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ORIGINAL PAPER

Clinicopathological heterogeneity in ovarian clear cell adenocarcinoma: a study on individual therapy practice

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Abstract Ovarian clear cell adenocarcinoma (CCA) has been believed to be a lethal histological subtype of an epithelial ovarian adenocarcinoma (EOA); its precursor has been assumed to be endometriosis. However, it has been reported that CCAs occasionally exhibit different clinical behaviors, suggesting that CCAs might not belong to a single category. We focused on CCAs combined with other histological types of EOAs; we re-evaluated the pathology of 46 CCAs and divided them into two subgroups: 35 CCAs alone (pure-type CCAs); and 11 CCAs with other histological types, endometrioid adenocarcinomas (EAs) or/and serous adenocarcinomas (SAs) (mixed-type CCAs). Immunohistochemical analysis for expression of ARID1A, p53, PTEN, Annexin 4, hepatocyte nuclear factor-1β (HNF-1β), and WT-1 was employed. We identified that patients with endometriosis were younger than those without endometriosis in pure-type CCAs ($P < 0.005$). In mixed-type CCAs, the immunohistochemical-staining patterns revealed internal transition of each histological component. In pure-type CCAs, expressions of ARID1A and p53 were mutually altered, and altered expression of p53 was associated with worse prognosis than that of ARID1A ($P < 0.001$). Our results provide evidence that CCAs would have clinicopathological heterogeneity, determining the patient's prognosis. Furthermore, immunohistochemical analysis may shed light on the selection of appropriate treatment, including chemotherapy.

Keywords Ovarian clear cell adenocarcinoma · Molecular pathology · Endometriosis · Heterogeneity · Prognosis · Therapeutic strategy

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Introduction

Ovarian clear cell adenocarcinomas (CCAs) were initially reported in 1899 by Peham [1]. They closely resembled renal cell carcinomas and were thought to be mesonephric in origin; therefore, in 1939, Schiller stated that ovarian tumors comprised clear and hobnail cells as mesonephromas [2]. Subsequently, Scully et al. [3] described a frequent association of CCAs with endometriosis in 1967 and suggested that CCAs originated from a Müllerian duct, similar to other major histological types of epithelial ovarian adenocarcinomas (EOAs). Consequently, in 1973, the World Health Organization (WHO) recognized CCAs as a distinct histological entity in the classification of EOAs [4].

Although comprising fewer than 3.7 % of EOAs worldwide [5], the prevalence of CCAs is 25 % in Japan [6]. CCAs have received much attention owing to their poor prognosis. Combination chemotherapy, with platinum plus paclitaxel, has been adopted as the standard regimen for front-line treatment of CCAs; this treatment is similar to that for serous adenocarcinomas (SAs) and endometrioid adenocarcinomas (EAs). In several retrospective studies [5, 7–10], the response rate (RR) to first-

line therapy with this regimen ranged between 22 and 56 %, compared with RR >70 % for patients with SAs. Recio et al. [11] showed that platinum-based chemotherapy did not improve 5-year overall survival. However, the mechanism underlying the chemoresistance of CCAs to platinum-based therapy is not well understood.

Morphologic studies over the past four decades showed an association of CCAs with endometriosis, and recent studies suggested that endometriosis was considered to be a precursor lesion of CCAs [12–14]. However, it has been reported that CCAs occasionally exhibit different clinical behaviors with better or worse prognoses [15, 16]. Because of these distinctive clinical and pathological features, the correct classification of CCAs is of critical importance. However, there is a difficulty of classification due to an occasional histological mixture of EOAs in CCAs. The WHO limits their classification to those mixed carcinomas in which one or more component other than the predominant component account for at least 10 % of the tumor on histopathological examination. Kurman and Craig reported that CCAs were found in association with other types of EOAs, although the most common mixtures were EAs and SAs [17]. In contrast, mixed carcinomas composed of CCAs and mucinous adenocarcinomas (MAs) were rare. Among mixed carcinomas of EOAs, mixed carcinomas composed of SAs and CCAs were indistinguishable from SAs with respect to clinical features. Some investigators suggested that they represented a variant of SAs and were not related to CCAs [18]. In regard to mixed carcinomas composed of CCAs and EAs, Köbel et al. [19] reported that CCAs and EAs tended to be observed together. Although mixed carcinomas have not been discussed in detail until the present, it is important to comprehensively investigate the clinical characteristics of CCAs.

We conducted a pathological re-evaluation of CCAs treated at Kumamoto University Hospital from 1990 to 2008, and investigated the association with endometriosis and patient age to determine the clinicopathological features. We performed immunohistochemical study for expression of ARID1A, p53, and PTEN as tumor suppressor genes for CCAs, SAs, and EAs, respectively. It was reported that alternative expression of ARID1A, p53, and PTEN corresponded to the mutation and loss of heterozygosity in each gene [20–27]. We included the immunohistochemical study for expression of Annexin 4, hepatocyte nuclear factor-1β (HNF-1β) as characterized genes for CCAs, and WT-1 as a distinctive gene for SAs [28–30]. Furthermore, we examined the heterogeneity of CCAs, including mixed carcinoma composed of CCAs and other histological types of EOAs.

Materials and methods

Human samples

Samples of CCAs were collected from 55 patients who underwent complete surgery at Kumamoto University Hospital from 1990 through 2008. Tissue blocks were prepared at a rate of one sample/cm according to the size of the tumor. They were routinely fixed in 10 % neutral buffered formalin and then embedded in paraffin blocks. These blocks were sectioned at 3 μm and stained by hematoxylin and eosin. Two independent gynecological pathologists determined the tumor type according to the WHO histological classification of ovarian tumors [4], and 46 cases were consistent with the original diagnosis. Furthermore, we divided 46 cases into two subgroups consisting of either CCA component alone (pure-type CCAs) or CCAs together with endometrioid, serous, or mucinous components (mixed type CCAs); in this study, mixed-type CCAs were defined when CCAs coexisted with other histological types of EOAs even to just a small degree. We investigated age and clinical stage according to the International Federation of Obstetrics and Gynecology (FIGO) system. Follow-up data were available for all 46 patients to evaluate the prognosis. We obtained consent from all the patients in this study.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens were cut into 3-μm sections and mounted on silanized glass slides. These slides were deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed in Tris-buffered saline (0.05 M Trizma base, 0.9 % NaCl, pH 7.4), according to the supplier's recommended protocol. These sections underwent antigen retrieval in a citrate buffer (0.01 M; pH 6.0) by microwave, or proteinase K and warm bath. They were stained with antibodies: p53, ARID1A, PTEN, HNF-1β, Annexin 4, and WT-1. Details of antibodies used and staining conditions were presented in Table 1. They were counterstained with hematoxylin. The immunoreaction was visualized using diaminobenzidine.

Evaluation of immunostaining

Immunostained slides were evaluated. The p53 staining was given an immunoreactive score obtained by multiplication based on the intensity of nuclear staining and quantity of cells stained according to the previously reported grading system [25]. The staining intensity was

Table 1 Details of antibodies used in this study

Antigen	Clone	Product code	Supplier	Dilution	Antigen retrieval
p53	Mouse monoclonal DO-7	N1581	Dako cytomation	1:1	Microwave
ARID1A	Mouse monoclonal	sc-32761	Santa cruz biotechnology	1:200	Microwave
PTEN	Rabbit monoclonal, 138G6	#9559	Cell signaling	1:200	Microwave
HNF-1β	Goat polyclonal	sc-7411	Santa cruz biotechnology	1:400	Microwave
Annexin4	Rabbit polyclonal	ab33009	Abcam	1:400	Microwave
WT-1	Mouse monoclonal, 6F-H2	M3561	Dako cytomation	1:25	Proteinase K, warm bath

Table 2 Clinical data of the 46 patients

Age (y)		
Median (range)	51.1 ± 1.8 years (range 28–91 years)	
Subgroup, *n* (%)		
Pure-type CCAs	35 (76.1%)	
Mixed-type CCAs	11 (23.9%)	
Histological pattern of mixed-type CCAs, *n* (%)		
CCAs and EAs	2 (18.2%)	
CCAs and SAs	8 (72.7%)	
CCAs and EAs and SAs	1 (9.1%)	
FIGO stage, *n* (%)		
I	33 (71.7%)	
II	4 (8.7%)	
III	7 (15.2%)	
IV	2 (4.3%)	
Endometriosis, *n* (%), median age (y)		
with endometriosis	34 (73.9%), 49.4 ± 1.7 years	*
without endometriosis	12 (26.1%), 55.5 ± 4.7 years	*

CCAs clear cell adenocarcinomas, *EAs* endometrioid adenocarcinomas, *SAs* serous adenocarcinomas

* $P < 0.012$

divided into four categories: 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive. The most intensely staining slides were deemed to be the upper limit. The quantity of cells stained was scored as follows: 0, no staining; 1, 1–10 %; 2, 11–50 %; 3, 51–80 %; 4 >80 % of tumor nuclei stained. With p53 staining, the multiplied immunoreactive score of 8–12 was considered strong immunoreactivity, 4–6 was moderate, 1–3 was weak, and 0 was negative. It was previously indicated that all carcinomas with strong immunoreactivity (score 8–12) showed p53 missense mutations, although some negative carcinomas (score 0) also revealed p53 frameshift mutations [25]. For ARID1A and PTEN, we defined loss of function as negative nuclear staining by the multiplied immunoreactive score, inversely with p53, because previous studies showed that negative staining of ARID1A and PTEN demonstrated gene mutation [20–24, 26, 27]. HNF-1β, Annexin 4, and WT-1 were binarized as negative or positive, and immunostaining in >50 % of tumor cells was positive [28–30].

Statistical analysis

The χ^2 test and Student's *t* test for unpaired data were used for statistical analysis. Patient survival distribution was calculated using the Kaplan–Meier method. The significance of the survival distribution in each group was tested by the log rank test. $P < 0.05$ was considered to be statistically significant. All values were given as the mean ± SD.

Results

Patient characteristics

The characteristics of the study population are presented in Table 2. Median age at diagnosis was 51.1 ± 1.8 years (range 28–91 years). Of the 46 tumors, 71.7 % were stage I, 8.7 % were stage II, 15.2 % were stage III, and 4.3 % were stage IV (FIGO classification). Endometriosis was histologically observed in 34/46 (73.9 %) of the cases. Patients with endometriosis were aged 49.4 ± 1.7 years, whereas those without endometriosis were aged 55.5 ± 4.7 years, showing that those with endometriosis were significantly younger ($P < 0.012$).

We identified 35 pure-type CCAs and 11 mixed-type CCAs coexisting with serous or/and endometrioid elements. Mixed-type CCAs coexisting with mucinous

Table 3 Characteristics of pure-type and mixed-type CCAs

Pure-type CCAs (total number: 35)		
FIGO stage, *n* (%)		Recurrence and/or dead, n
I	29 (82.9%)	3
II	3 (8.6%)	0
III	2 (5.7%)	1
IV	1 (2.9%)	0
Existence of endometriosis, *n* (%), median age (y)		
with endometriosis	29 (82.9%), 49.1±1.8 years **	4
without endometriosis	6 (17.1%), 63.4±6.2 years **	0
Mixed-type CCAs (total number: 11)		
FIGO stage, *n* (%)		
I	4 (36.4%)	0
II	1 (9.1%)	0
III	5 (45.5%)	3
IV	1 (9.1%)	0
Existence of endometriosis, *n* (%), median age (y)		
with endometriosis	5 (45.5%), 51.0±4.2 years	2
without endometriosis	6 (54.5%), 46.3±5.4 years	1

CCAs clear cell adenocarcinomas, *EAs* endometrioid adenocarcinomas, *SAs* serous adenocarcinomas

** $P < 0.005$

adenocarcinoma were not observed in our cases. The characteristics of pure-type and mixed-type CCAs are presented in Table 3. In pure-type CCAs, patients with endometriosis were younger than those without endometriosis ($P < 0.005$). In contrast, in mixed-type CCAs, there was no correlation between age and endometriosis.

Immunohistochemical findings

In 46 cases, negative staining (score 0) of ARID1A, strong positive staining (score 8–12) of p53, and negative staining (score 0) of PTEN were observed in 28 (60.9 %), 10 (21.7 %), and 2 (4.3 %) cases, respectively.

Positive staining of Annexin 4, HNF-1β, and WT-1 was found in 38 (82.6 %), 36 (78.3 %), and 3 (6.5 %) of 46 cases.

In pure-type CCAs, the immunohistochemical results are shown in Table 4. The negative staining of ARID1A, strong positive staining of p53, and negative staining of PTEN were observed in 23 (65.7 %), 5(14.3 %) and 0 (0.0 %) of 35 cases, respectively. Positive expressions of Annexin 4, HNF-1β, and WT-1 were observed in 35 (100.0 %), 34 (97.1 %), and 0(0.0 %) 35 cases, respectively. Endometriosis was observed in 18 (78.3 %) of 23 cases with negative staining of ARID1A, and in 4 (80.0 %) of five cases with strong positive staining of p53.

The immunohistochemistry results of mixed-type CCAs are demonstrated in Figs. 1 and 2. Two cases composed of CCAs and EAs had negative staining of PTEN in both areas of the CCAs and EAs (Fig. 2a–d). Eight cases composed of CCAs and SAs had various expression patterns. Negative staining of ARID1A in areas of the CCAs

Table 4 Number of cases observed with altered expression by immunohistochemical analysis in pure-type CCAs

Total number of pure-type CCAs 35				
Markers	Altered expression, *n* (%)	Endometriosis, *n* (%) in cases of altered expression	Recurrences and/or deaths, *n* (%) in cases of altered expression	Altered expression, *n* (%) in 4 recurrence and/or dead cases
ARID1A	23 (65.7 %)	18/23 (78.3 %)	0/23 (0.0 %)	0/4 (0.0 %)
p53	5 (14.3 %)	4/5 (80.0 %)	3/5 (60.0 %)	3/4 (75.0 %)
PTEN	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0/4 (0.0 %)
ARID1A and p53	1 (2.9 %)	0/1 (0.0 %)	0/1 (0.0 %)	0/4 (0.0 %)
Annexin4	35 (100.0 %)	29/35 (82.9 %)	4/35 (11.4 %)	4/4 (100.0 %)
HNF-1β	34 (97.1 %)	29/34 (85.3 %)	4/34 (11.8 %)	4/4 (100.0 %)
WT-1	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0/4(0.0 %)

CCAs clear cell adenocarcinomas

Case	Age (years)	Hystological type	PTEN		ARID1A (Baf250a)		Annexin4		HNF-1β		p53		WT-1		En
			CCA	EA	CCA	EA	CCA	EA	CCA	EA	CCA	EA	CCA	EA	
1	59	CCA+EA	-	-	+	+	-	-	-	-	-	-	-	-	+
2*	60	CCA+EA	-	-	+	+	-	-	-	-	-	-	-	-	+
			CCA	SA	CCA	SA	CCA	SA	CCA	SA	CCA	SA	CCA	SA	
3*	51	CCA+SA	+	+	+	+	+	+	-	-	-	-	-	-	+
4	48	CCA+SA	+	+	-	-	-	-	-	-	-	-	-	-	+
5	37	CCA+SA	+	+	-	-	-	-	+	-	-	-	-	-	+
6	32	CCA+SA	+	+	-	-	+	-	+	-	-	-	-	-	-
7	56	CCA+SA	+	+	-	-	-	-	-	-	-	+	-	-	-
8	49	CCA+SA	+	+	+	+	-	-	-	-	-	+	-	+	-
9*	28	CCA+SA	+	+	+	+	-	-	-	-	-	+	+	+	-
10	61	CCA+SA	+	+	-	+	-	-	-	-	+	+	+	+	-
			CCA	EA	SA	CCA	EA	SA	CCA	EA	SA	CCA	EA	SA	CCA	EA	SA	
11	52	CCA+EA+SA	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-

Fig. 1 Immunohistochemical characteristics in mixed-type CCAs. Positive immunostaining is demonstrated as *plus*, and negative as *minus*. Abnormal expression is surrounded by red. *EA* endometrioid adenocarcinoma, *SA* serous adenocarcinoma, respectively. *En* endometriosis; *plus* surrounded with *yellow* indicates that endometriosis was observed; *minus* surrounded with *green* indicates that endometriosis was not found. *Asterisk* recurrence and/or death

was observed in 5/8 (62.5 %), and in one case among these five cases positive staining of ARID1A was found in SAs (Fig. 2e–h). Strong positive staining of p53 in areas of the SAs was found in 4 (50.0 %) of eight cases, and in one case among of these four cases positive staining was observed in both areas of the CCAs and SAs (Fig. 2i–l). The negative staining of PTEN was not observed in all eight cases. One case composed of CCA, EA, and SA had altered expression of Annexin 4 and p53 without endometriosis.

Overall, in pure-type CCAs, the frequency of endometriosis was high. There was no significant difference between the involvement of endometriosis and expression of each protein. However, in mixed-type CCAs, endometriosis was not found in any of the p53 strong positive staining cases.

Patient prognosis

Of 35 patients with pure-type CCAs, 29 (82.9 %) had stage I disease, 3 (8.5 %) had stage II, 2 (5.7 %) had stage III, and 1 (2.9 %) had stage IV. In pure-type CCAs, the median survival time was 65.5 months (range 31–194 months), and recurrence and/or death occurred in 4/35 (11.4 %). In these 4 cases, negative staining of ARID1A was not found; however, strong positive staining of p53 was observed 3/4 (75.0 %) (Table 4). This indicated that altered expression of ARID1A and p53 showed contrary prognosis ($P < 0.001$).

Of 11 patients with mixed-type CCAs, 4 (36.4 %) had stage I disease, 1 (9.1 %) had stage II, 5 (45.4 %) had stage III, and 1 (9.1 %) had stage IV. In mixed-type CCAs, the median survival time was 60.4 months (range 1–160 months) and recurrence and/or death occurred in 3/11 (27.3 %). In patients with recurrence and/or death, a significant difference between prognosis and expression of p53 and ARID1A was not found.

Discussion

Recent pathological and molecular evidences have suggested that endometriosis serves as a precursor of CCAs [21, 31, 32]. In approximately 60 % of endometriosis-associated EOAs including CCAs, the carcinomas are adjacent to endometriosis or arise directly from atypical endometriosis, suggesting that malignant transformation

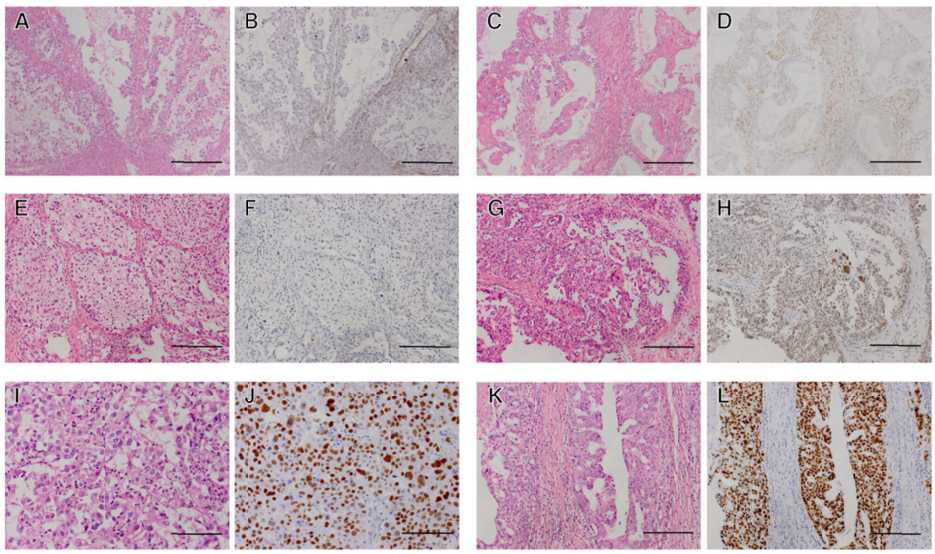

Fig. 2 Immunohistochemistry of mixed-type CCAs. **a**–**d** Case 1 of Fig. 2 is presented. **a**, **b** In the area of CCAs, PTEN was negative in the nucleus or cytoplasm of glandular cells. **c**, **d** In the area of EAs, PTEN was negative in the nucleus or cytoplasm of glandular cells. **e**–**l** Case 10 of Fig. 2 is presented. **e**, **f** In the area of CCAs, ARID1A was negative in the nucleus of glandular cells. **g**, **h** In the area of SAs, ARID1A was positive in the nucleus of glandular cells. **i**, **j** In the area of CCAs, p53 was positive in the nucleus of glandular cells. **k**, **l** In the area of SAs, p53 was positive in the nucleus of glandular cells. **a**–**l** ×100. *Scale bar* 200 μm

occurs in a subset of patients with ovarian endometriosis. In our current study, endometriosis was also histopathologically observed in 73.9 % of CCAs. Among pure-type CCAs selected in this study, endometriosis was observed in 82.9 % of cases, and patients with endometriosis were significantly younger than those without endometriosis. These results show that endometriosis represents an important site of the origin of pure-type CCAs. However, endometriosis was observed in 45.5 % of mixed-type CCAs, and there was no significant difference between the ages of patients with and without endometriosis. Furthermore, as for the prognosis, the ratio of cases of recurrences and/or deaths of mixed-type CCAs was higher than pure-type CCAs. This demonstrated that difference of characteristic between pure-type and mixed-type CCAs.

The p53 gene is the most frequently altered gene in human cancer [25]. Recently, it was reported that mutation of p53 was an important determinant of aggressive biological behavior, resulting in poor outcome of patients with several types of cancer [33]. In contrast, changes in chromatin can influence the epigenetic regulation of many genes, inducing transcription, DNA replication, and DNA damage repair in cancer. Chromatin remodeling is essential for all nuclear activities, and ARID1A is a chromatin remodeling factor [24, 25]. ARID1A is recognized as a tumor suppressor gene and also provides a potential approach to determine which of the numerous epigenetic changes in cancer confer a selective growth advantage. It was previously reported that the AIRD1A mutation is an early event in neoplastic transformation as well as p53 [22, 23, 34]. Importantly, the regulation of p53-related genes by ARID1A raises the possibility that ARID1A molecularly cooperates with p53 to inhibit tumor growth. Therefore, it is possible that in non-transformed cells, ARID1A and p53 collaborate as a pair of gatekeepers that prevent carcinogenesis by transcriptional activation of tumor-inhibiting downstream genes. Furthermore, it is thought that concurrent mutations in ARID1A and p53 are not required for carcinogenesis; in other words, both genes are mutually exclusive in tumor [22].

With the exception of one case, our immunohistochemical analysis demonstrated that in pure-type CCAs, tumors with mutated ARID1A contain wild-type p53 and tumors with mutated p53 harbor wild-type ARID1A. Both ARID1A and p53 appear to be essential for tumor suppression of pure-type CCAs, and concurrent mutations in

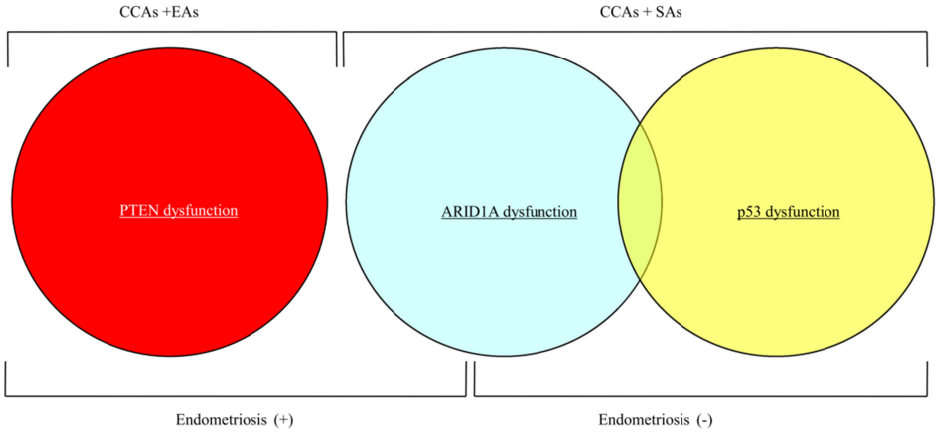

Fig. 3 Association between dysfunction of tumor suppressor genes and endometriosis in mixed-type CCAs. This figure presents a concise summary of mixed-type CCAs with altered expression of PTEN, p53, and/or ARID1A. Mixed-type CCAs lacking PTEN and ARID1A function were associated with endometriosis. Mixed-type CCAs lacking p53 function were not associated with endometriosis

both genes are not required for their carcinogenesis. Our results also suggest that ARID1A and p53 genes were mutually exclusive in CCAs [22]. Interestingly, we found altered expression of p53 and normal expression of ARID1A in pure-type CCAs with recurrence or death. Conversely, we found altered expression of ARID1A and normal expression of p53 in pure-type with good prognosis. This result indicated that pure-type CCAs may be divided into two subgroups with mutation in either ARID1A or p53; these subgroups correlate with prognosis. Henceforward, recognition of this subgroup of CCAs may play an indispensable role in determining patient prognosis and selecting the appropriate chemotherapy.

In this study, 11 cases were categorized by the present pathological review into mixed-type CCAs. Immunohistochemical results indicated that mixed-type CCAs could be divided into at least four groups accompanied with dysfunction of tumor suppressor genes, PTEN, ARID1A and/or p53, correlated with endometriosis: (1) In mixed-type CCAs coexisting with EAs lacking PTEN function, endometriosis was observed. This type of CCAs may have the characteristics of EAs, indicating intratumoral heterogeneity of CCAs. (2) In mixed-type CCAs coexisting with SAs lacking ARID1A function, endometriosis was observed. Altered expression of Annexin 4 or HNF-1β was found. These characteristics of CCAs were observed in the area of SAs, indicating intratumoral heterogeneity. (3) In mixed-type CCAs coexisting with SAs lacking p53 function, endometriosis was not observed. In these CCAs, ARID1A function was preserved. Altered expression of WT-1 was found in the area of CCAs. These results implied the intratumoral heterogeneity of CCAs showing the characteristics of SAs. (4) In mixed-type CCAs coexisting with SAs lacking both ARID1A and p53 function, endometriosis was not observed. These findings indicated intratumoral heterogeneity of CCAs that exhibited the characteristics of both CCAs and SAs (Fig. 3). These immunohistochemical results demonstrated that mixed-type CCAs containing SAs with altered expression of p53 exhibited different carcinogenesis from other mixed-type CCAs, independent of endometriosis. Furthermore, the rates of altered expression of Annexin 4 and HNF-1β were lower, and expression of WT-1 was higher than in pure-type CCAs. These immunostaining results also led us to infer that mixed-type CCAs have different characteristics from pure-type CCAs.

Conclusion

We revealed that CCAs should be first divided into two subgroups: pure type and mixed type. Furthermore, in clinical practice, we must consider that it is necessary to examine the molecular characteristics of individual CCAs. CCAs must be categorized into three subtypes: pure-type CCAs, CCAs with serous characteristics, and CCAs with other histological characteristics. We must deal with CCAs according to intratumoral heterogeneity, and these findings may be applied to clinical treatment, including chemotherapy. Moreover, we should consider intratumoral

heterogeneity because the difference in expression between ARID1A and p53 can influence the prognosis in pure-type CCAs. A new chemotherapy strategy is necessary, based on the expression of ARID1A and p53, including molecular targeting therapy. Detailed pathological review is important to acknowledge the existence of other histological types of EOAs. To the best of our knowledge, this is the first study to show the immunohistochemical differences between pure-type and mixed-type CCAs, and the first to mention the need for classification of CCAs to apply to appropriate chemotherapy according to clinicopathological heterogeneity.

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Conflict of interest The authors have no conflict of interest.

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