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ARID1A Deficiency Is Associated With High Programmed Death Ligand 1 Expression in Hepatocellular Carcinoma

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The clinicopathological features of carcinomas expressing AT-rich interaction domain 1a (ARID1A) and programmed death ligand 1 (PD-L1) in HCC are poorly understood. Here, we examined ARID1A and PD-L1 expression in surgically resected primary hepatocellular carcinoma (HCC) and the association of ARID1A and PD-L1 expression with clinicopathological features and patient outcomes. Their association with ARID1A expression and tumor-associated CD68-positive macrophage was further explored. Using a database of 255 patients who underwent hepatic resection for HCC, immunohistochemical staining of ARID1A, PD-L1, and CD68 was performed. We also analyzed the expression PD-L1 after ARID1A knockdown in HCC cell lines. Samples from 81 patients (31.7%) were negative for ARID1A. Negative ARID1A expression was significantly associated with male sex, high alpha-fetoprotein, high des-gamma-carboxyprothrombin, large tumor size, high rate of poor differentiation, microscopic intrahepatic metastasis, and PD-L1 expression. In addition, negative ARID1A expression was an independent predictor for recurrence-free survival, overall survival, and positive PD-L1 expression. Stratification based on ARID1A and PD-L1 expression in cancer cells was also significantly associated with unfavorable outcomes. PD-L1 protein expression levels were increased through phosphoinositide 3-kinase/AKT signaling after ARID1A knockdown in HCC cells. HCC with ARID1A-low expression was significantly correlated with high levels of tumor-associated CD68-positive macrophage. *Conclusion:* Our large cohort study showed that ARID1A expression in cancer cells was associated with a poor clinical outcome in patients with HCC, PD-L1 expression in cancer cells, and tumor microenvironment. Therefore, ARID1A may be a potential molecular biomarker for the selection of patients with HCC for anti-programmed death 1/PD-L1 antibody therapy. (*Hepatology Communications* 2020;0:1-14).

Primary liver cancer is the fourth most common tumor worldwide. HCC occurs primarily in patients with cirrhosis, hepatitis B or C virus infection, or nonalcoholic steatohepatitis. In addition, underlying liver diseases limit therapeutic efficacy.[1] Hepatic resection has been established as a safe and effective treatment in patients with HCC. However, the number of patients who develop recurrence remains high.[2,3] The currently available treatment for advanced HCC is molecular targeted therapy

Abbreviations: AFP, alpha-fetoprotein; ARID1A, AT-rich interaction domain 1a; CI, confidence interval; DCP, des-gamma-carboxyprothrombin; HBsAg, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; HR, hazard ratio; IFN-γ, interferon-γ; IL, interleukin; JCRB, Japanese Cancer Research Resources Bank; mTOR, mammalian target of rapamycin; OS, overall survival; PI3K, phosphoinositide 3-kinase; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; RFS, recurrence-free survival; RNAi, RNA interference; SWI/SNF, switch/sucrose nonfermentable.

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using sorafenib or lenvatinib, but the median overall survival (OS) time in drug-treated patients is only slightly improved compared with those given the placebo.[4]

Recently, immune checkpoint blockade using anti-programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) antibodies has been a topic of high interest in oncology fields. PD-L1 expression in cancer cells is associated with clinical outcomes in patients with HCC.[5,6] The immunohistochemical expression of PD-L1 in tumor cells or tumor-associated stromal cells is the most accurate predictive biomarker of patient response to PD-1/PD-L1-targeted therapy.[7] Although anti-PD-1/PD-L1 treatment provides promising outcomes for cancer patients, only a proportion of patients respond to the treatment.[8] Thus, the response to anti-PD-1/PD-L1 therapy cannot be predicted based on PD-L1 expression alone.

AT-rich interaction domain 1a (ARID1A) is a key component of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex and is involved in cancer cell development, differentiation, proliferation, and DNA repair.[9,10] It was reported that ARID1A expression is related to PD-L1 levels in various cancers.[11-13] However, the association between ARID1A and PD-L1 expression in HCC is not yet fully understood.

In this study, we investigated ARID1A and PD-L1 expression by immunohistochemistry and examined the prognostic significance of ARID1A and PD-L1 in patients with HCC. We evaluated the mechanism by which ARID1A regulates PD-L1 protein expression. Additionally, we showed the association between ARID1A expression and tumor-associated CD68-positive macrophage in the tumor microenvironment.

Patients and Methods

PATIENTS

A total of 255 patients with HCC who underwent hepatic resection at the Department of Surgery and Science, Kyushu University Hospital, between January 2002 and December 2015 were enrolled in this study. The details of our surgical techniques and patient selection criteria for hepatic resection in HCC have been previously reported.[14] Twenty-two patients had undergone transarterial chemoembolization preoperatively. Patients were followed up as outpatients every 1 to 3 months after discharge. Dynamic computed tomography was performed by radiologists every 3 months, and magnetic resonance imaging was performed if recurrence was suspected. Clinical information and follow-up data were obtained from medical records. No patients underwent immune checkpoint inhibitor treatment for recurrence. This study was approved by the Ethics Committee of Kyushu University (approval codes 30-454 and 2020-68).

IMMUNOCYTOCHEMICAL STAINING

Immunohistochemical staining for ARID1A, PD-L1, or CD68 was performed on 4-μm formalin-fixed and paraffin-embedded sections. Sections were first deparaffinized. After the inhibition of endogenous peroxidase activity for 30 minutes with 10% or 3% hydrogen peroxidase in methanol, the sections were pretreated with Target Retrieval Solution (Dako, Glostrup, Denmark) in a microwave oven at 99°C for 44 minutes for ARID1A, decloaking chamber at 110°C for 15 minutes for PD-L1, or microwave oven at 99°C for 20 minutes for CD68, and

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then incubated with monoclonal antibodies at 4°C overnight. The immune complex was detected with a Dako EnVision Detection System. The sections were finally incubated in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted.

The primary antibodies used were an ARID1A rabbit polyclonal antibody (dilution 1:100; Sigma-Aldrich, St. Louis, MO), PD-L1 rabbit monoclonal antibody (clone 28-8, dilution 1:450; Abcam, Cambridge, United Kingdom), and CD68 mouse antibody (PG-M1, dilution 1:50; Dako). Stained slides were scanned using the NanoZoomer (Hamamatsu Photonics KK, Shizuoka, Japan). Immunohistochemical data for ARID1A, PD-L1, or CD68 staining were evaluated by three experienced researchers (N.I., S.I., and K.K. or S.I., K.Y., and K.K.), who were blinded to the clinical status of the patients. The final assessments were achieved by consensus. Cancer cells with nuclear staining for ARID1A or membranous staining for PD-L1 were considered positive staining. Cells revealed cytoplasm or membranous staining for CD68. The number of cells with cytoplasm or membrane staining in three high-power fields. The proportion of ARID1A-positive cancer cells was estimated as the percentage of total cancer cells. We used cutoff values of 30% and 70% for cancer cells, as in a previous report.[15] The proportion of PD-L1-positive cancer cells was estimated as the percentage of total cancer cells. The percentage of PD-L1-positive cells was estimated as <1% (negative) or ≥1% (positive), as in previous reports.[5] Sections from human colons or placentas were used as positive controls.

CELLS, CELL CULTURE, AND GENERATION OF AN ARID1A KNOCKDOWN CELL LINE

HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (JCRB) Cell Bank (Osaka, Japan) and KAC (Kyoto, Japan), and were confirmed to be free from mutation in ARID1A and PD-L1 genes using the database of the Cancer Dependency Map (https://demap.org/portal/), Hep3B (Cell ID ECACC 86062703; Depmap ID ACH-000625; http://demap.org/potral/cell_line/ACH-000625?tab=mutaion), HuH7 (Cell ID JCRB 0403; Depmap ID ACH-000480; http://demap.org/potral/cell_line/ACH-000480?tab=mutaion), and PLC/PRF5 (Cell ID JCRB 0406; Depmap ID ACH-001318; http://demap.org/potral/cell_line/PLCPRF5_LIVER?tab=mutaion). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C at 10% CO_2. To generate ARID1A knockdown cells, transient gene suppression was performed in Hep3B and HuH7 HCC cells using Stealth RNA interference (RNAi; Thermo Fisher Scientific, Waltham, MA). Cells were prepared in complete growth medium without antibiotics to obtain a 500-μL suspension containing 25,000 cells (30%-50% confluent 24 hours after plating). Then, reverse transfection was performed using 10 nM Stealth RNAi with Opti-MEM I Reduced Serum Medium and Lipofectamine RNAiMAX for 48 hours at 37°C in a CO_2 incubator.

WESTERN BLOTTING

Samples were lysed in lysis buffer containing 50 mmol/l Tris HCl (pH 6.8) and 10% sodium dodecyl sulfate, and the protein concentration in each sample was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Samples were heated at 95°C for 5 minutes and subjected to electrophoresis using SuperSep Ace 12% gels (Fujifilm, Tokyo, Japan) at 20 mA for 80 minutes. The Trans-Blot Turbo Transfer System (Bio-Rad) was used to transfer proteins onto a polyvinylidene fluoride membrane (Bio-Rad). The membrane was incubated in iBind solution (Invitrogen, Carlsbad, CA) with primary and secondary antibodies diluted in iBind solution. Primary antibodies included anti-rabbit ARID1A (dilution 1:1,000, Sigma-Aldrich), anti-rabbit Akt (clone pan, dilution 1:1,000, Cell Signaling Technology, Danvers, MA), anti-rabbit phospho-Akt (clone Ser473, dilution 1:2,000, Cell Signaling), anti-rabbit mammalian target of rapamycin (mTOR) (dilution 1:1,000, Cell Signaling), anti-rabbit phospho mTOR (clone Ser2448, dilution 1:1,000, Cell Signaling), anti-rabbit phospho-P70 S6 kinase (clone Thr389, dilution 1:1,000; Cell Signaling), anti-rabbit PD-L1 antibody (clone 28-8, dilution 1:500; Abcam), and anti-rabbit glyceraldehyde 3-phosphate dehydrogenase (dilution 1:5,000; GeneTex, Irvine, CA). The secondary antibody was goat anti-rabbit immunoglobulin G H&L (1:5,000, Abcam). The membrane was incubated with Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Metuchen, NJ) and imaged using an Amersham Imager 600 (GE Healthcare, Chicago, IL) as previously reported.[16]

IMMUNOFLUORESCENCE

Treated cells were first incubated with 4% paraformaldehyde for 10 minutes at room temperature and then blocked with 5% goat serum for 60 minutes at room temperature. Cells were then incubated with primary antibodies (ARID1A, dilution 1:2,000, Sigma-Aldrich; PD-L1, dilution 1:1,000, Cell Signaling) at 4°C overnight, followed by incubation with Alexa Fluor 647-conjugated or Alexa Fluor 488-conjugated secondary antibodies (1:250, Thermo Fisher Scientific) for 1 hour at room temperature. Cells were then stained with diamidino-phenylindole for 10 minutes at room temperature. After washes, cells were observed using a fluorescence microscope (Biorevo BZ-9000; Keyence, Osaka, Japan).

STATISTICAL ANALYSIS

Standard statistical analyses were used to evaluate descriptive statistics, such as medians, frequencies, and percentages. Continuous variables without a normal distribution and variables, such as the data obtained using cell lines, were compared with the Mann-Whitney U test. A logistic regression analysis was performed to identify variables for ARID1A and PD-L1 expression. Categorical variables were compared using the $\chi 2$ test or Fisher's exact test. Survival data were used to establish a univariate Cox proportional hazards model. Covariates that were significant at $P < 0.05$ were included in the multivariate Cox proportional hazards model. Cumulative OS and recurrence-free survival (RFS) rates were calculated using the Kaplan-Meier method, and differences between the curves were evaluated using the log-rank test. Differences were considered to be significant at $P < 0.05$. All statistical analyses were performed using JMP14 software (SAS Institute Inc., Cary, NC).

Results

ASSOCIATION BETWEEN ARID1A AND PD-L1 EXPRESSION AND CLINICOPATHOLOGICAL CHARACTERISTICS IN PATIENTS WITH HCC

In our cohort of 255 patients with HCC, 197 patients (77.2%) were males. The median age of the patients was 68 years (range 17-87 years). In the total patient group, 45 (17.6%) and 138 (54.1%) patients showed positive hepatitis B surface antigen (HBsAg) and hepatitis C virus antibody (HCV-Ab) expression, respectively. The median observation period was 5.5 years (range 0.1-18.6 years).

Figure 1 shows representative immunohistochemical staining for ARID1A and PD-L1 in HCC tissues. ARID1A expression was observed in the nucleus, and PD-L1 expression was detected at the plasma membranes of cancer cells. We observed positive nuclear staining for ARID1A at a proportion of more than 70% (Fig. 1A), closer to 40% (Fig. 1B), and closer to 10% (Fig. 1C). A histogram of the percentage of ARID1A-positive cancer cells in all cases is shown in Supporting Fig. S1. Representative samples with positive and negative membrane staining for PD-L1 are shown in Fig. 1D,E, respectively.

The associations between ARID1A expression and patient clinicopathological characteristics are given in Table 1. Eighty-one patients (31.7%) were negative for ARID1A expression when the cutoff value was set at 30%, whereas 108 patients (42.3%) were negative for ARID1A expression when the 70% cutoff value was used. When the 30% cutoff was applied, low ARID1A expression was observed in male patients ($P = 0.0339$) and patients with high alpha-fetoprotein (AFP) concentration ($P = 0.0096$), high des-gamma-carboxyprothrombin (DCP) concentration ($P = 0.0055$), large tumor size ($P = 0.0001$), multiple cancers ($P = 0.0361$), high rate of poorly differentiated HCC ($P < 0.0001$), microscopic intrahepatic metastasis ($P = 0.0009$), and PD-L1-positive expression ($P = 0.0255$). When the 70% cutoff was applied, low ARID1A expression was observed in patients with low albumin concentrations ($P = 0.0038$), high AFP concentration ($P = 0.0486$), high DCP concentration ($P = 0.0049$), large tumor size ($P = 0.0006$), high rate of poorly differentiated HCC ($P = 0.0025$), and microscopic intrahepatic metastasis ($P = 0.0087$). HBsAg positivity and HCV-Ab positivity showed no association with ARID1A expression at either the 30% or 70% cutoff. Among 165 cases with recurrence, the rate of postoperative intrahepatic metastasis with multiple nodules was higher in patients with low ARID1A expression than in those with high ARID1A expression (33 of 59, 55.9% vs. 42 of 106, 39.6%; $P = 0.0437$). The rate of repeat hepatic resection or

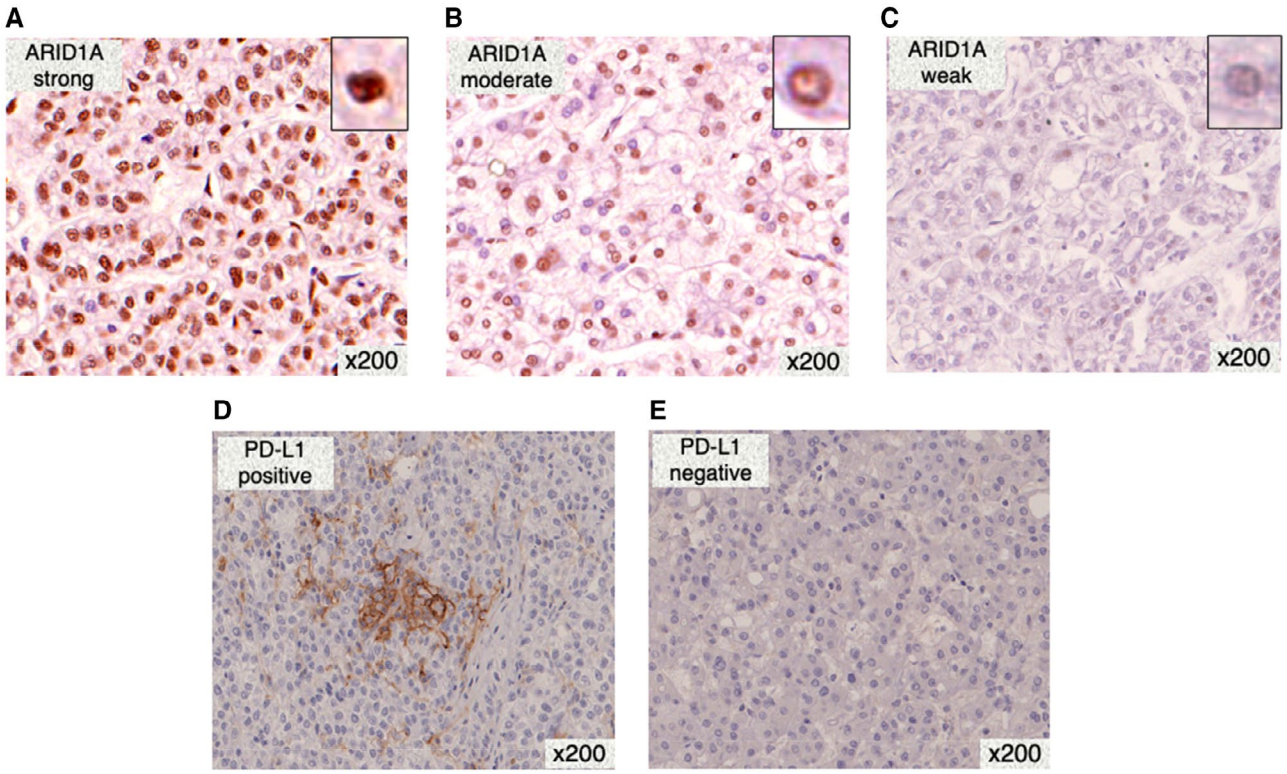

FIG. 1. Immunohistochemical staining of ARID1A and PD-L1 in patients with HCC. (A) Positive nucleus staining for ARID1A with a proportion of more than 70%. (B) Positive nucleus staining for ARID1A with a proportion closer to 40%. (C) Positive nucleus staining for ARID1A with a proportion closer to 10%. (D) Positive membrane staining for PD-L1. (E) Negative staining for PD-L1.

TABLE 1. ASSOCIATION BETWEEN ARID1A EXPRESSION AND TUMOR AND PATIENT CLINICOPATHOLOGICAL FACTORS

	ARID1A Expression at 30% Cutoff by Cancer Cells			ARID1A Expression at 70% Cutoff by Cancer Cells		
Factors	Positive (n = 174)	Negative (n = 81)	*P* Value	Positive (n = 147)	Negative (n = 108)	*P* Value
Age (years)	69 (17-87)	68 (29-85)	0.2268	69 (17-87)	68 (29-85)	0.4124
Sex, male/female	128/46	69/12	0.0339	111/36	86/22	0.4363
BMI (kg/m^2)	22.76 (15.74-32.60)	22.46 (16.42-32.17)	0.2524	22.49 (15.74-32.60)	22.92 (16.42-32.17)	0.7398
HBsAg-positive	27 (15.5%)	18 (22.2%)	0.1978	26 (17.69%)	19 (17.59%)	0.9844
HCV-Ab-positive	101 (58.0%)	37 (45.6%)	0.0652	86 (58.50%)	52 (48.15%)	0.1010
Albumin (g/dL)	4.0 (2.6-5.1)	3.9 (2.7-4.8)	0.1035	4.1 (2.8-5.1)	3.9 (2.6-4.8)	0.0038
AFP (ng/mL)	10.0 (0.8-66,825)	35.05 (0.5-577,660.0)	0.0096	10.1 (1-66,825)	17 (0.5-577,660.0)	0.0486
DCP (mAU/mL)	87 (8-75,000)	319 (2-75,000)	0.0055	82 (8-75,000)	307 (2-75,000)	0.0049
Tumor size (cm)	3.2 (1.0-20.0)	4.0 (1.5-16.5)	0.0001	3.0 (1.0-20.0)	3.75 (1.3-17.0)	0.0006
Solitary/multiple	143/31	57/24	0.0361	120/27	80/28	0.1488
Poor differentiation	40 (22.9%)	40 (49.3%)	<0.0001	35 (23.8%)	45 (41.6%)	0.0025
Microscopic vascular invasion	60 (34.4%)	34 (41.9%)	0.2503	52 (35.3%)	42 (38.8%)	0.5657
Microscopic intrahepatic metastasis	21 (12.0%)	24 (29.6%)	0.0009	18 (12.2%)	27 (25.0%)	0.0087
F3 or F4	70 (40.2%)	32 (39.5%)	0.9125	59 (40.1%)	43 (39.8%)	0.9587
PD-L1-positive	37 (21.2%)	28 (34.5%)	0.0255	32 (21.7%)	33 (30.5%)	0.1130

Note: Data are presented as n (%) or median (range).
Abbreviation: BMI, body mass index.

local ablation therapy for intrahepatic recurrence was higher in patients with high ARID1A expression than in those with low ARID1A expression (69 of 106, 65.0% vs. 21 of 59, 35.5%; $P = 0.0003$). There was no significant difference in the use of molecular target agents between high and low ARID1A expression groups (30 of 106, 28.3% vs. 10 of 59, 17.0%; $P = 0.1029$).

The associations between PD-L1 expression and patient clinicopathological characteristics are found in Table 2. Sixty-five patients (25.4%) were positive for PD-L1, with a cutoff value set at 1%. Negative PD-L1 expression was associated with DCP concentration ($P = 0.0094$), large tumor size ($P = 0.0001$), high rate of poorly differentiated HCC ($P < 0.0001$), microscopic vascular invasion ($P = 0.0078$), and microscopic intrahepatic metastasis ($P = 0.0064$). HBsAg positivity and HCV-Ab positivity were not associated with PD-L1 expression.

UNIVARIATE SURVIVAL ANALYSIS OF PATIENTS WITH SURGICALLY RESECTED HCC ACCORDING TO ARID1A AND PD-L1 EXPRESSION

Next, we assessed the associations between ARID1A protein expression and patient postoperative survival using the Kaplan-Meier method. The results showed that patients with ARID1A-negative expression in cancer cells had significantly shorter RFS (log-rank $P < 0.0001$) and shorter OS (log-rank $P < 0.0001$) after surgery compared to those with ARID1A-positive expression (Fig. 2A,B). There was a significant difference in OS ($P = 0.0003$) and RFS ($P = 0.0068$) in ARID1A-negative and ARID1A-positive groups at the 70% cutoff value (Fig. 2C,D).

To determine the preferable ARID1A cutoff levels for prognostic analysis, we then conducted forest plot analyses for both cutoff levels, assessing the RFS and OS of each subgroup (Supporting Fig. S2A,B). The forest plot analyses revealed that the 30% cutoff value was a more sensitive value for the prediction of postoperative prognosis in terms of both OS and RFS in each subgroup. Survival analyses using the Kaplan-Meier method showed that patients with positive PD-L1 expression had significantly shorter RFS (log-rank $P < 0.0001$) and shorter OS (log-rank $P < 0.0001$) after surgery than patients without PD-L1 expression (Fig. 2E,F).

UNIVARIATE AND MULTIVARIATE ANALYSES OF PROGNOSIS FACTORS FOR RFS AND OS

Table 3 lists the univariate and multivariate analysis results associated with RFS and OS in patients with HCC after hepatic resection. Cox proportional hazard regression models with multivariate analysis

TABLE 2. CHARACTERISTICS OF PATIENTS WITH HCC WHO UNDERWENT HEPATIC RESECTION

Variable	PD-L1-Negative (n = 190)	PD-L1-Positive (n = 65)	*P* Value
Age (years)	68 (17-87)	69 (39-86)	0.5550
Sex, male/female	148/42	49/16	0.6787
BMI (kg/m^2)	22.95 (15.74-32.60)	22.29 (16.42-31.34)	0.0586
HBsAg-positive	33 (17.3%)	12 (18.4%)	0.8424
HCV-Ab-positive	106 (55.7%)	32 (49.2%)	0.3602
Albumin (g/dl)	4.0 (2.6-5.0)	3.9 (2.7-5.1)	0.0344
AFP (ng/ml)	9.0 (1.0-383,541.0)	82.3 (0.5-577,660.0)	0.0549
DCP (mAU/ml)	101 (2-75,000)	266 (13-75,000)	0.0094
Tumor size (cm)	3.3 (1.0-20.0)	4.0 (1.2-17.0)	0.0001
Solitary/multiple	151/39	49/16	0.4935
Poor differentiation	44 (23.1%)	36 (55.3%)	<0.0001
Microscopic vascular invasion	61 (32.1%)	33 (50.7%)	0.0078
Microscopic intrahepatic metastasis	26 (13.6%)	19 (29.2%)	0.0064
F3 or F4	73 (38.4%)	29 (44.6%)	0.3806

Note: Data are presented as n (%) or median (range).
Abbreviation: BMI, body mass index.

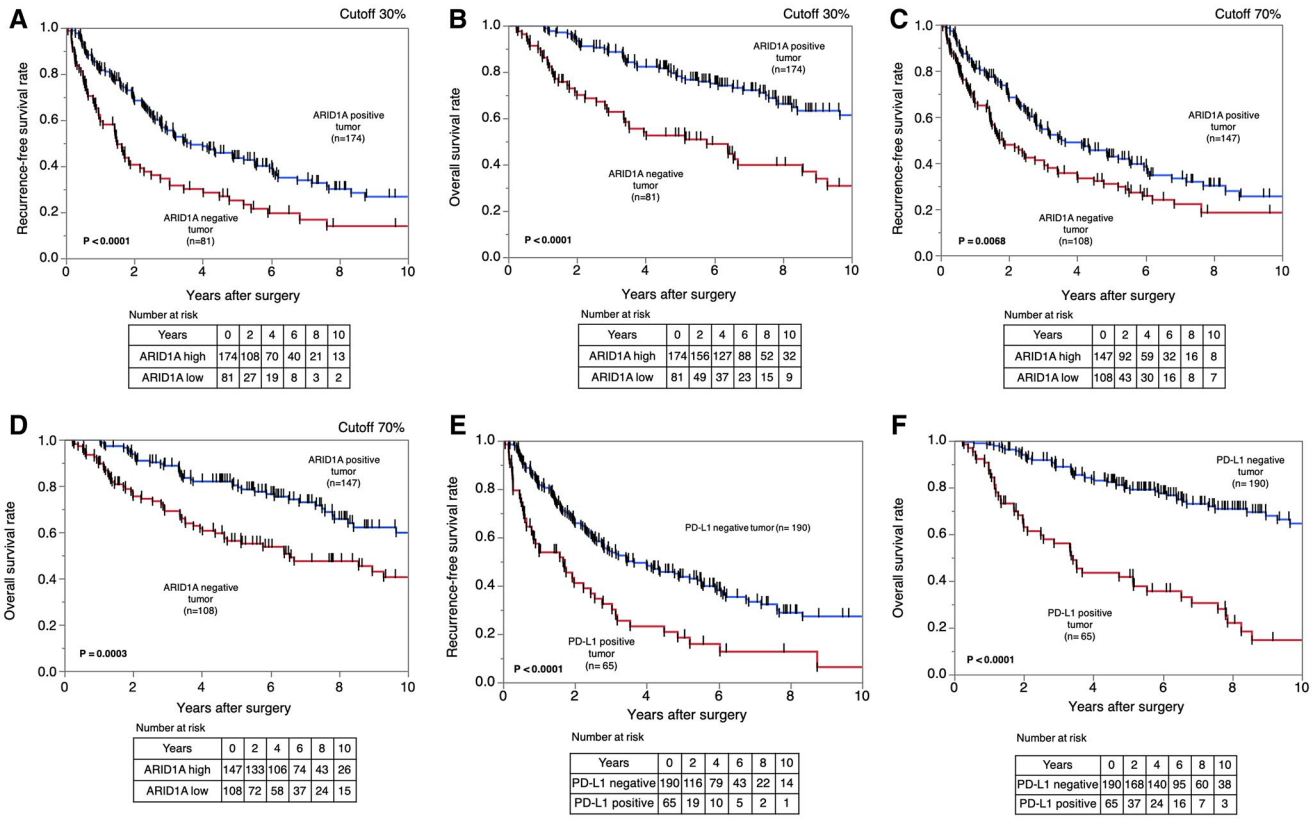

FIG. 2. Kaplan-Meier curves showing the survival of patients with HCC according to expressions of ARID1A or PD-L1. RFS (A) and OS (B) in all patients according to ARID1A expression using the 30% cutoff value. RFS (C) and OS (D) in all patients according to ARID1A expression using the 70% cutoff value. RFS (E) and OS (F) in all patients according to PD-L1 expression using the 1% cutoff value.

showed that ARID1A-negative expression in cancer cells was associated with significantly worse RFS and OS (hazard ratio [HR] 1.49, 95% confidence interval [CI] 1.05-2.13, P = 0.0245; and HR 2.20, 95% CI 1.43-3.38, P = 0.0003), and that PD-L1-positive expression in cancer cells was related to significantly worse RFS and OS (HR 1.73, 95% CI 1.17-2.56, P = 0.0056; and HR 3.48, 95% CI 2.24-5.40, P < 0.0001).

COMBINATION OF ARID1A EXPRESSION AND PD-L1 EXPRESSION IN HCC

Next, we evaluated the significance of ARID1A and PD-L1 expression in predicting OS and RFS. Patients were divided into the following three groups: ARID1A-positive/PD-L1-negative (n = 137); ARID1A-positive/PD-L1-positive or ARID1A-negative/PD-L1-negative (n = 90); and ARID1A-negative/PD-L1-positive (n = 28). We found that both RFS (log-rank P < 0.0001) and OS (log-rank P < 0.0001) were significantly different among the three groups. ARID1A-negative/PD-L1-positive patients showed a significantly worse RFS (log-rank P < 0.0001) and OS (log-rank P < 0.0001) compared with the other groups (Fig. 3A,B).

The associations between ARID1A-negative/PD-L1-positive expression and patient clinicopathological characteristics are reported in Supporting Table S1. ARID1A-negative/PD-L1-positive patients showed low albumin concentration (P = 0.0230), high AFP concentration (P = 0.0010), high DCP concentration (P = 0.0014), large tumor size (P < 0.0001), high rate of poorly differentiated HCC (P < 0.0001), and microscopic intrahepatic metastasis (P = 0.0002).

TABLE 3. UNIVARIATE AND MULTIVARIATE ANALYSES OF FACTORS RELATED TO RFS AND OS IN PATIENTS WITH HCC WHO UNDERWENT HEPATIC RESECTION (COX PROPORTIONAL HAZARDS ANALYSIS)

		RFS		OS	
		Univariate Analysis	Multivariate Analysis	Univariate Analysis	Multivariate Analysis
		HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Factors		*P* Value	*P* Value	*P* Value	*P* Value
Age (years)		1.009 (0.996-1.023)		1.020 (1.001-1.041)	1.011 (0.992-1.032)
		0.147		0.0351	0.2465
Sex	Male	1.723 (1.146-2.590)	1.948 (1.242–3.056)	1.568 (0.929-2.648)	
	Female	0.0056	0.0037	0.0773	
HBsAg	Positive	1.061 (0.720-1.564)		1.001 (0.605-1.656)	
	Negative	0.7638		0.9967	
HCV-Ab	Positive	0.936 (0.689-1.272)		1.150 (0.770-1.717)	
	Negative	0.6760		0.4913	
Albumin		0.531 (0.365-0.778)	0.618 (0.417-0.917)	0.366 (0.225-0.602)	0.496 (0.283-0.877)
		0.0013	0.0171	<0.0001	0.0162
AFP		1.000 (1.000-1.000)	1.000 (0.999-1.000)	1.000 (0.999-1.000)	
		0.0126	0.4383	0.0968	
DCP		1.000 (1.000-1.000)	1.000 (0.999-1.000)	1.000 (1.000-1.000)	0.999 (0.999-1.000)
		0.0041	0.4388	0.0397	0.7489
Tumor size		1.079 (1.034-1.120)	1.035 (0.975-1.094)	1.098 (1.045-1.147)	1.021 (0.945-1.095)
		0.0007	0.2442	0.0004	0.5797
Macroscopic tumor number	Multiple	2.157 (1.523-3.055)	1.636 (1.041-2.572)	2.087 (1.350-3.228)	1.042 (0.589-1.842)
	Single	<0.0001	0.0325	0.0017	0.8865
Poor differentiation	Present	1.581 (1.146-2.180)	0.924 (0.626-1.363)	1.984 (1.329-2.962)	0.9180 (0.567-1.486)
	Absent	0.0064	0.6914	0.0011	0.7279
Microscopic vascular invasion	Present	1.381 (1.010-1.888)	0.874 (0.600-1.274)	2.101 (1.412-3.126)	1.2884 (0.811-2.046)
	Absent	0.0450	0.4863	0.0003	0.2830
Microscopic intrahepatic metastasis	Present	3.225 (2.221-4.683)	2.276 (1.375-3.765)	3.406 (2.171-5.345)	2.520 (1.342-4.734)
	Absent	<0.0001	0.0014	<0.0001	0.0040
Microscopic liver fibrosis	F3 or F4	1.255 (0.923-1.708)		1.169 (0.785-1.741)	
	F0 or F1 or F2	0.1491		0.4420	
ARID1A expression	Negative	1.888 (1.369-2.603)	1.4979 (1.053-2.130)	2.643 (1.774-3.937)	2.206 (1.438-3.385)
	Positive	0.0002	0.0245	<0.0001	0.0003
PD-L1 expression	Positive	2.156 (1.530-3.037)	1.734 (1.175-2.560)	4.347 (2.896-6.526)	3.480 (2.241-5.406)
	Negative	<0.0001	0.0056	<0.0001	<0.0001

ARID1A REGULATES PD-L1 EXPRESSION IN HCC CELLS

To test whether PD-L1 expression was related to active phosphoinositide 3-kinase (PI3K)/AKT/mTOR/pS6k signaling induced by a loss of ARID1A, we first evaluated ARID1A protein levels in HCC cell lines by western blot and found that PLC/PRF5 cells showed a loss of ARID1A expression (Fig. 4A). Therefore, we selected ARID1A-expressing Hep3B and HuH7 cells for subsequent experiments. Next, we confirmed ARID1A, AKT/mTOR/pS6k, and PD-L1 protein levels in control and ARID1A-knockdown HCC cell lines using western blot analysis. We found that PD-L1 protein expression levels were increased after ARID1A knockdown through PI3K/AKT signaling in cells treated with interferon-γ (IFN-γ) (Fig. 4B). We also showed that PD-L1 protein expression in ARID1A knockdown cells was higher than in control cells using immunofluorescent double staining (Supporting Fig. S3).

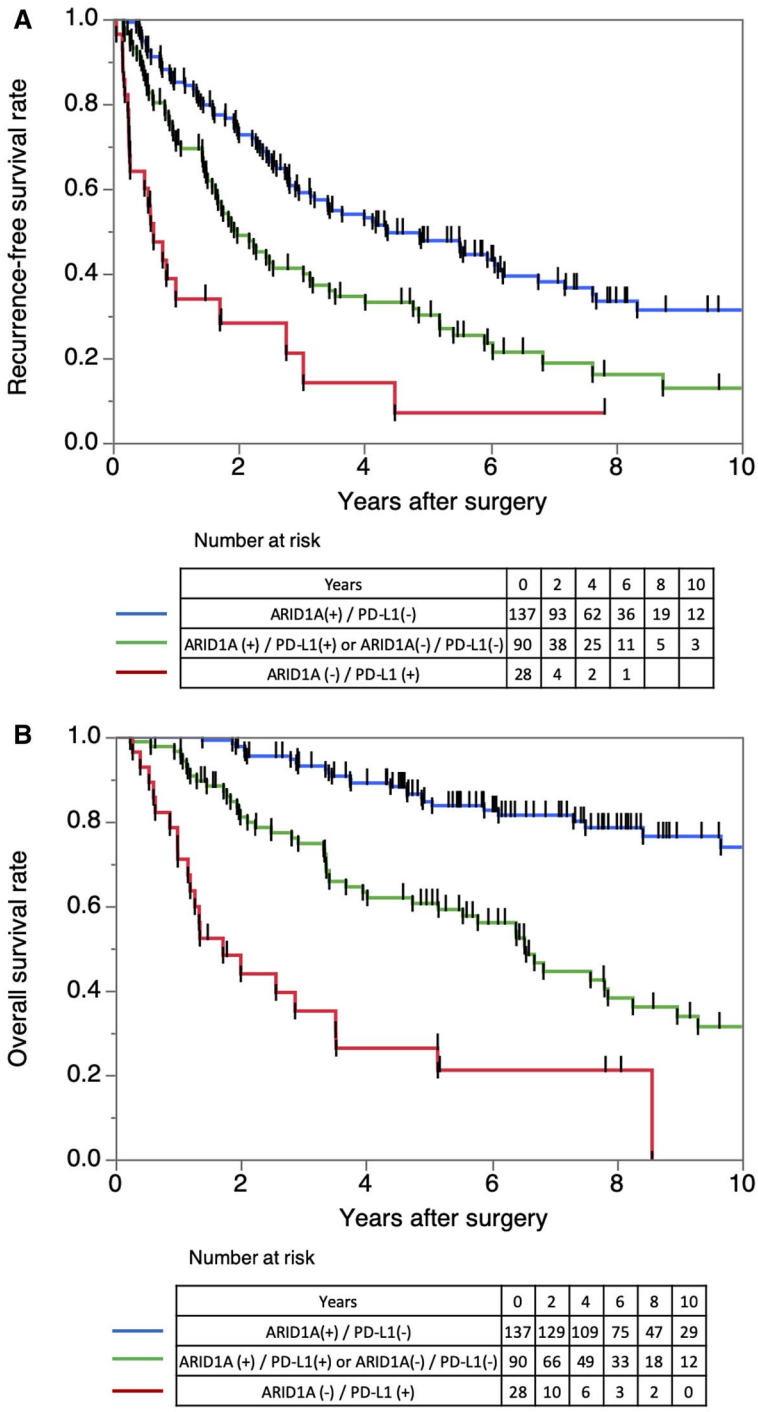

FIG. 3. Kaplan-Meier curves for RFS (A) and OS (B) in patients with HCC according to expressions of tumor ARID1A and PD-L1.

ARID1A AND IMMUNE CELL

We next performed immunocytochemical staining for CD68 in human HCC tissue. CD68 immunocytochemical expression was tested (Fig. 5A,B). The tumor-associated CD68-positive macrophage median was 104 (range, 2.7-206). ARID1A negative expression in cancer cells was associated with a significantly high level of tumor-associated CD68-positive macrophage compared with

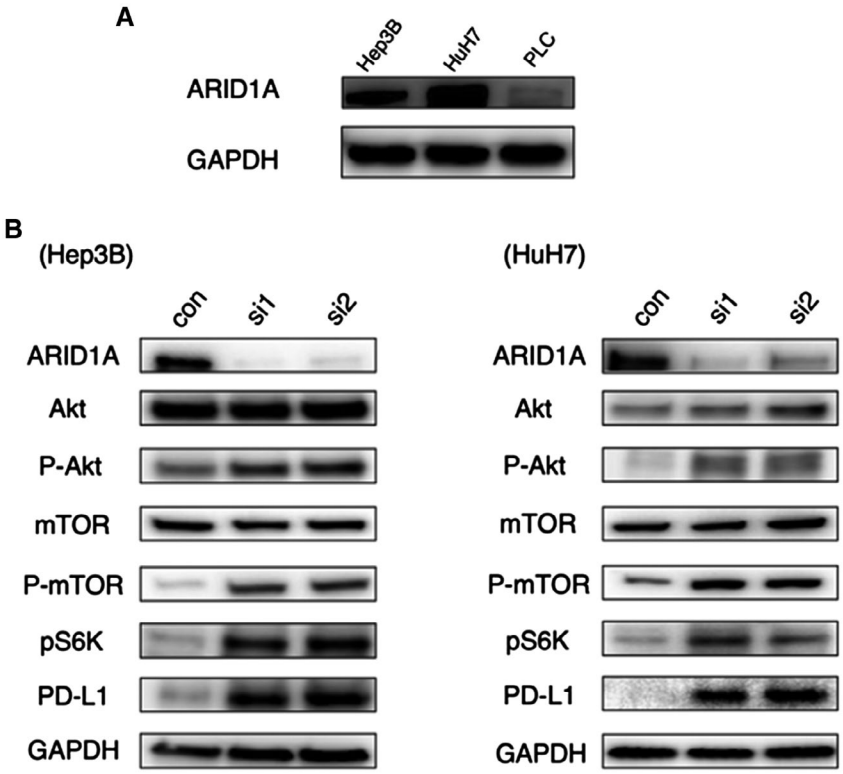

FIG. 4. Knockdown of ARID1A increases the protein levels of PD-L1 through PI3K/AKT signaling. (A) Protein levels of ARID1A in HCC cell lines. (B) Control and ARID1A-knockdown Hep3B and HuH7 cell lines were treated with IFN-γ. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

ARID1A-positive expression (median, 111.6 [range, 34.7-206] and median, 97.7 [range 2.7-195], respectively; $P = 0.0388$) (Fig. 5C).

Discussion

In the present article, we analyzed the expression of ARID1A in several patients with HCC who had undergone hepatic resection. We demonstrated that low ARID1A expression and high PD-L1 expression in cancer cells were poor prognostic factors, and we were able to stratify patient prognosis based on ARID1A and PD-L1 expression. We showed that ARID1A expression in cancer cells regulated PD-L1 expression through PI3K/AKT/mTOR/S6k signaling in HCC cells. We also found that cancer-cell ARID1A expression in tissue sample was correlated with tumor-associated CD68-positive macrophage.

Genes encoding components of the SWI/SNF chromatin remodeling complex have been identified in genome-sequencing studies as some of the most commonly mutated genes in cancer, with ARID1A being the most frequently mutated SWI/SNF gene.[17] ARID1A mutations were observed in 10%-16.8% of the studied tumors and 13% of hepatitis B virus–associated HCCs.[18-20] Previous research has shown that ARID1A deficiency in advanced human HCC is associated with increased vessel density. Mechanistically, loss of ARID1A causes aberrant histone H3K27ac deposition at the angiopoietin-2 (Ang2) enhancer and promoter, which eventually leads to ectopic expression of Ang2 and promotes HCC development.[15] Another study revealed that ARID1A up-regulates its downstream target CDKN1A and suppresses HCC cell proliferation and migration by inhibiting microvascular invasion in HCC.[21] He at al. showed that decreased expression of ARID1A is associated with poor prognosis in a limited sample size of 64 patients with HCC, and ARID1A protein expression was significantly correlated with lymph node metastasis,

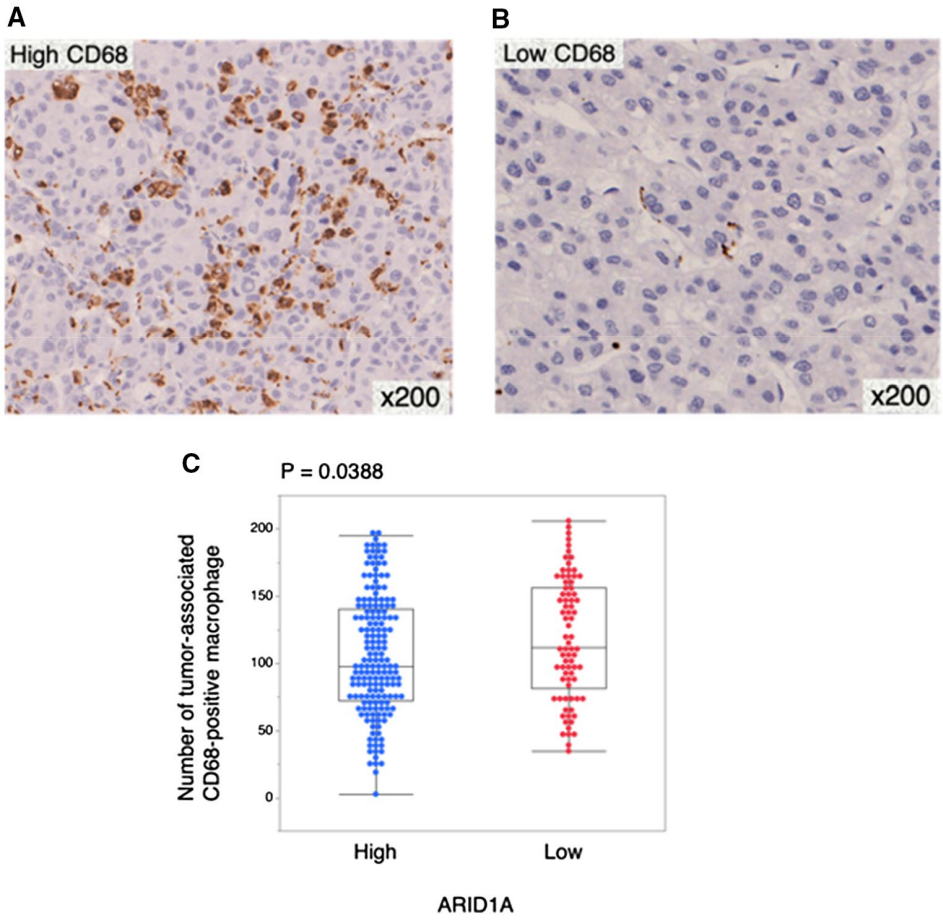

FIG. 5. Immunocytochemical staining of CD68 in patients with HCC. (A,B) High and low CD68 expression in tumor-associated macrophage. (C) Relation between expression of ARID1A and number of tumor-associated CD68-positive macrophage.

distant metastasis, and poor prognosis.[22] In the current study, we demonstrated that low ARID1A expression was associated with tumor size, number of tumors, poor differentiation, and microscopic intrahepatic metastasis. Our findings are consistent with the previous results. The present report used a large sample size to reveal the critical stratification of prognosis in patients with HCC based on ARID1A expression. We also showed that ARID1A deficiency was related to proliferation and migration in HCC cells (data not shown), which may be a contributing factor to the poor prognosis in patients with HCC.

In this study, we set the ARID1A immunohistochemical staining cutoff point at 30% of cells with nuclear staining. In a recent report, Hu et al. used cutoff values of 30% and 70%.[15] In this study, the 30% cutoff value was more sensitive than the 70% cutoff value for the prediction of postoperative prognosis in terms of both RFS and OS.

Recently, PD-L1 expression in tumor cells has received increasing attention. Many recent reports, including the present study, have evaluated the prognosis impact of PD-L1 protein expression in HCC.[5,23-25] We previously showed the significant stratification of prognosis based on PD-L1 expression and CD8 status and the association between PD-L1 expression in cancer cells and vascular formation in patients with HCC after resection.[5] Another report revealed that the expression of PD-L1 in either tumor or intratumoral inflammatory cells in patients with HCC was correlated with biological and pathological markers of aggressiveness.[23] In this study, a high level of PD-L1 was also an independent poor prognostic factor in 255 patients with HCC (Fig. 2E,F). Liu et al. showed

the wide heterogeneity in PD-L1 cutoff values for HCC in a meta-analyses of 17 studies; some studies used cutoff values of 1% or 5%.[26] We previously demonstrated that the 1% cutoff value was more sensitive than the 5% cutoff value for the prediction of postoperative prognosis in terms of both RFS and OS.[5] Therefore, in this study, we used a cutoff value of 1%.

Our data further revealed that low ARID1A expression in cancer cells was related to high PD-L1 expression in cancer cells, and among ARID1A-negative patients, and PD-L1-positive patients had a significantly worse RFS and OS compared with PD-L1-negative patients. However, the association of ARID1A and PD-L1 expression in HCC is not fully understood. We hypothesized that ARID1A regulates the expression of PD-L1, and that PD-L1 protein expression levels increase after ARID1A knockdown through PI3K/AKT/mTOR/S6k signaling. Previous studies showed that ARID1A activates the AKT pathway.[27,28] In addition, activation of AKT-mTOR signaling regulates PD-L1 expression *in vitro* and *in vivo*, and the oncogenic-mediated and IFN-γ-mediated expression of PD-L1 is dependent on mTOR in lung cancer.[29] Parsa et al. reported that loss of phosphatase and tensin homolog function increased PD-L1 expression by activating S6K1 in glioma.[30] Therefore, ARID1A knockdown increases PD-L1 protein expression through PI3K/AKT signaling in various cancer types. In our previous study, we demonstrated that ARID1A expression in cancer cells modulated PD-L1 expression in cancer cells through PI3K/AKT/mTOR/S6K signaling in HCC.

In the current study, we observed that HCC with low expression of ARID1A cancer cell was significantly correlated with tumor-associated CD68-positive macrophage. The *ARID2* gene encodes components of the SWI/SNF chromatin remodeling complex. The estimated fraction of macrophages M2 was significantly increased in *ARID2*-mutated tumors.[31] In ARID1A knockout mice, the innate immune cells, including macrophage and neutrophil cells, infiltrated into the liver parenchyma and accompanied by the increased interleukin (IL)-6.[32] Exposure of tumor-associated macrophage to tumor-derived cytokines such as IL-4 and IL-10 is able to convert them into polarized type 2 or M2 macrophages with immune-suppressive activities and proangiogenic effects, resulting in tumor progression.[33] Our results suggest that low expression of ARID1A might contribute to immune suppressivity in HCC cells. Therefore, the role of ARID1A interaction in the tumor microenvironment requires further investigation.

The recent large phase 3 study IMbrave150 evaluated atezolizumab + bevacizumab versus sorafenib as the first treatment for patients with unresectable HCC. The study demonstrated statistically significant and clinically meaningful improvements in both OS and RFS for atezolizumab + bevacizumab compared with sorafenib in patients with HCC. Therefore, atezolizumab + bevacizumab has the potential to be a practice-changing treatment in HCC.[34] Inhibition of vascular endothelial growth factor (VEGF) by bevacizumab modulates the immune environment, including enhancing T-cell priming and activation through promotion of dendritic cell maturation, increasing T-cell tumor infiltration by normalizing the tumor vasculature, and establishing an immune-permissive tumor microenvironment by decreasing myeloid-derived suppressor cell and regulatory T-cell populations.[35-39] Therefore, T cell–mediated cancer cell killing by atezolizumab could be enhanced through reversal of VEGF-mediated immunosuppression mechanisms by the addition of bevacizumab.[40] Our results show that patients with PD-L1-positive/ARID1A-negative expression have the worst prognosis compared with other patient groups, and that the expression of PD-L1 in cancer cells is mediated by ARID1A. Accordingly, we speculate that patients with HCC with high PD-L1 expression and low ARID1A expression may be more suitable for anti-PD-1/PD-L1 therapy.

In conclusion, we demonstrated that loss of ARID1A was significantly associated with high PD-L1 expression in HCC through activated PI3K/AKT signaling. Low expression of ARID1A and high expression of PD-L1 were found to be independent prognostic factors for OS and RFS, and patients with a loss of ARID1A and high PD-L1 expression showed the worst prognosis compared with other patients. HCC with ARID1A-low expression was significantly correlated with high levels of tumor-associated CD68-positive macrophage. Therefore, ARID1A may be a potential molecular biomarker for the selection of patients for anti-PD-1/PD-L1 therapy.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1659/suppinfo.