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

Norifumi Iseda,1 Shinji Itoh,1 Tomoharu Yoshizumi,1 Kyohei Yugawa,1 Akinari Morinaga,1 Takahiro Tomiyama,1 Takeo Toshima,1 Kenichi Kohashi,2 Yoshinao Oda,2 and Masaki Mori1

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 of 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 2021;5:675–688).

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.
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
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. 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. 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/portal/cell_line/ACH-000625?tab=mutation), HuH7 (Cell ID JCRB 0403; Depmap ID ACH-000480; http://demap.org/portal/cell_line/ACH-000480?tab=mutation), and PLC/PRF5 (Cell ID JCRB 0406; Depmap ID ACH-001318; http://demap.org/portal/cell_line/PLCPRF5_LIVER?tab=mutation). Cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C at 10% CO2. To generate ARID1A knockout 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 CO2 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 (BioRad). 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.
**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 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.

| Factors                        | ARID1A Expression at 30% Cutoff by Cancer Cells | ARID1A Expression at 70% Cutoff by Cancer Cells |
|--------------------------------|-------------------------------------------------|-------------------------------------------------|
|                                | 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/m²)                    | 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) | 0.0086 |
| DCP (mAU/mL)                   | 87 (87-75,000)     | 319 (2-75,000)    | 0.0055 | 82 (87-75,000)     | 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        | 21 (12.0%)         | 24 (29.6%)        | 0.0009 | 18 (12.2%)        | 27 (25.0%)        | 0.0087 |
| metastasis                     |                    |                  |        |                    |                    |        |
| 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% cut-off 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/m²)                  | 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 (2.7-5.00)           | 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.
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).
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).

### 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)

| Factors                        | RFS                  | OS                  |
|--------------------------------|----------------------|---------------------|
|                                | Univariate Analysis  | Multivariate Analysis | Univariate Analysis  | Multivariate Analysis |
|                                | HR (95% CI)          | P Value             | HR (95% CI)          | P Value             |
| Age (years)                    | 1.009 (0.996-1.023)  | 0.147               | 1.020 (1.001-1.041)  | 0.0351               |
|                                |                      |                     | 1.011 (0.992-1.032)  | 0.2465               |
| Sex                            |                      |                     |                     |                     |
| Male                           | 1.723 (1.146-2.590)  | 0.0037              | 1.568 (0.929-2.648)  | 0.0773               |
| Female                         | 0.0056               |                     | 1.001 (0.605-1.656)  | 0.9967               |
| HbsAg                          |                      |                     |                     |                     |
| Positive                       | 1.061 (0.720-1.564)  | 0.7638              |                      |                     |
| Negative                       |                      |                     |                      |                     |
| HCV-Ab                         |                      |                     |                     |                     |
| Positive                       | 0.936 (0.689-1.272)  | 0.6760              | 1.150 (0.770-1.717)  | 0.4913               |
| Negative                       |                      |                     |                      |                     |
| Albumin                        | 0.531 (0.365-0.778)  | 0.013               | 0.618 (0.417-0.917)  | <0.0001              |
|                                |                      |                     | 0.366 (0.225-0.602)  | 0.496 (0.283-0.877)  |
|                                |                      |                     |                      | 0.0162               |
| AFP                            | 1.000 (1.000-1.000)  | 0.0126              | 1.000 (0.999-1.000)  | 0.9968               |
|                                | 0.0365-1.000        |                     | 0.438              |                      |
| DCP                            | 1.000 (1.000-1.000)  | 0.041               | 1.000 (1.000-1.000)  | 0.999 (0.999-1.000)  |
|                                | 0.034-1.120         |                     | 0.438              |                      |
| Tumor size                      | 1.079 (1.034-1.120)  | 0.0007              | 1.098 (1.045-1.147)  | 0.0004               |
|                                | 0.2442              |                     | 0.0004             | 0.5797               |
| Macroscopic tumor number       | 2.157 (1.523-3.055)  | <0.0001             | 2.087 (1.350-3.228)  | 1.042 (0.589-1.842)  |
|                                |                      |                     | 0.0017             | 0.8865               |
| Poor differentiation            | 1.581 (1.146-2.180)  | 0.024               | 1.984 (1.329-2.962)  | 0.918 (0.567-1.486)  |
|                                |                      | 0.626-1.363         | 0.9911              | 0.7279               |
| Microscopic vascular invasion  | 1.381 (1.010-1.888)  | 0.874               | 2.101 (1.412-3.126)  | 1.2884 (0.811-2.046) |
|                                |                      | 0.600-1.274         | 0.0003              | 0.2830               |
| Microscopic intrahepatic metastasis | 3.225 (2.224-4.683)  | 0.276               | 3.406 (2.171-5.345)  | 2.520 (1.342-4.734)  |
|                                |                      | 1.375-3.765         | 0.0004              | 0.0040               |
| Microscopic liver fibrosis     | 1.255 (0.923-1.708)  | <0.0001             | 1.169 (0.785-1.741)  | 1.169 (0.785-1.741)  |
|                                |                      |                     | 0.4420              |                      |
| ARID1A expression              | 1.888 (1.369-2.603)  | 1.4979              | 2.643 (1.774-3.937)  | 2.206 (1.438-3.385)  |
|                                |                      | 1.053-2.130         | <0.0001             | 0.0033               |
| PD-L1 expression               | 2.156 (1.530-3.037)  | 1.734               | 4.347 (2.896-6.526)  | 3.480 (2.241-5.406)  |
|                                |                      | 1.175-2.560         | <0.0001             | <0.0001              |
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...
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 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,
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

---

**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.
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%.

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. 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. 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. Parsa et al. reported that loss of phosphatase and tensin homolog function increased PD-L1 expression by activating S6K1 in glioma. 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. 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. 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. 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. 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. Therefore, T cell–mediated cancer cell killing by atezolizumab could be enhanced through reversal of VEGF-mediated immunosuppression mechanisms by the addition of bevacizumab. 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.

Acknowledgments: The authors thank Ms. Saori Tsurumaru, Ms. Asuka Nakamura, Ms. Yuko Kubota,
and Ms. Miki Nakashima for their technical support. We thank Melissa Crawford, Ph.D., and Gabrielle White Wolf, Ph.D., from Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript.

REFERENCES

1) Villanueva A. Hepatocellular Carcinoma. N Engl J Med 2019;380:1450-1462.
2) Itoh S, Morita K, Ueda S, Sugimachi K, Yamashita Y-I, Gion T, et al. Long-term results of hepatic resection combined with intraoperative local ablation therapy for patients with multinodular hepatocellular carcinomas. Ann Surg Oncol 2009;16:3299-3307.
3) Itoh S, Shirabe K, Taketomi A, Morita K, Harimoto N, Tsujita E, et al. Zero mortality in more than 300 hepatic resections: validity of preoperative volumetric analysis. Surg Today 2012;42:435-440.
4) Kudo M, Finn RS, Qin S, Han K-H, Ikeda K, Piscaglia F, et al. ARID1A is tightly associated with high PD-L1 expression in hepatocellular carcinoma. J Hepatol 2018;68:465-475.
5) Itoh S, Yoshizumi T, Yugawa K, Imai D, Yoshiya S, Takeishi K, et al. Impact of immune response on outcomes in hepatocellular carcinoma: association with vascular formation. Hepatology 2020 Feb 29. https://doi.org/10.1002/hep.32106. [Epub ahead of print]
6) Itoh S, Yugawa K, Shimokawa M, Yoshiya S, Mano Y, Takeishi K, et al. Prognostic significance of inflammatory biomarkers in hepatocellular carcinoma following hepatic resection. BJU Int 2019;3:500-508.
7) Herbst RS, Soria J-C, Poddanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014;515:563-567.
8) Chen J, Jiang CC, Jin L, Zhang XD. Regulation of PD-L1: a novel role of pro-survival signalling in cancer. Ann Oncol 2016;27:409-416.
9) Reisman D, Giraldo S, Thompson EA. The SWI/SNF complex and cancer. Oncogene 2009;28:1653-1668.
10) Shen J, Ju Z, Zhao W, Wang L, Feng Y, Ge Z, et al. ARID1A deficiency promotes mutability and potentiates therapeutic anti-tumor immunity unleashed by immune checkpoint blockade. Nat Med 2018;24:556-562.
11) Fukumoto T, Fatkhutdinov N, Zundell JA, Tsyganov EN, Nacarelli T, Karakashev S, et al. HDAC6 inhibition synergizes with Anti-PD-L1 therapy in ARID1A-inactivated ovarian cancer. Cancer Res 2019;79:5482-5489.
12) Naito T, Udagawa H, Unemura S, Sakai T, Zenke Y, Kirita K, et al. Non-small cell lung cancer with loss of expression of the SWI/SNF complex is associated with aggressive clinicopathological features, PD-L1-positive status, and high tumor mutation burden. Lung Cancer 2019;138:35-42.
13) Kim YB, Ahn JM, Bae WJ, Sung CO, Lee D. Functional loss of ARID1A is tightly associated with high PD-L1 expression in gastric cancer. Int J Cancer 2019;145:916-926.
14) Itoh S, Shirabe K, Matsumoto Y, Yoshiya S, Muto J, Harimoto N, et al. Effect of body composition on outcomes after hepatic resection for hepatocellular carcinoma. Ann Surg Oncol 2014;21:3063-3068.
15) Hu C, Li WT, Tian F, Jiang K, Liu X, Cen J, et al. Arid1A regulates response to anti-angiogenic therapy in advanced hepatocellular carcinoma. J Hepatobiliary Pancreat Sci 2018;25:465-473.
16) Shimokawa M, Yoshizumi T, Itoh S, Iseda N, Sakata K, Yugawa K, et al. Modulation of Nqo1 activity intercepts anokisia resistance and reduces metastatic potential of hepatocellular carcinoma. Cancer Sci 2020;111:1228-1240.
17) Kadoc H, Crabtree GR. Reversible disruption of mSWI/SNF (BAF) complexes by the SS18-SSX oncogenic fusion in synovial sarcoma. Cell 2013;153:71-85.
18) Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 2012;44:694-698.
19) Fujimoto A, Totoki Y, Abe T, Boroevich KA, Hoosoda F, Nguyen HH, et al. Whole-genome sequencing of liver cancers identifies etiological influences on mutation patterns and recurrent mutations in chromatin regulators. Nat Genet 2012;44:760-764.
20) Toftanin S, Cornella H, Harrington A, Llovet JM. Next-generation sequencing: path for driver discovery in hepatocellular carcinoma. Gastroenterology 2012;143:1391-1393.
21) Cheng S, Wang L, Deng CH, Du SC, Han ZG. ARID1A represents hepatocellular carcinoma cell proliferation and migration through IncRNA MVHI. Biochem Biophys Res Commun 2017;491:178-182.
22) He F, Li J, Xu JF, Zhang S, Xu YP, Zhao WX, et al. Decreased expression of ARID1A associates with poor prognosis and promotes metastases of hepatocellular carcinoma. J Exp Clin Cancer Res 2015;34:47.
23) Calderaro J, Rousseau B, Amaddeo G, Mercey M, Charpy C, Costentin C, et al. Programmed death ligand 1 expression in hepatocellular carcinoma: relationship with clinical and pathological features. Hepatology 2016;64:2038-2046.
24) Liu C-Q, Xu J, Zhou Z-G, Jin L-L, Yu X-J, Xiao G, et al. Expression patterns of programmed death ligand 1 correlate with different microenvironments and patient prognosis in hepatocellular carcinoma. Br J Cancer 2018;119:80-88.
25) Chang B, Huang T, Wei H, Shen L, Zhu D, He W, et al. The correlation and prognostic value of serum levels of soluble programmed death protein 1 (sPD-1) and soluble programmed death-ligand 1 (sPD-L1) in patients with hepatocellular carcinoma. Cancer Immunol Immunother 2019;68:353-363.
26) Liu GM, Li XG, Zhang YM. Prognostic role of PD-L1 for HCC patients after potentially curative resection: a meta-analysis. Cancer Cell Int 2019;19:22.
27) Lee D, Yu EJ, Han IH, Hur H, Kim YS. AKT inhibition is an effective treatment strategy in ARID1A-deficient gastric cancer cells. Onco Targets Ther 2017;10:4153-4159.
28) Samartzis EP, Gutsche K, Dedes KJ, Fink D, Snucik M, Imesch P, et al. Loss of ARID1A expression sensitizes cancer cells to PI3K- and AKT-inhibition. Oncotarget 2014;5:5295-5303.
29) Lastwika KJ, Wilson W, Li QK, Norris J, Xu H, Ghazarian SR, et al. Control of PD-L1 expression by oncogenic activation of the AKT-mTOR pathway in non-small cell lung cancer. Cancer Res 2016;76:227-238.
30) Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of tumor suppressor PTEN function increases B7–H1 expression and immunoresistance in glioma. Nat Med 2007;13:84-88.
31) Fujita M, Yamaguchi R, Hasagawa T, Shimada S, Aritoshi K, Hayashi S, et al. Classification of primary liver cancer with immunoresistance and reduced metastatic potential of hepatocellular carcinoma. PLoS One 2015;10:e0143042.
32) Mantovani A, Locati M. Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization: lessons and open questions. Arterioscler Thromb Vasc Biol 2013;33:1478-1483.
34) Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim T-Y, et al. Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. N Engl J Med 2020;382:1894-1905.
35) Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med 1996;2:1096-1103.
36) Oyama T, Ran S, Ishida T, Nadaf S, Kerr L, Carbone DP, et al. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. J Immunol 1998;160:1224-1232.
37) Hodi FS, Lawrence D, Lezcano C, Wu X, Zhou J, Sasada T, et al. Bevacizumab plus ipilimumab in patients with metastatic melanoma. Cancer Immunol Res 2014;2:632-642.
38) Wallin JJ, Bendell JC, Funke R, Sznol M, Korski K, Jones S, et al. Atezolizumab in combination with bevacizumab enhances antigen-specific T-cell migration in metastatic renal cell carcinoma. Nat Commun 2016;7:12624.
39) Hegde PS, Wallin JJ, Mancao C. Predictive markers of anti-VEGF and emerging role of angiogenesis inhibitors as immunotherapeutics. Semin Cancer Biol 2018;52:117-124.
40) Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity 2013;39:1-10.

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