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*Original Article*

# Altered expression of AT-rich interactive domain 1A in hepatocellular carcinoma

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**Abstract:** AT-rich interactive domain 1A (ARID1A) is a subunit of the Switch/Sucrose non-fermentable (SWI/SNF) chromatin remodeling complex. Recently, genome-wide whole exome sequencing revealed frequent mutations of ARID1A in hepatocellular carcinoma, but clinicopathological significance of ARID1A alteration has not been clarified yet. In this study, expression of ARID1A was investigated immunohistochemically in 290 cases of hepatocellular carcinomas. In the evaluation of tissue microarrays, cases of ARID1A alteration (63 total cases, 21.7%) consisted of 11 (3.8%) cases showing loss of expression and 52 (17.9%) with weak expression. Alteration of ARID1A was correlated with larger tumor size ($P$ = 0.034) and well or moderate differentiation of tumor histology ($P$ = 0.035). There was no significant correlation with age, sex, cirrhosis, TNM stage, tumor size, number of tumors, vascular invasion, patient survival, HBV infection, HCV infection, heavy use of alcohol, nor diabetes mellitus. EBER *in situ* hybridization was negative in all 11 cases with loss of ARID1A. Altered expression of ARID1A was inversely correlated with nuclear expression of p53 (P = 0.018) or beta-catenin ($P$ = 0.025). There was some heterogeneity of ARID1A alteration within each case, and immunohistochemistry of the whole sections demonstrated that four of 11 cases with loss of ARID1A in TMA analysis showed localized positive area within the tumor. Alteration of ARID1A may accelerate tumor growth in a subset of hepatocellular carcinoma, and this pathway may be distinct from p53 and beta-catenin pathways.

**Keywords:** ARID1A, hepatocellular carcinoma, p53, beta-catenin, immunohistochemistry

## Introduction

AT-rich interactive domain 1A (ARID1A) is a subunit of the Switch/Sucrose non-fermentable (SWI/SNF) chromatin remodeling complex that regulates chromatin structure in an ATP-dependent manner. Mutations in the genes of ARID1A and other component molecules of the SWI/SNF complex have been reported in various malignancies and are generally believed to cause impairment of the tumor-suppressive function of the SWI/SNF complex [1]. Recent studies of genome-wide whole exome sequencing revealed that mutations in ARID1A accumulate in certain subsets of each cancer category, such as clear cell carcinoma of ovarian cancer and subtypes of gastric cancer, which show microsatellite instability (MSI) or infection with Epstein-Barr virus (EBV) [2-5]. Clear cell carcinoma of the ovary is closely related with endometriosis, and ARID1A expression is frequently lost in atypical endometriosis [4, 6]. Alteration of ARID1A expression is also observed in early stages of EBV-associated gastric cancer, in contrast to its occurrence in much later stages of cancer progression in EBV-negative gastric cancer [7]. These facts suggest that ARID1A mutation has specific significance, probably in a context-dependent manner, in the development and progression of these subtypes of cancer.

ARID1A mutations have been also identified in hepatocellular carcinoma (HCC) at relatively high frequencies, of 10-16% [8-10]. HCC is one of the major causes of cancer-related death in the world [11], and is etiologically related with viral infection, high alcohol intake, and various metabolic abnormalities, including obesity and insulin resistance. However, these etiologies are interrelated, and there has been a few defi-

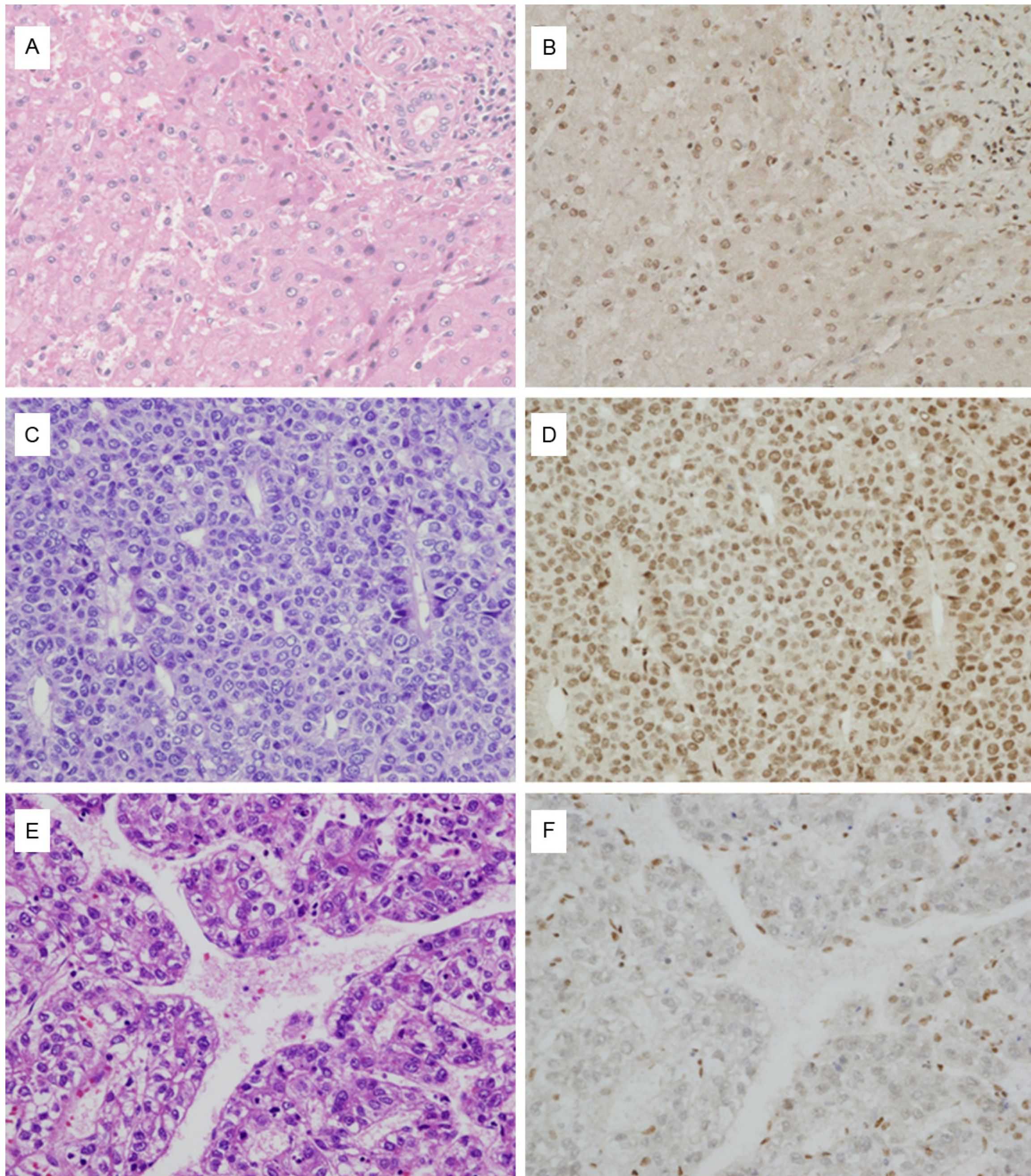


**Figure 1.** Immunohistochemistry of ARID1A. A, B: Non-neoplastic liver tissue; C, D: ARID1A-normal case: E, F: ARID1A-lost case. ARID1A expression was noted in the nuclei. In non-neoplastic liver, hepatocytes, biliary epithelial cells, endothelial cells, fibroblasts and inflammatory cells were all positive for ARID1A. In tumor tissue, stromal cells such as endothelial cells were used as internal positive controls. A, C, E: Hematoxylin and eosin stain; B, D, F: ARID1A staining.

nite subgroups showing common molecular (e.g. p53 and beta catenin) or morphological abnormalities in relation to certain specific etiology. Because genetic or molecular classification of HCC is in the early stages, it is worth investigating clinicopathological features of HCC cases that harbor ARID1A abnormalities. In the present study, we evaluated ARID1A by immunohistochemistry. Because most ARID1A mutations are truncation mutations and closely correlate with loss or decrease of protein expression, alteration of ARID1A expression includes genetic and epigenetic abnormalities of ARID1A [4, 12]. We also evaluated p53 and beta catenin abnormalities and clonal evolution of ARID1A alteration.

**Table 1.** Correlation between ARID1A expression and clinicopathological factors

| | | Cases | ARID1A expression | | *P* value |
|---|---|---|---|---|---|
| | | | Normal (n = 227) | Altered (n = 63) | |
| Age | | | | | |
| | ≤ 60 | 90 | 66 | 24 | 0.17 |
| | > 60 | 200 | 161 | 39 | |
| Sex | | | | | |
| | Male | 229 | 180 | 49 | 0.79 |
| | Female | 61 | 47 | 14 | |
| Cirrhosis | | | | | |
| | Present | 165 | 127 | 38 | 0.54 |
| | Absent | 125 | 100 | 25 | |
| TNM Stage | | | | | |
| | Stage 1 | 29 | 22 | 7 | 0.97 |
| | Stage 2 | 127 | 100 | 27 | |
| | Stage 3 | 108 | 84 | 24 | |
| | Stage 4 | 26 | 21 | 5 | |
| Tumor size | | | | | |
| | ≤ 50 mm | 206 | 168 | 38 | 0.034* |
| | > 50 mm | 84 | 59 | 25 | |
| Histological differentiation | | | | | |
| | Well | 92 | 69 | 23 | 0.035* |
| | Moderate | 161 | 123 | 38 | |
| | Poor | 37 | 35 | 2 | |
| Number of tumors | | | | | |
| | Single | | 146 | 38 | 0.56 |
| | Multiple | | 81 | 25 | |
| Vascular invasion | | | | | |
| | Present | 92 | 75 | 17 | 0.36 |
| | Absent | 198 | 152 | 46 | |

*$P < 0.05$.

## Material and methods

### *Tissue samples*

A total of 290 formalin-fixed and paraffin-embedded HCC tissues resected from 1995 to 2006 at the University of Tokyo Hospital were obtained. Tissue microarrays (TMAs) were constructed from paraffin tissue blocks of HCCs, each made by punching out 2-mm-diameter cores of tumors from the block. At least two cores were obtained from each case. Cases with loss of ARID1A expression in TMA were also examined by immunohistochemistry of whole sections, and distribution of ARID1A-positive and -negative cells within the tumor was analyzed.

Correlations of ARID1A expression status were evaluated with the following clinical and pathological variables: age, gender, HBV and HCV infection, history of heavy use of alcohol, diabetes mellitus, cirrhosis, TNM stage, tumor size, histology, number of tumor, vascular invasion, nuclear expression of p53 or beta-catenin, and prognosis. All data were collected from clinical record and pathological report of the hospital. Virus infection is assessed by serum HBs antigen and HCV antibody, respectively. None of the cases received neoadjuvant chemotherapy. The study was approved by the ethics committee of the institute.

### *Immunohistochemistry and Epstein-Barr encoding region (EBER) in situ hybridization*

Immunohistochemistry of ARID1A was performed on TMAs and whole sections. All specimens were deparaffinized and rehydrated. Antigen retrieval was performed by autoclave at 120°C for 10 min. Samples were incubated with primary antibody (rabbit polyclonal anti-ARID1A, HPA005456, dilution 1:200; Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. After a wash with Tris-buffered saline, the slides were incubated with biotinylated goat polyclonal anti-rabbit IgG antibody (BA-1000, dilution 1:200; Vector Laboratories) for 30 min at room temperature. For detection, the peroxidase method was used with the VECSTATIN ABC kit (Vector Laboratories, Burlingame, CA, USA) and DAB+ chromogen (Dako, Glostrup, Denmark).

Immunohistochemistry of p53 and beta-catenin was performed using the Ventana BenchMark automated immunostainer (Roche, Basel, Switzerland) with the labeled streptavidin-biotin method and visualized with DAB. The primary antibodies used were mouse monoclo-

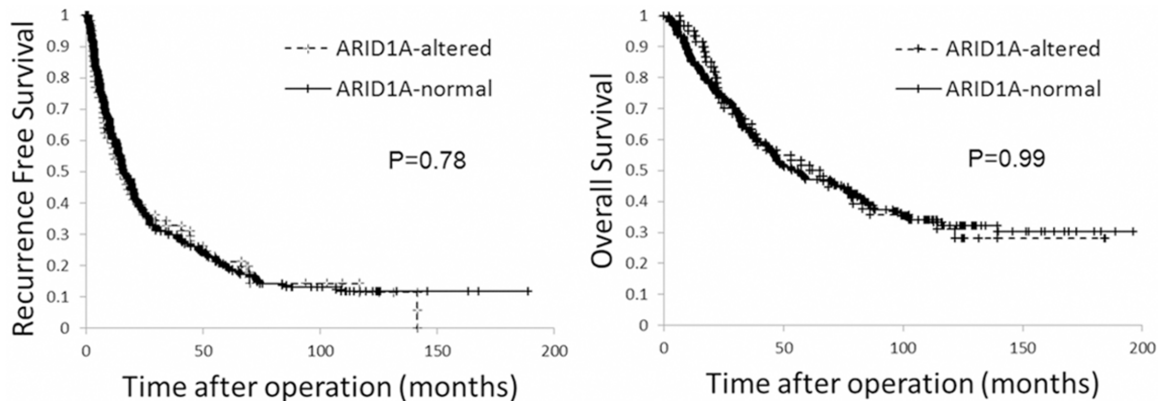


**Figure 2.** Kaplan-Meier analysis of recurrence free survival and overall survival in ARID1A-positive and -altered hepatocellular carcinoma. There was no significant correlation between ARID1A expression and survival.

**Table 2.** Correlation between ARID1A expression and etiologic factors

| | Cases | ARID1A expression | | *P* value |
|---|---|---|---|---|
| | | Normal (n = 227) | Altered (n = 63) | |
| HBs Antigen | | | | |
| Positive | 63 | 48 | 15 | 0.65 |
| Negative | 227 | 179 | 48 | |
| HCV Antibody | | | | |
| Positive | 191 | 152 | 39 | 0.45 |
| Negative | 99 | 75 | 24 | |
| Diabetes Mellitus | | | | |
| Present | 8 | 6 | 2 | 0.84 |
| Absent | 218 | 170 | 48 | |
| Heavy alcohol drinking | | | | |
| Present | 41 | 31 | 10 | 0.70 |
| Absent | 185 | 145 | 40 | |

nal anti-TP53 (clone DO7, dilution 1:50; Novocastra, Newcastle, UK) and mouse monoclonal anti-beta-catenin (clone 14, dilution 1:300; BD Biosciences, Franklin Lakes, NJ, USA). EBER *in situ* hybridization was performed on whole sections using a fluorescein isothiocyanate (FITC)-labeled peptide nucleic acid probe (Y5200; Dako) and anti-FITC antibody (V0403, dilution 1:200; Dako).

ARID1A expression was determined by nuclear staining, and non-neoplastic cells such as fibroblasts and endothelial cells served as internal positive controls for ARID1A. Expression of ARID1A was assessed as lost, weak, or normal in comparison with endothelial cells within the same core. When two cores of the same case showed different expression status, the lower expression was adopted. Expression of p53 and beta-catenin were also determined by nuclear staining.

*Statistical analysis*

The chi-square test was used to examine the distribution of two variables. Overall survival and disease-free survival data were plotted by Kaplan-Meier methods, and *P* values were calculated by the log-rank test. *P* values less than 0.05 were considered statistically significant.

**Results**

*Alteration of ARID1A in HCC and clinicopathological factors*

Representative images of immunohistochemistry for ARID1A are shown in **Figure 1**. Loss of ARID1A expression was observed in 11 (3.8%) of 290 cases, while 52 (17.9%) showed weak expression of ARID1A. Expression of ARID1A was normal in the other 227 (78.3%) cases. Lost or weak expression was regarded as “altered” expression. We next analyzed the relationships between ARID1A expression and clinicopathological factors (**Table 1**). Altered ARID1A expression was associated with larger tumor size (*P* = 0.034) and well or moderately differentiated histology (*P* = 0.035). There was no significant correlation with age, sex, cirrho-

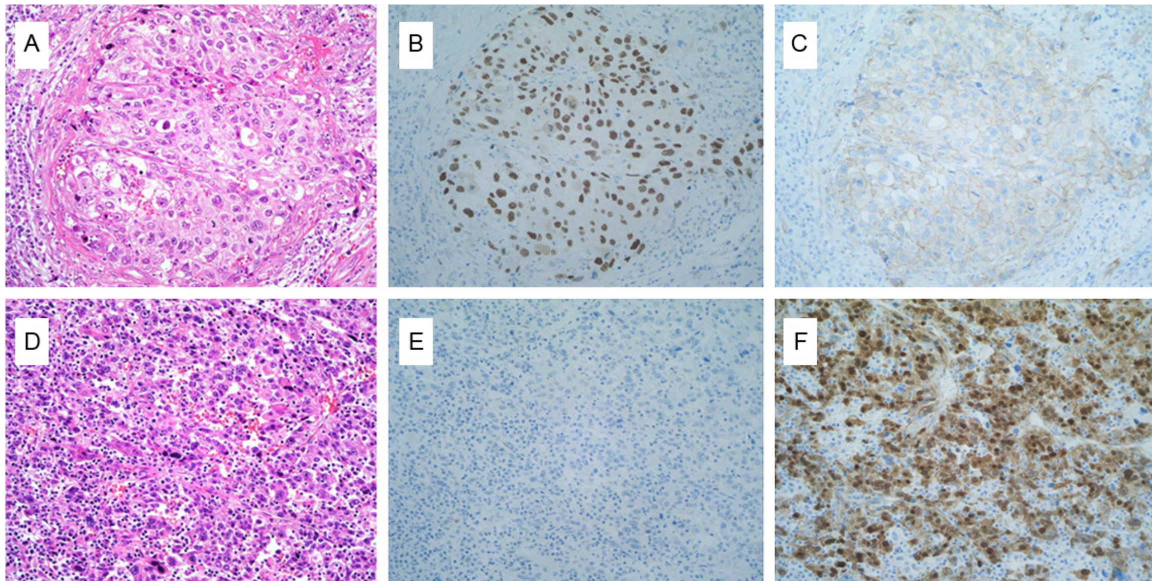


**Figure 3.** Immunohistochemistry of p53 and beta-catenin. A-C: p53-positive and beta-catenin-negative case; D-F: p53-negative and beta-catenin-positive case. Positive expression of p53 and beta-catenin was confirmed by nuclear staining. Membranous staining of beta-catenin was regarded as negative. A, D: Hematoxylin and eosin stain; B, E: p53; C, F: beta-catenin.

**Table 3.** Correlation of ARID1A expression with p53 and beta-catenin

| | Cases | ARID1A expression | | *P* value |
|---|---|---|---|---|
| | | Normal (n = 226) | Altered (n = 63) | |
| p53 | | | | |
| Positive | 80 | 70 | 10 | 0.018* |
| Negative | 209 | 156 | 53 | |
| Beta-catenin | | | | |
| Positive | 56 | 50 | 6 | 0.025* |
| Negative | 233 | 176 | 57 | |

*P < 0.05.

sis, TNM stage, number of tumors, or vascular invasion.

Analyses of recurrence free survival and overall survival failed to show significant correlation with ARID1A expression status (**Figure 2**).

*Etiologic factors including viral infection (HBV, HCV and EBV)*

Altered ARID1A expression was not statistically correlated with complication of diabetes mellitus or history of heavy use of alcohol. No correlation was observed with HBV or HCV infection (**Table 2**). EBER *in situ* hybridization was performed on whole sections of 11 cases with complete loss of ARID1A in TMA analysis, and no case showed positive signals.

*Molecular abnormalities (p53 and beta-catenin)*

Abnormalities of p53 and beta catenin are involved in independent pathways of molecular abnormalities in HCC. We performed immunohistochemistry of p53 and beta catenin (**Figure 3**), and found altered ARID1A expression was significantly correlated with normal expression pattern of p53 and beta-catenin (**Table 3**).

*Distribution of ARID1A-lost carcinoma cells in tumors*

In the TMA analysis, lost or weak expression of ARID1A was consistent in the two cores of TMA in 37 cases, but inconsistent in 26 cases. In the 11 cases with loss of ARID1A in TMA, whole sections of the tumor were also analyzed by immunohistochemistry. In four of the 11 cases, localized areas of the tumor showed preserved expression of ARID1A (**Figure 4**).

## Discussion

In the present study, lost or weak expression of ARID1A was noted in 22% of the total 290 examined cases, and this frequency is comparable to the mutation rates of ARID1A in previ-

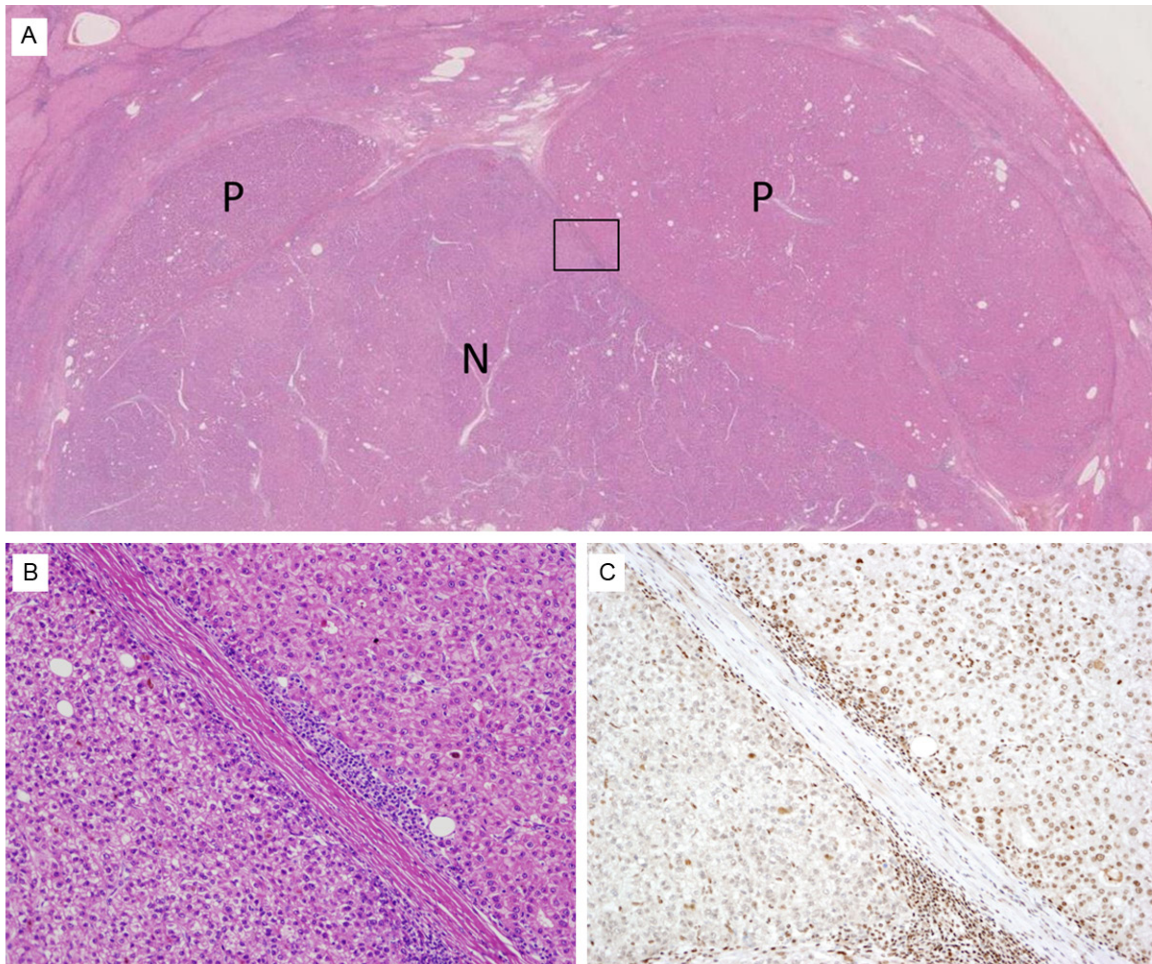


**Figure 4.** Distribution of ARID1A-lost carcinoma cells. A: A whole section of hepatocellular carcinoma at low magnification. "P" indicates ARID1A-positive area, and "N" indicates ARID1A-negative area. In this case, ARID1A-normal and -lost areas were separated by fibrous septum. The tumor had "nodule-in-nodule" appearance, and the ARID1A-lost area was in the center of the tumor. ARID1A-normal cells were distributed in the periphery. B, C: High power view of the border of the ARID1A-normal and -lost areas corresponding to the square in A. The border was clearly defined. In this case, the ARID1A-lost area showed smaller tumor cell size and higher nucleus/cytoplasm ratio than the ARID1A-normal area. However, there was no consistent tendency of morphological change among the cases. A, B: Hematoxylin and eosin stain; C: ARID1A immunohistochemistry.

ous exome sequencing studies (10-16%). In the present study, cases with altered expression of ARID1A showed inverse correlations with nuclear localization of p53 and beta-catenin. Because the p53 and WNT/beta-catenin pathways are two major pathways in liver carcinogenesis [13], loss of ARID1A might represent an alternative pathway to p53 or beta-catenin in HCC. In support of this possibility, the previous whole exome sequence study showed that p53 and ARID1A mutations were mutually exclusive, although the sample number was relatively small [9].

HCC tumors with altered expression of ARID1A were significantly larger in size than those with normal expression, although alteration of ARID1A was not correlated with tumor stage or prognosis. In the present study, nearly half the cases with ARID1A alteration showed a discrepancy in expression status between the two cores of the TMA. Immunohistochemistry of whole sections revealed that there was localized area where ARID1A was preserved in some of the cases showing negative results in TMA. These facts suggest that alteration of ARID1A is a late stage event in cancer progression in HCC, and loss of ARID1A might accelerate tumor progression. The pattern of ARID1A alteration was clonal in distribution, as observed in uterine endometrioid adenocarcinoma and colorectal carcinoma [14, 15], indicating that

alteration of ARID1A drives the clonal growth of cancer cells.

Viral and non-viral etiologies are both important in the development of HCC. The simple idea is that a specific combination of etiology, molecular mechanism, and morphology comprises a subtype of cancer. In the present study, there was no correlation of ARID1A alteration with complication of diabetes mellitus or history of heavy use of alcohol or viral infection of HBV and HCV. Sugawara et al. reported that more than one copy of EBV DNA per 100 cells was detected in 56 (33%) of the HCC sections in HCC tissues from HCV-positive patients [16]. ARID1A alteration occurs frequently in EBV-associated gastric cancers. Thus, we investigated EBV infection in the cases of HCC with ARID1A-lost expression by EBER *in situ* hybridization, and confirmed that no etiological relation with EBV was present, at least in this subset of HCC.

In conclusion, alteration of ARID1A was observed in 22% of HCC cases, and it may accelerate tumor growth of a subset of well and moderately differentiated HCC cases. Furthermore the ARID1A pathway in HCC may be distinct from p53 and beta-catenin pathways. Thus, ARID1A may be a potential therapeutic target for a subset of HCC.

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#### Disclosure of conflict of interest

None.

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