平成３０年度シニア・リサーチフェロー

研究成果報告書

令和元年 6 月 28 日 提出

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研究課題：ARID1A 変異がんに対する個別化治療法の開発

研究期間：　自　平成　30　年　4　月　1　日

至　平成　31　年　3　月　31　日

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(1)シニア・リサーチフェロー期間中の研究について

1）要旨

卵巣明細胞がんは殺細胞性抗がん剤による標準治療法に対して抵抗性を示す。また、卵巣明細胞がんの約50%においてSWI/SNFクロマチン制御遺伝子のサブユニットの一つである*ARID1A*（AT-rich interactive domain 1A）の変異を有する特徴がある。今回の研究では、ARID1A欠損卵巣明細胞がん患者に対する殺細胞性抗がん剤を用いた個別化治療法の開発を目的とした。卵巣がんの化学療法の中で標準的に用いられている殺細胞性抗がん剤に対してスクリーニングを行ったところ、薬剤Xで*ARID1A*ノックアウト細胞株に対して選択的感受性を示した。ARID1A発現卵巣明細胞がん細胞株とARID1A欠損卵巣明細胞がん細胞株において薬剤Xの50%阻害濃度（IC50）を比較したところ、ARID1A欠損卵巣明細胞がん細胞株群で有意に低かった（$p = 0.0001$）。また、マウス異種移植腫瘍モデルにおいて、ARID1A欠損卵巣明細胞がんでは薬剤Xの投与により、腫瘍抑制効果を認めた。さらに、薬剤Xの投与により、ARID1A欠損卵巣明細胞がん細胞株ではアポトーシスが誘導されていることを確認した。これらの研究結果より、ARID1A欠損卵巣明細胞がんでは薬剤X投与が有効であると考えられた。

2) 序

卵巣明細胞がんは卵巣がんの一つに分類され、病因，分子・遺伝子的背景、臨床像を含め、卵巣漿液性がんとは区別される[1-3]。卵巣明細胞がんの頻度は欧米では卵巣がん全体の約10%と報告されているが、日本を含めた東アジアでは約30%と高い[4-7]。卵巣がんは白金製剤を含めた化学療法が標準治療とされているが、卵巣明細胞がんではそれらを用いた標準治療の奏功率は約30%と報告されており、卵巣漿液性がんにおける奏功率（約70%）と比較し、低い[6, 8-10]。卵巣明細胞がんはPARP阻害剤が有効とされるBRCA1/2変異等を有さず、標準化学療法だけでなく、分子治療標的薬であるキナーゼ阻害剤等にも耐性を示す[2, 11−13]。

卵巣明細胞がんは頻度が低いことから、卵巣漿液性がんと異なり大規模臨床試験に含まれる割合が少なく、個別化治療は開発が不十分で、アンメット・メディカル・ニーズである[2, 3, 10]。卵巣明細胞がんは分子生物学的・臨床的特徴から、その他の卵巣がんとは区別されるべきであり、個別の治療法が考慮される[14]。

また、卵巣明細胞がんの約50%で、*ARID1A*遺伝子の機能喪失型変異を有する。*ARID1A*遺伝子はSWI/SNFクロマチンリモデリング複合体のサブユニットの一つであり、複数のがん種において変異が認められ、遺伝子発現を制御している[15-18]。最近の研究では、ARID1A欠損は発癌を促進し、代謝経路を含む生物学的特徴に対して影響することがわかってきた[19,20]。高頻度でのARID1A欠損は卵巣明細胞がんにおいて、個別化治療の指標となる可能性を示唆している[21,22]。いくつかの報告ではARID1A欠損は予後不良因子とされている[22-26]。しかし、ARID1A欠損と特定の殺細胞性抗がん剤の有効性との関連に関する報告はなく、*ARID1A*変異の有無により選択される治療法はまだ確立されていない。今回、我々はARID1A欠損に基づいた卵巣明細胞がんの有効な治療法の開発のために、*ARID1A*ノックアウト卵巣明細胞がん細胞株と、複数の卵巣明細胞がん細胞株を用いて薬剤スクリーニングを行った。

3）実験方法

細胞株

細胞株は、37℃、5% CO2 インキュベーターで培養した。培地には DMEM/F-12（Wako）に 10% ウシ胎児血清（FBS; Gibco/Life Technologies）、100 U/ml ペニシリン、100 mg/ml ストレプトマイシン（Wako）を添加した。TOV-21G と ES-2 は American Type Culture Collection（ATCC）より入手した。RMG-I、RMG-V、HAC-2 は JCRB 細胞バンクより入手した。JHOC-9 は理研バイオリソースセンターより入手した。HCT116 及び、*ARID1A* ノックアウト（KO）細胞株 HCT116-*ARID1A* Q456*/Q456*を Horizon Discovery より購入した。細胞は入手後継代して 3 ヶ月以内に機能解析実験を行った。MycoAlert（Lonza）によりすべての細胞株がマイコプラズマ陰性であることを確認した。

CRISPR/Cas9 を用いた *ARID1A* ノックアウト（*ARID1A*-KO）細胞の作成

RMG-I 細胞株と HEK293T 細胞株を hCMV-PuroR-Cas9 ユニットを含むレンチウィルスに感染させ、2 ug/ml ピューロマイシン（Sigma-Aldrich）を含む培地で培養した。*ARID1A*（Dharmacon、017263-03-0005 TATGGGTTAGTCCCGCCATA）を標的とした gRNA と tracrRNA を DharmaFECT Duo(Dharmacon、T-2010-03)を用いて細胞にトランスフェクションした。翌日、培地を増殖培地に交換した。薬剤耐性のクローンを選択し、スケールアップした。標的遺伝子はウェスタンブロッティング解析とサンガー法シーケンシングで確認した。

cDNA 発現レンチウィルスとウィルス感染細胞の作成

cDNA の恒常的発現のために発現レンチウィルスベクター（pLenti-puro-*ARID1A*、#39478）（Addgene）とパッケージプラスミド（psPAX2:#12260、pMD2.G:#12559）を用いた。293LTV 細胞にレンチウィルスプラスミドとパッケージプラスミドを Lipofectamine 3000（Invitrogen/ThermoFisher Scientific）でトランスフェクションし、ウィルスを作成した。翌日培地を交換し、レンチウィルスを含む培地を遠心にて濃縮した。ウィルス感染細胞を樹立するために TOV-21G 細胞株にウィルスベクターを形質導入し、2 ug/ml ピューロマイシン（Sigma-Aldrich）を含む増殖培地中で 7-14 日間かけて培養した。

ウェスタンブロッティング解析

細胞回収後に、PBS で洗浄し、NETN420 緩衝液に Protease inhibitor cocktail(Active Motif、37491)を加え、遠心分離後に上澄みを SDS サンプル緩衝液に加えた。タンパク質を SDS-PAGE によって分離し、PVDF メンブレンに転写し、抗体で免疫ブロットした。メンブレンを 4℃（一晩）または 25℃（30 分）で PVDF ブロッキング試薬 Can Get Signal(TOYOBO、NYPBR01)を用いてブロッキングした。次いで一次抗体を含む Can Get Signal Solution 1(TOYOBO、NKB-201)で認識した。TBS（0.1% Tween20、1% BSA）で洗浄した。洗浄後、HRP(ホースラディッシュペルオキシダーゼ)法に基づいた抗ラビット二次抗体を含む TBS で標識した。TBS で洗浄後、Western Lightning ECL Pro（PerkinElimer）によって検出した。化学発光シグナルは、LAS-3000 Imaging System(Fujifilm)を用い、シグナル強度は Multi Gauge software を用いて解析した。使用した抗体は ARID1A(Sigma-Aldrich、5456)、β-actin(Cell Signaling Technology、4790)である。

細胞生存率の測定

細胞生存率の測定には細胞内の ATP 量を CellTiter-Glo Luminescent Cell Viability Assay(Promega)を用い、ルシフェラーゼ反応により生じる発光を用いた。発光の測定には Envision Multi-label plate reader(PerkinElmer)を用いた。細胞生存率を測定するために各プレートに対して 3 回測定を行った。各薬剤に対して生存曲線の作成と 50%阻害濃度算出（IC50）を GraphPad Prism version 7 を用いて行った。再現性の確認のため、同様の実験を 3 回以上行った。

マウス異種移植腫瘍モデル

全てのマウス実験は国立がん研究センターの動物実験倫理委員会の承認を得て、ヘルシンキ宣言に則り行った。細胞数をカウントし、氷上で 100 ul の培地と 100 ul のマトリゲル（BD Biosciences）を懸濁した。細胞は（ES2: 1.0 x 10^6 cells/mouse; JHOC-9: 2.0 x 10^6 cells/mouse）を 6 週齢のメスの BALB/c-nu/nu mice（日本クレア）に皮下移植した。皮下移植モデルでは移植後 7-18 日に腫瘍が触知可能になってから、マウスのグループ分けを行った。薬剤投与の実験では PBS もしくは薬剤 X（25mg/kg）を 3-4 日ごとに 3 回投与を行った。腫瘍の増殖は数日おきにノギスを用いて計測した。移植腫瘍の容積（mm^3）は、（長径（mm）x 短径^2（mm））/2 で求めた。マウスは実験終了後、プロトコールに従って安楽死した。

Annexin V/Propidium Iodide アッセイ

Annexin V-FITC/PI Apoptosis Detection Kit(Sigma-Aldrich　11858777001)を用いてアポトーシス細胞の検出を行った。Annexin V-FLUOS と PI を混注し、発光は Guava flow cytometer(Millipore)で計測し、GuavaSoft software(ver2.7)で解析を行った。Sub-G1 と Annexin V 陽性細胞に関しては非薬剤暴露サンプルをコントロールとした。再現性確認のため、同様の実験を 3 回行った。

統計解析

統計解析は GraphPad Prism software (ver 7.02)　(GraphPad Software) を用いて Student's t-test、Mann-Whitney U test を行った。得られた結果は平均±標準誤差または標準偏差で示し、図の説明文に記載されている。統計学的有意差はアスタリスクを用いて表示され、$^{**}p < 0.01$、$^{***}p < 0.001$ である。

4）結果

***ARID1A* 欠損がん細胞株では選択的に薬剤 X に感受性を示す**

NCCN ガイドラインの卵巣がん治療において実臨床で使用されている既存の標準治療薬を用いて卵巣明細胞がん細胞株（RMG-I）において、ARID1A 欠損と感受性の関連を調べた。CRISPR/Cas9 システムを用いてノックアウトし、発現消失が確認されている RMG-I *ARID1A*-KO 細胞において、*ARID1A* 野生型（*ARID1A*-WT）でタンパク質発現が確認できている RMG-I 親細胞と比較し、薬剤 X 投与により約 100 倍の感受性増加を認めたが、その他の薬剤では感受性に差はなかった（Fig1. B-C）。大腸がん由来の HCT116 と、ヒト胎児腎由来の HEK293T 細胞においても、*ARID1A*-KO 細胞は親細胞に比較して、薬剤 X の感受性が高かった（Fig. S1A-B）。これらの結果は ARID1A 欠損により薬剤 X の選択的感受性が増加することが示された。

***In vitro* と *in vivo* において薬剤 X は *ARID1A* 欠損卵巣明細胞がんに対して腫瘍増殖抑制効果を示す**

我々は一般的使用されている卵巣明細胞がん細胞株において *ARID1A* のステータスによる薬剤 X の選択的感受性について調べた。*ARID1A* の変異がなく、タンパク質発現が確認できている RMG-I と ES-2、*ARID1A* 遺伝子変異があり、タンパク質発現の消失している TOV-21G、JHOC-9、HAC-2、RMG-V の 6 種類の細胞株を用いた（Fig. 2A, 2B）。RMG-I と ES-2 は ARID1A 発現（ARID1A-proficient）細胞株に、TOV-21G、JHOC-9、HAC-2、RMG-V を *ARID1A* 欠損（ARID1A-deficient）細胞株に分類した。ARID1A 欠損卵巣明細胞がん細胞株では ARID1A 発現卵巣明細胞がん細胞株に比較して、薬剤 X の IC50 が有意に低かった（Fig. 2C、$p = 0.0001$）。ARID1A 欠損細胞株における薬剤 X の増殖抑制効果は ARID1A の安定的発現により解除されることが示され、薬剤 X の感受性に関して ARID1A 欠損が関連していることが確認された（Fig.2D）。

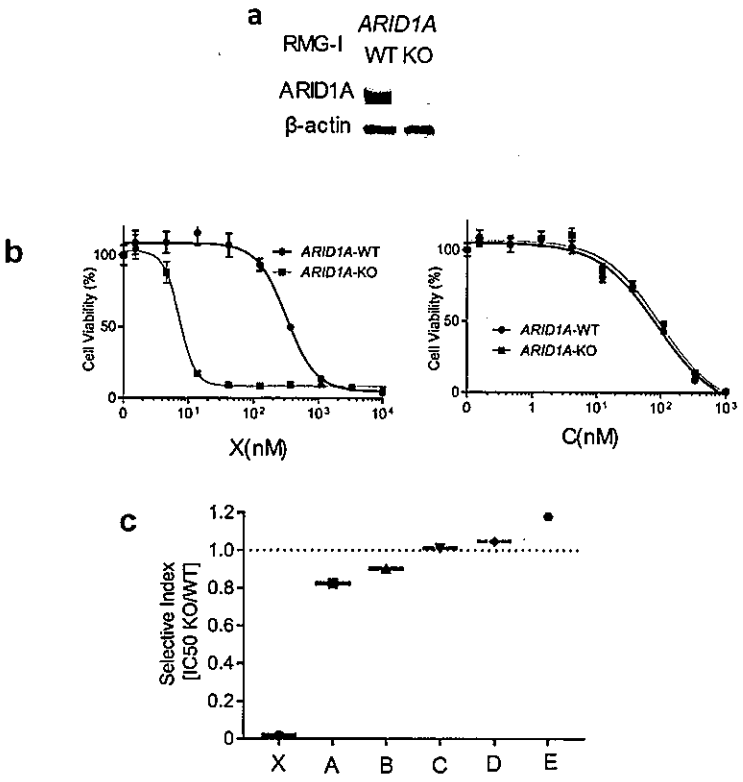
また、薬剤 X 投与により、ARID1A 欠損細胞株 JHOC-9 の移植腫瘍モデルでは有意に腫瘍増殖抑制効果があったが、ARID1A 発現細胞株 ES-2 では薬剤 X 投与による腫瘍増殖抑制効果は確認できなかった。このように ARID1A 欠損卵巣明細胞がんにおいて、薬剤 X 治療は有効であることが示唆された。

薬剤 X は *ARID1A* 欠損卵巣明細胞がんにおいてアポトーシスを誘導する

ARID1A のステータスの違いによる卵巣明細胞がん細胞株の薬剤 X 投与での細胞周期への影響を調べた。薬剤 X 投与により、RMG-I *ARID1A*-KO 細胞株においては sub-G1 が増加したが、RMG-I *ARID1A*-WT 細胞株では変化がなかった（Fig. 3A）。また、その sub-G1 は、時間依存性に増加していた（Fig. 3B）。また、ARID1A 欠損細胞株 RMG-V では薬剤 X 投与により sub-G1 の割合が増加したが、ARID1A 発現細胞株 ES-2 では増加はみられなかった（Fig. 3C）。さらに、薬剤 X 投与によりアポトーシスの指標である Annexin V 陽性細胞は ARID1A 欠損細胞株 RMG-V では増加していたが、ARID1A 発現細胞株 ES2 では増加していなかった（Fig. 3D）。

つまり、薬剤 X は ARID1A 発現卵巣明細胞がん細胞株と比較し、ARID1A 欠損卵巣明細胞株でより効果的にアポトーシスを誘導され、結果として、腫瘍増殖が抑制されていることが示唆された。

Figure 1. ***ARID1A*-KO 細胞株は薬剤 X に対して選択的感受性を示す**

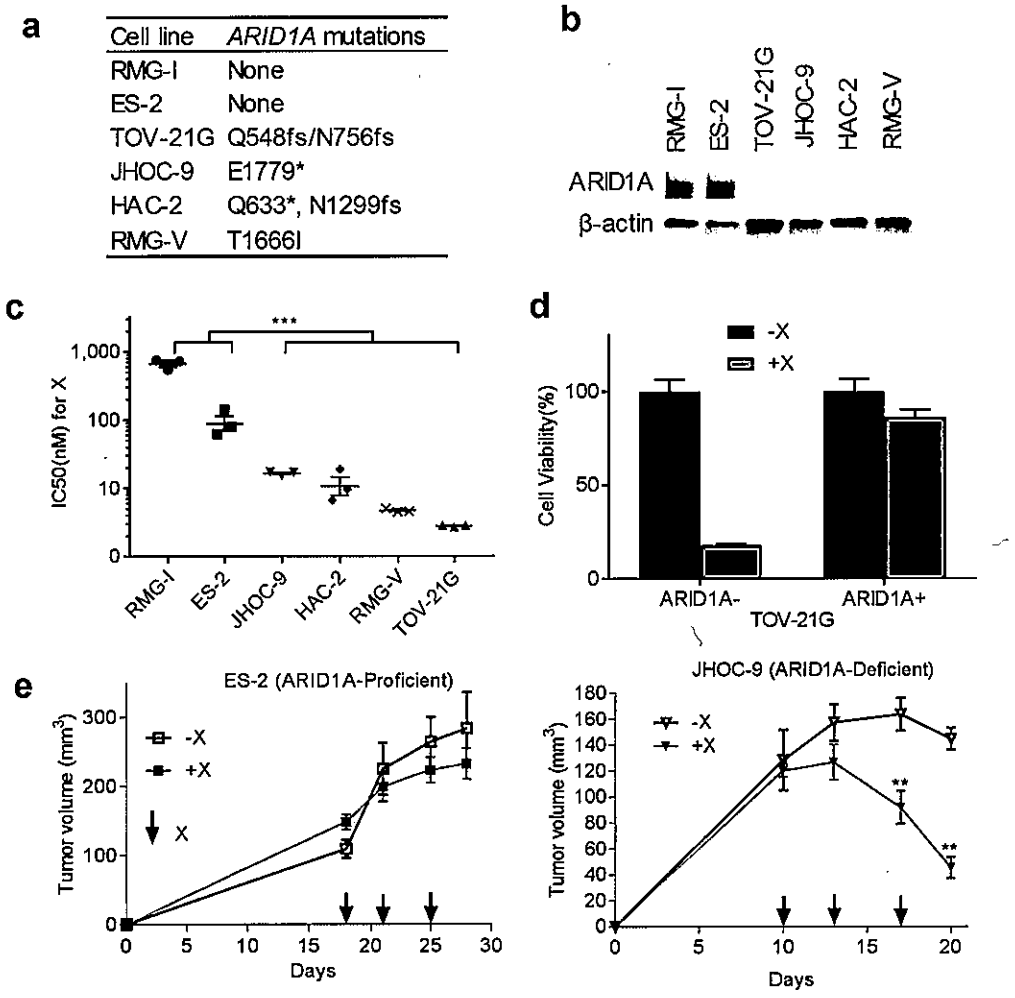


(a) 卵巣明細胞がん細胞株 RMG-I *ARID1A*-WT と *ARID1A*-KO の全細胞画分の *ARID1A* と β-actin のウェスタンブロット。

(b) 卵巣明細胞がんの標準治療で使用されている薬剤 X と C に対する RMG-I *ARID1A*-WT と *ARID1A*-KO 細胞株の細胞生存率。平均値±標準偏差で表記。

(c) 標準治療薬剤処理後の細胞生存率から算出した IC50 の RMG-I *ARID1A*-WT に対する *ARID1A*-KO の選択指数（selective index）。

Figure 2. 薬剤 X 投与により **ARID1A** 欠損卵巣明細胞がん細胞株の増殖を抑制する



(a) 卵巣明細胞がん細胞株の *ARID1A* 遺伝子の変異。

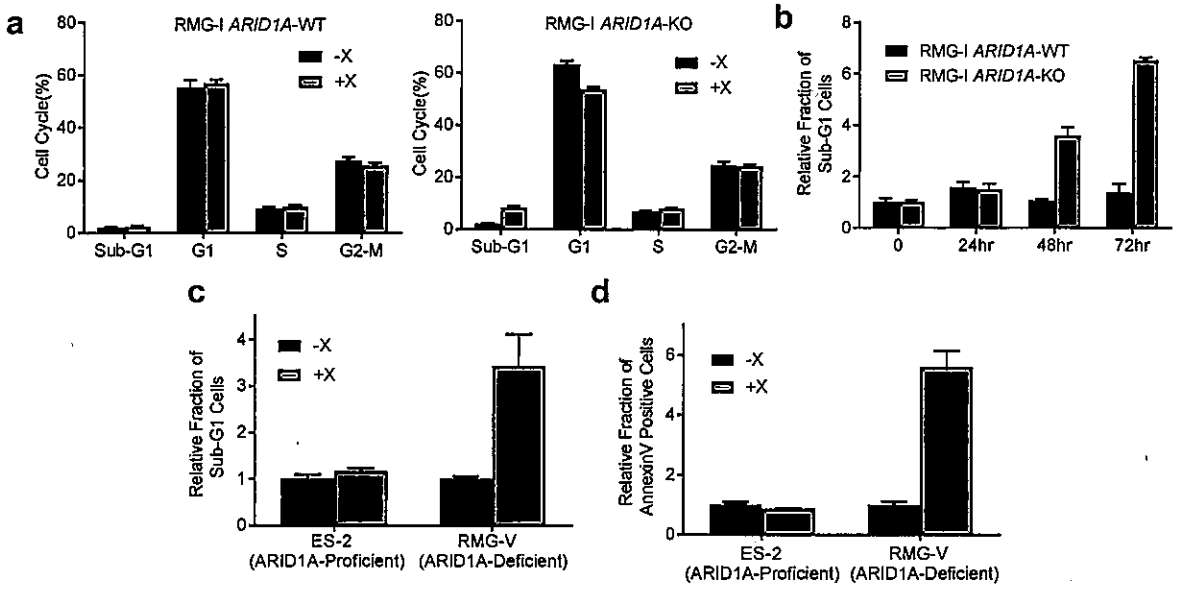
(b) 卵巣明細胞がん細胞株の全細胞画分の ARID1A と β-actin のウェスタンブロット。

(c) 薬剤 X 処理後の細胞生存率から算出した IC50 値（ARID1A 発現細胞株；黒、ARID1A 欠損細胞株；灰色）。平均値±標準誤差で表記（n = 3）。（***p < 0.001; Mann-Whitney U test)。

(d) TOV-21G 親細胞株（MOCK）と ARID1A 発現細胞株の無処理と薬剤 X 処理時の細胞生存率。平均値±標準偏差で表記。

(e)ARID1A 発現細胞株 ES-2 と ARID1A 欠損細胞株 JHOC-9 のマウス異種移植腫瘍モデルにおける薬剤 X 投与群（25 mg/kg 腹腔内投与）と非投与群の腫瘍の大きさ。平均値±標準誤差で表記 (n = 5) (**p < 0.01; Student's t-test)。

Figure 3. ARID1A 欠損卵巣明細胞がんにおいて、薬剤 X はアポトーシスを誘導する



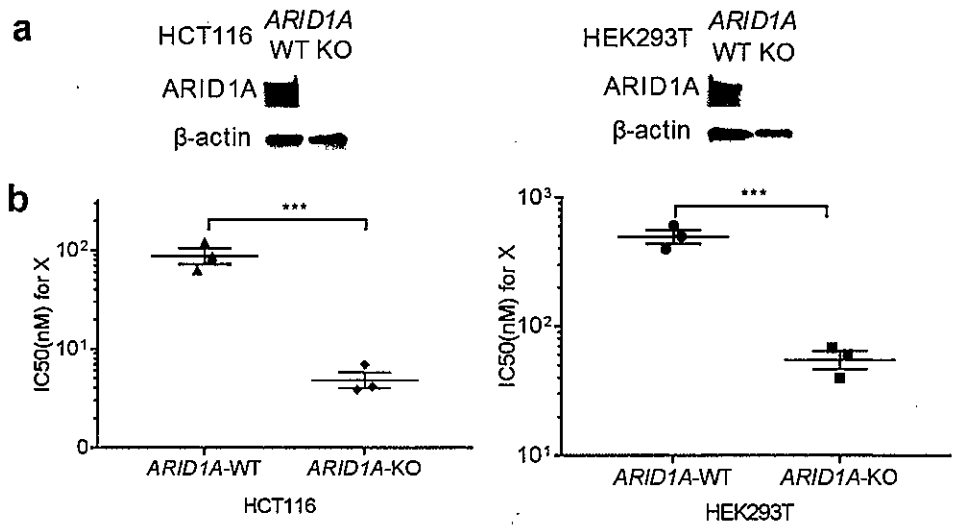
(a) 薬剤 X 非処理と薬剤 X 処理 48 時間後の RMG-I *ARID1A*-WT と *ARID1A*-KO の細胞周期の分布。平均値±標準偏差で表記。

(b) 無処理に対する薬剤 X 処理 24、48、72 時間後の RMG-I *ARID1A*-WT と *ARID1A*-KO における sub-G1 の比。平均値±標準偏差で表記。

(c) 薬剤 X 非処理と処理 48 時間後の ARID1A 発現細胞株 ES-2 と ARID1A 欠損細胞株 RMG-V における sub-G1 の比。平均値±標準偏差で表記。

(d) 薬剤 X 非処理と処理 48 時間後の ARID1A 発現細胞株 ES-2 と ARID1A 欠損細胞株 RMG-V における Annexin V 陽性細胞の比。平均値±標準偏差で表記。

Figure S1. ***ARID1A*-KO 細胞株は薬剤 X に選択的感受性を示す**



(a)全細胞画分の ARID1A と β-actin のウェスタンブロット（HCT116 *ARID1A*-WT、*ARID1A*-KO；左と HEK293T *ARID1A*-WT、*ARID1A*-KO；右）。
(b) 薬剤 X 処理後の細胞生存率から算出した IC50 値（*ARID1A*-WT and *ARID1A*-KO HCT116；左、*ARID1A*-WT and *ARID1A*-KO HEK293T；右）。 平均値±標準誤差で表記。(***p < 0.001; Student's t-test)。

5)考察

今回の研究は、卵巣明細胞がんの個別化治療の開発を目的とした。卵巣明細胞がんは、既存の白金製剤を中心とした標準治療法に対して抵抗性を示す卵巣がんの一つであり[2, 3]、PARP 阻害剤の効果と関連する BRCA 1 /BRCA2 変異を有しない[11, 12]。今回、卵巣明細胞がんで高頻度に認められ ARID1A 欠損に着目して研究を行った[15-18]。ARID1A 欠損は腫瘍細胞において生物学的・代謝的な特徴において、多くの遺伝子発現の制御に関与していると考えられており[19, 20]、我々は ARID1A 欠損が特定の抗がん剤に対して感受性を増強するかもしれないと仮説を立てた。複数の *ARID1A* ノックアウト細胞株、一般的に用いられる卵巣がん細胞株、マウス異種移植腫瘍モデルを用いて薬剤 X で感受性が増加することを示した。

ARID1A 欠損の頻度は高いものの、薬剤 X の感受性との関連に関しては報告がない。これは、卵巣がんにおいて卵巣明細胞がんは約 10%と希少であることによるものと考えられる。卵巣がんにおける大規模臨床試験において、卵巣明細胞がんは約 7%程度しか含まれていない。今後は、再発卵巣明細胞がんに対して薬剤 X を使用した症例を抽出し、奏功率、予後と ARID1A ステータスとの関連を調べていく。

薬剤 X は広く実臨床で使用されている薬剤であり、ARID1A 欠損と薬剤 X 感受性の関連を示唆する今回の研究は卵巣明細胞がんの標準治療における個別化治療法の確立に寄与する可能性がある。

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(2) シニア・リサーチフェロー期間中の研究成果を、今後の研究にどのように役立てたいと考えているか

もともと産婦人科医である経験を生かし、日本を含むアジアでは頻度が高いが、欧米では頻度が低く、大規模臨床試験でのデータに乏しく、既存の抗がん剤に抵抗性を示す卵巣明細胞癌に着目して研究を行った。特に卵巣明細胞癌の特徴である *ARID1A* 変異に対して、既存の抗がん剤の中で、薬剤 X に選択的感受性を示すことを確認した。

今後は、卵巣明細胞がんに対して薬剤 X を使用した症例を後方視的に集積し、ARID1A のステータスと、奏功率を比較し、リサーチフェロー期間中の研究成果と一致するかを確認する。確認できた場合、次に、前向き観察研究を計画していく。薬剤 X は主に再発後の 2 次治療以降に用いられることが多いが、現在卵巣がん治療において、既に使用できる薬剤である。現在保険適応となった「NCC オンコパネル検査」の 114 個の遺伝子に *ARID1A* も含まれていることから、現在の標準治療に対して治療抵抗性を示す ARID1A 欠損卵巣明細胞がんへ薬剤 X を早期に投与し、個別化治療の実臨床への応用を目指す。

今回のリサーチフェロー期間中に学んだ技術・知識を活かし、更なる進歩ができるように努力していきたい。

ARTICLE IN PRESS

Gynecologic Oncology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

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.

ARTICLE INFO

Article history:
Received 2 August 2019
Received in revised form
29 September 2019
Accepted 3 October 2019
Available online xxx

Keywords:
ARID1A
Ovarian clear cell carcinoma (OCCC)
Gemcitabine
Precision medicine
Predictive biomarker

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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https://doi.org/10.1016/j.ygyno.2019.10.002
0090-8258/

Please cite this article as: T. Kuroda et al., Therapeutic preferability of gemcitabine for ARID1A-deficient ovarian clear cell carcinoma, Gynecologic Oncology, https://doi.org/10.1016/j.ygyno.2019.10.002

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).

Please cite this article as: T. Kuroda et al., Therapeutic preferability of gemcitabine for ARID1A-deficient ovarian clear cell carcinoma, Gynecologic Oncology, https://doi.org/10.1016/j.ygyno.2019.10.002

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

Please cite this article as: T. Kuroda et al., Therapeutic preferability of gemcitabine for ARID1A-deficient ovarian clear cell carcinoma, Gynecologic Oncology, https://doi.org/10.1016/j.ygyno.2019.10.002

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.

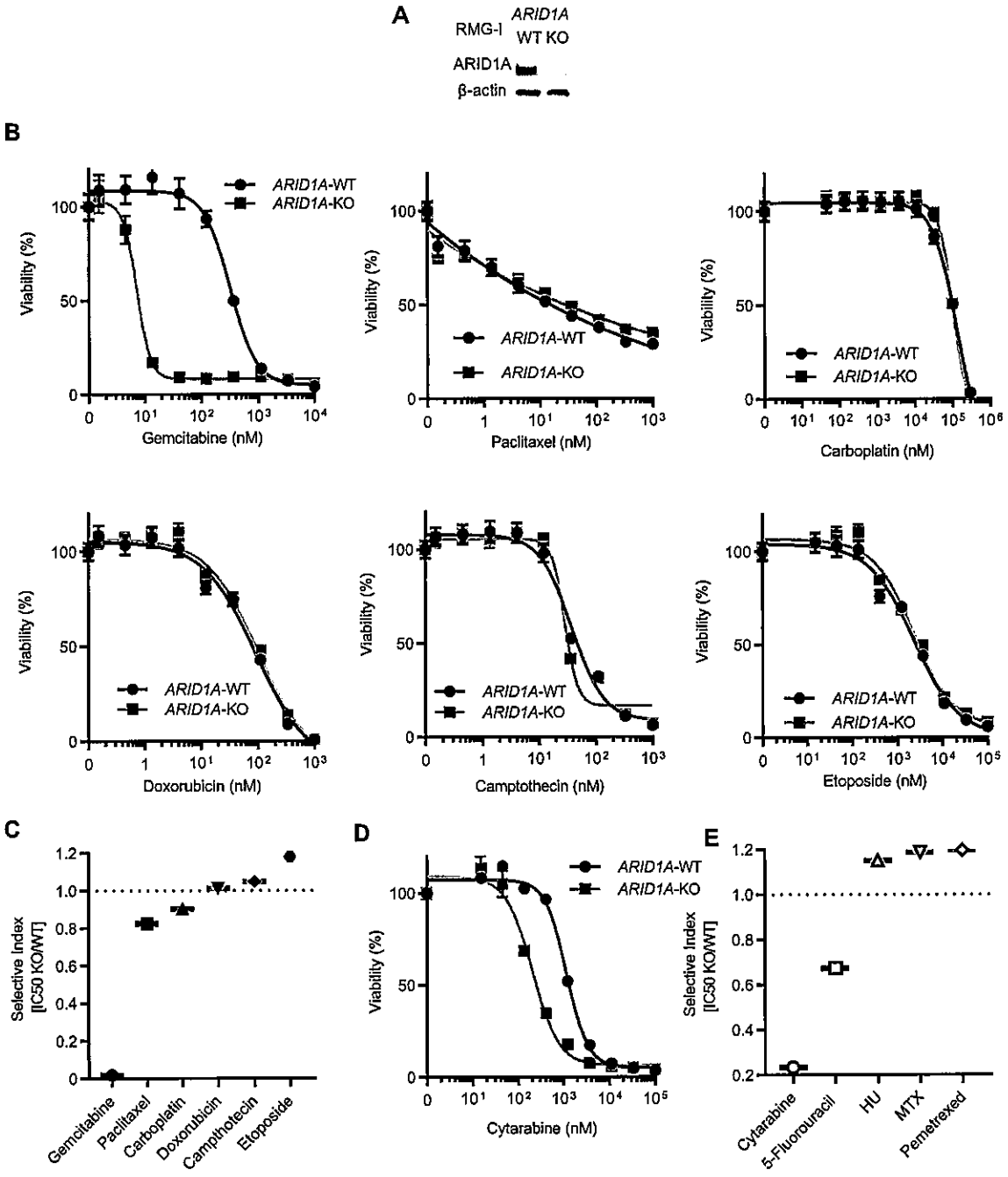

Fig. 1. *ARID1A*-KO cells are selectively sensitive to gemcitabine
(A) Immunoblotting of whole-cell extracts of *ARID1A*-WT and *ARID1A*-KO RMG-I OCCC cells for ARID1A and β-actin.
(B) Viability of *ARID1A*-WT and *ARID1A*-KO RMG-I cells after treatment with standard chemotherapeutic drugs used for OCCC. 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.

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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.

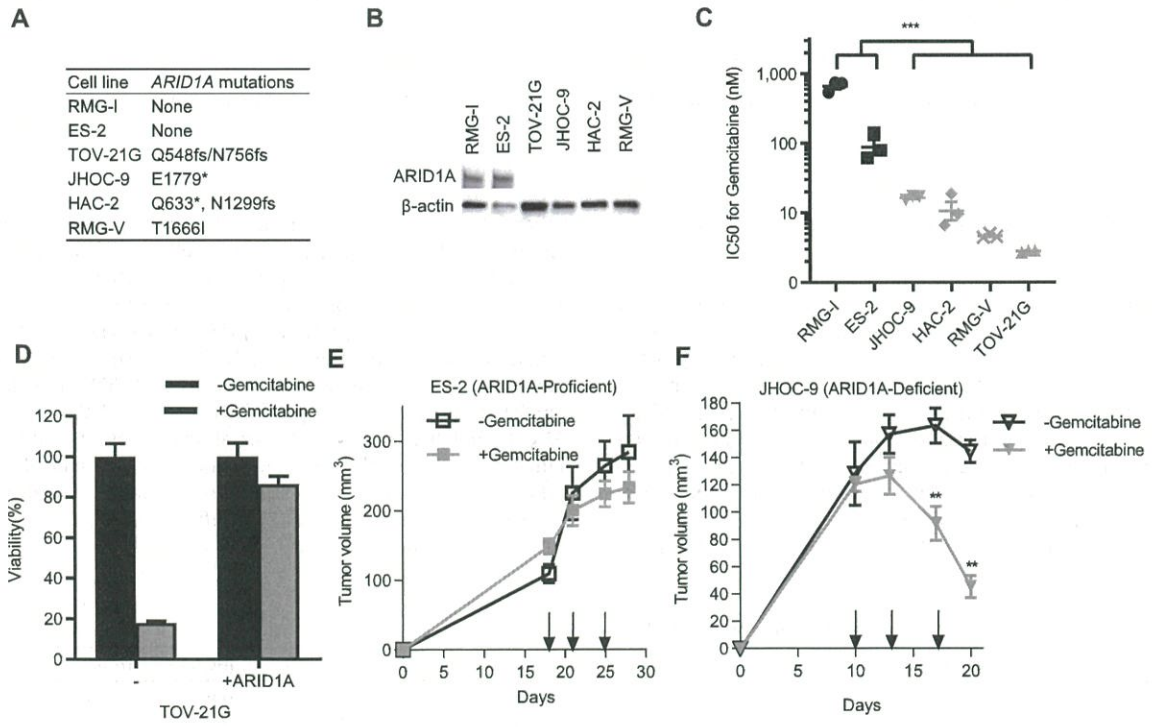

Fig. 2. Gemcitabine suppresses OCCC cell growth *in vitro* and *in vivo*

(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).

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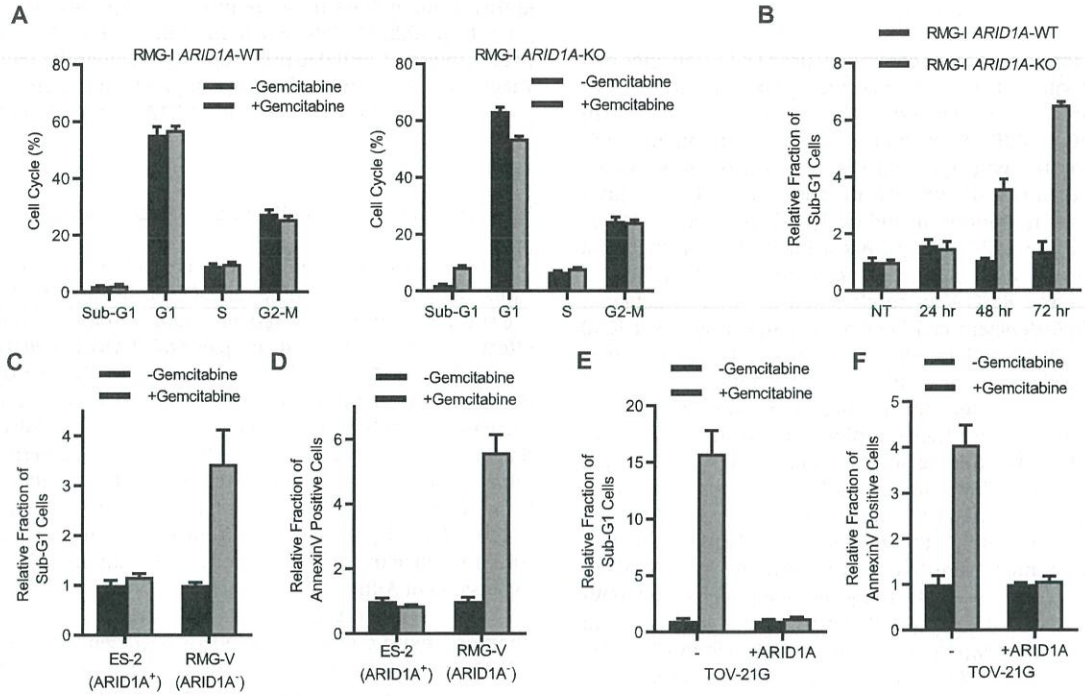

Fig. 3. Gemcitabine causes apoptosis in ARID1A-deficient OCCC cells

(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.

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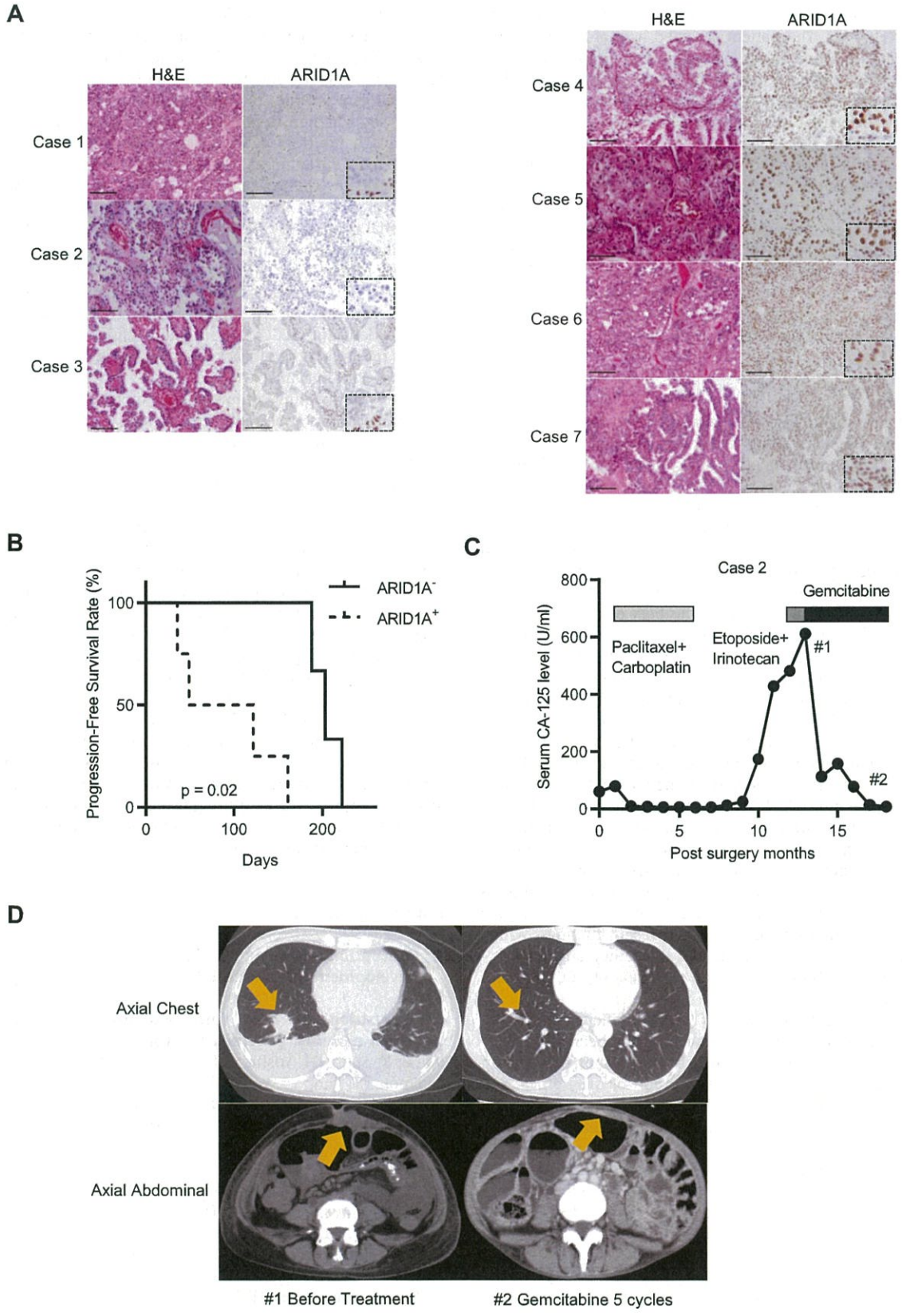

Fig. 4. Gemcitabine treatment response in ARID1A-deficient and ARID1A-proficient OCCC patients
(A) Immunohistochemical analysis of ARID1A protein in OCCC specimens. Scale bar, 100 μm
(B) Kaplan-Meier curves for PFS of ARID1A-proficient and ARID1A-deficient patients (*p = 0.02; log-rank test).
(C) Serum 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.

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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

Conception and design: T. Kuroda., H. Ogiwara.

Development of methodology: T. Kuroda., H. Ogiwara., M. Sasaki.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Kuroda., H. Ogiwara., K. Sudo., H. Yoshida., K. Tamura., T. Kiyokawa., T. Kato., A. Okamoto., T. Kohno.

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

Writing, review, and/or revision of the manuscript: T. Kuroda., H. Ogiwara., M. Sasaki., K. Takahashi., H. Yoshida., T. Kiyokawa., K. Sudo., K. Tamura., T. Kato., A. Okamoto., T. Kohno.

Administrative, technical, or material support **(i.e., reporting or organizing data, constructing databases):** T. Kuroda., H. Ogiwara., M. Sasaki., K. Takahashi., K. Sudo., H. Yoshida., T. Kohno.

Study supervision: H. Ogiwara., T. Kohno.

Declaration of competing interest

None.

Please cite this article as: T. Kuroda et al., Therapeutic preferability of gemcitabine for ARID1A-deficient ovarian clear cell carcinoma, Gynecologic Oncology, https://doi.org/10.1016/j.ygyno.2019.10.002

Acknowledgments

We thank Daito Noguchi, Sou Hirose, Ayako Kawabata, Toshiyuki Seki, Masataka Takenaka, Nozomu Yanaihara, Yoko Shimada, Hitoshi Ichikawa, and Naoto Tsuchiya for technical assistance and helpful comments. This work was supported in part by grants-in-aid from the Japan Society for the Promotion of Science (JSPS) KAKENHI [17K11310 to T. Kato and H. Ogiwara] and by the Practical Research for Innovative Cancer Control from the Japan Agency for Medical Research and Development [JP18ck0106374 to H. Ogiwara]. The National Cancer Center Biobank is supported by the National Cancer Center Research and Development Fund. This work was also supported in part by the Foundation for Promotion of Cancer Research in Japan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.10.002.

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Please cite this article as: T. Kuroda et al., Therapeutic preferability of gemcitabine for ARID1A-deficient ovarian clear cell carcinoma, Gynecologic Oncology, https://doi.org/10.1016/j.ygyno.2019.10.002

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Please cite this article as: T. Kuroda et al., Therapeutic preferability of gemcitabine for ARID1A-deficient ovarian clear cell carcinoma, Gynecologic Oncology, https://doi.org/10.1016/j.ygyno.2019.10.002