Significance of PD-L1 clones and C-MET expression in hepatocellular carcinoma

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Received September 14, 2018; Accepted March 29, 2019

DOI: 10.3892/ol.2019.10222

Abstract. Programmed cell death ligand 1 (PD-L1) is an essential immune checkpoint protein implicated in immune evasion by malignant tumors. Overexpression of programmed cell death protein 1 (PD-1) and its ligand PD-L1 is associated with poor prognosis in various types of cancer. Recently, multiple advances have occurred in the area of cancer immunotherapy. Inhibiting the ligation of PD-1 by PD-L1 has been the major focus of anti-tumor immunotherapy. In diagnostic pathology, it has become crucial to detect PD-L1$^+$ tumor cases using a validated immunohistochemistry (IHC) approach. Preliminary data demonstrate that C-MET promotes survival of some (e.g., renal) cancer types through regulation of PD-L1. However, C-MET expression, and its association with PD-L1, has not been well-characterized in the context of hepatocellular carcinoma (HCC), and no anti-HCC immunotherapy is currently available in Korea. Therefore, it is crucial to investigate the expression of C-MET and PD-L1, and their association with clinicopathologic factors, to facilitate the development of targeted treatments for HCC. PD-L1 expression was examined in tumor cells (TC) and immune cells (IC) of 70 patient-derived HCC specimens using IHC. Two anti-PD-L1 monoclonal antibodies (MAbs), SP263 and SP142, were utilized. Additionally, TC C-MET expression was assessed. Correlations between PD-L1 expression (as identified by both MAbs), C-MET expression and clinicopathologic factors were assessed. More PD-L1$^+$ cases were identified via SP263 than via SP142 when assessing both TC and IC; in the former group, SP236 identified 14/70 positive cases, while SP142 identified only 2/70. In the latter group, SP236 identified 49/70 positive cases, while SP142 identified 30/70. Both MAbs demonstrated a higher frequency of PD-L1 expression by IC than TC. The Edmondson-Steiner grade statistically correlated with a higher frequency of SP236-detected TC PD-L1 expression. C-MET was significantly associated with advanced tumor size and was positively correlated with SP263-detected PD-L1 expression in TC. These results suggest that C-MET may serve a role in regulating PD-L1 expression in HCC. Furthermore, while SP263 generally exhibited a higher sensitivity for PD-L1 detection, concordance in PD-L1$^+$ case detection between the two different MAbs was generally good. These background data may be helpful in the development of targeted anti-HCC immunotherapy focused on PD-L1 or C-MET, and in evaluating selection criteria for target populations best suited to such treatments.

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Key words: programmed cell death ligand 1, C-MET, immunotherapy, hepatocellular carcinoma, immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive malignant tumors, causing more than one million deaths annually (worldwide) and representing the second leading cause of death from tumors (1,2). It is highly correlated with hepatitis B or C virus (HBV or HCV) infection-associated chronic hepatitis (3), and exhibits a high prevalence rate (24.5 per 100,000) in Korea (4). Various local therapies, including resection, transplantation, and carotid chemoembolization, have been optimized for the treatment of HCC, but prognosis remains poor in patients with late-stage or relapsing disease (4). The majority of HCC patients are treated with chemoembolization or Nexavar® (sorafenib) (5). However, Nexavar® treatment is limited by high cost and an unfavorable adverse effect profile, and-while it has been reported to inhibit the growth of HCC-it is known to be not induce necrosis (6). Thus, new approaches for HCC treatment are required (4). One recent significant advance in tumor therapy is the targeting of genes associated with important immune system checkpoints (7). The most notable of these is programmed cell death ligand 1 (PD-L1), which was thought to be expressed only by inactivated immune cells, but was then also found to be expressed by various activated immune cell types (including T cells, B cells, natural killer cells, dendritic cells, and monocytes) (8,9). PD-L1 is a ligand for programmed cell death protein 1 (PD-1), which is expressed by various human cell types-including antigen-presenting cells, parenchymal cells, and endothelial cells-and this ligand/receptor pair plays an important role in inhibiting antitumor immunity.

Signal transduction initiated by PD-1/PD-L1 bonding mediates dephosphorylation of key molecules, thereby inhibiting T cell function, reducing production of inflammatory cytokines such as IFN-γ, and inhibiting T cell proliferation (9). The PD-1/PD-L1 signaling pathway is reportedly activated in various types of carcinoma, including renal cell carcinoma, gastric cancer, ovarian cancer, and hematological malignancies (10-15). Activation of this pathway is associated with an unfavorable prognosis in a variety of malignant tumors, including ovarian epithelial malignancy, pulmonary non-small cell carcinoma, gastric cancer, and nasopharyngeal cancer (16-21).

C-MET is a known receptor for Hepatocyte Growth Factor/Scatter Factor (HGF/SF), and has been reported to be directly involved in mediating invasive and metastatic capacity of non HCC cancer cells through the activation of various intracellular pathways; it is thus is a potential target for the development of novel anticancer drugs (22). HGF-induced C-MET activation occurs during activation of the PD-1/PD-L1 signaling pathway, and C-MET has various regulatory effects on this pathway (22). C-MET binds to HGF to form a dimer, leading to C-MET carboxy-terminus tyrosine phosphorylation, which in turn results in activation of MAPK and PI3K, ultimately impacting cell proliferation, survival, and angiogenesis. In cancer cells, the C-MET/HGF signaling pathway becomes hyper-activated, leading to uncontrolled cell proliferation and angiogenesis (23,24). One drug targeting C-MET is cabozantinib, which has been reported to reduce tumor reactivation and α-fetoprotein (AFP) levels in HCC patients. Another anti-HCC MET-inhibitor, Tivantinib, is currently undergoing clinical trials and may increase overall survival rate when administered to patients exhibiting high levels of C-MET expression (25,26).

In Korea, immunotherapeutic agents have been approved for use in small cell lung cancer, bladder carcinoma, and metastatic melanoma. In order to develop and approve novel HCC-appropriate immunotherapies, an improved understanding of PD-L1 and C-MET expression patterns in HCC patients, as well as of their involvement in the mechanisms of growth and inhibition in HCC, is required.

The current study analyzed and compared the expression pattern and level of PD-L1 in HCC patient-derived samples. This was achieved using the anti-PD-L1 monoclonal Abs (MAbs) SP263 and SP142, which are employed in immunohistochemical assays to determine PD-L1 positivity as part of standard treatment guidelines (in order to determine whether treatment with Opdivo® (nivolumab; targeting tumor cells (TC)) or and Ticentriq® (atezolizumab; targeting immune cells (IC) and TC) is indicated.) Additionally, the correlation of PD-L1 expression with various clinicopathologic factors was analyzed. Finally, because C-MET inhibition-via modulation of the PD-L1 pathway-is expected to have an anticancer effect, we performed a preliminary study examining the correlation between expression patterns of C-MET and PD-L1 in HCC.

The present study provides important data which will contribute towards the development of anticancer drugs and immunotherapeutic agents for improved treatment of HCC, and towards determination of future Korean prescription standards.

The study was approved by the ethics committee of Chosun University Hospital (Institutional review Board of Chosun university hospital, Gwangju, Korea), who waived the requirement for written informed consent due to the nature of the study (IRB no: 2018-04-003-001).

Materials and methods

Case selection. We evaluated the 70 cases of HCC using paraffin blocks and medical records, retrospectively. Among HCC patients who underwent lobectomy or segmentectomy at Chosun University Hospital during the period from February 2013 to December 2017, 70 patients with well-documented medical records were discontinuously selected.

Histopathology

Microscopic examination. Clinical records and tissue slides were retrospectively analyzed. Patient age and sex were confirmed, and the presence/absence of the HBV surface antigen (HBsAg, indicating current infection) and antibodies to the HBV surface antigen (HBsAb) were serologically confirmed. Slides were reviewed to select representative tissue sites corresponding to the study purpose. Paraffin-embedded tissues fixed in 10% neutral formalin buffer were cut into 4~5 μm-thick sections prior to hematoxylin and eosin (H&E) staining. The sections were examined under a light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan). By review of the H&E slides, Tumor stage (pT), histological classification, tumor number, Edmonson-Steiner (ES) grade, and the presence of portal vein invasion, bile duct invasion, and background sclerotic lesions were re-evaluated.

Immunohistochemistry (IHC). Expression of PD-L1 and C-MET were evaluated by immunohistochemical staining. For detection of PD-L1, we used two rabbit MAbs directed against PD-L1: SP-142 (cat. no. 740-4859; Ventana Medical Systems, Inc., Tucson, AZ, USA) and SP-263 (cat. no. 740-4907; Ventana Medical Systems). Similarly, detection of C-MET (cat. no. 790-4430; Ventana Medical Systems) was achieved using a rabbit MAb. All MAb assays were conducted according to the manufacturer's instructions. Immunohistochemical staining was performed using a Benchmark ULTRA (Ventana Medical Systems) slide-processing instrument. Expression and amplification of anti-PD-L1 MAb SP-142 was performed using an OptiView DAB IHC detection kit (cat. no. 760-700/0639650000) and an OptiView amplification kit (cat. no. 760-099/06396518001). Immunolocalization of PD-L1 (using SP-263) was performed using a haptenated secondary antibody and a multimeric anti-hapten-horseradish peroxidase (HRP) conjugate, and Ab-enzyme complexes were visualized via consequent production of a fluorescent enzyme reaction product (Optiview DAB IHC detection kit; cat. no. 760-700). C-MET was stained and detected using a commercial detection kit (Ventana Medical Systems). Ab-stained non-HCC tissues acted as expression controls: Normal tonsil (SP-142-detected PD-L1 expression negative control), placenta (SP-263-detected PD-L1 expression positive control), and colon cancer (C-MET expression positive control).

Briefly, staining proceeded as follows: Paraffin-embedded fixed tissue was cut into 4~5 μm-thick sections, adhered to

X-tra™ slides (Surgipath, Richmond, USA), deparaffinized with xylene, treated with anhydrous alcohol (90, 75 and 50%), and stained using a standard labeled streptavidin biotin (LSAB) method. To recover antigenicity, slides were boiled in citrate buffer (10 mM, pH 6.0) for 15 min in an electronic oven, cooled for 20 min at room temperature, and washed with 50 mM Tris buffer (TBS, pH 7.5). In order to inhibit the activity of endogenous peroxidases, slides were treated with 0.3% hydrogen peroxide-methanol solution for 10 min, washed with distilled water, reacted with blocking antibody for 10 min at room temperature, and coated with Ab (SP-142, SP-263, and anti-C-MET) for 32 min. Contrast staining was performed with hematoxylin (catalog no. 760-2021; Ventana Medical Systems) and tissue sections were sealed with Clearmount TM Mounting solution (Zymed Laboratories; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Determination of immunohistochemical staining. Staining results were interpreted by a pathologist blinded to the clinical course of the corresponding patient. Tissue sections were read as positive for PD-L1 (as detected by SP-142 or SP-263) staining if ≥5% of ICs (intra- and peritumoral lymphocytes, macrophages, dendritic cells, and granulocytes) exhibited a dark brown punctate staining pattern in the cell membrane. TC are stained with dark brown cell membrane pattern, and read as positive when ≥5% of TCs are stained. Positive C-MET staining appeared as yellow to dark brown staining in the cell membrane and/or cytoplasm of TC, and was graded according to intensity (0: Negative, 1: Weak, 2: Moderate, and 3: Intense; Fig. 1).

Statistical analysis. Statistical analysis was performed using the STAT View software package (Abacus Concepts, Piscataway, NJ, USA). We examined expression levels of PD-L1 (as detected by SP-142), PD-L1 (as detected by SP-263), and C-MET protein in HCC. Correlation between the expression of each protein, as well as between the expression of each protein and various clinicopathologic factors, was analyzed by the χ^2-test, respectively. Additionally, the following comparisons were made: Stage T1 with T2-4, low (grades 1 and 2) with high (grades 3 and 4) ES grade, negative (scores 0 and 1) with positive (scores 2 and 3). PD-L1 expression, and low-grade (scores 0 and 1) with high-grade (scores 2 and 3) C-MET expression. P<0.05 was considered to indicate a statistically significant difference.

Table I. Clinicopathologic factors.

Factors	N (%)
Age (year)	
<62	33 (47.1)
≥62	37 (52.9)
Sex	
Male	60 (85.7)
Female	10 (14.3)
Tumor size (cm)	
≤2	31 (44.3)
>2	39 (55.7)
T stage (pT)	
pT1	54 (77.1)
pT2-4	16 (22.9)
Portal vein invasion	
Absent	67 (95.7)
Present	3 (4.3)
Cirrhosis	
Absent	17 (24.3)
Present	53 (75.7)
Tumor histology	
Trabecular	54 (77.1)
Glandular	4 (5.7)
Mixed	12 (17.1)
Edmonson-Steiner grade	
1 and 2	45 (64.3)
3 and 4	25 (35.7)
Bile duct invasion	
Absent	69 (98.6)
Present	1 (1.4)
HBsAg	
Absent	27 (38.6)
Present	43 (61.4)
HBsAb	
Absent	57 (81.4)
Present	13 (18.6)

HBsAg, Hepatitis B surface antigen; HBsAb, Hepatitis B surface antibody.

Results

Distribution of clinicopathologic factors. We examined the distribution of various clinicopathologic factors among a total of 70 HCC patients (Table I). Age distribution ranged from 33 to 80 years (mean age 61 years). Overall ratio of males to females was 6:1 (85.7:14.3%). Regarding tumor size, 31 cases (44.3%) exhibited tumors of less than <2 cm diameter, and 39 cases (55.7%) exhibited tumors of >2 cm diameter. Regarding T stage distribution, 54 cases (77.1%) were staged as T1, 11 cases (15.7%) were staged as T2, 3 cases (4.3%) were staged as T3, and 2 cases (2.9%) were staged as T4. Portal vein involvement was observed in 3 cases (4.3%). Regarding histological classification of the tumor, 54 cases (77.1%) exhibited a tumor of trabecular type, 4 cases (5.7%) exhibited a tumor of pseudoglandular type, and 12 cases (17.1%) exhibited a tumor of mixed type. Bile duct invasion was observed in 1 case (1.4%). Regarding ES grade, 45 cases (64.3%) were low-grade (grades 1 and 2), and 25 cases (35.7%) were high-grade (grades 3 and 4). The presence of HBsAg or HBsAb was noted in 43 cases (61.4%) and 13 cases (18.6%), respectively. In 53 (75.7%) of tissue samples, a cirrhotic background was evident.

Association between PD-L1 expression and clinicopathologic factors. We examined the expression patterns of PD-L1 using two MAbs (SP263 and SP142), and the relationship between these

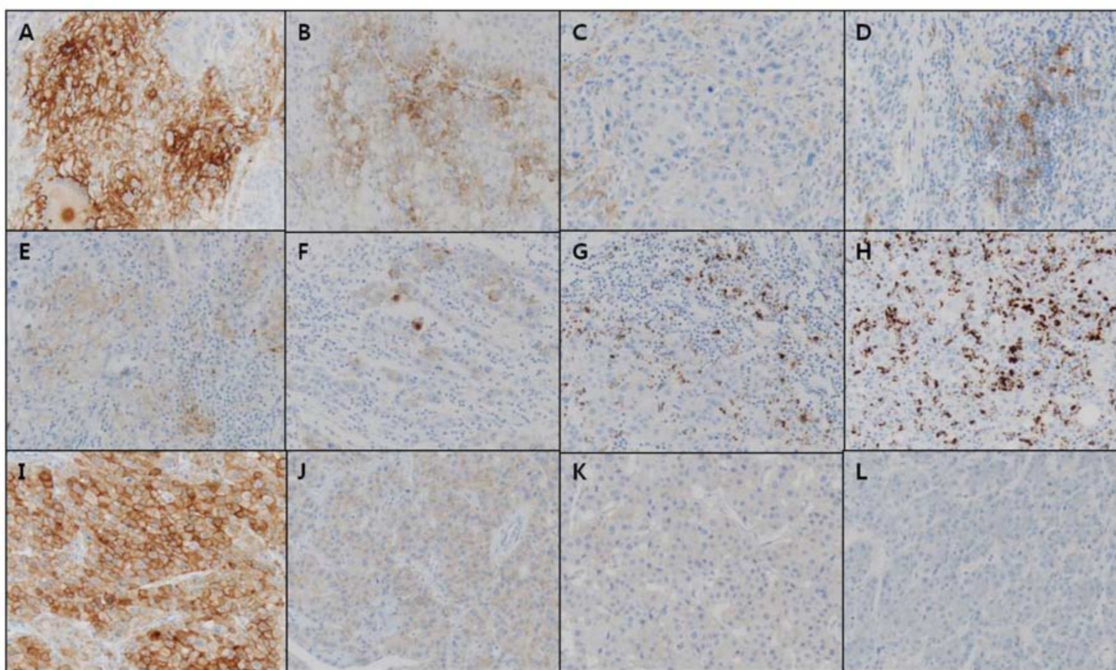

Figure 1. Immunostaining pattern using 3 antibodies (magnification, x20). (A-D) SP263 (anti-PD-L1 MAb); (A and B) positive staining in TC, (C) staining in <5% of TC and (D) expression in peritumoral IC. (E-H) SP142 (anti-PD-L1 MAb); (E and F) positive staining in TC and (G and H) positive staining in peri- and intratumoral IC. (I-L) Anti-C-MET MAb; (I) 3+ staining, (J) 2+ staining, (K) 1+ staining and (L) negative staining. PD-L1, Programmed cell death-ligand 1; MAb, monoclonal antibody; TCs, tumor cells; ICs, immune cells.

and various clinicopathologic factors (Tables II, III, and IV). Fig. 2 is representative staining pattern of each antibodies. (Fig. 2) As detected by SP263, PD-L1 was expressed in 20% (14/70) of TC and 70% (49/70) of IC. As detected by SP142, PD-L1 was expressed in 2.9% (2/70) of TC and 42.9% (30/70) of IC. In TC, PD-L1 expression detected by the different antibodies overlapped in both cases. In IC, PD-L1 expression detected by the different antibodies overlapped with the exception of 2 cases. Expression of PD-L1 (as detected by SP263) in TC exhibited a statistically significant (positive) correlation only with ES grade (among all clinicopathologic factors) (Table II, $P<0.01$). The high-grade (grades 3 and 4) ES group accounted for 71.4% (10/14) of the PD-L1-expressing group and only 26.8% (15/56) of the non-PD-L1 expressing group. Although no significant correlation existed between TC PD-L1 expression (as detected by SP263) and other clinical factors, pseudoglandular and mixed type tumors showed a higher frequency of PD-L1 expression than trabecular type tumors ($P=0.09$). Neither TC expression of PD-L1 (as detected by SP142) nor IC expression of PD-L1 (as detected by SP263) correlated with any clinicopathologic factors.

Association between C-MET expression and clinicopathologic factors. High-grade C-MET expression was low overall (10/70 cases, 14.3%) (Table V), and was found to be significantly positively correlated only with higher T stage ($P=0.042$). No other statistically significant association with clinicopathologic features was observed (Table V).

Comparison of PD-L1 expression as measured by SP263 and SP142. When comparing PD-L1 expression as measured by two MAbs, SP263-mediated detection reported higher levels of PD-L1 expression than SP142-mediated detection. Most cases (51/70, 72.9%) exhibited positive IC PD-L1 expression, and in all of these this expression was detected in peritumoral IC (SP263-detected: 49 cases, SP142-detected: 30 cases). Interestingly, 28/30 PD-L1 positive cases (as detected by SP142) also exhibited high PD-L1 expression as detected by SP263, and only two cases exhibited IC PD-L1 expression detectable only by SP142. When comparing PD-L1 expression levels of TC and IC, the latter exhibited significantly higher expression. Overall, PD-L1 expression was as follows: IC (49/70, 70%) as detected by SP263, IC (30/70, 42.9%) as detected by SP142, TC (14/70, 20%) as detected by SP263, and TC (2/70, 2.86%) as detected by SP142. Thus, the frequency of PD-L1 expression in IC was high (and was comparably detected by both MAbs). However, the frequency of PD-L1 expression in TC was low (14/70, 20%), and only two of these also exhibited PD-L1 expression as detected by SP142. In summary, IC PD-L1 expression results-as detected by both MAbs-significantly corresponded to each other: Most SP263-detectable PD-L1 expression was also detectable by SP142. Furthermore, statistically significant positive correlations between PD-L1 expression (as detectable by either MAb, and as detectable in either cell type) were observed (Tables IV and VI). The higher the IC PD-L1 positive expression rate (as detected by SP263), the higher the IC PD-L1 positive expression rate (as detected by SP142) ($P<0.001$; Table IV). The number of cases in which both MAbs were able to detect PD-L1 are as follows: 2 cases within TC ($P=0038$), 28 cases within IC ($P<0.001$), and 11 cases between TC and IC ($P=0.005$). The expression of SP263 PD-L1 in the IC and SP142 PD-L1 in the ICS were not significantly related ($P=1.000$; Table IV). Expression of PD-L1 as detected by

Table II. Association of clinicopathologic factors with SP263-detected expression of PD-L1 in TCs and immune cells.

	PD-L1 (SP263), TC (n=70)			PD-L1 (SP263), IC (n=70)		
Factors	(-) n (%)	(+) n (%)	P-value	(-) n (%)	(+) n (%)	P-value
Age (year)						
<62	28 (50.0)	5 (35.7)	0.38	12 (57.1)	21 (42.9)	0.31
≥62	28 (50.0)	9 (64.3)		9 (42.9)	28 (57.1)	
Sex						
Male	48 (85.7)	12 (85.7)	1.00	2 (9.5)	8 (16.3)	0.71
Female	8 (14.3)	2 (14.3)		19 (90.5)	41 (83.7)	
Tumor Size (cm)						
≤2	22 (39.3)	9 (64.3)	0.13	9 (42.9)	22 (44.9)	1.00
>2	34 (60.7)	5 (35.7)		12 (57.1)	27 (55.1)	
T stage (pT)						
pT1	43 (76.8)	11 (78.6)	1.00	18 (85.7)	36 (73.5)	0.36
pT2-4	13 (23.2)	3 (21.4)		3 (14.3)	13 (26.5)	
PV invasion						
Absent	55 (98.2)	12 (85.7)	0.10	21 (100.0)	46 (93.9)	0.55
Present	1 (1.8)	2 (14.3)		0 (0.0)	3 (6.1)	
Cirrhosis						
Absent	14 (25.0)	3 (21.4)	1.00	5 (23.8)	12 (24.5)	1.00
Present	42 (75.0)	11 (78.6)		16 (76.2)	37 (75.5)	
Histology						
Trabecular	46 (82.1)	8 (57.1)	0.09	17 (81.0)	37 (75.5)	0.42
Glandular	2 (3.6)	2 (14.3)		2 (9.5)	2 (4.1)	
Mixed	8 (14.3)	4 (28.6)		2 (9.5)	10 (20.4)	
ES grade						
1 and 2	41 (73.2)	4 (28.6)	<0.01[a]	15 (71.4)	30 (61.2)	0.59
3 and 4	15 (26.8)	10 (71.4)		6 (28.6)	19 (38.8)	
BD invasion						
Absent	56 (100)	13 (92.9)	0.20	21 (100.0)	48 (98.0)	1.00
Present	0 (0)	1 (7.1)		0 (0.0)	1 (2.0)	
HBsAg						
Absent	21 (37.5)	6 (42.9)	0.76	8 (38.1)	19 (38.8)	1.00
Present	35 (62.5)	8 (57.1)		13 (61.9)	30 (61.2)	
HBsAb						
Absent	46 (82.1)	11 (78.6)	0.72	18 (85.7)	39 (79.6)	0.74
Present	10 (17.9)	3 (21.4)		3 (14.3)	10 (20.4)	

Statistical analysis was performed using the χ^2 test. [a]P<0.05. TCs, tumor cells; PV, portal vein; BD, bile duct; ES grade, Edmondson-Steiner grade; IC, immune cells; PD-L1, programmed death-ligand 1.

SP263 was significantly positively associated between cell types (IC and TC; Table VI). The number of cases in which PD-L1 expression was positive or negative in both cell types (IC and TC) were 14/70 and 21/70, respectively (P=0.007; Table VI). Expression of PD-L1 as detected by SP142 did not correlate between IC and TC (Table VII).

Correlation of C-MET and PD-L1 expression. A statistically significant positive correlation was observed between SP263-detected TC PD-L1 expression and C-MET expression (P=0.022; Table VIII). No statistically significant correlation existed between IC PD-L1 expression (as detected by either MAb) and C-MET expression.

Discussion

In the immune system, T cells are activated via T cell receptor-mediated recognition of MHC-antigen complexes, and this activation is modulated through the integration of both co-stimulatory and co-inhibitory signals. However,

Table III. Association of clinicopathologic factors with SP142-detected expression of PD-L1 in TCs and immune cells.

	PD-L1 (SP142), TC (n=70)			PD-L1 (SP142), IC (n=70)		
Factors	(-) n (%)	(+) n (%)	P-value	(-) n (%)	(+) n (%)	P-value
Age (year)						
<62	33 (48.5)	0 (0.0)	0.49	21 (52.5)	12 (40.0)	0.34
≥62	35 (51.5)	2 (100.0)		19 (47.5)	18 (60.0)	
Sex						
Male	9 (13.2)	1 (50.0)	0.27	6 (15.0)	4 (13.3)	1.00
Female	59 (86.8)	1 (50.0)		34 (85.0)	26 (86.7)	
Tumor Size (cm)						
≤2	30 (44.1)	1 (50.0)	1.00	14 (35.0)	17 (56.7)	0.09
>2	38 (55.9)	1 (50.0)		26 (65.0)	13 (43.3)	
T stage (pT)						
pT1	52 (76.5)	2 (100.0)	1.00	31 (77.5)	23 (76.7)	1.00
pT2-4	16 (23.5)	0 (0.0)		9 (22.5)	7 (23.3)	
PV invasion						
Absent	65 (95.6)	2 (100.0)	1.00	39 (97.5)	28 (93.3)	0.57
Present	3 (4.4)	0 (0.0)		1 (2.5)	2 (6.7)	
Cirrhosis						
Absent	15 (22.1)	2 (100.0)	0.06	8 (20.0)	9 (30.0)	0.40
Present	53 (77.9)	0 (0.0)		32 (80.0)	21 (70.0)	
Histology						
Trabecular	53 (77.9)	1 (50.0)	0.85	32 (80.0)	22 (73.3)	0.53
Glandular	3 (4.4)	1 (50.0)		2 (5.0)	2 (6.7)	
Mixed	12 (17.6)	0 (0.0)		6 (15.0)	6 (20.0)	
ES grade						
1 and 2	45 (66.2)	0 (0.0)	0.12	26 (65.0)	19 (63.3)	1.00
3 and 4	23 (33.8)	2 (100.0)		14 (35.0)	11 (36.7)	
BD invasion						
Absent	67 (98.5)	2 (100.0)	1.0	40 (100.0)	29 (96.7)	0.43
Present	1 (1.5)	0 (0.0)		0 (0.0)	1 (3.3)	
HBsAg						
Absent	27 (39.7)	0 (0.0)	0.52	14 (35.0)	13 (43.3)	0.62
Present	41 (60.3)	2 (100.0)		26 (65.0)	17 (56.7)	
HBsAb						
Absent	55 (80.9)	2 (100.0)	1.00	33 (82.5)	24 (80.0)	1.00
Present	13 (19.1)	0 (0.0)		7 (17.5)	6 (20.0)	

Statistical analysis was performed using the χ^2 test. PV, portal vein; BD, bile duct; ES grade, Edmondson-Steiner grade; TCs, tumor cells; IC, immune cells; PD-L1, programmed death-ligand 1.

cancer cells are often able to evade immunity by means of various mechanisms, such as expression of proteins (or other molecules) that interfere with induction of immune responses or elimination of cancer cells (8,27). Immunologic anticancer drugs, which are immunoprotein-based therapeutic agents that induce host IC to selectively attack cancer cells, include immune checkpoint inhibitors (CTLA4-, PD-1-, or PD-L1 inhibitors), cell-based immunotherapy, and viral vector-based immunotherapy. Immune-based anticancer therapeutics offer an improved adverse effect profile compared to first-generation chemotherapeutic drugs, are not subject to acquired resistance (as are second-generation targeted anticancer drugs), and possess additional advantages such as long-term efficacy, long-term survival, and broad-spectrum anticancer effects (8,27,28). Such benefits have generated increasing research interest in immunotherapy, and since approval of the first-in-class drug Provenge® (sipuleucel-T; an autologous tumor vaccine) for the treatment of prostate adenocarcinoma in 2010, a number of additional immune checkpoint inhibitors have been approved (27). Exploitation of the PD-1/PD-L1

Table IV. Comparison of PD-L1 expression as detected by 2 MAbs (SP263 and SP142).

	SP263, TC			SP263, IC		
Variables	(-) n (%)	(+) n (%)	P-value	(-) n (%)	(+) n (%)	P-value
SP142, TC						
-	56 (100.0)	12 (85.7)	0.04[a]	21 (100.0)	47 (95.9)	1.00
+	0 (0.0)	2 (14.3)		0 (0.0)	2 (4.1)	
SP142, IC						
-	37 (66.1)	3 (21.4)	<0.01[a]	19 (90.5)	21 (42.9)	<0.001[a]
+	19 (33.9)	11 (78.6)		2 (9.5)	28 (57.1)	

Statistical analysis was performed using the χ^2 test. [a]P<0.05. TC, tumor cells; IC, immune cells; PD-L1, Programmed death-ligand 1.

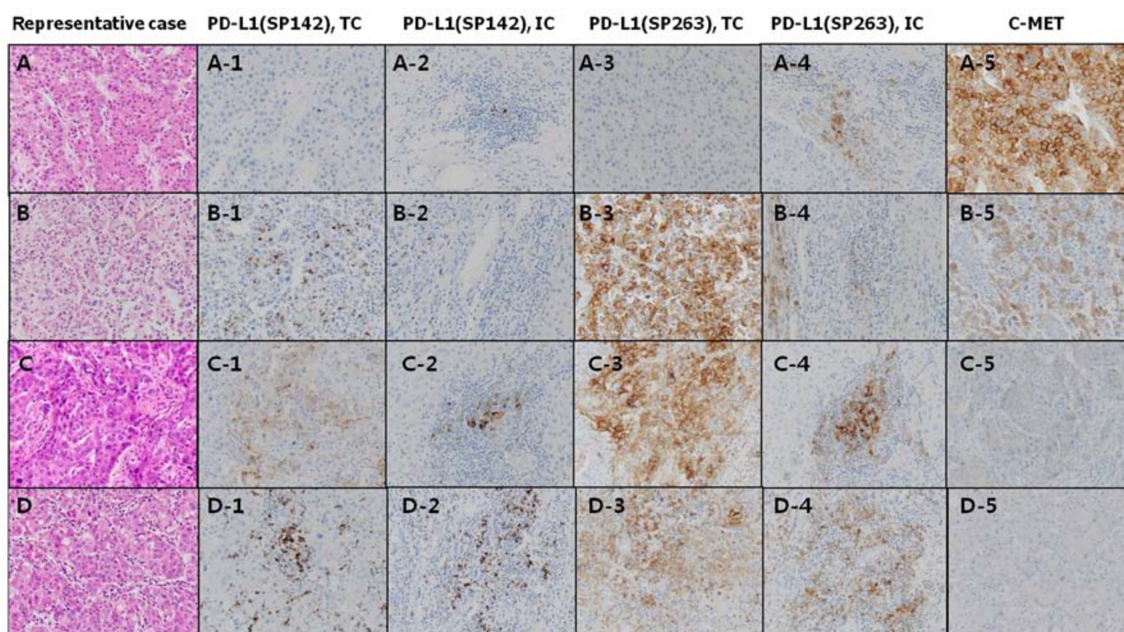

Figure 2. Immunostaining scores of each antibodies in representative cases. (A) [N,N,N,N,3+], (B) [N,N,P,N,2+], (C) [P,N,P,P,1+], (D) [N,P,P,P,N]. 1, expression of PD-L1 (SP142) in TCs; 2, expression of PD-L1 (SP142) in ICs; 3, expression of PD-L1 (SP263) in TCs; 4, expression of PD-L1 (SP263) in ICs; and 5, expression of C-MET in TCs. N, negative; P, positive; TCs, tumor cells; ICs, immune cells; PD-L1, Programmed cell death-ligand 1.

pathway is one mechanism by which cancer cells evade T cell immunity: PD-L1 expressed on the cancer cell surface ligates T cell PD-1, thereby inhibiting T cell anti-tumor immunity (27). In recent years, PD-1 and PD-L1 inhibitors-which target the PD-1/PD-L1 signaling pathway in order to induce T cell-mediated cancer cell apoptosis-have been developed and approved (24).

Keytruda® (pembrolizumab), the first PD-1 inhibitor approved by the United States Food and Drug Administration (US FDA) in 2014 (29), was also subsequently (in 2015) approved for the treatment of metastatic melanoma and non-small cell lung cancer (NSCLC) in Korea. Another PD-1 inhibitor, Opdivo® (nivolumab), was approved by the US FDA in 2014 (29), and in Korea has since been approved for the treatment of metastatic melanoma, NSCLC, lymphoma, squamous cell carcinoma of the head and neck, and urothelial carcinoma. The development of PD-1 inhibitors naturally led to the development of PD-L1 inhibitors, the first of which, Tecentriq® (atezolizumab), was approved by the US FDA in 2016 (29), and in Korea has since (in 2017) been approved for the treatment of locally advanced or metastatic NSCLC, and urothelial carcinoma. Also in 2017, the US FDA approved a second PD-L1 inhibitor, Imfinzi® (durvalumab), for the treatment of severe bladder cancer (i.e. progressing even after surgery or chemotherapy) (29).

Immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway have achieved good clinical results in the treatment of early melanoma, and have been approved for the treatment of NSCLC and renal cancer, with indications now expanding to include various additional cancers such as lymphoma and urothelial carcinoma (29). In 2018, the US FDA further approved Opdivo as a second-line treatment (regardless of PD-L1 expression status) for HCC patients not responding to standard first-line treatment with Nexavar® (29). According to the US Checkmate-040 clinical trial, Opdivo exhibited efficacy against HCC regardless of PD-L1 expression status,

Table V. Association of clinicopathologic factors with C-MET expression.

	C-MET (n=70)		
Factors	Low n (%)	High n (%)	P-value
Age (year)			1.00
<62	28 (46.7)	5 (50.0)	
≥62	32 (53.3)	5 (50.0)	
Sex			1.00
Male	9 (15.0)	1 (10.0)	
Female	51 (85.0)	9 (90.0)	
Tumor Size (cm)			0.32
≤2	25 (41.7)	6 (60.0)	
>2	35 (58.3)	4 (40.0)	
T stage (pT)			0.04[a]
pT1	49 (81.7)	5 (50.0)	
pT2-4	11 (18.3)	5 (50.0)	
PV invasion			0.38
Absent	58 (96.7)	9 (90.0)	
Present	2 (3.3)	1 (10.0)	
Cirrhosis			0.43
Absent	16 (26.7)	1 (10.0)	
Present	44 (73.3)	9 (90.0)	
Histology			0.18
Trabecular	3 (5.0)	1 (10.0)	
Glandular	9 (15.0)	3 (30.0)	
Mixed	48 (80.0)	6 (60.0)	
ES grade			0.15
1 and 2	41 (68.3)	4 (40.0)	
3 and 4	19 (31.7)	6 (60.0)	
BD invasion			1.00
Absent	59 (98.3)	10 (100.0)	
Present	1 (1.7)	0 (0)	
HBsAg			0.17
Absent	21 (35.0)	6 (60.0)	
Present	39 (65.0)	4 (40.0)	
HBsAb			1.00
Absent	49 (81.7)	8 (80.0)	
Present	11 (18.3)	2 (20.0)	

Statistical analysis was performed using the χ^2 test. [a]P<0.05. PV, portal vein; BD, bile duct; ES grade, Edmondson-Steiner grade.

Table VI. Agreement of SP263-detected PD-L1 expression between tumor and immune cells.

	TC (SP263)		
Factors	(-) n (%)	(+) n (%)	P-value
IC (SP263)			
(-)	21 (37.5)	0 (0.0)	0.02[a]
(+)	35 (62.5)	14 (100.0)	

Statistical analysis was performed using the χ^2 test. [a]P<0.05. TC, tumor cells; IC, immune cells; PD-L1, Programmed death-ligand 1.

Table VII. Agreement of SP142-detected PD-L1 expression between tumor and immune cells.

	TC (SP142)		
Factors	(-) n (%)	(+) n (%)	P-value
IC (SP142)			
- (n=40)	39 (57.4)	1 (50.0)	1.00
+ (n=30)	29 (42.6)	1 (50.0)	

Statistical analysis was performed using the χ^2 test. TC, tumor cells; IC, immune cells; PD-L1, Programmed death-ligand 1.

and regardless of the presence/absence of active hepatitis B or C (28).

As mentioned, tumor cell-expressed PD-L1 ligates immune cell PD-1 receptors, leading to inhibition of T cell-mediated anti-tumor immunity; high PD-L1 expression is therefore correlated with poorer prognosis. For example, Gao *et al* (25) reported that PD-L1 over-expression and tumor size correlates with tumor recurrence. In the current study, HCC patients exhibiting high PD-L1 expression also had a worse prognosis (relative to those with low PD-L1 expression). Furthermore, multivariate analysis demonstrated that the PD-L1 expression status could be used as an independent marker for postoperative HCC recurrence. Another recent study suggested that PD-L1 expression in combination with $CD4^+$ and $CD8^+$ T cells may have utility as an HCC prognostic indicator (e.g. that positive Ab-mediated staining may indicate a higher risk for recurrence) (30).

Chang *et al* (7) demonstrated that PD-L1 predicts a poorer prognosis in patients exhibiting $CD8^+$ tumor infiltrating lymphocytes (TILs), and independently predicts poorer survival. Jung *et al* (4) analyzed the correlation between poor prognosis and over-expression of PD-L1 and PD-L2 in HCC patients, and demonstrated that PD-L1 over-expression correlated with tumor size, recurrence, and survival. However, PD-L1 expression in esophageal cancer tissue exhibits no correlation with prognosis (31). Nevertheless, poor outcomes in esophageal cancer are largely due to metastasis, and Miao *et al* (22) predicted that PD-L1 expression would play an important role in this process. In light of the above findings, PD-L1 expression is expected to be an important index for the prediction of tumor recurrence.

Another study by Gao *et al* (25) demonstrated that over-expression of PD-L1 and PD-L2 is closely linked to poorer survival, but that the correlation with recurrence rate was not statistically significant. Thus, inhibiting PD-1 expression may be a more effective anticancer strategy than inhibiting expression of PD-L1 or PD-L2. In addition to clinical prognostic studies, IHC-based examination of liver tissue expression of

Table VIII. Correlation of C-MET and PD-L1 expression.

	C-MET			
Factors	n (%)	Low n (%)	High n (%)	P-value
IC (SP263)				
(-)	21 (30.0)	19 (31.7)	2 (20.0)	0.712
(+)	49 (70.0)	41 (68.3)	8 (80.0)	
TC (SP263)				
(-)	56 (80.0)	51 (85.0)	5 (50.0)	0.022[a]
(+)	14 (20.0)	9 (15.9)	5 (50.0)	
IC (SP142)				
(-)	68 (97.1)	58 (96.7)	10 (100.0)	1.000
(+)	2 (2.9)	2 (3.3)	0 (0.0)	
TC (SP142)				
(-)	40 (57.1)	36 (60.0)	4 (40.0)	0.308
(+)	30 (42.9)	24 (40.0)	6 (60.0)	

Statistical analysis was performed using the χ^2 test. [a]P<0.05. TC, tumor cells; IC, immune cells; PD-L1, Programmed death-ligand 1.

PD-1 and PD-L1 in patients with hepatitis and HCC demonstrated that while PD-L1 expression in HBV hepatitis and HCC was high during the early (proliferative) stages of HCC, this level became progressively lower as HCC progressed toward the terminal stage (26). HBV infection did not impact PD-L1 expression in liver cancer tissues. However, a study investigating expression of PD-1 and PD-L1 in HBV-induced HCC patients treated with cryoablation demonstrated that PD-L1 expression correlates with poor prognosis (32). Such results suggest that further studies are required to clarify the relationship between PD-L1 expression and survival rate in HBV hepatitis-induced HCC.

The anti-PD-L1 MAb SP142 is raised against rabbit serum, and is used when determining whether prescription of Ticentriq is appropriate (33,34). Similarly, it has been validated for use in determining whether atezolizumab treatment for advanced urothelial carcinoma and NSCLC is indicated (35-37). In these clinical trials, positive PD-L1 expression by TC and IC was an indication for treatment (38-39). The anti-PD-L1 MAb SP263 is used to determine whether the PD-1 inhibitor Opdivo is indicated. MAb SP263 directly targets an intracellular portion of human PD-L1 (40). It was optimized for use in NSCLC tissue samples, and its diagnostic value has been validated. It has also been validated for use in clinical trials for the establishment of nivolumab treatment guidelines (41,42).

In a study using SP142-based IHC to compare TC and IC PD-L1 expression between NSCLC biopsies and surgical resection specimens from 160 patients, results were inconsistent, with an overall discordance rate (non-agreement of PD-L1 expression between the two samples) of 48% (κ=0.218) (43). However, another NSCLC study which retrospectively performed the same comparison using the same technique (n=79 patients) demonstrated that 38.0% of surgical resection specimens and 35.4% of biopsy specimens exhibited PD-L1 expression, with a concordance rate of 92.4% (κ=0.8366) (44). Although this retrospective study was limited by a relatively small sample size and unavailability of the entire biopsy specimen (44-46), it is significant that SP142-based confirmation of PD-L1 expression is accurate

Many previous studies have suggested that PD-L1 expression correlates with a variety of oncogenic signaling pathways. For example, Azuma *et al* (45) demonstrated that over-expression of mutant EGFR correlates with high PD-L1 expression in surgically resected NSCLC specimens. In addition, Tang *et al* (46) demonstrated that an EGFR mutation correlated with PD-L1 expression during treatment of progressive NSCLC with a tyrosine phosphorylase inhibitor. Gabrielson *et al* (47), reported that low HCC PD-L1 expression is significantly associated with a higher density of tumor-infiltrating $CD3^+$ T cells and $CD8^+$ T cells, consistent with an increased survival rate and a low tumor recurrence rate (suggesting PD-L1 expression as a useful prognostic indicator in HCC patients who have undergone tumor resection).

In the current study, HCC patient TC and IC PD-L1 expression was determined. The frequency of positive expression was highest in IC as detected by SP263 (70%), followed by IC as detected by SP142 (), then in TC as detected by SP263 (), followed by TC as detected by SP142 (). In all except 2 cases, there was concordance between positive IC PD-L1 expression as detected by either MAb (i.e. the MAbs demonstrated positive correlation between detected IC PD-L1 expression patterns). In addition, both MAbs demonstrated a higher frequency of positive PD-L1 expression in IC than TC, with SP263 exhibiting higher sensitivity than SP142. If these results accurately represent positive PD-L1 expression frequencies, SP263 may be a better candidate for IC PD-L1 expression detection, and such PD-L1 detection in IC (rather than TC) may be a better prognostic indicator candidate. These data are expected to contribute towards selection of the ideal MAb for PD-L1 detection, as well as determination of the $PD\text{-}L1^+$ case rate in HCC, which will facilitate development of HCC immunotherapy prescription standards in Korea.

Although Jung *et al* (4) have reported that PD-L1 expression, histological findings, and overall tumor size are predictors of poor prognosis in HCC patients, to date no study has examined TC and IC PD-L1 and C-MET expression patterns, and their correlation with prognostic factors in HCC. C-MET is a type of tyrosine kinase which becomes mutated and/or over-expressed on the surface of cancer cells. It has been reported that C-MET is over-expressed in HCC, gastric cancer, rectal cancer, and breast cancer, which are common human carcinomas (48,49). Activation of C-MET is known to promote tumor cell survival, proliferation, invasion, and metastasis (50). Ligation of C-MET by hepatocyte growth factor (HGF) triggers initiation of signaling (51) which culminates in cancer cell growth and proliferation. Overactive C-MET/HGF signaling has been shown to be associated with invasion and proliferation of small cell lung cancer (30). Many studies have demonstrated that activation of C-MET-mediated intracellular signaling pathways may be associated with poor prognosis in lung cancer and other solid tumors (52-55). Several clinical NSCLC studies have reported that C-MET over-expression correlates with poor survival (56-59). Miao *et al* (22) studied PD-1 and C-MET expression relative to survival in small cell lung cancer patients and suggested that C-MET over-expression is an important prognostic factor during the early stages. Activated C-MET signaling has also been identified as a potential therapeutic target, given its involvement in cancer cell proliferation and invasion (60,61).

Similarly, many studies have demonstrated C-MET expression in HCC lesions. However, the value of C-MET expression as a prognostic factor in this context remains unclear. In a recent meta-analysis of 1,480 HCC patients undergoing surgical resection it was suggested that C-MET over-expression (when comparing high- and low-expression groups) is a prognostic indicator of recurrence and survival (33). It is likely that tumor PD-L1 and/or C-MET expression is correlated with poor prognosis due to involvement of these proteins in mechanisms of immune evasion. Over-expression of C-MET in HCC is reportedly associated with tumor progression (49,62), central venous invasion or thrombosis (49,51), intrahepatic metastasis (63,64), tumor recurrence (63,64) and survival (63). Wang *et al* (63) found that high C-MET expression in HCC patients (with lesions of less than 5 cm) undergoing surgical resection was independently correlated with shorter survival intervals. Although some studies have shown that C-MET over-expression is of prognostic value in early-stage HCC in patients who have undergone a partial hepatectomy, C-MET over-expression has also been shown to be of limited prognostic value since it does not appear to be an obvious indicator of end-stage HCC (62,64).

The current study investigated the correlation between C-MET and PD-L1 expression (the latter as determined by two MAb types), and clinicopathologic factors. Expression of TC PD-L1 (as detected by SP263) positively correlated with C-MET expression, indicating that C-MET-mediated regulation of PD-L1 pathways may be involved in HCC. Furthermore, statistically significant correlations were observed between TC PD-L1 expression (as detected by SP263) and ES grade, as well as between C-MET expression and T stage. Such correlation between PD-L1 and C-MET expression and clinicopathological parameters suggests that expression of these proteins may be of utility as potential prognostic factors in HCC. The enrolled patient had surgery recently (2013~2017), so we cannot analyzed survival of patients. I will do survival analysis later. And, molecular study will be the best and I also have the plan about the molecular experiment of PD-L1 sub-molecules after the IHC experiment.

Results of the current study are expected to be of use in the future approval of immunotherapeutic agents and determination of a prescription standard for HCC. Data also demonstrate the value of PD-L1 and C-MET as prognostic factors through their correlation with clinicopathologic factors. Correlation between PD-L1 (as detected by SP263) and C-MET expression provides baseline data for future development of C-MET-targeting immunotherapeutic interventions.

Acknowledgements

Not applicable.

Funding

The present study was supported by research funds from Chosun University, Republic of Korea, 2016 (grant no. 2016-01).

Availability of data and materials

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

Authors' contributions

RH designed the study, performed the experiments, provided financial support, revised the manuscript and gave final approval of the version to be published. HWC provided financial support, interpreted data and wrote the paper.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Chosun University Hospital (Institutional review Board of Chosun University Hospital, Gwangju, Korea), who waived the requirement for written informed consent due to the nature of the study (IRB No.: 2018-04-003-001).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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