Abstract. Background/Aim: Gastric cancer is a common cancer worldwide. Chromatin remodeling complexes have emerged as tumor suppressors and include AT-rich interaction domain-containing proteins (ARIDs) 1A, 1B, and 2. We examined their expression and clarified their roles in gastric carcinogenesis. Materials and Methods: The expression of ARIDs was studied by immunohistochemistry in 469 gastric carcinoma and 47 adenoma samples and was analyzed according to clinicopathological factors. Results: Low expression rates of ARID1A, 1B, and 2 in gastric carcinoma were 20%, 10%, and 15% respectively. ARIDs are correlated to each other. Low expression of ARID1A was related to advanced tumor and vessel infiltration. Loss of ARID1B and ARID2 was also related to tumor progression, but their relationship was weaker than that of ARID1A. Conclusion: ARID1A is the strongest tumor suppressor in gastric carcinogenesis among ARIDs. Their aberration might be caused by shared mechanisms such as mutation and methylation.

Gastric cancer (GC) is one of the most common cancers worldwide and the second leading cause of cancer-related death globally (1). Although prognosis for early-stage GC is good, 80-90% of patients are diagnosed at an advanced stage. The 5-year survival rate for advanced gastric cancer is less than 10% (2). The mechanisms of gastric carcinogenesis and cancer progression should be further clarified.

Chromatin-remodeling factors play essential roles in many biological processes, including gene expression, DNA replication and repair, and cell division. Switch/sucrose non-fermentable (SWI/SNF) is a family of chromatin remodeling complexes. The SWI/SNF complex, conserved from yeast to humans, is composed of 10-15 biochemically-distinct subunits, including the AT-rich interaction domain-containing protein (ARID) family. The ARID family is a superfamily of 15 members. ARID1A, 1B, and 2 have been shown to be widely distributed in tissues. In addition, ARID1A and 2 are highly mutated and show loss of expression in certain types of cancer.

Mutations of SWI/SNF are widespread across a variety of human cancers (3, 4). Mutation of the ARID1A gene or loss of ARID1A has been reported in ovarian cancer, endometrial carcinoma, gastric cancer, hepatocellular carcinoma, lung adenocarcinoma, clear cell renal cell carcinoma (4), cervix cancer (5), colon cancer (6), non-small cell lung cancer (7), breast cancer (8), prostate cancer (9), neuroblastoma (10), glioblastoma (11), intrahepatic cholangiocarcinoma (12) and urinary bladder cancer (13). ARID1B is also lost in certain cancers, such as colorectal (14), pancreatic (15), hepatocellular carcinoma, clear cell renal cell carcinoma (4), cervix cancer (5), colon cancer (6), non-small cell lung cancer (7), breast cancer (8), prostate cancer (9), neuroblastoma (10), glioblastoma (11), intrahepatic cholangiocarcinoma (12) and urinary bladder cancer (13). ARID1B is also lost in certain cancers, such as colorectal (14), pancreatic (15), hepatocellular carcinoma, clear cell renal cell carcinoma (4), cervix cancer (5), colon cancer (6), non-small cell lung cancer (7), breast cancer (8), prostate cancer (9), neuroblastoma (10), glioblastoma (11), intrahepatic cholangiocarcinoma (12) and urinary bladder cancer (13).

Mutations of ARIDs in human cancers have been mostly identified by whole-exome surveys (3). Methylation also suppresses the expression of ARID1A (17). In the present study, we investigated the expression of ARID1A, 1B, and 2 in gastric cancer immunohistochemically on a large scale, compared the expression with clinicopathological data in detail, and revealed their function in gastric carcinogenesis.

Materials and Methods

IHC on conventional sections. Conventional sections of five tumors were tested for check of intra-tumor heterogeneity. Three early GC and two advanced GC were used. One representative section was selected for each tumor and stained using all three ARIDs antibodies, as described below.

Tissue arrays. Four hundred and sixty-nine primary GCs and 47 gastric adenomas were obtained from the archives of the...
Department of Pathology, Teikyo University Hospital from 2000 to 2011. The series of GC used in the study is the same as the set used in our previous report (18). Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were used for staining. This study was approved by the Ethics Committee of the Teikyo University School of Medicine.

Histological and pathological data were evaluated according to 7th edition of the International Union Against Cancer (UICC)/TNM system (19). The Lauren classification (20) was also adopted for histological classification.

Tissue cores, 2 mm in diameter, from FFPE blocks were arrayed using a manual device, KIN-1 (Azumaya, Japan). Two representative cores were obtained from each tissue block. Suitable cases were defined as tumors occupying more than 50% of the core area. The arrayed tissues were sliced at 4 μm thickness and placed on slides. One mucinous adenocarcinoma is excluded from the study due to paucity of tumor cells in its cores. Clinicopathological data of the examined tumors are summarized in Table I.

Staining. ARID expression was immunohistochemically determined with the following antibodies: anti-ARID1A rabbit antibody (Sigma-Aldrich, St. Louis, MO, U.S.A.; dilution 1:500), anti-ARID1B mouse antibody clone 2D2 (Abnova, Taiwan; dilution 1:500), and anti-ARID2 rabbit antibody (Bethyl Laboratories, Montgomery, TX, USA; dilution 1:750). The sections were autoclaved for 10 minutes in citrate buffer, pH 6, for antigen retrieval. The slides were incubated with primary antibodies for 30 min at room temperature and the sections were then stained by a detection method using EnVision FLEX (Dako, Glostrup, Denmark) according to the manufacturer’s protocol and counterstained with hematoxylin. The scoring for IHC staining was performed as previously described (21, 22). Briefly, the expression level of immunostaining was evaluated based on the intensity (categorized as 0 [ absent], 1 [ weak], 2 [ moderate], or 3 [ strong]) and the percentage of positively stained cancer cells (scored as 0 [ 0%-5% positive], 1 [ 6%-25%], 2 [ 26%-50%], 3 [ 51%-75%], or 4 [ 75%-100%]). Nuclear immunoreactivity was considered a positive expression. For the immunostaining score, the intensity and positivity scores were multiplied, resulting in a value between 0 and 12. All of the tumors were independently evaluated by two trained pathologists (TA and KB) with antigen retrieval by autoclaving. The slides were incubated with primary antibodies for 30 min at room temperature and the sections were then stained by a detection method using EnVision FLEX (Dako, Glostrup, Denmark) according to the manufacturer’s protocol and counterstained with hematoxylin.

After scoring, the samples were divided into high- and low-expression groups. The threshold was determined by observation of non-tumorous tissues. The groups with high expression of ARID1A and ARID1B had a score of 8 or higher. The group with high expression of ARID2 had a score of 3 or higher.

\[ \text{P53 and hMLH1 were also immunostained using antibodies; anti-p53 mouse antibody (clone DO-7, Dako; dilution 1:100) and anti-hMLH1 mouse antibody (clone G168-15, Biocare Medical, Concord, CA, U.S.A.; dilution 1:50) with antigen retrieval by autoclaving. The immunostaining of p53 and hMLH1 was categorized into 1 of 5 grades according to the positivity rate: 0 [0%], 1 [0-10%], 2 [10-30%], 3 [30-70%], and 4 [70-100%].} \]

\[ \text{After survey of non-tumorous tissue, the groups with high expression of p53 had a score of 2 or higher. The group with high expression of hMLH1 had a score of 4.} \]

Epstein-Barr virus (EBV) status was determined by EBER in situ hybridization (ISH), as reported previously (18).

Cell line study. We studied four GC cell lines, AGS, NUGC3, MKN45, and MKN74. GC cells were grown in RPMI-1640 with 10% FBS in the absence and presence of 5-aza-2’-deoxycytidine (5-Aza-dC) at concentrations of 3 μM for three days, respectively.

\[ \text{RF-real time PCR. Total tissue RNA was extracted with RNeasy Mini Kit (QIAGEN, Venlo, Netherlands). First-strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative real time PCR was performed using a SYBR Green method with Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and Applied Biosystems 7300 Real Time PCR system (Life Technologies), according to the manufacturer's instructions. The primer sets are ARID1A 5'-CAGTAAAGGGAGGCGCAAGAG-3' and 5'-GAGGAGAGAAAGGAGACTGTA-3'; ARID1B 5'-CGGAATGCAGTACCCCTCAGC-3' and 5'-GGTGGGATCAACCAGAATGTGT-3'; ARID2 5'-AGCTCTTGCCAGCTATCGTG-3' and 5'-ACAGGGTCTCCATGAAAGCCTCAG-3'; GAPDH 5'-GAAGGTTAGGTCAAGTC-3' and 5'-GAAGATGGTGATGTGGATTTC-3'.} \]

Each PCR assay was performed in triplicate for each sample. Estimated quantities of ARIDs mRNA were standardized as the ratio to GAPDH mRNA for each sample.

Statistics. The correlations between ARID positivity and clinicopathological factors were tested by the Chi-square test and unpaired t-test. The correlation of ARIDs was tested by chi-square test. Differences were considered significant in a two-tailed test at \( p<0.05 \).

Results

IHC on conventional sections. No heterogeneity of ARID1A, 1B, and 2 positivity in each tumor was observed in the preliminary study of five tumors. Non-tumorous epithelium showed strong positivity for each ARID. After intra-tumor homogeneity was confirmed, we used tissue arrays for further studies.

IHC expression. All positivity for ARID, p53, and hMLH1 immunostaining was located in the nuclei (Figure 1). Low-expression groups constituted 20% for ARID1A, 10% for ARID1B, and 15% for ARID2. A summary of the evaluation is shown in Table II.

Relationships among ARIDs. The relationships among the ARIDs were examined. The levels of all ARIDs in GC were significantly related to each other in Chi-square tests (Table III).

Relationship between ARID status and clinicopathological factors. We explored the association of ARID loss with the clinicopathological factors (Table IV). Loss of ARID1A was related to advanced tumor stage, venous infiltration, nodal involvement, and EBV infection. Loss of ARID1B was related to lymphatic infiltration and nodal involvement. Loss of ARID2 was related to older age, advanced tumor stage, lymphatic and venous infiltration, and nodal involvement. All
ARIDs were related to loss of hMLH1, but not to p53 status. The rates of high ARIDs expression during gastric carcinogenesis are summarized in Figure 2A. Adenomas are regarded as precursor lesions of differentiated-type GC (23). Therefore, the positive rates of adenoma are compared to those of intestinal type GC (Figure 2B). All ARIDs gradually decreased during tumor progression.

**ARIDs expression after de-methylation.** After de-methylation by 5-Aza-dC, all four GC cells increased all ARIDs expression (Figure 3, **ARID1A**: 1.28-2.72 fold, **ARID1B**: 1.19-3.21 fold, **ARID2**: 1.11-2.13 fold). MKN45 showed marked increase among four cell lines.

**Discussion**

**Aberration of ARIDs in GC.** Abnormalities of ARID1A in GC have been reported in several studies. Mutations of **ARID1A** are reported in 8-27% of GC (3, 9, 24-26). Immunohistochemical loss of ARID1A is reported in 9-51% of GC (6, 27-30). In the present study, loss of ARID1A was found in 20% of GC. The varying rates of loss among published reports might be because of the antibodies and the threshold of positivity used. We employed immunostaining score and decided on a threshold after the observation of non-tumorous mucosa.

The mutation rates of **ARID1B** and **ARID2** in GC were 4-9% and 0-2% respectively in previous reports (3, 26) using 22 GCs and 50 GCs. Those results are obtained in studies of relatively small sizes, because of the costs and limited materials for the analysis. In the present study, we analyzed ARIDs expression among gastric cancer in a large scale. As results, the proportions of those in the low group for **ARID1B** and **ARID2** were 10% and 15%, respectively. The low-expression rates in this study were higher than previously reported mutation rates. Mutations of ARIDs have been studied using whole-genome sequencing among small number of cancers. The rates of **ARID** mutations are lower than the depletion rate in immunohistochemical studies, including this one. Other mechanisms including epigenetic regulation of genes might also contribute to ARID depletion.

**Significance of ARIDs in gastric carcinogenesis.** Our study revealed that **ARID1B** and **ARID2** are decreased during progression of GC as well as **ARID1A** reported previously (28). This tendency is particularly clear in intestinal-type GC. Gastric adenoma is thought to be involved in the progression of intestinal-type GC (Figure 2B). In the stage of adenoma, ARIDs are slightly decreased. After progression to early cancer, ARIDs are decreased during tumor progression.
Figure 1. Immunohistochemical staining of GCs. (A, B) ARID1A, (C, D) ARID1B, and (E, F) ARID2 staining. (A) Score is 12=Intensity 3x positivity 4, (B) score is 6=2x3, (C) score is 6=2x3, (D) score is 2 =1x2, (E) score is 3=1x3, and (F) score is 1=1x1. Magnifications are identical. The bar in (A) represents 200 micrometers.
progression. SWI/SNF complex has a tumor-suppressive function (4). In our study, ARIDs, which are components of the SWI/SNF complex, were indicated to have tumor-suppressive functions. Loss of ARID1A has been reported to be a poor prognostic factor in GC (28, 31, 32).

The low-expression group might have been involved in haploinsufficiency, down-regulation by DNA methylation and other factors. The expression of ARIDs mRNA was increased after de-methylation in our cell-culture study. DNA methylation is related to the decrease of ARIDs expression. ARID1A has been reported to be effected by promoter hypermethylation in breast cancer (17).

The function of mutated ARIDs is controversial in terms of whether these mutations act in a dominant-negative or gain-of-function manner on ARID function. In a study of congenital abnormalities due to SWI/SNF mutation, haploinsufficient effects have been observed in a mice study (33). Congenital anomalies and mutations of ARID1A are associated with the severest phenotypes (34). Almost all ARID1A mutations in cancers are in a hemiallele. Therefore, haploinsufficiency is sufficient to induce carcinogenesis.

ARIDs, hMLH1, and p53. Low expression of ARIDs was correlated with each other in this study. ARID1A and ARID1B are alternative subunits in the SWI/SNF complex (35), but their complementarity was not observed in our study. They were positively correlated with each other.

Microsatellite instability (MSI) is closely related to aberration of hMLH1. The loss of ARID1A was shown to be related to the loss of hMLH1 or MSI in previous studies (6, 28, 36, 37). In our study, hMLH1 loss was related not only to ARID1A loss but also to ARID1B and ARID2 losses. This might be explained by hMLH1 function, as a gene guardian. Aberration of hMLH1 is caused by mutation or hypermethylation of the promoter region. Mechanism of hypermethylation might affect not only hMLH1 but also ARIDs.

Several studies have reported that p53 aberration and ARID1A aberration are exclusive or negatively correlated (3, 38). However, p53 and ARID1A statuses were not related to each other, as determined by the Chi-square test in our study. We further tested them by Spearman’s correlation test, and obtained a correlation coefficient between ARID1A score and p53 score of 0.15 (p=0.0005). The trend in this correlation test was correspondent with previous reports.

ARIDs and EBV. EBV-associated GC is a distinct group of GC. EBV status was shown to be related only to ARID1A abnormality as a previous report (25). EBV-associated GC shows prominent methylation in the promoters of cancer-related genes. hMLH1 is also depressed by methylation in EBV-associated GC, followed by MSI features. ARID1A abnormalities might be connected to EBV-associated GC through hMLH1 status and hypermethylation. Meanwhile, ARID1B and ARID2 abnormalities were not related to EBV status. The effects of EBV on ARID differ among ARIDs.
Conclusion

ARID1A, 1B, and 2 showed a tumor-suppressive function in gastric carcinogenesis. ARID1A is the strongest suppressor among these three ARIDs. These ARIDs might have different functions, so they might also show different levels of tumor-suppressive function. In gastric carcinoma, they might share mutual mechanisms of decreased expression, such as gene
mutations and methylation. Further studies of ARID function are required to obtain an understanding of their function in a healthy state and in carcinogenesis.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 26670180. The Authors have declared that no competing interest exists.

References

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Figure 3. Changes of ARIDs mRNA expression after de-methylation. (A) Relative expression of ARID1A mRNA to GAPDH mRNA. Bars show standard deviations. All the cell lines increase ARID1A expression after 5-Aza-dC treatment. (B) Relative expression of ARID1B mRNA to GAPDH mRNA. (C) Relative expression of ARID2 mRNA to GAPDH mRNA. ARID1B and ARID2 also increase after 5-Aza-dC treatment as well as ARID1A in all four cell lines.


