

Loss of ARID1A protein expression occurs as an early event in ovarian clear-cell carcinoma development and frequently coexists with *PIK3CA* mutations

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ARID1A is a recently identified tumor suppressor gene that is mutated in ~50% of ovarian clear-cell carcinomas. This mutation is associated with loss of ARID1A protein expression as assessed by immunohistochemistry. The present study aimed at determining the timing of the loss of ARID1A protein expression during the development of ovarian clear-cell carcinoma and assessing its relevance in correlation to *PIK3CA* gene mutations. A total of 42 clear-cell carcinoma cases with adjacent putative precursor lesions (endometriosis-associated carcinoma cases ($n=28$) and (clear-cell) adenofibroma-associated carcinoma cases ($n=14$)) were selected and subjected to immunohistochemical analysis for ARID1A protein expression and direct genomic DNA sequencing of exons 9 and 20 of the *PIK3CA* gene. ARID1A immunoreactivity was deficient in 17 (61%) of the 28 endometriosis-associated carcinomas and 6 (43%) of the 14 adenofibroma-associated carcinomas. Among the precursor lesions adjacent to the 23 ARID1A-deficient carcinomas, 86% of the non-atypical endometriosis (12 of 14) and 100% of the atypical endometriosis (14 of 14), benign (3 of 3), and borderline (6 of 6) clear-cell adenofibroma components were found to be ARID1A deficient. In contrast, in the 19 patients with ARID1A-intact carcinomas, all of the adjacent precursor lesions retained ARID1A expression regardless of their types and cytological atypia. Analysis of 22 solitary endometrioses and 10 endometrioses distant from ARID1A-deficient carcinomas showed that all of these lesions were diffusely immunoreactive for ARID1A. Among the 42 clear-cell carcinomas, somatic mutations of *PIK3CA* were detected in 17 (40%) tumors and majority (71%) of these were ARID1A-deficient carcinomas. These results suggest that loss of ARID1A protein expression occurs as a very early event in ovarian clear-cell carcinoma development, similar to the pattern of *PIK3CA* mutation recently reported by our group, and frequently coexists (not mutually exclusive) with *PIK3CA* mutations.

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Among ovarian carcinomas, clear-cell carcinoma has been recognized as a distinct clinicopathological

entity in view of its characteristic histology, frequent concurrence of endometriotic lesions, and highly chemoresistant nature, resulting in an extremely poor prognosis when surgical cytoreduction is insufficient.^{1–4} Although little is known about the molecular genetic alterations underlying tumor development, a hypothesis of multistep tumorigenesis of ovarian clear-cell carcinoma, starting with histologically benign-appearing precursor lesions

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(ie, endometriosis and benign clear-cell adenofibroma), progressing to their atypical counterparts (ie, atypical endometriosis and borderline clear-cell adenofibroma), and ultimately to clear-cell carcinoma, has been proposed and widely considered.^{5–11} To establish novel therapeutic strategies for ovarian clear-cell carcinoma, it is crucial to elucidate the molecular aberrations that are characteristic of this type of carcinoma during tumor development.

Although little is known about the pathogenesis of ovarian clear-cell carcinoma, somatic mutations of the *ARID1A* (the AT-rich interactive domain 1A) and *PIK3CA* genes are the most common molecular genetic alterations identified thus far in ovarian clear-cell carcinomas.^{12–15} *ARID1A* (also known as BAF250a), a protein encoded by the *ARID1A* gene located on chromosome 1p36, is a key component of the multi-protein SWI/SNF chromatin-remodeling complex present in all eukaryotes and is believed to confer specificity in the regulation of gene expression.¹⁶ The chromatin-remodeling activity of SWI/SNF has an integral role in controlling gene expression and is critical in tissue development, cellular differentiation, and tumor suppression.^{16–20} Inactivation of *ARID1A* is thought to enhance cell-cycle progression by potentially involving c-myc, thereby contributing to uncontrolled cellular proliferation in cancer cells.^{21–23} Recently, with the use of whole-exome sequencing and transcriptome sequencing, two independent studies showed novel somatic mutations of the *ARID1A* gene in 43 and 56% of ovarian clear-cell carcinomas, suggesting a major role of *ARID1A* in the pathogenesis of this carcinoma type.^{12,13} However, to date, it is unclear whether the alteration of *ARID1A* is an early or late event in the development and progression of ovarian clear-cell carcinoma.

The *PIK3CA* gene, encoding the catalytic subunit p110a of phosphatidylinositol-3 kinases (PI3K), is located on chromosome 3q26.3, and both somatic mutations and gene amplification of *PIK3CA* increase PI3K activity and activate the downstream Akt signaling pathway.^{24–26} Somatic activating mutations of *PIK3CA* have been reported in ovarian clear-cell carcinomas with high frequencies of 32–46%.^{14,15} Our recent study showed that somatic mutations of *PIK3CA* were also present in the coexisting endometriotic epithelium, adjacent to the *PIK3CA*-mutation-positive clear-cell carcinomas, even in those lacking cytological atypia.¹⁵ These findings suggest that mutations of the *PIK3CA* gene occur before the development of the atypical precancerous lesions (ie, atypical endometriosis) and that *PIK3CA* mutations are very early events in the development of endometriosis-associated ovarian clear-cell carcinoma. It is therefore of interest to determine whether *PIK3CA* mutations and *ARID1A* could coexist or be mutually exclusive in the pathogenesis of ovarian clear-cell carcinoma.

In the present study, the timing of the alterations of *ARID1A* expression during the development and progression of ovarian clear-cell carcinoma was examined. For this purpose, 42 patients with clear-cell carcinoma belonging to a group of tumors with adjacent putative precursor lesions were selected. As the immunohistochemical analysis of clear-cell and endometrioid carcinomas of the ovary showed that loss of *ARID1A* protein expression was highly correlated with the presence of *ARID1A* mutations,^{12,27} and as the *ARID1A* mutations are randomly distributed in 20 exons and most of these are the insertion/deletion type of mutations, making it very difficult to be detected in formalin-fixed paraffin-embedded specimens,^{12,13} we used immunohistochemistry to assess possible alterations of *ARID1A*. *ARID1A* expression was specifically examined in the benign-appearing putative precursor lesions (ie, non-atypical endometriosis and benign clear-cell adenofibroma), their atypical forms (atypical endometriosis and borderline clear-cell adenofibroma), and the corresponding invasive carcinoma components. Moreover, to assess the possible relationships between *ARID1A* and *PIK3CA* alterations, immunoreactivity for *ARID1A* was compared with the mutational status of *PIK3CA*. In ovarian carcinomas, >80% of mutations in *PIK3CA* have been found in the helical (exon 9) and kinase domains (exon 20).^{14,24,25} Therefore, we restricted our analyses to these regions of the gene.

Materials and methods

Cases and Histological Components Analyzed

Hematoxylin- and eosin-stained sections from a consecutive series of 90 primary clear-cell adenocarcinoma cases, retrieved from the files of the Department of Laboratory Medicine; National Defense Medical College Hospital; Japan, were histologically reviewed. On the basis of the histopathological criteria described previously,^{5,7–9} of the 90 patients, 28 patients with synchronous endometriotic lesions (endometriosis-associated carcinomas) and 14 patients with adjacent clear-cell adenofibroma components (adenofibroma-associated carcinomas) were identified. All these 42 patients had undergone surgical resection between 1986 and 2007, and none had undergone chemotherapy or radiation therapy before surgery. All specimens analyzed were formalin-fixed and paraffin-embedded tissue sections. There was no overlap between the endometriosis-associated and adenofibroma-associated carcinoma cases. The diseases were clinically staged according to the International Federation of Gynecology and Obstetrics system. Of the 28 patients with endometriosis-associated carcinoma, 18 (64%) had stage I, 3 (11%) had stage II, 5 (18%) had stage III, and 2 (7%) had stage IV carcinomas. Of the 14 patients

with adenofibroma-associated carcinoma, 10 (71%) had stage I, 1 (7%) had stage II, 2 (14%) had stage III, and 1 (7%) had stage IV carcinoma. Twenty-two solitary endometrioses were also identified from salpingo-oophorectomy specimens. The research protocol was approved by the ethics committee of the National Defense Medical College, Tokorozawa, Japan.

On the basis of the previously described histological criteria for 'atypical endometriosis',^{5,7,8} endometriotic lesions adjacent to clear-cell adenocarcinoma were classified into non-atypical and atypical forms. Of the 28 patients with endometriosis-associated carcinoma, 15 had both non-atypical and atypical endometriosis, 6 had only non-atypical endometriosis, and 7 had only atypical endometriosis. Therefore, 21 non-atypical and 22 atypical endometriotic lesions as well as 28 endometriosis-associated invasive carcinoma components were analyzed by immunohistochemistry.

With reference to the histopathological criteria of benign and borderline clear-cell adenofibroma described previously,^{7,9} adenoma lesions in each adenofibroma were histologically subclassified into benign and borderline categories. Consequently, 9 of the 14 adenofibroma-associated patients had both the evaluable benign and borderline (clear-cell) adenofibroma components, 4 had the borderline adenofibroma component only, and 1 had the benign adenofibroma only. Therefore, 10 benign adenofibroma components, 13 borderline components, and 14 adenofibroma-associated invasive carcinoma components were analyzed by immunohistochemistry.

Immunohistochemistry for ARID1A Detection

All the selected formalin-fixed and paraffin-embedded specimens were cut into 4 μ m thick serial

sections and analyzed by immunohistochemistry. A commercially available polyclonal rabbit anti-ARID1A antibody (HPA005456; Sigma-Aldrich; diluted 1:50) was used as the primary antibody for ARID1A protein detection, as described previously.²⁸ Sections were deparaffinized and boiled in an autoclave at 121 °C for 15 min in 0.01 mol/l citrate buffer (pH 6.0) and then allowed to cool at room temperature. Endogenous peroxidase activity was blocked using 5% hydrogen peroxide. The slides were incubated at 4 °C overnight with primary antibodies and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase for 1 h at room temperature. Specific antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide, and counterstaining was performed using Mayer's hematoxylin. Non-neoplastic cells, including endothelial cells, fibroblasts, and lymphocytes, normally show ARID1A nuclear immunoreactivity, and therefore, they served as positive internal controls. As negative controls, sections without the primary antibody were used.

Previous studies have shown that inactivating mutations of *ARID1A* are associated with the loss of protein expression.^{12,27} Therefore, we focused our attention on the lesions with undetectable ARID1A immunoreactivity in the nucleus and used a scoring system to classify all lesions into ARID1A deficient (immunoreactions undetectable) and ARID1A intact (immunoreactions detectable); any level of ARID1A immunoreactivity was considered ARID1A intact. As the majority of the carcinomas showed an ARID1A immunoreaction that was diffusely (>80% of the cells of interest) immunoreactive or homogeneously undetectable, we did not consider the percentage of the immunoreactive cells in the examined lesions.

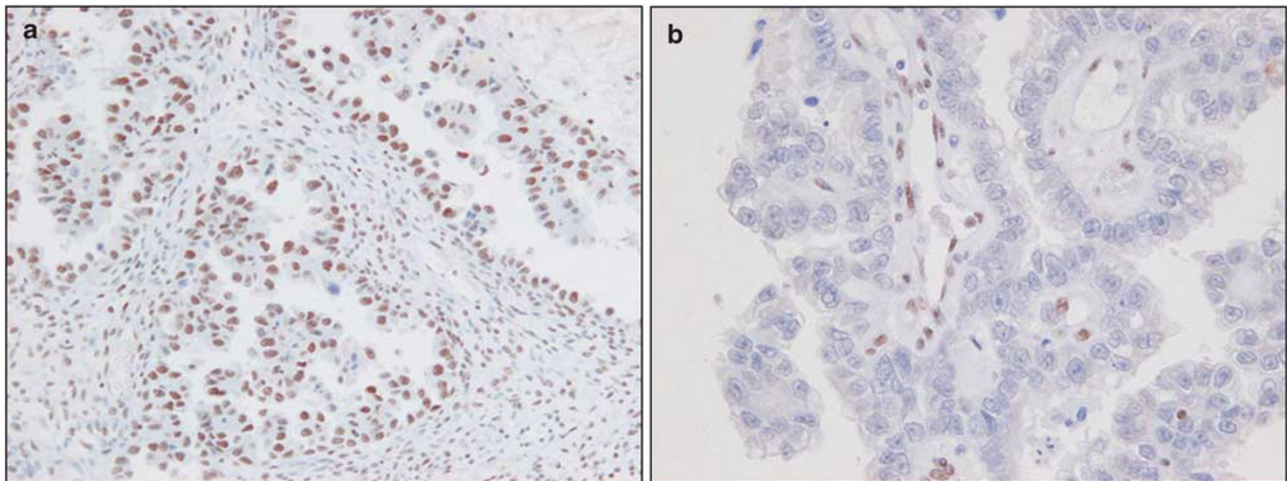


Figure 1 ARID1A immunoreactivity in two representative ovarian clear-cell carcinoma cases. (a) A clear-cell carcinoma showing diffuse and strong nuclear immunoreactivity for ARID1A. (b) A clear-cell carcinoma with undetectable ARID1A immunoreactivity. Stromal cells of both the samples show moderate to strong immunoreactivity and serve as internal positive controls. The carcinomas shown in panels a and b were defined as ARID1A-intact and ARID1A-deficient carcinomas, respectively. Immunoperoxidase stain, original magnification $\times 200$ for (a) and $\times 400$ for (b).

Mutation Assay of the *PIK3CA* Gene

Invasive carcinoma components from the enrolled 42 cases were analyzed for somatic mutations of the *PIK3CA* gene using direct genomic DNA sequencing. Details of these methods have been described in our previous study.¹⁵ Briefly, exons 9 and 20 of the *PIK3CA* gene were amplified using PCR for genomic DNA. PCR was conducted using AmpliTaq Gold (Applied Biosystems, Foster City, CA). The primers for PCR and sequencing conditions have been described previously.¹⁵ The PCR products were subsequently subjected to direct sequencing PCR with BigDye terminator V 3.1/1.1 cycle sequencing reagents (Applied Biosystems), and samples were analyzed on an ABI PRISM 3130

Genetic Analyzer (Applied Biosystems) with DNA Sequencing Analysis Software v5.2 (Applied Biosystems). For 23 of the 28 patients with endometriosis-associated carcinomas, we used the required data from our previous study.¹⁵ The remaining 5 patients with endometriosis-associated carcinomas and 14 with adenofibroma-associated carcinomas were analyzed for the first time in the present study.

Statistical Analysis

Statistical analyses were performed using StatMate III software (ATMS, Tokyo, Japan). Comparisons between parameters were computed using the χ^2 test or Student's *t*-test for unpaired data.

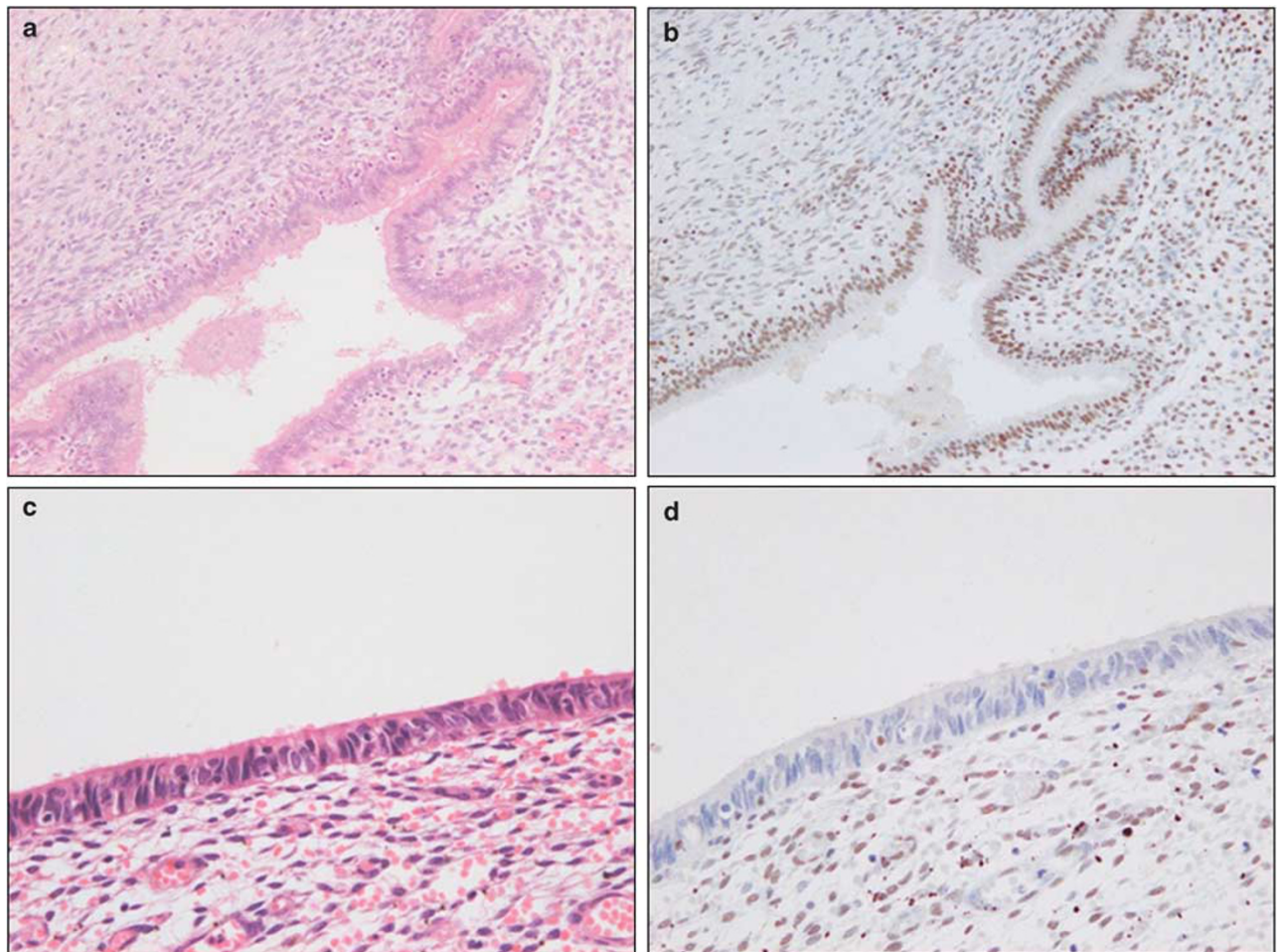


Figure 2 Representative histological features and ARID1A immunoreactions in solitary endometriosis (a, b), non-atypical endometriosis adjacent to clear-cell carcinoma (c, d), and atypical endometriosis adjacent to clear-cell carcinoma (e–h). (a) Hematoxylin and eosin (HE) staining of a solitary endometriosis sample. (b) Epithelial cells and stromal cells show diffusely immunoreactive for ARID1A. (c) A case of non-atypical endometriosis lacking evident cytological or structural atypia in the epithelial cells. (d) Stromal cells are diffusely immunoreactive for ARID1A, while the immunoreaction is undetectable in the endometriotic epithelia. (e) A case of atypical endometriosis showing moderate degrees of cytological atypia and cellular stratification. (f) Similar to panel d, the ARID1A immunoreaction is undetectable in epithelia. (g) HE staining of another case of atypical endometriosis. (h) ARID1A immunostaining of the samples shown in (g). Epithelial cells, as well as stromal cells, show diffuse immunoreactivity for ARID1A. (a, c, e, and g) HE stain, original magnification $\times 200$ for (a, e, and g) and $\times 400$ for (c). (b, d, f, and h) Immunoperoxidase stain, original magnification $\times 200$ for (b, f, and h) and $\times 400$ for (d).

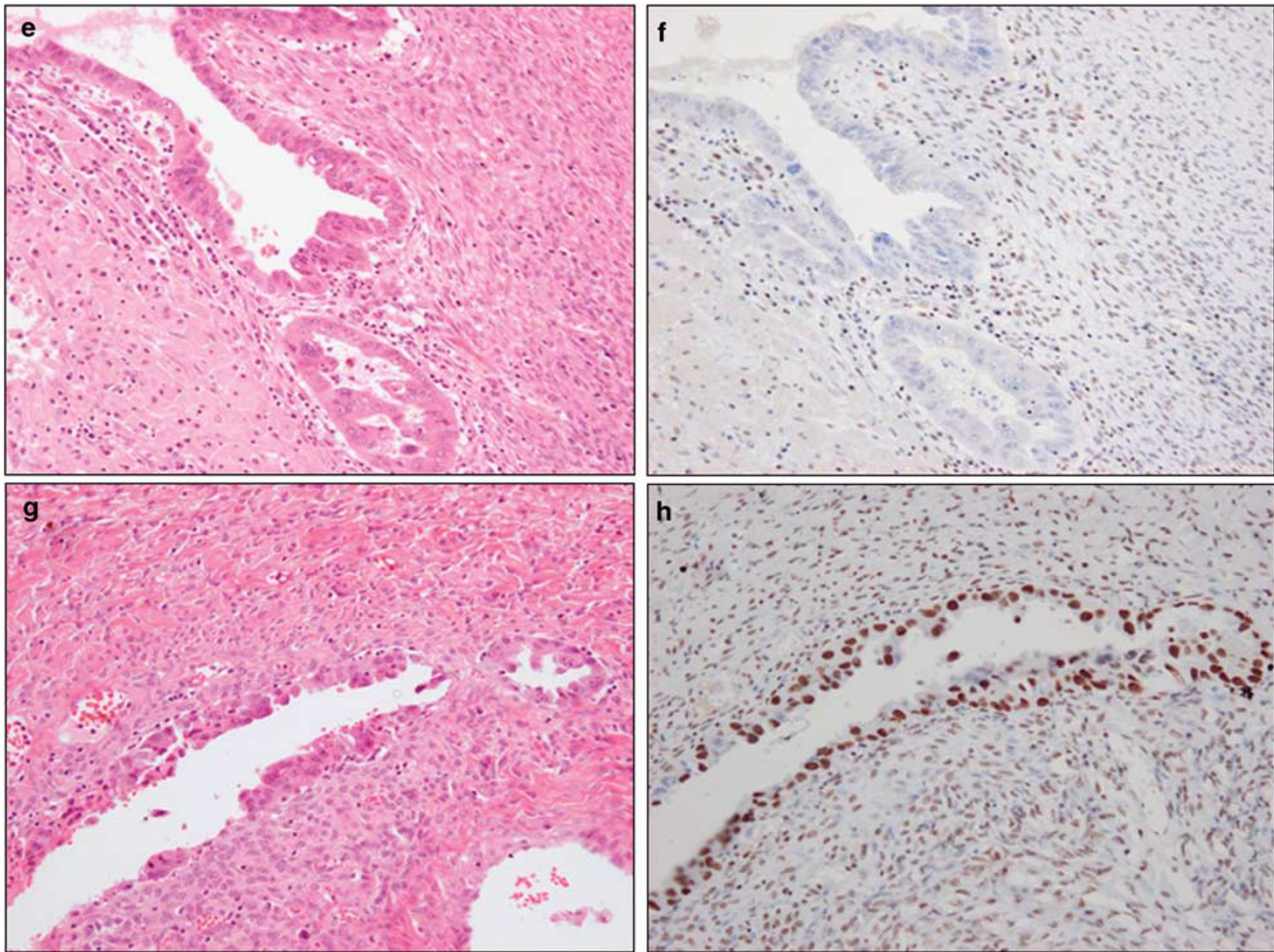


Figure 2 Continued.

Values of $P < 0.05$ were considered to be statistically significant.

Results

ARID1A Expression in Invasive Carcinoma Components

Analysis of invasive carcinoma components showed that immunoreactivity was always in a diffusely (not heterogeneously) detectable or homogeneously undetectable pattern. Of the 28 endometriosis-associated carcinomas, 17 (61%) and 11 (39%) were judged as the ARID1A-deficient and the ARID1A-intact tumors, respectively (Figure 1). Of the 14 adenofibroma-associated carcinomas, 6 (43%) and 8 (57%) were judged as the ARID1A-deficient and the ARID1A-intact tumors, respectively. Overall, 23 (55%) of the 42 clear-cell carcinomas analyzed were determined as ARID1A deficient. Comparison between the endometriosis-associated carcinomas and adenofibroma-associated carcinomas showed no statistically significant differences in the frequency of deficiency of ARID1A immunoreaction ($P = 0.273$).

ARID1A Expression in Endometriosis

All of the 22 solitary endometriosis samples showed diffuse and strong immunoreactions for ARID1A (Figures 2a and b; Table 1).

Among the 17 patients with endometriosis-associated carcinomas that were ARID1A-deficient tumors, 14 non-atypical endometrioses and 14 atypical endometrioses were histologically identified. Of the 14 non-atypical endometrioses, 12 (86%) and 2 (14%) endometrioses were judged as ARID1A deficient and ARID1A intact, respectively (Figures 2c and d), and all of the 14 atypical endometrioses were ARID1A deficient (Figures 2e and f). Three (25%) of the 12 ARID1A-deficient non-atypical endometrioses showed front formation during immunoreactivity, ie, the areas of ARID1A-deficient cells were adjacent to the ARID1A-intact areas, giving the impression of an 'abrupt loss' of ARID1A expression (Figure 3).

Among the 11 endometriosis-associated carcinoma cases with an ARID1A-intact carcinoma, 7 non-atypical and 8 atypical endometrioses lesions were identified, and all 15 lesions showed diffusely immunoreactivity for ARID1A (Figures 2g and h).

Table 1 The ARID1A immunoreactivity in the putative precursor lesions of ovarian clear-cell carcinomas

	Number of cases (%)	
	ARID1A	ARID1A
Type of precursor lesion	Deficient	Intact
<i>In the endometriosis-associated carcinomas</i>		
<i>In the ARID1A-deficient carcinomas</i>		
Non-atypical endometriosis (distant; n = 10)	0	10 (100)
Non-atypical endometriosis (adjacent; n = 14)	12 (86) ^a	2 (14)
Atypical endometriosis (adjacent; n = 14)	14 (100)	0
<i>In the ARID1A-intact carcinomas</i>		
Non-atypical endometriosis (adjacent; n = 7)	0	7 (100)
Atypical endometriosis (n = 8)	0	8 (100)
<i>In the adenofibroma-associated carcinomas</i>		
<i>In the ARID1A-deficient carcinomas</i>		
Benign clear-cell adenofibroma (adjacent; n = 3)	3 (100)	0
Borderline clear-cell adenofibroma (adjacent; n = 6)	6 (100)	0
<i>In the ARID1A-intact carcinomas</i>		
Benign clear-cell adenofibroma (adjacent; n = 10)	0	7 (100)
Borderline clear-cell adenofibroma (adjacent; n = 14)	0	7 (100)

^aThree of these 12 lesions exhibited a pattern of immunoreactivity in which areas of ARID1A-deficient cells were adjacent to the ARID1A-intact areas.

Of the 23 patients with endometriosis-associated carcinomas that were ARID1A deficient, 10 lesions of non-atypical endometriosis distant from carcinoma components were also identified, and all 10 lesions were diffusely immunoreactive for ARID1A.

Consequently, with the exception of three ARID1A-deficient non-atypical endometrioses showing heterogeneous immunoreaction (area of ARID1A-deficient cells was adjacent to the area of ARID1A-intact cells), all of the endometriotic lesions analyzed showed diffusely detectable or homogeneously undetectable immunoreaction patterns for ARID1A.

ARID1A Expression in Clear-Cell Adenofibroma

Among the six cases of adenofibroma-associated carcinomas that were ARID1A-deficient tumors, three benign clear-cell adenofibroma and six clear-cell borderline adenofibroma components were histologically identified (Table 1). All three benign adenofibromas and six borderline adenofibroma components were judged as ARID1A deficient (Figures 4a–d).

Among the eight cases of adenofibroma-associated carcinomas that were ARID1A-intact tumors, seven benign adenofibromas and seven borderline adeno-

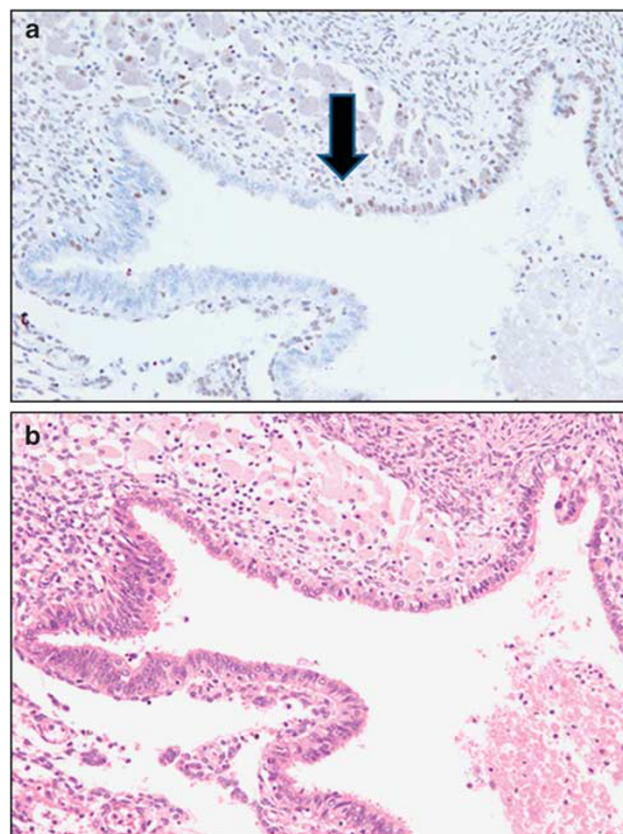


Figure 3 Representative case of a non-atypical endometriosis lesion adjacent to clear-cell carcinoma, showing a pattern of heterogeneous immunoreactions for ARID1A. (a) The area of ARID1A-deficient cells is adjacent to the ARID1A-intact area and shows front formation, namely the 'abrupt loss of ARID1A expression' (arrow), suggesting that mutations arose in clones within this lesion. (b) Hematoxylin and eosin (HE) staining corresponding to the samples shown in panel a. Regarding the histomorphology or the extent of cellular atypia, the ARID1A-intact and ARID1A-deficient epithelia show similar appearance. Original magnification, $\times 200$ each.

fibroma components were histologically identified, and all 14 lesions were diffusely immunoreactive for ARID1A (Figures 4e and f).

Consequently, all the clear-cell adenofibroma components analyzed showed a diffusely detectable or homogeneously undetectable immunoreaction pattern for ARID1A.

Relationship Between ARID1A Immunoreactivity and PIK3CA Mutations

Sequencing chromatograms for *PIK3CA* exons 9 and 20 were obtained for all the 42 carcinomas analyzed. Direct sequencing showed mutations in 17 (40%) carcinomas: 14 (83%) were found in exon 20 (all of these were H1047R substitutions); and the remaining 3 (17%) were in exon 9 (two were E545K substitutions and one was an E545V substitution). Of the 17 *PIK3CA*-mutation-positive carcinomas, 12 (71%) tumors were ARID1A-deficient carcinomas;

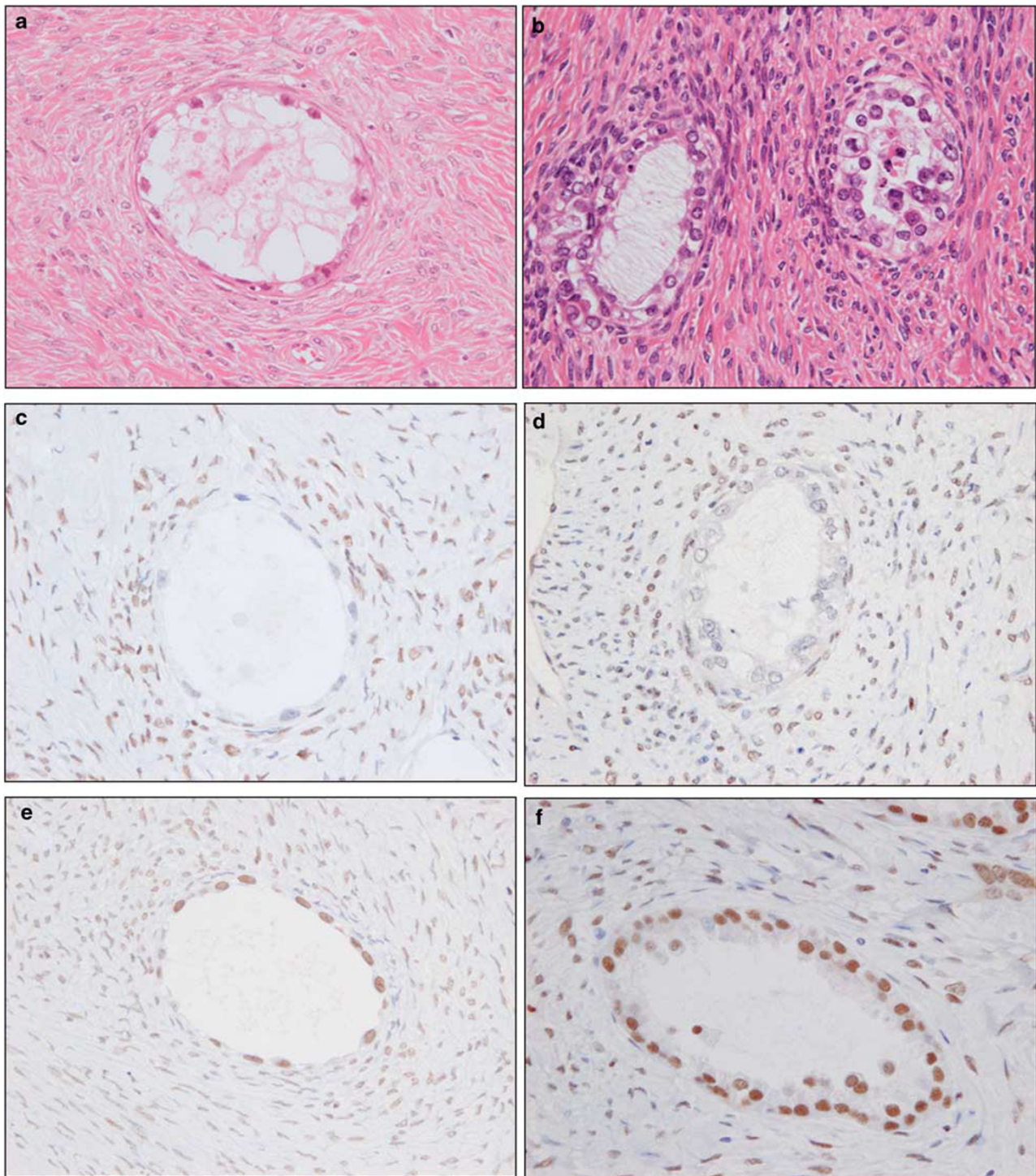


Figure 4 Representative histological features and ARID1A immunoreactions in benign clear-cell adenofibroma (**a**, **c**, and **e**) and borderline clear-cell adenofibroma (**b**, **d**, and **f**). (**a**) A case of benign clear-cell adenofibroma lacking cytological or structural atypia. (**b**) A case of borderline clear-cell adenofibroma. Epithelial cells show a moderate degree of cytological atypia. (**c**, **d**) Stromal cells are diffusely immunoreactive for ARID1A, while the immunoreaction is undetectable in epithelial cells. (**e**, **f**) Epithelial cells, as well as stromal cells, show diffuse immunoreactivity for ARID1A. (**a**, **b**) Hematoxylin and eosin (HE) staining, original magnification $\times 400$ each. (**c**–**f**) Immunoperoxidase stain, original magnification $\times 400$ each.

of the 19 ARID1A-intact carcinomas, *PIK3CA* mutations were detected in only 5 (26%) tumors (Table 2). The frequency of *PIK3CA* mutations in the

ARID1A-deficient carcinomas was higher than that in the ARID1A-intact carcinomas, but not significantly different ($P = 0.083$).

The ten endometriosis-associated carcinoma cases, in which our previous study analyzed the status of *PIK3CA* mutations both in clear-cell carcinoma and coexisting endometriotic lesions,¹⁵ were combined with the data obtained from the present study (Table 3). Of these 10 carcinoma components harboring *PIK3CA* mutations, all but one carcinomas (case no. 12) were ARID1A deficient. All of the six *PIK3CA*-mutation-positive non-atypical endometrioses (case no. 5, 8, 14, 17, 18, and 20) were immunohistochemically ARID1A deficient. Moreover, except for case no. 12, all of the six atypical endometrioses harboring *PIK3CA* mutations were of ARID1A deficient. In case no. 6, *PIK3CA* mutation was firstly documented in the invasive carcinoma component, but ARID1A immunoreactions were already deficient in the adjacent atypical endometriosis.

Discussion

In recent years, genome-wide sequencing analyses have made substantial progress in cataloging molecular genetic alterations in human cancers. One of the most significant recent findings is the identification of somatic mutations of several chromatin-remodeling genes in certain tumor types. These

genes include the *JARID1C* gene in renal cell carcinomas,²⁹ *SMARCA4/BRG1* (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4) in lung carcinomas,^{30,31} and most recently, *ARID1A* in ovarian and endometrial cancers.^{12,13,28} The mutations in *ARID1A* were widely distributed throughout the coding region and all were predicted to truncate the protein through a base substitution, resulting in a stop codon or an out-of-frame insertion or deletion. These alterations resulted in the loss of ARID1A protein expression, suggesting that somatic mutations inactivate the gene product as a tumor suppressor.^{12,13}

The main findings of our investigation can be summarized as follows: (a) loss of ARID1A expression in the putative precursor lesions of ovarian clear-cell carcinoma occurred specifically in cases in which the corresponding carcinoma was ARID1A deficient. (b) In majority of the ARID1A-deficient carcinoma cases, the deficiency of ARID1A immunoreaction was already evident at the stage of precursor lesions lacking cytological atypia (ie, non-atypical endometriosis adjacent to carcinoma or benign clear-cell adenofibroma), suggesting that loss of ARID1A protein occurs as a very early event in tumorigenesis. (c) Even in patients with ARID1A-deficient clear-cell carcinoma, endometriosis distant from the carcinoma was always ARID1A intact. (d) Loss of ARID1A expression and *PIK3CA* mutations frequently coexisted and were not mutually exclusive. Although in the current study the mutational status of the *ARID1A* gene was not known, in ovarian clear-cell carcinomas, the presence of *ARID1A* mutations were adequately, but not perfectly, correlated with the loss of ARID1A immunoreactivity.^{12,27} Therefore, the data obtained in the present study may indicate how early *ARID1A* is mutated during the development of ovarian clear-cell carcinoma.

Table 2 Comparison between the ARID1A immunoreactivity and *PIK3CA* mutations in ovarian clear-cell carcinomas

<i>PIK3CA</i> mutations	Number of cases (%)		P-value
	<i>ARID1A</i>	<i>ARID1A</i>	
In exon 9 or 20	Deficient	Intact	0.083
Present (n = 17)	12 (71)	5 (29)	
Absent (n = 25)	11 (44)	14 (56)	

Table 3 Frequent concurrence of *PIK3CA* mutations and loss of ARID1A immunoreactivity in the endometriosis-associated ovarian clear-cell carcinoma development

Case no. (patient's age (years))	Status of <i>PIK3CA</i> /immunoreaction for <i>ARID1A</i>			
	Clinical stage of disease ^a	Non-atypical endometriosis	Atypical endometriosis	Clear-cell carcinoma
5 (49)	I	Mut/deficient	Mut/deficient	Mut/deficient
6 (48)	I	Wt/intact	Wt/deficient	Mut/deficient
8 (56)	IV	Mut/deficient	—/—	Mut/deficient
12 (53)	I	Wt/intact	Mut/intact	Mut/intact
13 (41)	IV	—/—	Mut/deficient	Mut/deficient
14 (51)	I	Mut/deficient	Mut/deficient	Mut/deficient
15 (58)	II	—/—	Mut/deficient	Mut/deficient
17 (48)	I	Mut/deficient	Mut/deficient	Mut/deficient
18 (53)	I	Mut/deficient	Mut/deficient	Mut/deficient
20 (50)	III	Mut/deficient	—/—	Mut/deficient

^aClinical stages of disease defined by the International Federation of Gynecology and Obstetrics.

Mut, somatic mutations (+); Wt, wild type for exon 9 and 20; —/—, corresponding components were not histologically identified. The data of this study combined with those obtained from our previous study.¹⁵

Several recent studies have strongly indicated that loss of ARID1A protein expression and/or its mutations has an important role in the pathogenesis of certain ovarian cancers thought to be derived from endometriosis.^{12,13,28} However, in the present study, in 43% (6 of 14) of the cases, loss of ARID1A expression was also associated with another putative ovarian clear-cell carcinogenic pathway, namely, the adenofibroma-associated pathway. These results suggest that regardless of the pathogenic pathway involved (ie, endometriosis-associated vs adenofibroma-associated pathways), ARID1A alterations might be commonly associated with the development of ovarian clear-cell carcinoma.

In the present study, excluding the cases of non-atypical endometriosis adjacent to the ARID1A-deficient carcinomas, all lesions analyzed showed diffusely detectable or homogeneously undetectable ARID1A immunoreactivity. These findings contrast those of the study by Guan *et al*,²⁸ in which a fraction of uterine endometrioid carcinomas with *ARID1A* mutations were characterized by a heterogeneous immunostaining pattern for ARID1A. However, this type of heterogeneous loss of ARID1A immunoreactions was only detected in tumors with *ARID1A* mutations and not in those without mutations,²⁸ suggesting that *ARID1A* mutations occurred in the subclones of cells within these endometrial carcinomas. Wiegand *et al*³² reported that a loss of ARID1A protein expression was not detected in any of the nine lesions of endometrial complex atypical hyperplasia considered as putative precursors of uterine endometrioid carcinoma. On the basis of the complete data available, it is likely that (1) *ARID1A* mutations occur as very early events in ovarian clear-cell carcinoma development, creating ARID1A-deficient subclones of a neoplastic nature in the endometriosis tissues or facilitating tumor initiation in clear-cell adenofibroma and (2) the biological significance or timing of occurrence of alterations of ARID1A expression might differ between ovarian clear-cell carcinoma and endometrial carcinoma; in the latter, ARID1A alterations may be a late event in tumorigenesis. Moreover, the present analysis showed that, in the three non-atypical endometrioses that showed a pattern of heterogeneous ARID1A immunoreactivity, areas of ARID1A-deficient cells were detected adjacent to those of the ARID1A-intact areas, suggesting that mutations might arise in clones within these lesions.

Another interesting finding of the present study was the frequent coexistence of ARID1A alterations with the *PIK3CA* gene mutations. Guan *et al*²⁸ showed that the loss of ARID1A immunoreactivity and *ARID1A* somatic mutations were frequently (26 and 40%, respectively) detected in uterine endometrioid carcinoma in comparison with other histological subtypes of uterine carcinomas. Endometrioid carcinoma of the uterus frequently harbors sequence mutations in *PIK3CA* (estimated 24–39%

of cases) as well as in the *CCNB1* and *PTEN* genes, and these mutations are rarely found in uterine serous carcinoma.^{33–36} Moreover, in a previous comparative study of 44 endometrioid adenocarcinomas and 29 precursor lesions (complex atypical hyperplasias), *PIK3CA* mutations were identified in 39% of the former but only 7% of the latter, suggesting that such mutations represent a late event in endometrial carcinogenesis, which is similar to the data on ARID1A alterations in this type of tumor.^{32,36} In contrast to endometrial carcinomas, *PIK3CA* mutations can occur as an early event in ovarian clear-cell carcinoma development, usually appearing at the stage of non-atypical endometriosis.¹⁵ Moreover, in the present data combined with our previous study,¹⁵ concurrence of *PIK3CA* mutations and loss of ARID1A immunoreactions occurred at the very early stage of the endometriosis-associated clear-cell carcinoma development (ie, at the non-atypical endometriosis). These findings suggest the possibility of cross-talk between the ARID1A and PI3K signaling pathways. Further investigation of this possible cross-talk and assessment of the contribution of ARID1A inactivation to tumor initiation and progression in these tumors should be conducted. Somatic mutations of tumor suppressor genes such as *ARID1A* could directly lead to epigenetic changes in tumor cells through specific modifications of chromatin proteins. Therefore, by altering the accessibility of transcription factors to chromatin, additional mutations in the PI3 kinase pathway (ie, *PIK3CA* mutation) would promote its oncogenic properties.

In conclusion, loss of ARID1A protein expression (and possible *ARID1A* mutations) appears to be an early molecular event in ovarian clear-cell carcinogenesis, regardless of the carcinogenic pathway involved. Moreover, the coexistence of the loss of ARID1A expression and *PIK3CA* mutations suggested a possible cross-talk between the regulation of ARID1A expression and the PI3K signaling pathway during ovarian clear-cell carcinoma development. An increased understanding of the molecular events involved in the initiation and development of ovarian clear-cell carcinoma will provide a basis for developing novel forms of early diagnosis and therapy.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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