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Therapeutic preferability of gemcitabine for ARID1A-deficient ovarian clear cell carcinoma

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HIGHLIGHTS

- *ARID1A*-Deficient Ovarian Clear Cell Carcinoma (OCCC) cells were selectively sensitivity to gemcitabine.
- Growth of xenograft derived from *ARID1A*-Deficient OCCC cells was suppressed by treatment with gemcitabine.
- Gemcitabine treatment induced apoptosis in ARID1A-deficient OCCC cells.
- Response to gemcitabine in ARID1A-deficient OCCC patients got better than that in ARID1A-proficient OCCC patients.

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ABSTRACT

Objective: Ovarian clear cell carcinoma (OCCC) is often resistant to conventional, standard chemotherapy using cytotoxic drugs. OCCC harbors a unique genomic feature of frequent (approximately 50%) ARID1A deficiency. The present study was performed to investigate standard chemotherapeutic options suitable for ARID1A-deficient OCCC patients.

Methods: Drugs with selective toxicity to ARID1A-deficient OCCC cells were identified among six cytotoxic drugs used in standard chemotherapy for OCCC by employing multiple *ARID1A*-knockout cell lines and an OCCC cell line panel. Anti-tumor effects of drug treatment were assessed using a xenograft model. To obtain proof of concept in patients, seven OCCC patients who received single-agent therapy with gemcitabine were identified in a retrospective cohort of 149 OCCC patients. Patient samples and cases were analyzed for association between therapeutic response and ARID1A deficiency.

Results: *ARID1A*-knockout and *ARID1A*-deficient OCCC cells had selective sensitivity to gemcitabine. IC50 values for gemcitabine of ARID1A-deficient cells were significantly lower than those of ARID1A-proficient cells ($p = 0.0001$). Growth of OCCC xenografts with ARID1A deficiency was inhibited by administration of gemcitabine, and gemcitabine treatment effectively induced apoptosis in ARID1A-deficient OCCC cells. Three ARID1A-deficient OCCC patients had significantly longer progression-free survival after gemcitabine treatment than four ARID1A-proficient OCCC patients ($p = 0.02$). An ARID1A-deficient case that was resistant to multiple cytotoxic drugs, including paclitaxel plus carboplatin in the adjuvant and etoposide plus irinotecan in the first-line treatment, exhibited a dramatic response to gemcitabine in the second-line treatment.

Conclusion: ARID1A-deficient OCCC patients could benefit from gemcitabine treatment in clinical settings.

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1. Introduction

Ovarian clear cell carcinoma (OCCC) is a subtype of ovarian cancer with distinct characteristics from those of high-grade serous carcinoma (HGSC), including etiologies and molecular, genetic, and clinical characteristics [1–3]. The incidence of OCCC among ovarian cancer patients is higher in East Asia (approximately 30%) than in Europe and the United States (approximately 10%) [4–7]. OCCC has a response rate of approximately 30% to conventional, standard platinum-based chemotherapy established for ovarian cancers, which is significantly lower than that of HGSC, which has a response rate higher than 70% [6,8–10]. In addition, loss-of-function mutations in the BRCA1/BRCA2 gene observed in 15% of HGSC patients [11,12] has opened the prospect of developing new therapeutic options based on PARP inhibitors [13]. On the other hand, efficient therapeutic options for OCCCs remain limited due to the low frequency of BRCA1/BRCA2 mutations [2,14].

Due to the rarity of OCCC, only a small portion of OCCC cases have been included in large clinical trials of investigative drugs, while HGSC cases are frequently included [3,10]. Therefore, precision medicine is not established for OCCC, making this disease an unmet clinical need [2]. Because the biological and clinical characteristics of OCCC are distinct from those of other types of ovarian cancers, OCCC-specific therapeutic strategies should be considered independently from other types of ovarian cancers [15]. Gemcitabine is a deoxycytidine analogue that inhibits ribonucleoside reductase, resulting in depletion of deoxyribonucleotide pools necessary for DNA synthesis and induction of apoptotic cell death via signaling pathways activated by AKT and GSK3 [16–20]. Gemcitabine is sometimes used in late lines of treatment for OCCC after platinum-resistant recurrence [21]. Interestingly, a few platinum-resistant OCCC cases have been reported to respond more effectively to gemcitabine than to other cytotoxic drugs, although only 20% of patients with OCCC recurrence have received gemcitabine treatment [22–24]. Therefore, some therapeutic options may be more suitable for OCCC than for other types of ovarian cancer, and these modalities would contribute to precision medicine for OCCC.

Development of OCCC is characterized by a high frequency of loss-of-function mutations in the *ARID1A* gene (approximately 50%) [25–27]. *ARID1A* encodes a subunit of the SWI/SNF chromatin-remodeling complex, which regulates expression of multiple genes, and is mutated in a variety of human cancers [28]. Recent studies, including our own, have revealed that ARID1A deficiency promotes carcinogenesis and affects biological characteristics, including metabolism, in multiple manners [29,30]. The high prevalence of ARID1A deficiency suggests that it may be a biomarker for precision medicine of OCCC [30,31]. For instance, several reports indicate that ARID1A deficiency is linked to poor prognosis in OCCC [32–36]. However, to the best of our knowledge, there have been no reports that ARID1A deficiency is linked to the efficacy of specific chemotherapeutic drugs. Consequently, therapeutic selection based on ARID1A status has not been established. Therefore, we investigated the possibility of therapeutic selection for OCCC based on ARID1A deficiency/proficiency by performing drug screening using *ARID1A*-knockout (KO) OCCC cells and other ovarian cancer cells, and a panel of commonly used OCCC cell lines. Furthermore, we retrospectively analyzed therapeutic effects in a cohort of 149 OCCC patients.

2. Materials and methods

2.1. Reagents

Gemcitabine (G6423), paclitaxel (T7402), doxorubicin (D1515), camptothecin (C9911), carboplatin (C2538), etoposide (E1383), cytarabine (PHR1787), 5-fluorouracil (03738), hydroxyurea (H8627), methotrexate (M7824), and pemetrexed (SML1490) were purchased from Sigma-Aldrich.

2.2. Cell lines

Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 in DMEM/F-12 (Wako) supplemented with 10% fetal bovine serum (Gibco/Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako). TOV-21G and ES-2 cells were obtained from the American Type Culture Collection (ATCC). RMG-I, RMG-V, and HAC-2 cells were obtained from the Japanese Collection of Research Bioresources (JCRB). JHOC-9 cells were obtained from the Riken Bioresource Center (RBC). *ARID1A*-KO (Q456X/Q456X) and parental HCT116 cells were purchased from Horizon Discovery. Cell lines were authenticated by verifying alterations of multiple cancer-related genes via sequencing. Cells were used for functional experiments after less than 3 months of passaging post-receipt. All cell lines tested negative for mycoplasma, as tested by MycoAlert (Lonza, LT07-418). The genomic status of *ARID1A* in these OCCC cell lines was examined by targeted sequencing of genomic DNA according to a described previously method [30].

2.3. Generation of ARID1A-KO cell lines using CRISPR-Cas9

RMG-I and HEK293T cells were infected with a lentivirus containing a hCMV-PuroR-Cas9 unit, and infected cells were subsequently selected with medium containing 2 μg/mL puromycin. A gRNA to target ARID1A (Dharmacon, 017263-03-0005 TATGGGTTAGTCCCGCCATA) and tancrRNA were transfected into the cells using DharmaFECT Duo (Dharmacon, T-2010-03). On the following day, the medium was replaced with fresh growth medium. The drug-resistant clones were selected and scaled up. Gene targeting was confirmed by immunoblot analysis and Sanger sequencing of genomic DNA as recently described [30].

2.4. Generation of ARID1A-Expressing lentiviruses and virus-Infected cells

cDNA-expressing lentiviral vectors (pLenti-puro-ARID1A, #39478) (Addgene) and packaging plasmids (psPAX2: #12260 and pMD2.G: #12259) (Addgene) were used for constitutive expression of cDNAs. To generate viruses, 293LTV cells were transfected with lentiviral plasmids and packaging plasmids using Lipofectamine 3000 (Invitrogen/ThermoFisher Scientific). On the following day, the medium was replaced with fresh growth medium and lentivirus-containing supernatants were harvested and concentrated by centrifugation. To establish cells infected with viral constructs, cells were transduced with lentiviral vectors and then incubated for 7–14 days in growth medium containing 2 mg/ml puromycin (Sigma-Aldrich).

2.5. Immunoblot analysis

Cells were lysed in NETN420 buffer supplemented with a protease inhibitor cocktail (Active Motif, 37491). The soluble fractions of whole-cell lysates were mixed with SDS sample buffer. Proteins were separated by SDS-PAGE and subsequently transferred to PVDF membranes. Membranes were blocked overnight at 4 °C or for 0.5 h at 25 °C with PVDF Blocking Reagent for Can Get Signal (TOYOBO, NYPBR01), and subsequently probed with Can Get Signal Solution 1 (TOYOBO, NKB-201) containing primary antibodies. The membranes were washed and incubated with TBS containing 0.1% Tween 20, 1% BSA, and horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and visualized using Western Lightning ECL Pro (PerkinElmer, NEL121001EA). Chemiluminescent signals were measured using a LAS-3000 Imaging System (Fujifilm).

Signal intensities were measured using Multi Gauge software. The following antibodies were used for immunoblotting: ARID1A (Sigma-Aldrich, 5456) and β-actin (Cell Signaling Technology, 4790).

2.6. Cell viability assay

Cells were seeded in 96-well plates, incubated for 24 h, and subsequently treated with serially diluted chemotherapeutics. Cell viability was assessed after 6 days using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7570). Luminescence was measured using an Envision Multi-label plate reader (PerkinElmer). The luminescence reading under each condition in triplicate plates was used to determine the cell viability relative to that of cells treated with the solvent. Viability curves and the IC50 (half maximal inhibitory concentration) of each compound were calculated using GraphPad Prism version 7. Reproducibility was confirmed by performing the experiment three or more times.

2.7. Mouse xenograft model

Cells were counted and re-suspended in a 1:1 mixture of 100 μl of culture medium and 100 μl of Matrigel (BD Biosciences) on ice. Thereafter, cells (ES-2: 1.0×10^6 cells/mouse; JHOC-9: 2.0×10^6 cells/mouse) were injected subcutaneously into the flank of 6-week-old female BALB/c-nu/nu mice (CLEA Japan) according to a protocol [T17-074] approved by the Ethical Committee on Animal Experiments at the National Cancer Center. The experiments were conducted according to the criteria set by the Declaration of Helsinki. In the subcutaneous model, once the tumors were palpable (7–18 days after implantation), ten mice were randomly divided into control and treatment groups. Mice were injected intraperitoneally with either phosphate-buffered saline or gemcitabine (25 mg/kg) three times every 3–4 days. Tumor growth was measured every few days using calipers. The volume of implanted tumors was calculated using the formula $V = L \times W^2/2$, where V is volume (mm^3), L is the largest diameter (mm), and W is the smallest diameter (mm). At the end of the experiment, mice were euthanized in accordance with standard protocols.

2.8. Annexin V/propidium iodide (PI) staining assay

An Annexin V-FITC/PI Apoptosis Detection Kit (Sigma-Aldrich, 11858777001) was used to detect apoptotic cells following the manufacturer's protocol. Fluorescence was analyzed with a Guava flow cytometer (Millipore). Cells were harvested and stained with annexin V-FLUOS and PI. Data were analyzed using GuavaSoft software (ver 2.7). Relative ratios of the sub-G1 and Annexin V-positive fractions in treated samples were normalized against untreated samples. Reproducibility was confirmed by performing the experiments in triplicate.

2.9. Retrospective analysis of OCCC patients

A cohort of 149 patients who underwent surgery and were diagnosed with OCCC at the National Cancer Center Hospital (NCCH) or the Jikei University Hospital (JUH) was prepared. Of the 149 patients, 28 relapsed, and of these seven were treated with no less than one cycle of gemcitabine single-agent chemotherapy as a second line treatment and subjected to analyses (see Fig. S3). Tumors were reviewed to confirm OCCC diagnosis by two of the authors (H. Yoshida and T. Kiyokawa) and pathologically staged according to the International Federation of Gynecology and Obstetrics (FIGO) classification (2014). The ARID1A status of tumor cells obtained at initial surgery was examined in the seven patients by performing immunohistochemistry (IHC). Patient characteristics, including age at diagnosis, surgical procedure, FIGO stages, recurrence site, chemotherapy treatments, progression-free survival (PFS), and response to chemotherapy, were retrospectively examined. This study was approved by the Institutional Review Board of the National Cancer Center (NCCH) [2017–190] and the Jikei University [30–446(9467)], and informed consent was obtained from the patients. This study was conducted according to the criteria set by the Declaration of Helsinki.

Disease status, based on imaging results and/or clinical evaluation, was monitored by the attending physicians on a daily basis. Response and progression after treatment were retrospectively evaluated using RECIST guidelines. TFI (treatment-free interval) was defined as time from primary surgery to first disease progression on or after first-line chemotherapy. PFS (progression-free survival) was defined as the time interval between the last date of the previous chemotherapy and the date of disease progression or recurrence.

2.10. Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor samples obtained at initial surgery of seven OCCC patients were deparaffinized, and representative whole 4-μm-thick sections were analyzed by IHC. Tumor sections were stained using an antibody against ARID1A (HPA005456, 1:2000 dilution; Sigma-Aldrich) and IHC was performed using a Dako autostainer Link48 (Dako) according to the manufacturer's instructions. Lack of nuclear immunoreactivity or weak nuclear immunoreactivity in the tumors was considered to show ARID1A deficiency, and definite nuclear staining was considered to show ARID1A proficiency in comparison with stromal cells as previously described [30].

2.11. Statistical analysis

Statistical analyses of differences were analyzed by the Student's t-test, Mann-Whitney *U* test, or log-rank test using GraphPad Prism software (ver 7.02) (GraphPad Software). Data are expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) as indicated in the figure legends. Statistical differences are indicated by asterisks, where $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

3. Results

3.1. ARID1A deficiency selectively increases sensitivity to gemcitabine

Six cytotoxic chemotherapeutic drugs used in standard therapy for OCCC belonging to six categories, including carboplatin (platinum), paclitaxel (taxane), gemcitabine (nucleoside analog), doxorubicin (anthracycline antibiotic), camptothecin (topoisomerase I inhibitor), and etoposide (topoisomerase II inhibitor), were selected according to NCCN clinical practice guidelines in oncology of ovarian cancer (version 4.2017). We first investigated the association of ARID1A deficiency with drug sensitivity in OCCC cells. RMG-I OCCC cells with *ARID1A*-KO and without ARID1A protein expression were approximately 100-fold more sensitive to gemcitabine than isogenic RMG-I *ARID1A*-WT cells with ARID1A protein expression (Fig. 1A–C). *ARID1A*-KO cells derived from HCT116 colon cancer cells and HEK293T human embryonic kidney cells were also significantly more sensitive to gemcitabine than corresponding isogenic *ARID1A*-WT cells (Figs. S1A–B). These results indicate that *ARID1A* deficiency selectively increases sensitivity to gemcitabine, irrespective of cell type.

Gemcitabine (difluorodeoxycytidine) is an analogue of deoxycytidine and is classified into a group of pyrimidine antimetabolites

among the antimetabolite group. To explore the specificity of our findings to gemcitabine, five other antimetabolites, including cytarabine (pyrimidine antimetabolite), 5-fluorouracil (uracil antimetabolite), hydroxyurea (urea antimetabolite), methotrexate (folate antimetabolite), and pemetrexed (folate antimetabolite), were examined for selective sensitivity according to ARID1A deficiency using *ARID1A*-WT and *ARID1A*-KO RMG-I cells. *ARID1A*-KO cells were markedly sensitive to cytarabine, another pyrimidine antimetabolite like gemcitabine, in comparison with other antimetabolites, while 5-fluorouracil had weaker selectivity (Fig. 1D–E, S1C). These results indicate that *ARID1A* deficiency is strongly associated with specific sensitivity to pyrimidine antimetabolite drugs.

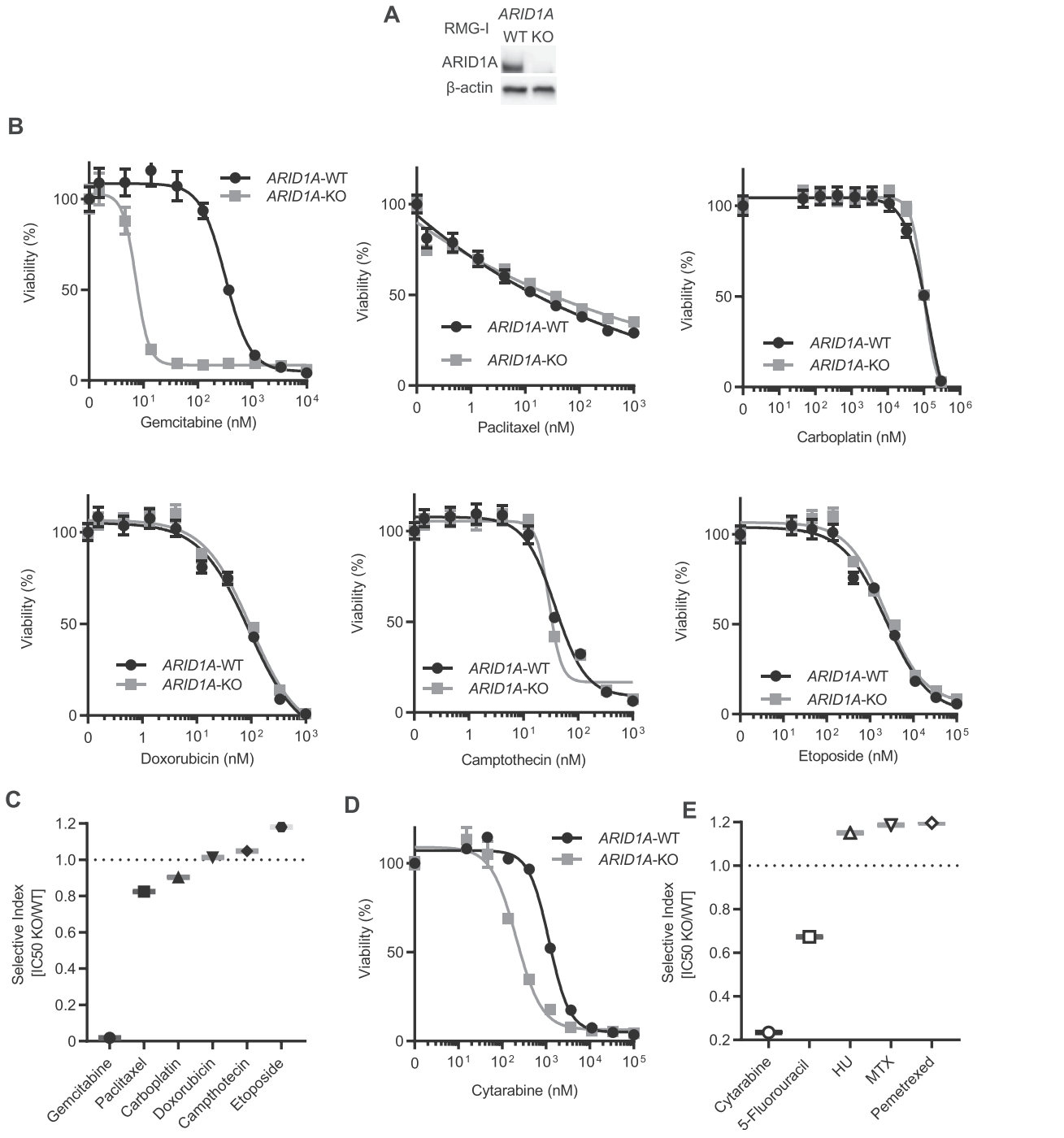

Fig. 1. *ARID1A*-KO cells are selectively sensitive to gemcitabine
(A) Immunoblotting of whole-cell extracts of *ARID1A*-WT and *ARID1A*-KO RMG-I OCCC cells for ARID1A and β-actin.
(B) Viability of *ARID1A*-WT and *ARID1A*-KO RMG-I cells after treatment with standard chemotherapeutic drugs used for OCCC. Data are expressed as mean ± SD.
(C) Selective indexes based on the IC50 values of ARID1A-KO cells relative to ARID1A-WT cells. IC50 values were based on the viability of *ARID1A*-WT and *ARID1A*-KO RMG-I cells after treatment with standard chemotherapeutic drugs used for OCCC.
(D) Viability of *ARID1A*-WT and *ARID1A*-KO RMG-I cells after treatment with cytarabine. Data are expressed as mean ± SD.
(E) Selective index based on IC50 values of ARID1A-KO cells relative to ARID1A-WT cells. IC50 values based on the viability of *ARID1A*-WT and *ARID1A*-KO RMG-I cells after treatment with the indicated drugs.

3.2. Gemcitabine suppresses growth of OCCC cells in vitro and in vivo.

Next, we examined whether commonly used OCCC cell lines had selective sensitivity to gemcitabine according to their ARID1A status. For this purpose, we prepared a panel of six OCCC cell lines. In addition to RMG-I, ARID1A protein expression was retained in ES-2 cells harboring the wild-type *ARID1A* gene. On the other hand, ARID1A protein expression was lost in four other OCCC cell lines with *ARID1A* gene mutations, including TOV-21G, JHOC-9, HAC-2, and RMG-V cells (Fig. 2A and B). Accordingly, RMG-I and ES-2 were classified as ARID1A-proficient cell lines, while TOV-21G, JHOC-9, HAC-2, and RMG-V were classified as ARID1A-deficient cell lines. ARID1A-deficient cell lines had significantly lower IC50 values for gemcitabine than ARID1A-proficient OCCC cell lines (Fig. 2C, $p = 0.0001$). Similar results were also obtained for cytarabine, consistent with the above studies of ARID1A-KO cells (Fig. S2A). Taken together, these findings demonstrated that selective sensitivity to pyrimidine antimetabolite drugs is a common feature of OCCC cells deficient for ARID1A. We also examined whether ovarian endometrioid carcinoma cells with ARID1A-deficiency were sensitive to gemcitabine. ARID1A-deficient A2780 cells were more sensitive to gemcitabine than ARID1A-proficient RMG-I cells (Fig. S2B). The growth suppression of TOV-21G ARID1A-deficient OCCC cells following gemcitabine treatment was rescued by stable expression of the *ARID1A* cDNA (Fig. 2D), confirming that ARID1A deficiency was responsible for gemcitabine sensitivity.

Because gemcitabine is one of the most commonly used pyrimidine antimetabolite drugs in oncology [21], we next investigated the anti-tumor efficacy of this drug in mouse xenografts. Administration of gemcitabine significantly suppressed growth of ARID1A-deficient JHOC-9 xenografts, but did not suppress growth of ARID1A-proficient ES-2 xenografts (Fig. 2E and F). Taken together, these findings suggest that gemcitabine is a promising drug for treatment of ARID1A-deficient OCCC.

3.3. Gemcitabine causes apoptosis in ARID1A-deficient OCCC cells

The effect of gemcitabine on the cell cycle according to the ARID1A status was examined in OCCC cells. In RMG-I *ARID1A*-KO, the sub-G1 fraction increased with gemcitabine treatment, but this effect was not observed in parental RMG-I *ARID1A*-WT cells (Fig. 3A). In RMG-I *ARID1A*-KO cells, the sub-G1 fraction increased in a time-dependent manner (Fig. 3B). Gemcitabine treatment also increased the sub-G1 fraction in ARID1A-deficient RMG-V cells, but not in ARID1A-proficient ES-2 cells (Fig. 3C). Furthermore, gemcitabine treatment increased the fraction of cells positive for annexin V, an apoptotic marker, among RMG-V cells, but not among ES-2 cells (Fig. 3D). In addition, gemcitabine-induced apoptosis in ARID1A-deficient TOV-21G cells was suppressed by ectopic expression of ARID1A (Fig. 3E and F).

Taken together, these results indicate that gemcitabine causes apoptosis more efficiently in ARID1A-deficient OCCC cells than in ARID1A-proficient OCCC cells, which is consistent with previous results showing that gemcitabine induces apoptosis by activating several signaling pathways [16–20]. These results also suggest that gemcitabine suppresses growth of ARID1A-deficient OCCC cells by causing apoptosis.

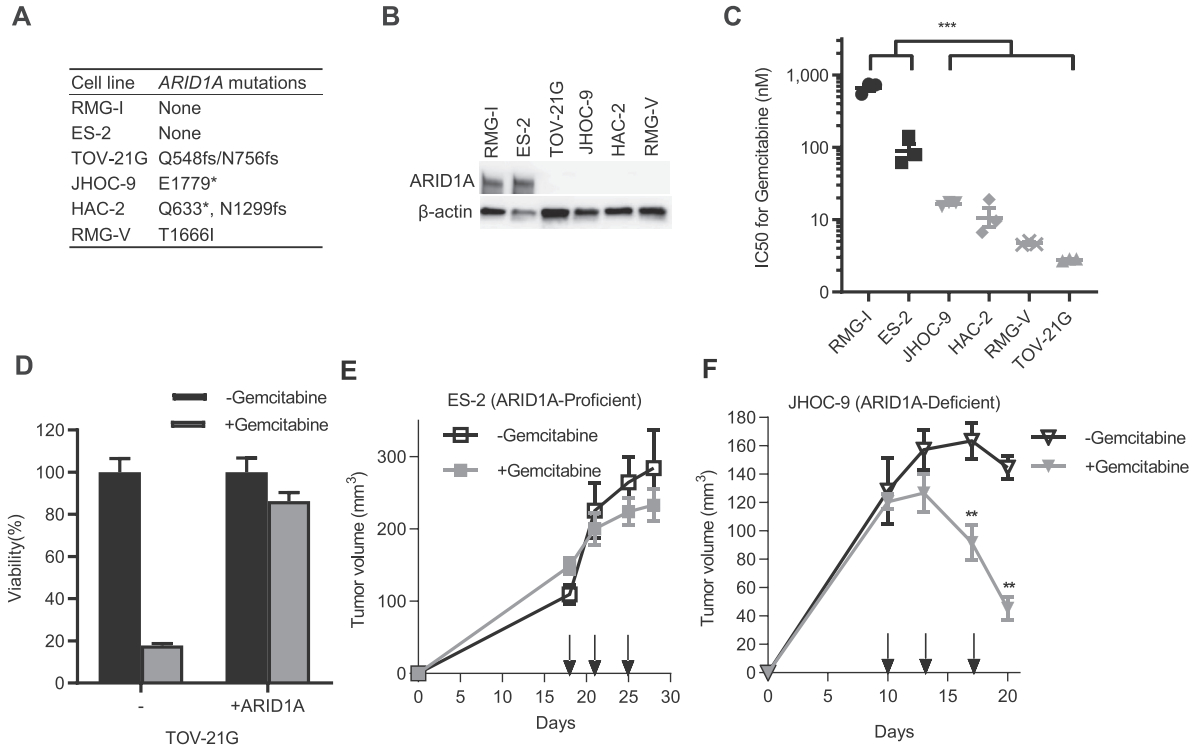

Fig. 2. Gemcitabine suppresses OCCC cell growth *in vitro* and *in vivo*
(A) *ARID1A* gene status in OCCC cell lines.
(B) Immunoblotting of whole-cell extracts of OCCC cell lines for ARID1A and β-actin.
(C) IC50 values based on the viability of ARID1A-proficient (black) and ARID1A-deficient (gray) cells treated with gemcitabine. Data are expressed as mean ± SEM ($n = 3$) (***$p < 0.001$; Mann-Whitney *U* test).
(D) Viability of parental and ARID1A-expressing TOV-21G cells after treatment with 5 nM gemcitabine. Representative data are expressed as mean ± SD.
(E, F) Tumor volume of xenografts derived from ARID1A-proficient ES-2 cells (E) and ARID1A-deficient JHOC-9 cells (F) in mice treated with gemcitabine. Arrows indicate administration of 25 mg/kg gemcitabine or vehicle intraperitoneally. Data are expressed as mean ± SEM ($n = 5$) (**$p < 0.01$; Student's t-test).

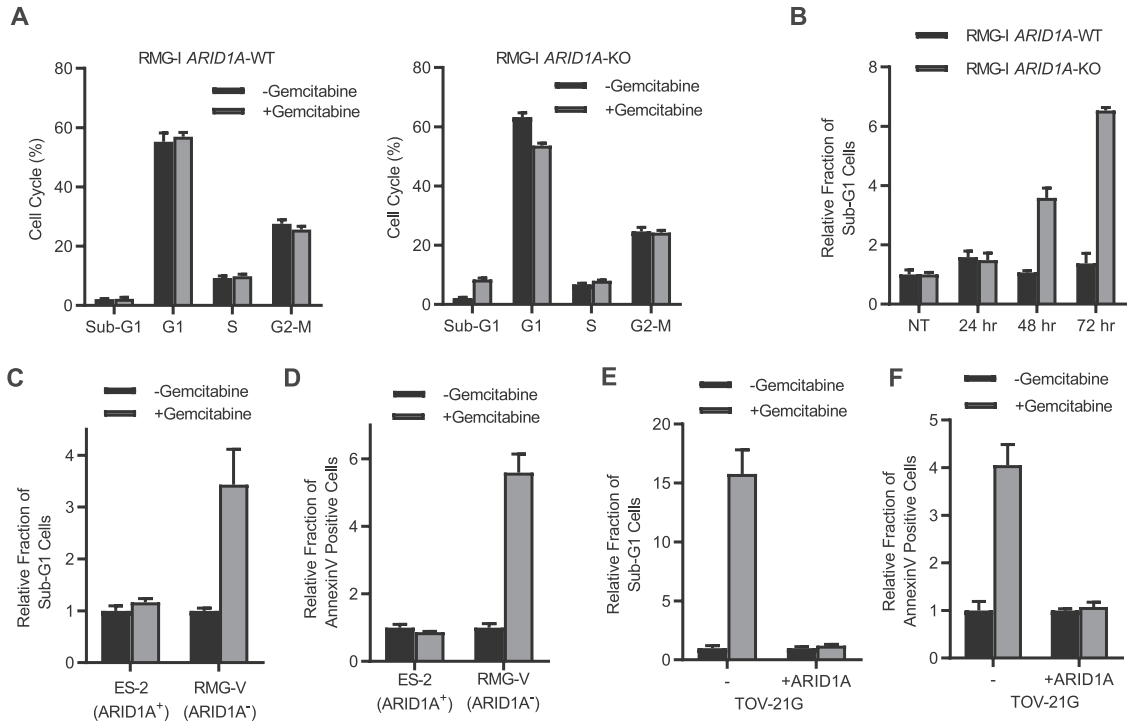

Fig. 3. Gemcitabine causes apoptosis in ARID1A-deficient OCCC cells
(A) Cell cycle profiles of *ARID1A*-WT and *ARID1A*-KO RMG-I cells treated with 400 nM gemcitabine for 48 h. Data are expressed as mean ± SD.
(B) Relative sub-G1 fraction of *ARID1A*-WT and *ARID1A*-KO RMG-I cells treated with 400 nM gemcitabine for 24, 48 and 72 h relative to the non-treated control (NT). Data are expressed as mean ± SD.
(C) Relative sub-G1 fraction of ARID1A-proficient ES-2 cells and ARID1A-deficient RMG-V cells treated with 200 nM gemcitabine for 48 h. Data are expressed as mean ± SD.
(D Relative annexin V-positive fraction of ARID1A-proficient ES-2 cells and ARID1A-deficient RMG-V cells treated with 200 nM gemcitabine for 48 h. Data are expressed as mean ± SD.
(E) Relative sub-G1 fraction of parental TOV-21G cells and TOV-21G expressing ARID1A cDNA (+ARID1A) cells treated with 100 nM gemcitabine for 48 h. Data are expressed as mean ± SD.
(F) Relative annexin V-positive fraction of parental TOV-21G cells and TOV-21G expressing ARID1A cDNA (+ARID1A) cells treated with 100 nM gemcitabine for 48 h. Data are expressed as mean ± SD.

3.4. Gemcitabine treatment is promising for ARID1A-deficient OCCC patients

Gemcitabine is recommended for the treatment of recurrent ovarian cancers, especially platinum-resistant ones, according to NCCN (National Comprehensive Cancer Network) clinical practice guidelines for ovarian cancers (version 4.2017). In our OCCC patient cohort receiving second line treatments, seven of 28 relapsed patients (25%) were treated with no less than one cycle of gemcitabine single-agent chemotherapy as a second line treatment (Fig. S3). These patients were retrospectively examined for response to gemcitabine therapy according to ARID1A status (Table 1). ARID1A expression was lost or reduced in three cases (cases 1–3), while the other four cases (case 4–7) retained ARID1A protein expression at comparable levels to that in stromal cells, which were used as an internal positive control (Fig. 4A). This result is consistent with previous reports that approximately 50% of OCCC cases have loss or reduction of ARID1A protein expression associated with loss-of-function *ARID1A* mutations [25,26,32–36]. The median PFS after gemcitabine treatment was 6.7 months in ARID1A-deficient cases and 2.9 months in ARID1A-proficient cases ($p = 0.02$, Fig. 4B). Three ARID1A-deficient cases had partial responses or stable disease, while only one of the ARID1A-proficient cases had SD, and the other cases exhibited progressive disease (Table 1). These findings suggest that specifically ARID1A-deficient OCCC patients benefit from gemcitabine treatment.

Table 1
Details of seven OCCC patients treated with gemcitabine single-agent chemotherapy.

Case	Age (years)	FIGO stage	Adjuvant CT	TFI (Mo)	First CT	Lesions	PFS (Mo)	Second CT (Cycles)	Lesions	PFS (Mo)	IHC for ARID1A	Best response of GEM
1	54	IIIC	PTX + CBDCA	9.3	PLD + CBDCA	Peri, LN	8	GEM (8)	Peri, LN	7.3	Low	SD
2	41	IC	PTX + CBDCA	3.5	ETP + CPT-11	Peri, lung	1.8	GEM (5)	Peri, lung	6.7	Low	PR
3	51	IIIB	PTX + CBDCA	0.4	PLD	Peri	7.8	GEM (6)	Liver, peri	6.2	Low	SD
4	69	IA	None	5.6	PTX + CBDCA	Peri	14.2	GEM (5)	LN	5.3	High	SD
5	50	IIIC	PTX + CBDCA	2.9	PTX + Bev	LN	5.3	GEM (3)	LN	4	High	PD
6	42	IC	PTX + CBDCA	9	PLD + CBDCA	Peri	1.8	GEM (2)	PE	1.8	High	PD
7	68	IIIC	PTX + CBDCA	3.6	PLD	LN	11.7	GEM (2)	LN	1.2	High	PD

FIGO; The International Federation of Gynecology and Obstetrics, CT; Chemotherapy, TFI; Treatment-free interval, PFS; Progression-free survival, PTX; Paclitaxel, CBDCA; Carboplatin, ETP; Etoposide, CPT-11; Irinotecan, PLD; Pegylated liposomal doxorubicin, Bev; Bevacizumab, GEM; Gemcitabine, Peri; Peritoneal.
LN; Lymph nodes, PE; Pleural effusion, PR; Partial response, SD; Stable disease, PD; Progressive disease, IHC; Immunohistochemistry.

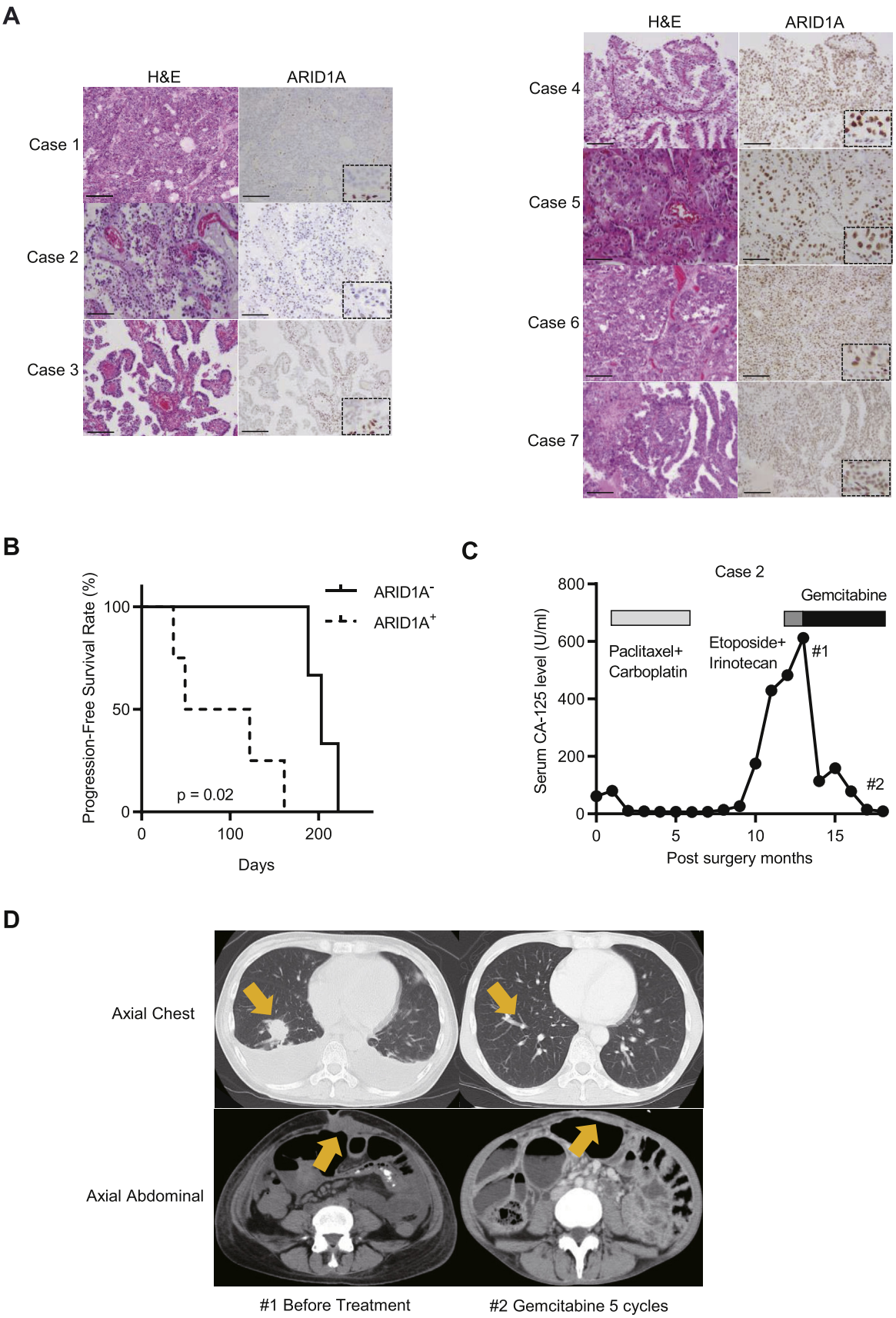

Fig. 4. Gemcitabine treatment response in ARID1A-deficient and ARID1A-proficient OCCC patients
(A) Immunohistochemical analysis of ARID1A protein in OCCC specimens. Scale bar, 100 μm
(B) Kaplan-Meier curves for PFS of ARID1A-proficient and ARID1A-deficient patients (*p = 0.02; log-rank test).
(C) Serum CA-125 levels during the clinical course for case 2.
(D) Axial chest and abdominal computed tomographic scans of case 2. Time points of CT scan images are indicated by #1 and #2. Yellow arrows indicate tumor lesions.

Case 2 with ARID1A deficiency had a dramatic response to gemcitabine (Fig. 4C). The patient received a total hysterectomy, bilateral salpingo-oophorectomy, partial omentectomy, and pelvic and para-aortic lymphadenectomy, and was diagnosed as stage IC3. Three months after receiving six cycles of paclitaxel (80 mg/m^2 weekly) and carboplatin (area under the blood concentration-time curve; 6 mg x hr/l every 3 weeks) as adjuvant chemotherapy, she was diagnosed with recurrent disease in her lung and peritoneum. Single-cycle treatment of chemotherapy using etoposide (50 mg/day × 21 days every 28 days) and irinotecan (70 mg/m^2 every 2 weeks) did not improve her CA-125 biomarker level or abdominal pain (Fig. 4C and D left). She then received five cycles of gemcitabine single-agent therapy (1,000 mg/m^2 on days 1, 8, and 15 every 28 days) in her second-line treatment. After the treatment, her CA-125 level and tumor size dramatically decreased, with a concurrent decrease of abdominal pain. (Fig. 4C and D Right).

4. Discussion

This study investigated a precision medicine strategy for OCCC, a malignant subtype of ovarian cancer resistant to conventional platinum-based chemotherapy [2,3] and lacking BRCA1/BRCA2 alterations linked to efficacy of PARP inhibitors [37,38]. This study focused on ARID1A deficiency, one of the most prevalent molecular alterations in OCCC [25–27]. Previously, ARID1A-deficiency was reported to be a negative prognostic factor in OCCC patients treated with platinum-based chemotherapy [35]. Immunotherapy was reported as the optimal therapeutic option for ARID1A-deficient stomach cancer [39,40]. However, to the best of our knowledge, there have been no reports showing that ARID1A deficiency is associated with the efficacy of specific chemotherapeutic drugs. ARID1A deficiency is thought to dysregulate expression of many genes involved in the biological and metabolic characteristics of tumor cells [29,30]. Therefore, we hypothesized that ARID1A deficiency may enhance sensitivity to some anticancer drugs. We demonstrated that ARID1A deficiency enhances sensitivity to pyrimidine antimetabolites, particularly gemcitabine, by performing experiments with multiple KO cells, commonly used OCCC cell lines, and xenografts as well as by conducting retrospective analysis of OCCC patients that received gemcitabine therapy. The data also support previous reports of a few platinum-resistant OCCC cases that responded to gemcitabine [22–24], although the ARID1A status was not examined in those cases. Because gemcitabine is a widely used chemotherapeutic drug, the present findings, which suggest an association between ARID1A deficiency and gemcitabine sensitivity, contribute to precision medicine of OCCC in standard treatments.

Despite the frequent occurrence of ARID1A deficiency, its association with sensitivity to gemcitabine had not been previously assessed. This might be due to the rarity of OCCC among all ovarian cancers (approximately 10%). Prior evaluation of drug efficacy in ovarian cancer has only been conducted in patient cohorts with only 7% of OCCC cases [41]. Further, gemcitabine has often been considered for late lines of treatment, after treatments with other multiple drugs, such as platinum and other cytotoxic drugs, have failed. Therefore, any association of gemcitabine efficacy with ARID1A deficiency may have been masked by the effects of pretreatments and the poor status of patients. Only 20% of recurrent OCCC patients received gemcitabine therapy at a second or earlier line of therapy in several previous cohorts [22,24,33,41]. Consistently, our retrospective cohort of 28 relapse cases included seven cases (25%) that were treated with single-agent gemcitabine as a second-line treatment, and no case received treatment with gemcitabine as an earlier line of therapy. Despite the small number of examined cases, the prognosis and response of ARID1A-deficient OCCCs were better than those of ARID1A-proficient OCCCs, as demonstrated by higher fractions of responsive cases among ARID1A-deficient patients. In addition, compared with the results of a randomized phase III trial of gemcitabine-single chemotherapy for all types of recurrent ovarian cancer [42], the response rates and PFS after gemcitabine treatment of ARID1A-deficient OCCCsin our cohort were higher. In particular, one ARID1A-deficient case was resistant to multiple cytotoxic drugs in adjuvant and first-line treatments, but had a marked response to gemcitabine in second-line treatment. The association of ARID1A deficiency with a preferable response to gemcitabine should be validated in larger or additional cohorts of OCCC. However, gemcitabine, as a precision medicine, might be a suitable option for ARID1A-deficient OCCC.

We propose that ARID1A deficiency may be a predictive biomarker for the response of OCCC to gemcitabine treatment. Because the response and prognosis after gemcitabine treatment of ARID1A-deficient OCCCs varied among cases in our cohort, other factors may affect the efficacy of gemcitabine. Due to limitations in tumor specimen availability, we did not search for other potential factors by performing comprehensive omics analyses. In addition, tumor specimens analyzed in the present study were obtained in the first diagnosis. Therefore, heterogeneity of tumors during treatments might have affected the response to gemcitabine. Prospective analysis of OCCC patients receiving gemcitabine therapy coupled with extensive molecular profiling is currently underway by our group to investigate these points.

Although the focus of our study was conventional cytotoxic drugs, bevacizumab, which reduces tumor growth by suppressing angiogenesis, may be another agent worth examining [45, 46]. Other models, such as those employing orthotopic implantation, might also be more suitable than conventional subcutaneous xenograft models, which is reproducibility and convenience [43,44]. Additionally, the mechanisms underlying the association of sensitivity to pyrimidine antimetabolites with ARID1A deficiency remains unclear. ARID1A-deficient OCCC cells may have vulnerabilities in nucleic acid metabolism in addition to glutathione metabolism [30]. These mechanisms should be investigated in future studies. This study originates from the urgent need of physicians to improve the poor prognosis of OCCC. The present findings may be translated to clinical benefit for OCCC patients, contributing to precision medicine in clinical settings.

Author contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Kuroda., H. Ogiwara., T. Kohno.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

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