT/β-catenin signal activation can be classified into two distinct types: inflamed tumors with CD8+ T cell infiltration and non-inflamed tumors without CD8+ T cell infiltration (26-28).

In this study, we investigated whether WNT/β-catenin signal activation and CD8+ TIL infiltration on HCC tissues could be useful biomarkers for predicting response to tremelimumab plus durvalumab.

Patients and Methods

Patients. This single-center prospective study analyzed the efficacy of tremelimumab plus durvalumab in HCC patients based on WNT/β-catenin signaling and CD8+ TIL infiltration. These parameters were evaluated by immunohistochemical staining of tumor tissues obtained by liver biopsy or lymph node metastasis biopsy using endoscopic ultrasound-guided fine needle aspiration at Aso Iizuka Hospital between April 2023 and May 2024. Thirty-two patients received tremelimumab plus durvalumab for advanced HCC. There were 10 patients who did not undergo tumor biopsy prior to chemotherapy and seven patients who were followed up within four weeks before evaluation of treatment response. We evaluated the remaining 15 patients. According to the Declaration

Table II. *Comparisons of responses between the groups*.

	All (n=15)	A (n=4)	B (n=7)	C (n=4)	*p*-Value
Overall response					0.0340
CR	1 (6.7%)	1 (25.0%)	0 (0%)	0 (0%)	
PR	5 (33.3%)	3 (75.0%)	2 (28.6%)	0 (0%)	
SD	3 (20.0%)	0 (0%)	1 (14.3%)	2 (50.0%)	
PD	6 (40.0%)	0 (0%)	4 (57.1%)	2 (50.0%)	
ORR (CR+PR)	6 (40.0%)	4 (100%)	2 (28.6%)	0 (0%)	0.0027
DCR (CR+PR+SD)	9 (60.0%)	4 (100%)	3 (42.9%)	2 (50.0%)	0.0787

Data are shown as n (%). Group A: WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration. Group B: WNT/β-catenin signal activation and high-level CD8+ TIL infiltration or WNT/β-catenin signal inactivation and low-level CD8+ TIL infiltration. Group C: WNT/β-catenin signal activation and low-level CD8+ TIL infiltration. CR: Complete response; PR: partial response; SD: stable disease; PD: progressive disease; ORR: objective response rate; DCR: disease control rate; TIL: tumor-infiltrating lymphocyte.

Table III. *Wnt/β-catenin signal activation and CD8+ TIL infiltration in the 15 patients*.

Case	Age, years	Sex	Etiology	β-catenin	GS	CD8	Group	Response
1	63	M	NBNC	1	1	0	C	PD
2	79	M	HCV	0	0	1	A	PR
3	79	M	HCV	1	1	1	B	PD
4	88	M	NBNC	0	0	0	B	PD
5	75	F	NBNC	0	0	1	A	CR
6	63	M	NBNC	1	1	1	B	PR
7	68	M	NBNC	1	1	1	B	SD
8	66	M	HCV	1	1	0	C	SD
9	53	M	HBV	1	0	0	C	PD
10	72	M	NBNC	1	1	0	C	SD
11	69	M	HCV	1	1	1	B	PD
12	65	M	HCV	0	1	1	B	PD
13	83	F	HCV	0	0	1	A	PR
14	71	M	NBNC	1	1	1	B	PR
15	82	M	HCV	0	0	1	A	PR

Group A: WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration. Group B: WNT/β-catenin signal activation and high-level CD8+ TIL infiltration or WNT/β-catenin signal inactivation and low-level CD8+ TIL infiltration. Group C: WNT/β-catenin signal activation and low-level CD8+ TIL infiltration. TIL: Tumor-infiltrating lymphocyte; HCV: hepatitis C; HBV: hepatitis B; NBNC: non-hepatitis B non-hepatitis C; GC: glutamine synthetase; PD: progressive disease; PR: partial response.

of Helsinki, the study was approved by the Ethics Committee of Aso Iizuka Hospital (approval no. 23056). Consent for the study was obtained using the opt-out method.

Treatment protocol. This regimen consisted of tremelimumab 300 mg and durvalumab 1,500 mg every four weeks (termed the STRIDE regimen) administered intravenously according to the HIMALAYA study conducted by AstraZeneca Co., Ltd. (8). The treatment was continued until the disease progressed or the side effects became intolerable.

Evaluation of efficacy. Every 4-12 weeks following treatment initiation, computed tomography or magnetic resonance imaging was performed to determine the treatment's effectiveness. Response Evaluation Criteria in Solid Tumors (RECIST) criteria were used by the treating physician to evaluate the antitumor response (29). A complete response (CR), partial response (PR), or stable disease

(SD) lasting at least four months was defined as the disease control rate (DCR). The objective response rate (ORR) was defined as PR+CR. Patients were followed up every two weeks.

Immunohistochemistry (IHC). We conducted immunostaining in accordance with our previous reports (25, 30). The presence of β-catenin, glutamine synthetase (GS), and CD8 was evaluated using the following primary antibodies: monoclonal mouse anti-human β-catenin (#610153; 1:300; BD Biosciences, Franklin Lakes, NJ, USA), monoclonal mouse anti-human GS (#GS-6; 1:500; Millipore, Burlington, MA, USA), and mouse anti-human monoclonal CD8 (clone C8/144B; 1:50; DAKO, Agilent, Santa Clara, CA, USA). The activation of WNT/β-catenin signaling was defined by β-catenin nuclear staining in ≥5% of cancer cells or diffuse GS staining in ≥50% of cancer cells, as previously reported (31-35). The high and low level of CD8+ cell infiltration in the tumors was defined using a cut off value of 15.9 cells/high-power field according to our previous report (25).
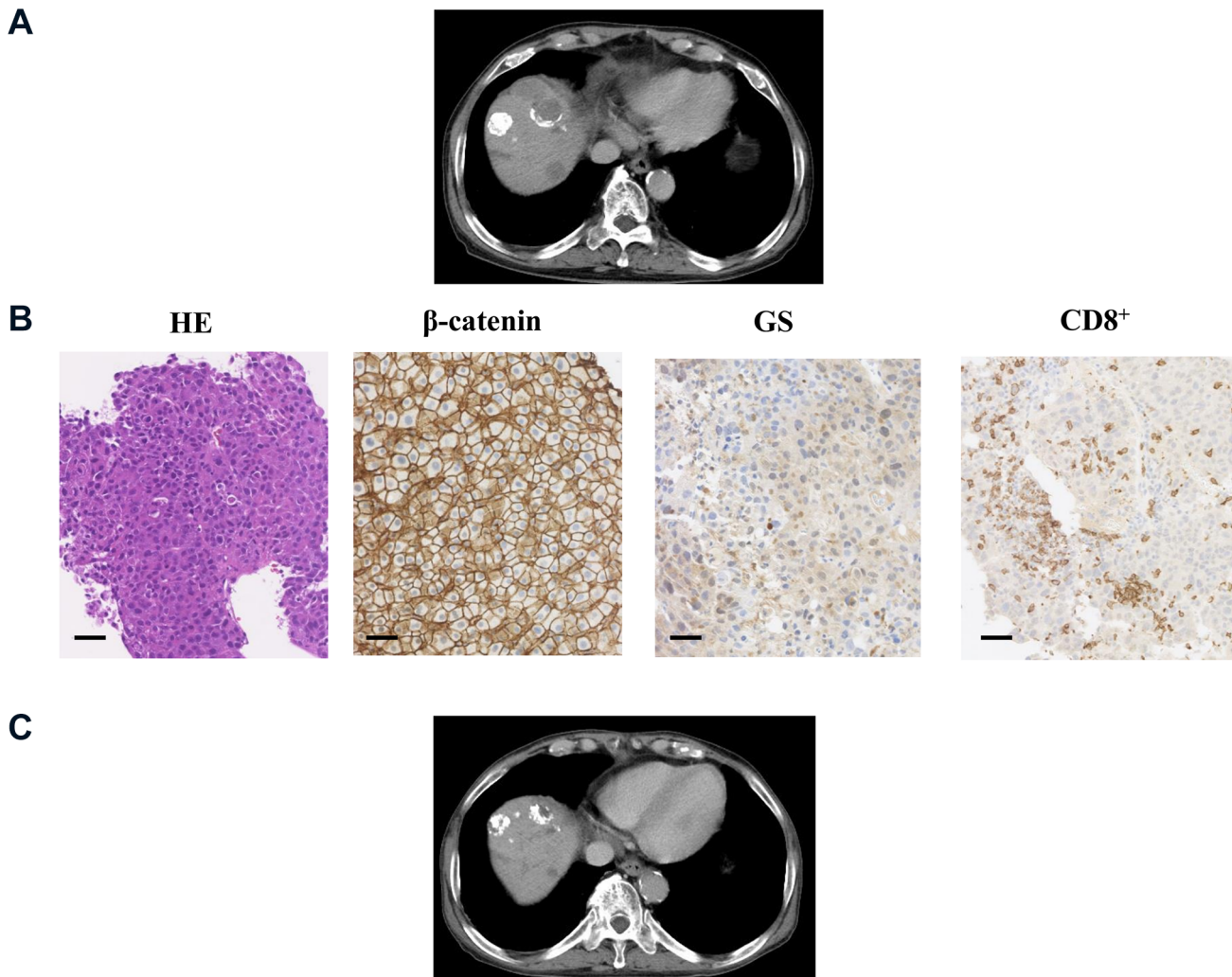
A

B

| HE | β-catenin | GS | CD8⁺ |

C

Figure 1. *Representative Case 2: WNT/β-catenin signal inactivation and high-level CD8+ tumor-infiltrating lymphocyte infiltration. (A) Computed tomography image of the early arterial phase prior to treatment. (B) Images of liver specimens subjected to hematoxylin-eosin (HE) staining (magnification: 100×; scale bar: 100 μm) and immunohistochemical staining for β-catenin, glutamine synthetase (GS), and CD8 (magnification: 100×, scale bar: 100 μm). (C) CT image of the early arterial phase to evaluate treatment efficacy.*

Statistical analysis. All statistical analyses were conducted using JMP Pro version 11 software (SAS Institute Inc., Cary, NC, USA). Data are presented as medians (interquartile ranges), and Fisher's exact test and Mann-Whitney *U*-tests are used to compare groups. Statistical significance was accepted at $p<0.05$.

Results

Patient characteristics. The characteristics of the 15 patients who received tremelimumab plus durvalumab are shown in Table I. We divided the patients into three groups based on WNT/β-catenin signaling and CD8+ T cell infiltration: Group A, WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration; Group B, WNT/β-catenin signal activation and high-level CD8+ TIL infiltration or WNT/β-catenin signal inactivation and low-level CD8+ TIL infiltration; Group C, WNT/β-catenin signal inactivation and low-level CD8+ TIL infiltration.

There were four patients in Group A, seven patients in Group B, and four patients in Group C. There were more female patients and more patients with Barcelona Clinic Liver Cancer stage C in Group A than in the other groups. The level of protein induced by vitamin K absence or antagonist-II was lower in Group C than in the other groups. Age, etiology, Child–Pugh grade, tumor size, number of intrahepatic lesions, microvascular invasion, extrahepatic spread, and serum α-fetoprotein (AFP) levels were similar among the groups.
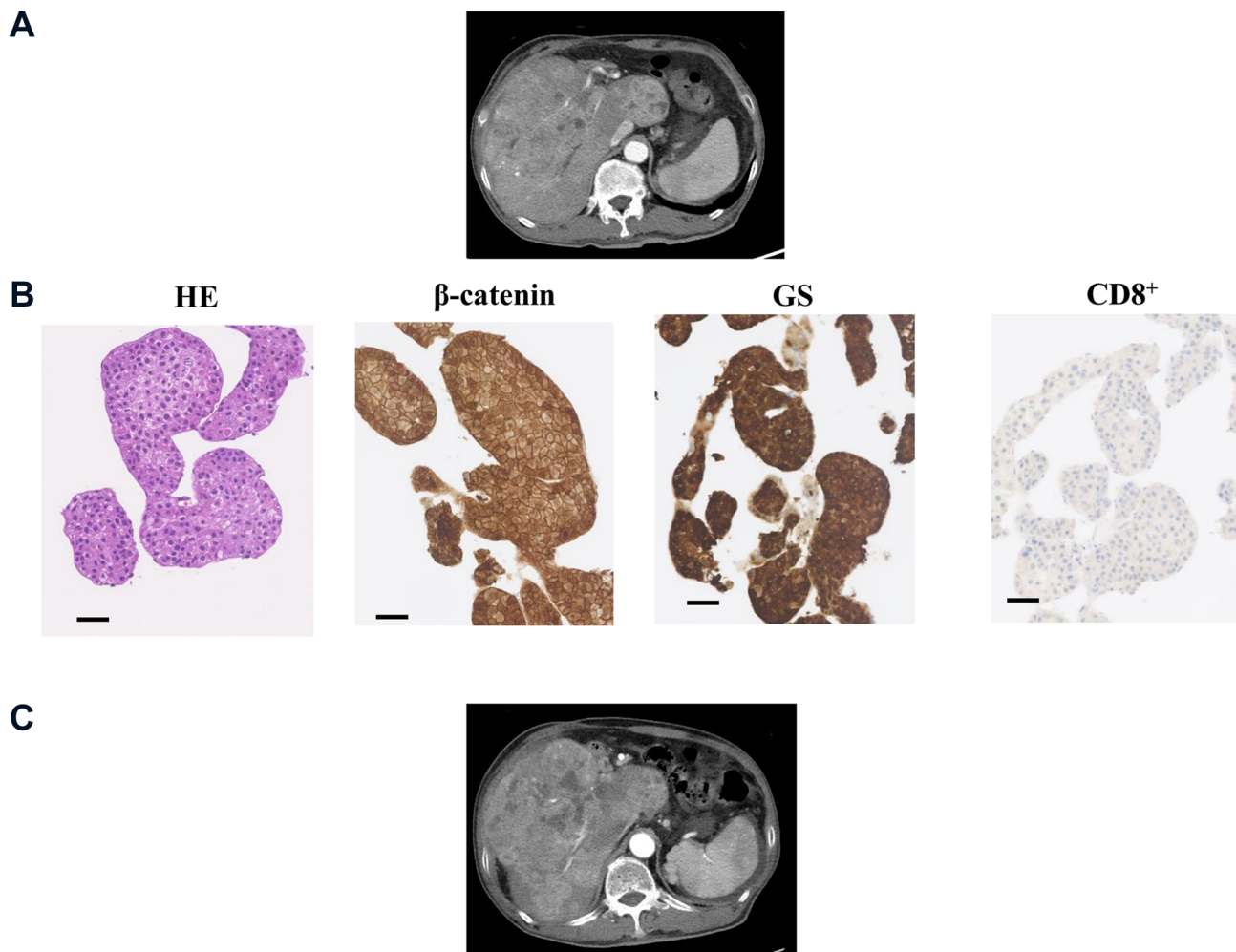
A

B

HE	β-catenin	GS	CD8+

C

Figure 2. *Representative Case 9: WNT/β-catenin signal activation and low-level CD8+ tumor-infiltrating lymphocyte infiltration. (A) Computed tomography image of the early arterial phase prior to treatment. (B) Images of liver specimens subjected to hematoxylin-eosin (HE) staining (magnification: 100×; scale bar: 100 μm) and immunohistochemical staining for β-catenin, glutamine synthetase (GS), and CD8 (magnification: 100×; scale bar: 100 μm). (C) CT image of the arterial phase to evaluate treatment efficacy.*

Effect of tremelimumab plus durvalumab. The ORR (CR+PR) was 4/4 (100%) in Group A, 2/7 (33.3%) in Group B, and 0/0 (0%) in Group C (*p*=0.0027). The DCR (CR+PR+SD) was 4/4 (100%) in Group A, 3/7 (42.9%) in Group B, and 2/4 (50%) in Group C (*p*=0.0787) (Table II). Therefore, WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration affected the efficacy of tremelimumab plus durvalumab.

IHC for β-catenin, glutamine synthetase and CD8 in HCC tissues. The expression levels of β-catenin, GS, and CD8 were assessed by IHC prior to tremelimumab plus durvalumab therapy (Table III). Representative cases are shown in Figure 1 and Figure 2. Case 2 was a 79-year-old man with hepatitis C virus-related multiple HCC (Figure 1). The immunostaining in this moderately differentiated HCC revealed WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration. After administration of tremelimumab plus durvalumab, CT imaging of the liver showed decreased tumor size and enhancement in the arterial phase, indicating PR. The serum AFP level was 142,562.5 ng/ml before treatment and decreased to 8,544.8 ng/ml after three cycles. Case 9 was a 53-year-old man with hepatitis B virus-related HCC (Figure 2). The biopsy specimen was diagnosed as moderately differentiated HCC, and immunostaining revealed WNT/β-catenin signal activation with low-level CD8+ TIL infiltration. CT imaging demonstrated increased tumor size and enhancement in the arterial phase, indicating PD. The serum AFP level was 1,141,188.0 ng/ml before treatment and increased to 2,206,150.0 ng/ml after three cycles.

Discussion

The immune response can play an important role in cancer progression. Based on the cellular and molecular characteristics, Llovet *et al.* (36) classified HCC into inflamed and non-inflamed classes. The inflamed class includes the immune-active, exhausted, and immune-like subtypes, while the non-inflamed class includes the intermediate and excluded subtypes.

The WNT/β-catenin cascade is a major signaling pathway for the regulation of liver carcinogenesis. Approximately 30%-40% of HCC cases were reported to show WNT/β-catenin signaling activation induced by gene mutations (37, 38). WNT/β-catenin activation was shown to inhibit cytotoxic TIL infiltration in the immune microenvironment of melanoma, resulting in resistance to anti-PD-L1/anti-CTLA-4 monoclonal antibody therapy (39). WNT/β-catenin activation had negative effects on the DCR and progression-free survival (PFS) in patients with HCC who received anti-PD-L1 antibody therapy (11-13). Taken together, these studies indicate that WNT/β-catenin activation may be a biomarker for the response to ICI therapy in patients with HCC. We previously reported that CD8+ TIL infiltration could be a biomarker for predicting the response to atezolizumab plus bevacizumab and that the efficacy of atezolizumab plus bevacizumab may be unaffected by WNT/β-catenin signal activation (25, 30). WNT/β-catenin signal activation causes immune exclusion, and HCC with WNT/β-catenin signal activation can be classified into two distinct types. Specifically, HCC with mutations causing WNT/β-catenin signal activation can be either inflamed with CD8+ T cell infiltration or non-inflamed without CD8+ T cell infiltration (26, 27). We evaluated whether IHC staining of tumor tissues to assess WNT/β-catenin activation and CD8+ TIL infiltration classified into three groups could predict response to tremelimumab plus durvalumab in patients with HCC. We found that tremelimumab plus durvalumab was remarkably effective in HCC patients with WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration.

In this study, PFS and overall survival (OS) were not evaluated because of the small number of cases. ORR has been shown to predict OS in individuals with unresectable HCC (40, 41). Regarding tremelimumab plus durvalumab therapy, the good response in HCC with WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration may result in long OS.

A limitation of the present study was the inclusion of only a small number of HCC patients who underwent tumor biopsies because of its nature as a single-center study. Furthermore, the study included advanced HCC cases of different stages. Finally, when analyzing a small number of cases, it is difficult to match groups according to liver function and tumor stage.

Conclusion

The present findings suggest that WNT/β-catenin signaling and CD8+ TIL infiltration, as evaluated by IHC staining of tumor biopsy tissues, may be useful biomarkers for predicting response to tremelimumab plus durvalumab therapy in patients with HCC. The findings suggest that tremelimumab plus durvalumab can be recommended for HCC with WNT/β-catenin signal inactivation and high-level CD8+ TIL infiltration based on IHC staining of tumor biopsy tissues. To improve the prognosis of patients with advanced HCC, further studies regarding chemotherapy selection are needed.

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Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

A.K., K.T., and K.M. designed the study. A.K., K.T., J.T., and H.S. assisted with data analyses. Y.O. performed pathological examinations, including immunostaining. A.K. wrote the initial draft of the manuscript. K.T. contributed to the analysis and interpretation of the data. K.T. and K.M. assisted in the preparation and critical review of the manuscript. All Authors approved the final version of the manuscript and have agreed to be accountable for all aspects of the work.

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