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Programmed death-ligand 1 expression is an unfavorable prognostic factor of hepatocellular carcinoma after archiving sustained virologic response for hepatitis C virus infection

REIICHIRO KONDO[1], JUN AKIBA[2], SACHIKO OGASAWARA[1], OSAMU NAKASHIMA[3], YOSHIKI NAITO[2], HIRONORI KUSANO[1], YUTARO MIHARA[1], MASAHIKO TANIGAWA[1] and HIROHISA YANO[1]

[1]Department of Pathology, Kurume University School of Medicine; Departments of [2]Diagnostic Pathology and [3]Clinical Laboratory Medicine, Kurume University Hospital, Kurume, Fukuoka 830-0011, Japan

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Abstract. The aim of the present study was to study the pathological prognostic factor of initial hepatocellular carcinoma (HCC) after archiving sustained virologic response (SVR) for hepatitis C virus (HCV) infection. A single-center retrospective analysis was performed for patients who underwent hepatectomy between 2003 and 2017. We studied clinico-pathological findings of resected liver tissues in 35 patients with HCC after SVR treated by interferon (IFN group) and 13 patients with HCC after SVR treated by direct acting antivirals (DAA group). We also performed immunohistochemical staining using antibodies against programmed death-ligand 1 (PD-L1), cytokeratin 19, epithelial cell adhesion molecule (EpCAM) and regulator of G-protein signaling 5 (RGS5). PD-L1 positive HCC was observed in 6 cases of the IFN group and 4 cases of the DAA group. In the IFN group, in univariate analysis of recurrence free survival after surgery (RFS), the PD-L1 expression had a statistically significant impact (HR=6.01; P=0.02). In the multivariate analysis of RFS, PD-L1 expression significantly remained (HR=5.01; P=0.03). For both RFS and overall survival, Kaplan-Meier curves confirmed that patients with PD-L1 expression showed significantly worse prognosis (log-rank test P<0.01). Nuclear grade, RGS5 expression, and EpCAM expression were significantly higher in the PD-L1-positive HCC group compared with the PD-L1-negative HCC group (P<0.05). Therefore, PD-L1 expression may be an independent prognostic factor of surgically resected HCC after achieving SVR.

Correspondence to: Dr Reiichiro Kondo, Department of Pathology, Kurume University School of Medicine, 67 Asahi-Machi, Kurume, Fukuoka 830-0011, Japan
E-mail: kondou_reiichirou@kurume-u.ac.jp

Key words: pathology, hepatocellular carcinoma, sustained virologic response, biomarker, programmed death-ligand 1

Introduction

Sustained virological response (SVR) is defined as undetectable serum hepatitis C virus (HCV) RNA 24 weeks after completion of antiviral therapy for chronic HCV infection. In the 1990's to the 2000's, Interferon (IFN) was used as the main antiviral therapy for HCV infection. About 50% of patients with HCV genotype type 1 have a SVR using peginterferon alfa-2a plus ribavirin (1). Recently, the development of an IFN-free regimen using direct acting antivirals (DAA) has been a revolution in the treatment of patients with chronic hepatitis C. More than 95% of patients achieved a SVR using DAA (2,3).

HCV infection is a significant risk factor for progressive hepatic fibrosis, subsequent liver cirrhosis and the development of hepatocellular carcinoma (HCC). Among HCV-infected patients, achieving SVR was associated with a reduced risk of the development of HCC (4). However, the risk of developing HCC does not completely disappear even after SVR. It was reported that the 5- and 10-year HCC incidence rates after achieving SVR by IFN-based therapy were 0.8-5.8% and 2.8-11.1%, respectively (5-10). There were differences in gene mutation between patients who developed HCC after achieving SVR and patients who developed HCC with chronic HCV infection (10,11). Many HCV-infected patients will be able to obtain SVR, so identification of biological characteristics in HCC after achieving SVR is very important. However, the molecular biological feature of HCC after achieving SVR was not yet clear.

In the present study, we aimed at characterizing molecular pathological feature of surgically resected HCC after achieving SVR and determining its relationship with prognosis and pathological features, as well as programmed death-ligand 1 (PD-L1) expression, markers of progenitor cells [cytokeratin 19 (CK19), epithelial cell adhesion molecule (EpCAM)], and portal vein invasion associated marker [regulator of G-protein signaling 5 (RGS5)].

Patients and methods

Patients. We studied the clinical and pathological findings of 372 patients associated HCV infection who were underwent hepatectomy for initial HCC at Kurume University Hospital

between January 2003 and April 2017. Of these, 48 patients who developed initial HCC after achieving SVR. In 48 patients, there are 35 patients with HCC after SVR treated by IFN (IFN group) and 13 patients with HCC after SVR treated by DAA (DAA group). They were underwent hepatectomy for initial HCC at the Kurume University Hospital between January 2003 and April 2017.

Histopathological examination. Each surgically resected liver tissue was fixed with 10% formalin, embedded in paraffin, cut into 5-µm sections, and then used for histological analyses. The specimens were stained with hematoxylin and eosin (H&E), reticulin, and Azan. We evaluated all specimens, histopathologically (e.g., HCC tissue: Size, tumor differentiation, vascular invasion, nuclear grade; non-HCC liver tissue: Fibrosis, inflammation, steatosis). The histological features of HCC were evaluated according to the World Health Organization classification (12). We classified the degree of nuclear grade into 3 levels according to nuclear atypia and mitosis (Table I). The degrees of liver fibrosis and hepatic inflammation were scored according to the New Inuyama Classification from F0 to F4, and A0 to A3 (13). The liver fibrosis stage was classified as follows: F0 (no fibrosis), F1 (fibrous portal expansion), F2 (bridging fibrosis), F3 (bridging fibrosis with lobular distortion) and F4 (liver cirrhosis), and the inflammatory grade was classified as A0 (no necro-inflammatory reaction), A1 (mild necro-inflammatory reaction), A2 (moderate necro-inflammatory reaction), or A3 (severe necro-inflammatory reaction) (13). Steatosis was defined as fat deposits of 5% or more hepatocytes. Histopathological diagnosis and classification were performed by three pathologists (R.K, J.A, and O.N).

Table I. Assessment of nuclear grade.

A, Nuclear grade

Grade/Score	Result
Grade 3	Atypia score + Mitosis score =5 or 6
Grade 2	Atypia score + Mitosis score =4
Grade 1	Atypia score + Mitosis score =2 or 3

B, Nuclear atypia score

Grade/Score	Result
Score 3	Multinucleated and pleomorphic cells
Score 2	Other than score 1 and 3
Score 1	Homogenous small round nuclear cells

C, Mitosis

Grade/Score	Result
Score 3	>10 mitotic cells/10 HPFs
Score 2	5-10 mitotic cells/10 HPFs
Score 1	<5 mitotic cells/10 HPFs

HPF, high power field.

Immunohistochemical analysis. Immunostaining was performed on paraffin-embedded materials. The following primary antibodies were used: Anti-PD-L1 (clone 28-8; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), anti-CK19 (clone RCK108; Dako; Agilent Technologies, Inc.), anti-EpCAM (clone VU1D9; Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-RGS5 (clone 1C1; Novus Biologicals, LLC, Littleton, CO, USA). Immunohistochemical examination of PD-L1 was performed using a fully automated DAKO system (Dako; Agilent Technologies, Inc.), according to the manufacturer's instructions. PD-L1 expression was observed in both neoplastic HCC cells and intratumoral inflammatory cells. For neoplastic HCC cells, the percentage of cells displaying unequivocal membranous staining was recorded. Immunohistochemical examination of CK19 and EpCAM were processed on an automated immunostainer (BenchMark ULTRA; Ventana Automated Systems, Inc., Tucson, AZ, USA), according to the manufacturer's instructions. Immunohistochemical examination of RGS5 was performed according to our previous report (14). The positive expression in tumor cells was shown in Fig. 1. The expression of tumor cells were scored as follows: No expression, score 0; 1 to 4% of positive cells, score 1+; more than 5% of positive cells, score 2+. In addition, we performed double immunostaining for CD34 and α-smooth muscle actin (αSMA). We used monoclonal antibodies against CD34 (mouse, clone QBEnd/10, 1:100; Leica Biosystems, Newcastle, United Kingdom) and αSMA (mouse, clone 1A4, 1:10; Dako; Agilent Technologies, Inc.). The primary antibody for CD34 labeled horseradish peroxidase was visualized using DAB resulting in a brown/black target signal. The second antibody for αSMA labeled alkaline phosphatase was visualized using Fast Red/Naphthol resulting in a bright red target signal. The CD34 and αSMA expression in sinusoidal mesenchymal cells was scored as follows: No positive sinusoidal mesenchymal cells, 0; positive sinusoidal mesenchymal cells in less than four fields under low power magnification (x4 objective), score 1+; positive sinusoidal mesenchymal cells in five or more fields under low power magnification (x4 objective), score 2+. In chronic liver disease, perisinusoidal mesenchymal cells express CD34 as transformed hepatic endothelial cells (HECs) and perisinusoidal mesenchymal cells express αSMA as transformed hepatic stellate cells (HSCs). The positive expression of perisinusoidal mesenchymal cells in non-cancerous liver tissue was shown in Fig. 1.

Statistical analysis. All data are expressed as the mean ± standard deviation (SD). Comparisons between 2 groups were performed using Welch's t-test for continuous variables, and the χ^2 test for discrete variables. Kaplan-Meier survival and tumor recurrence analyses were performed using the log-rank test. Multivariate analyses were performed using linear and logistic regressions. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using JMP software package (v.13.0; SAS Institute, Inc., Cary, NC, USA).

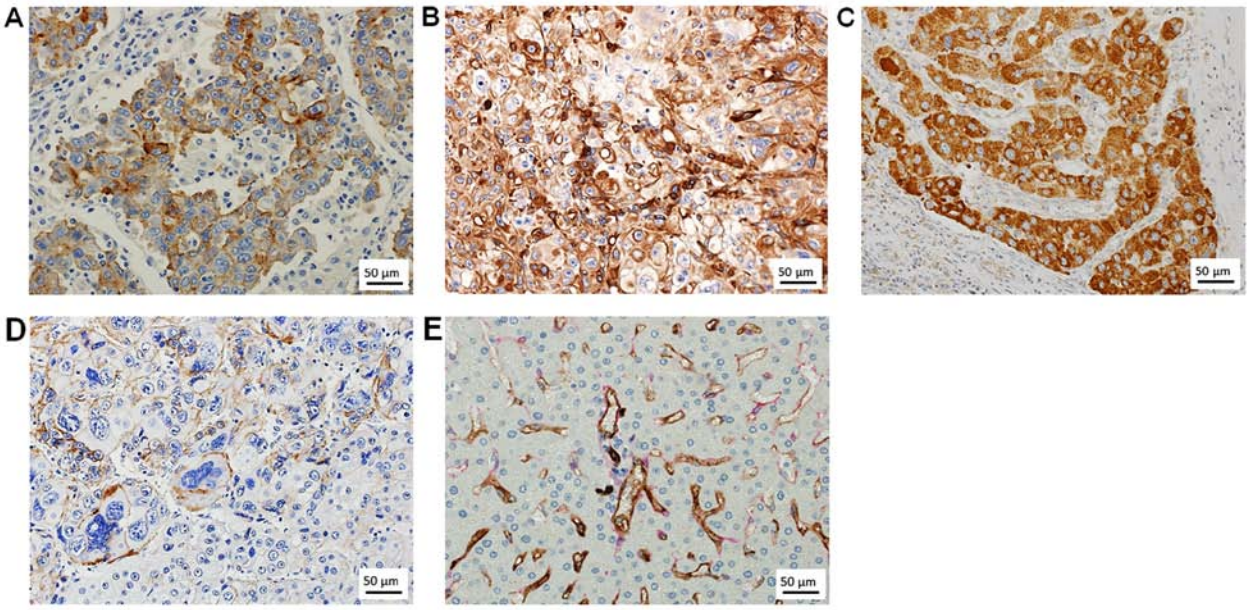

Figure 1. Immunohistochemical findings. (A) IHC of PD-L1. Unequivocal PD-L1 membranous staining of tumor cells. (B) IHC of CK19. Strong CK19 membranous or cytoplasmic staining of tumor cell. (C) IHC of RGS5. Strong RGS5 cytoplasmic staining of tumor cells. (D) IHC of EpCAM. Strong unequivocal EpCAM membranous or cytoplasm staining of tumor cells. (E) IHC of CD34 (brown) and αSMA (red). CD34 and αSMA expressions in sinusoidal mesenchymal cells of non-cancerous liver tissue are observed. The αSMA expression area is close to the CD34 expression area. IHC, immunohistochemistry; PD-L1, programmed death-ligand 1; CK19, cytokeratin 19; RGS5, regulator of G-protein signaling 5; EpCAM, epithelial cell adhesion molecule; αSMA, α-smooth muscle actin.

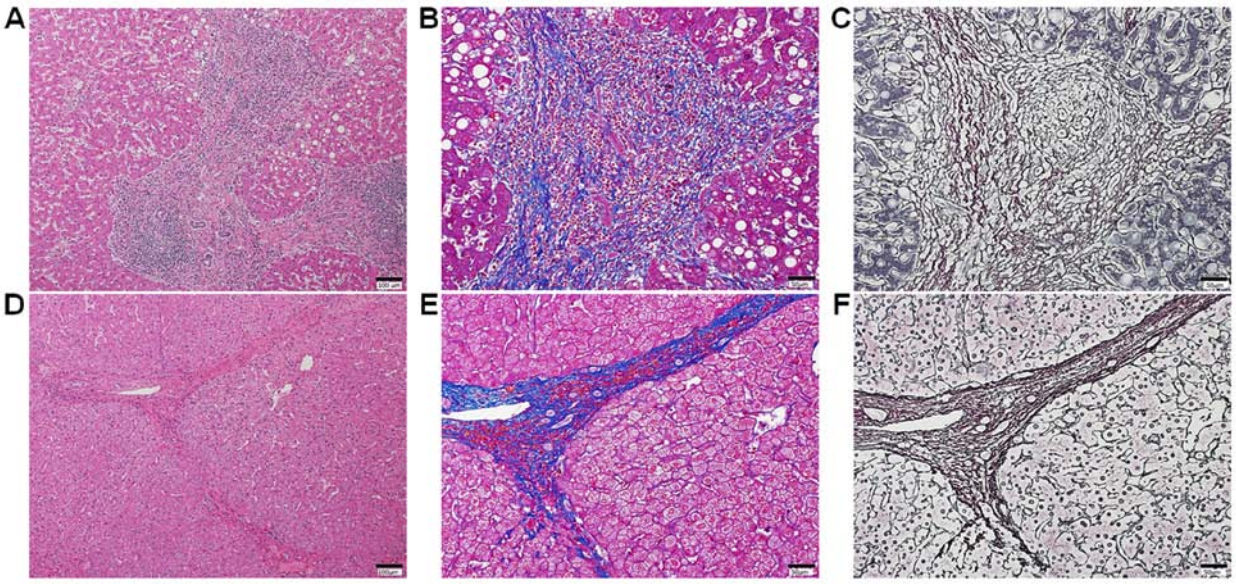

Figure 2. Liver fibrosis in non-cancerous liver tissue. (A) Stained with H&E. Mild fibrosis (New Inuyama Classification, F1) with moderately inflammatory reactions (New Inuyama Classification, A2) and mild steatosis is seen. There are no ballooning hepatocytes, or Mallory-denk bodies. Staining with (B) Azan and (C) reticulin. The fibrosis septa are wide, loosely aggregated collagen fibers. (D) Stained with H&E. Moderate fibrosis (New Inuyama Classification, F2) with mild inflammatory reactions (New Inuyama Classification, A1) is seen. There is no steatosis, ballooning hepatocytes, or Mallory-denk bodies. Staining with (E) Azan and (F) reticulin. The fibroseptal stroma showing features of thin, densely compacted stroma is present. H&E, hematoxylin and eosin.

Results

Pathological findings. The clinicopathological findings in the IFN group and the DAA group are shown in Table II. In all cases, there were solitary HCC without hepatic vein invasion, hepatic artery invasion, bile duct invasion, peritoneal dissemination, and lymph node metastasis. Surgical margin was negative in all cases.

In the IFN group, there were 24 men and 11 women. The mean age was 68.8±8 years. Diabetes was present in 11 (31%) patients of the IFN group, respectively. Histologically, among the HCC tissues in the IFN group, 2 cases were well differentiated HCCs, 30 cases were moderately differentiated HCCs, and 3 case was poorly differentiated HCC. The mean tumor size and nuclear grade were 29±19 mm and 2±0.9, respectively. Portal vein invasion was present in 21 cases (60%).

Table II. Summary of patients.

A, Clinical findings

Characteristics	IFN (n=35)	DAA (n=13)	P-value
Age (year)	68.8±8	68.1±7.4	0.76
Male/Female	24/11	5/8	0.1
Diabetes (prevalence rate, %)	11 (31%)	7 (54%)	0.32
Observation period after resection (median)	1,080 days	501 days	<0.01
Serum total bililubin (mg/dl)	0.8±0.3	0.7±0.3	0.12
Prothrombin activity (%)	90.1±15	92.7±12	0.54
Serum albumin (g/dl)	4.2±0.3	4.2±0.2	0.86
Serum AFP (ng/ml, median)	6.7	6.4	0.11
Serum PIVKA (mAU/ml, median)	118	32	0.93

B, HCC findings

Characteristics	IFN (n=35)	DAA (n=13)	P-value
Tumor size (mm)	29±19	19±4	<0.01
Tumor differentiation (well/moderate/poor)	2/30/3	3/9/1	0.23
Nuclear grade	2±0.9	1.9±0.9	0.79
Atypia score	2.3±0.5	2.5±0.5	0.39
Mitosis score	1.7±0.8	1.7±0.9	0.85
Portal vein invasion (prevalence rate, %)	21 (60%)	5 (38%)	0.21
PD-L1 score (0/1+/2+)	29/3/3	9/3/1	0.55
CK19 score (0/1+/2+)	35/0/0	12/1/0	0.34
EpCAM score (0/1+/2+)	29/4/2	9/2/2	0.33
RGS5 score (0/1+/2+)	6/22/7	5/7/1	0.11

C, Non-HCC findings

Characteristics	IFN (n=35)	DAA (n=13)	P-value
Fibrosis (New Inuyama Classification, F)	2.3±1.3	3.5±1	<0.01
Inflammation (New Inuyama Classification, A)	1.2±0.5	1.3±0.5	0.65
Steatosis (prevalence rate, %)	9 (26%)	6 (46%)	0.29
CD34 score (0/1+/2+)	0/4/31	0/2/11	0.74
αSMA score (0/1+/2+)	18/9/8	3/6/4	0.16

AFP, α-fetoprotein; αSMA, α-smooth muscle actin; CK19, cytokeratin 19; EpCAM, epithelial cell adhesion molecule; PD-L1, programmed death-ligand 1; PIVKA, protein induced by vitamin K absence or antagonist-II; RGS5, regulator of G- protein signaling 5.

In non-HCC tissues of the IFN group, the degree of hepatic inflammation (A) was 1.2±0.5, and the degree of liver fibrosis (F) was 2.3±1.3. Between the IFN group and the DAA group, there were significant differences in the observation period after resection, the tumor size, and the degree of liver fibrosis.

In non-cancerous liver tissues of both IFN group and DAA group, mixed with progressive fibrosis and regressive fibrosis were observed. The progressive fibrosis defined as fibroseptal stroma showing wide/broad, loosely aggregated collagen fibers, which are moderately to markedly cellular containing, variably, inflammatory cells, and ductular reactions (Fig. 2A-C) (15). The regressive fibrosis defined as fibroseptal stroma showing thin, densely compacted stroma, which are largely acellular (Fig. 2D-F) (15). Two patients (6%) of the IFN group and 2 patients (18%) of the DAA group were identified as having non-alcoholic steatohepatitis (NASH)-like pathological features, such as steatosis, ballooning hepatocytes and Mallory-Denk bodies.

Immunohistochemically, PD-L1 positive reaction was found inside the tumor not interface of the tumor. In the IFN group, 3, 3, and 29 cases showed a PD-L1 Score of 2+, 1+, and 0, respectively. All cases were negative for CK19. Two, 4, and 29 cases showed an EpCAM Score of 2+, 1+, and 0, respectively. In the DAA, 1, 3, and 9 cases showed a PD-L1 Score of

Table III. Clinicopathological findings of PD-L1 positive HCC.

A, Clinical findings

Characteristics	PD-L1 expression of HCC		P-value
	Positive (n=10)	Negative (n=38)	
Age (year)	71±8	68.3±8.1	0.69
Male/Female	4/6	25/13	0.16
IFN/DAA	6/4	29/9	0.43
Diabetes (prevalence rate, %)	1 (10%)	17 (45%)	0.06
Serum total bililubin (mg/dl)	0.8±0.3	0.9±0.2	0.21
Prothrombin activity (%)	85.9±10.8	92.2±14.7	0.15
Serum albumin (g/dl)	4.1±0.38	4.2±0.29	0.41
Serum AFP (ng/ml, median)	84	4.5	0.46
Serum PIVKA (mAU/ml, median)	63	100	0.11

B, HCC findings

Characteristics	PD-L1 expression of HCC		P-value
	Positive (n=10)	Negative (n=38)	
Tumor size (mm)	26±14	26±17	0.92
Tumor differentiation (well/moderate/poor)	0/8/2	5/31/2	0.06
Nuclear grade	2.7±0.5	1.8±0.8	<0.01
Atypia score	2.6±0.5	2.3±0.5	0.11
Mitosis score	2.5±0.7	1.5±0.7	<0.01
Portal vein invasion (prevalence rate, %)	5 (50%)	21 (55%)	1
CK19 score (0/1+/2+)	9/1/0	38/0/0	0.34
EpCAM score (0/1+/2+)	5/3/2	33/3/2	0.04
RGS5 score (0/1+/2+)	0/6/4	11/23/4	<0.01

C, Non-HCC findings

Characteristics	PD-L1 expression of HCC		P-value
	Positive (n=10)	Negative (n=38)	
Fibrosis (New Inuyama Classification, F)	3.3±0.9	2.4±1.3	0.03
Inflammation (New Inuyama Classification, A)	1.5±0.5	1.2±0.5	0.11
Steatosis (prevalence rate, %)	5 (50%)	10 (26%)	0.24
CD34 score (0/1+/2+)	0/1/9	0/5/33	0.79
αSMA score (0/1+/2+)	3/4/3	18/11/9	0.43

AFP, α-fetoprotein; αSMA, α-smooth muscle actin; CK19, cytokeratin 19; EpCAM, epithelial cell adhesion molecule; DAA, direct acting antivirals; HCC, hepatocellular carcinoma; IFN, interferon; PD-L1, programmed death-ligand 1; PIVKA, protein induced by vitamin K absence or antagonist-II; RGS5, regulator of G- protein signaling 5.

2+, 1+, and 0, respectively. In the IFN group, CD34 and αSMA expression in sinusoidal mesenchymal cells were present in 35 (100%) and 17 (49%) patients, respectively. The CD34 expressions in sinusoidal mesenchymal cells were observed in the periportal area with regionality (Rappaport zone 1 to zone 2). The αSMA expression area was close to the CD34 expression area (Fig. 1).

PD-L1 expression and pathological findings. The clinicopathological findings of the PD-L1-positive HCC and the PD-L1-negative HCC are shown in Table III. In the HCC tissues, mitosis was more frequently observed in the PD-L1-positive HCC than in the PD-L1-negative HCC (P<0.01). The Nuclear grade was higher in the PD-L1-positive HCC than in the PD-L1-negative HCC (P<0.01). RGS5

Table IV. Univariate and multivariate analysis for recurrence free survival in IFN group.

A, Clinical findings

	Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (>70 years)	3.96 (1.23-13.16)	0.02	3.07 (0.79-11.27)	0.1
Male	1.31 (0.51-3.79)	0.59		
Diabetes	0.37 (0.06-1.37)	0.15		
Serum T. Bililubin (>0.8 mg/dl)	1.14 (0.44-2.95)	0.78		
Prothrombin activity (<90%)	1.67 (0.59-4.53)	0.32		
Serum albumin (<4.2 g/dl)	0.92 (0.34-2.4)	0.87		
Serum AFP (>6.7 ng/ml)	1.46 (0.55-3.93)	0.44		
Serum PIVKA (>118 mAU/ml)	1.21 (0.46-3.09)	0.69		

B, HCC findings

	Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Tumor size (>30 mm)	1.72 (0.54-4.8)	0.34		
Poor differentiation	5.78 (0.83-26.96)	0.07	2.95 (0.39-16.19)	0.26
Nuclear grade (>grade 2)	1.61 (0.62-4.69)	0.34		
Portal vein invasion	1.57 (0.61-4.31)	0.34		
PD-L1 positive	6.01 (1.45-23.3)	0.02	5.01 (1.14-21.08)	0.03
CK19 or/and EpCAM positive	Unparsable			
RGS5 positive	3.46 (0.7-62.57)	0.15		

C, Non-HCC findings

	Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Liver fibrosis (>F3)	0.92 (0.35-2.35)	0.86		
Hepatic inflammation (>A2)	0.68 (0.15-2.13)	0.53		
Liver steatosis	0.52 (0.14-1.5)	0.24		
CD34 expression (>score 2+)	3.13 (0.63-56.68)	0.19		
αSMA positive (>score 2+)	2.21 (0.82-5.75)	0.11		

AFP, α-fetoprotein; αSMA, α-smooth muscle actin; CI, confidence interval; CK19, cytokeratin 19; EpCAM, epithelial cell adhesion molecule; HR, Hazard ratio; IFN, interferon; PD-L1, programmed death-ligand 1; PIVKA, protein induced by vitamin K absence or antagonist-II; RGS5, regulator of G- protein signaling 5; T. Bililubin, total bililubin.

and EpCAM expression levels were higher in the PD-L1 positive-HCC than that in the PD-L1-negative HCC (P<0.05). In the non-cancerous liver tissues, the degree of liver fibrosis was higher in the patients with PD-L1-positive HCC than in those with PD-L1-negative HCC.

Univariate and multivariate analysis for prognosis. The observation period after resection was longer in the IFN group than in the DAA group (P<0.01). In the IFN group, univariate analysis for recurrence free survival after surgery (RFS) revealed that PD-L1 expression was significant predictor for recurrence (positive vs. negative; HR=6.01, 95% CI=1.45-23.3, P=0.02) (Table IV). Multivariate analysis demonstrated that PD-L1 expression was an independent poor prognostic factor for recurrence in the IFN group (positive vs. negative; HR=5.01, 95% CI=1.14-21.08, P=0.03) (Table IV). In the DAA group, there were not significant prognostic factors by univariate and multivariate analyzes.

The survival curves. In the IFN group, the Kaplan-Meier curves demonstrate that both RFS and overall survival time were significantly shorter for the PD-L1-positive HCC, compared with the PD-L1-negative HCC (log-rank test P<0.01; Fig. 3).

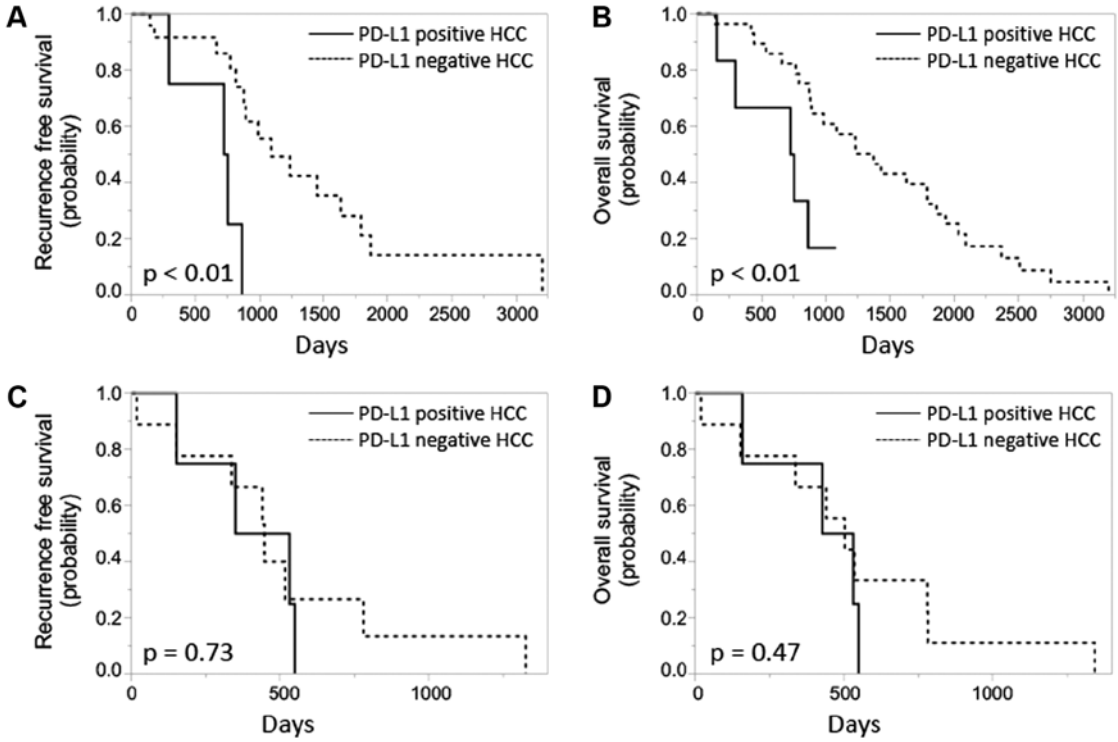

Figure 3. Survival curves. Kaplan-Meier analysis of the (A) recurrence free survival and (B) overall survival of patients with PD-L1 positive HCC or PD-L1 negative HCC in the interferon group. Significant differences were evaluated using a log-rank test. Kaplan-Meier analysis of the (C) recurrence free survival and (D) overall survival of patients with PD-L1 positive HCC or PD-L1 negative HCC in the direct acting antivirals group. PD-L1, programmed death-ligand 1; HCC, hepatocellular carcinoma.

Discussion

We studied the molecular pathological characteristics of patients who developed initial HCC after achieving SVR. The increasing understanding of HCC biology is necessary for future targeted therapies and personalized care.

We demonstrated that PD-L1 expression was an independent unfavorable prognostic factor of surgically-resected HCC after archiving SVR treated by IFN. Recent studies have demonstrated that PD-L1 positive HCC display biological and pathological markers of aggressiveness (e.g., high serum AFP levels, poor differentiation, and vascular invasion) (16). PD-L1, the major ligand for PD-1, plays a crucial role in PD-1-dependent immune suppression, which is mediated by an antigen-specific T-cell response. PD-L1 expression might contribute to aggressive behavior by blocking antitumor immunity, but the specific details remain unclear. There is very limited information on the expression of PD-L1 in HCC (16,17), and its relationship with the clinical and histopathological features remains unknown. In our study, PD-L1 positive HCC also display biological and pathological markers of aggressiveness (e.g., high mitotic activity, RGS5 expression, and EpCAM expression). RGS5 is a member of the RGS protein family and RGS proteins act GTPase-activating proteins for heterotrimeric G protein α subunit, negatively regulating G-protein signaling. With regard to RGS5 and HCC, RGS5 expression has been demonstrated to enhanced portal vein invasion (14,18). Tsujikawa *et al* (19), reported that immunohistochemical molecular analysis revealed B/S group expressing CK19, SALL4, and EpCAM shows frequent portal vein invasion, high Ki67 labeling index, and worse prognosis. HCCs with TP53 mutation are more proliferative and aggressive and show CK19 and EpCAM expressions, frequent microvascular invasion, and poor survival (20,21). Our data suggest that PD-L1 positive HCC may have higher malignant potential. The timing of PD-L1 positivity initiation is not clear. However, the PD-L1 positive reaction was observed in 0% (none of 5 cases) of well differentiated HCC, 21% (8 cases of 39 cases) of moderately differentiated HCC, and 50% (2 cases of 4 cases) in poorly differentiated HCC. According to these our results, we considered that the PD-L1 positivity initiation of HCC may be occurred during dedifferentiation process.

It has been reported that both hepatic inflammation and fibrosis are reversible and decrease after achieving SVR by IFN-based therapy. George *et al* (22), reported on 49 patients with HCV infection who had undergone liver biopsy before treatment and 4-years after achieving SVR by IFN-based therapy. Forty of these patients (82%) showed a decrease in fibrosis score, and 45 (92%) showed a decrease in inflammation score. Ten patients (20%) had healthy or nearly healthy livers on long-term follow-up biopsy. In this study, uncertain mixed both progressive fibrosis and regressive fibrosis were observed in non-cancerous liver tissues of patients with HCC after achieving SVR. Recently, regressive fibrosis in chronic hepatitis B has been reported (15). Predominately regressive was defined as most (more than 50%) fibroseptal stroma in the liver biopsy specimens showing features of thin, densely compacted stroma, largely darkly staining on trichrome, which are largely acellular (15). It has been reported that serum levels of Wisteria floribunda agglutinin-positive human

Mac-2 binding protein glycosylation isomer (M2BPGi) can be used to predict the development of HCC in patients with HCV SVR (23). M2BPGi is a serum marker of liver fibrosis that indicates the extent of liver fibrosis in patients with chronic liver disease. Bekki *et al* (24), reported that M2BP is secreted by activated HSCs. Liver fibrosis that does not decrease sufficiently after achieving SVR may be an important contributory factor to liver carcinogenesis in the patients after achieving SVR. However, in this study, the liver fibrosis was not associated with a poor prognosis in surgically resected HCC after archiving SVR.

In this study, the degree of inflammation and fibrosis was higher in the DAA group than those in the IFN group. In Japan, the IFN therapy cannot be used as an anti-HCV treatment for cirrhotic patients. On the other hand, the DAA therapy can be used as an anti-HCV treatment for cirrhotic patients. Advanced liver fibrosis cases were seen in the patients with achieving SVR treated by DAA more frequently than in the patients with achieving SVR treated by IFN. Besides, the observation period of the DAA group was shorter than those of the IFN group because DAA was new drug developed in 2011. Advanced liver fibrosis cases should require clinical follow-up even after achieving SVR.

Immunohistochemically, in patients developed HCC after achieving SVR, expression of CD34 and αSMA in sinusoidal mesenchymal cells of non-cancerous liver tissue were frequently observed. In chronic liver disease, hepatic fibrosis starts with the stimulation of HSCs. Activated HSCs transform into myofibroblasts and increase of the extracellular matrix. As a result, capillarization of the hepatic sinusoidal endothelial cells (HECs) occurs (25). αSMA is frequently used as a marker of activated and myofibroblast-like HSCs (26). Capillarized sinusoids are lined by a continuous endothelial lining surrounded by a complete basement membrane (27). Immunophenotyping studies of human HECs have demonstrated that HECs of healthy livers are characterized by the expression of the CD32 and CD16 for the Fc fragment of IgG, but lack CD34 (28). In liver cirrhosis, HECs of capillarized sinusoids displayed changes in the positive expression of the CD34 (29). It has been reported that capillarized sinusoids contribute to sinusoidal portal hypertension in liver cirrhosis (30). In capillarized sinusoids, metabolism between the blood circulation and hepatocytes is impaired, because the continuous endothelial lining is surrounded by a complete basement membrane. Further molecular biological study of sinusoidal capillarization and activation of HSCs after achieving SVR will contribute to improving the treatment of fibrosis after achieving SVR.

The current study has several limitations. First, this was a retrospective study. Second, while the statistical signification of this study was sufficient, the number of cases was relatively small. Third, we were not able to investigate the relationship of PD-L1 with prognosis of the DAA group enough, because the sample size was small and the observation period after resection was very short. These may affect the statistical results. We tried to add the samples in the DAA group, but we cannot add the samples because DAA was new drug developed in 2011. Further prospective studies with larger numbers of patients and long observation period is needed to confirm the results of this study.

In conclusion, we found that PD-L1 expression was associated with a poor prognosis in surgically resected HCC after archiving SVR. We considered that PD-L1 positive HCC had blocking antitumor immunity but also high mitotic activity. In addition, we demonstrated liver fibrosis in non-cancerous liver tissues of patients with HCC after achieving SVR. We consider that advanced liver fibrosis may be a contributory factor of liver carcinogenesis after achieving SVR. The mechanism of fibrosis and fibrinolysis in the liver tissue after achieving SVR is not clearly. Further molecular biological study of sinusoidal capillarization, i.e., CD34 expression, and activation of HSCs, i.e., CD34 expression, after achieving SVR is necessary. These may associate with hepatic fibrosis after achieving SVR.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

RK, JA, ON and HY designed the research. RK, SO, YN, HK, YM and MT performed the research. RK analyzed the data and wrote the paper. HY and JA critically revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee at Kurume University (approval no. 16060). The Ethical Committee waived the requirement for written informed consent for the cases as the data for these patients were retrospectively analyzed.

Patient consent for publication

Written informed consent was obtained from all participants for the publication of any data or associated images.

Competing interests

The authors declare that they have no competing interests.

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