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Anti-tumor activity of dual inhibition of phosphatidylinositol 3-kinase and MDM2 against clear cell ovarian carcinoma

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HIGHLIGHTS

- High expression of both *PIK3CA* and *MDM2* was associated with poor prognosis in clear cell ovarian carcinoma.
- Dual inhibition of PI3K/mTOR and MDM2 showed synergistic anti-proliferative effects in CCOC cells without *TP53* mutations.
- The dual inhibition significantly reduced tumor volumes in mice xenograft models.
- The dual inhibition reduced tumor vascularity and increased apoptotic cell death.

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ABSTRACT

Introduction: PI3K pathway signaling has received attention as a molecular target in clear cell ovarian carcinoma (CCOC). MDM2 is one of the AKT effectors in the PI3K pathway, which binds to and degrades p53. In this study, we aimed to clarify the prognostic significance of *PIK3CA* and *MDM2* expression, and potential therapeutic effect of a dual inhibition of the PI3K pathway and MDM2.

Materials and methods: cDNA expression was evaluated by using microarray data using 75 samples of CCOC. DS-7423 (dual inhibitor of pan-PI3K and mTOR) and RG7112 (MDM2 inhibitor) were used on CCOC cell lines to evaluate cell proliferation, expression level of MDM2 related proteins, and apoptosis by MTT assay, western blotting, and flow cytometry. DS-7423 (3 mg/kg) and/or RG7112 (50 mg/kg) were orally administrated every day for three weeks, and the anti-tumor effect was evaluated using tumor xenografts, along with immunohistochemistry.

Results: Tumors with high expression of both *PIK3CA* and *MDM2* showed significantly worse prognosis in expression array of 71 CCOCs ($P = 0.013$). Dual inhibition of the PI3K pathway by DS-7423 and MDM2 by RG7112 showed synergistic anti-proliferative effect in 4 CCOC cell lines without *TP53* mutations. The combination therapy more robustly induced pro-apoptotic proteins (PUMA and cleaved PARP) with increase of sub G1 population and apoptotic cells, compared with either single agent alone. The combination therapy significantly reduced tumor volume in mice ($P < 0.001$ in OVISE, and $P = 0.038$ in RMG-I) without severe body weight loss. Immunohistochemistry from the xenograft tumors showed that the combination treatment significantly reduced vascularity and cell proliferation, with an increase of apoptotic cell death.

Conclusion: A combination therapy targeting the PI3K pathway and MDM2 might be a promising therapeutic strategy in CCOC.

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1. Introduction

The incidence of clear cell ovarian carcinoma (CCOC) represents approximately 25% of all epithelial ovarian cancers in Japan, although it does compared with only 4.8% in the United States [1]. CCOC is commonly endometriosis-associated and characterized by lower chemosensitivity, similar to those with other histological subtypes [2]. Patients with CCOC are conventionally treated with surgery and/or combination chemotherapy with paclitaxel and carboplatin, as well as those with other histological subtypes. However, the 3-year survival rate of CCOCs with residual tumors over 2 cm was only 10.2% [3]. Development of a novel therapeutic strategy is warranted to improve the prognosis of CCOC.

The Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway and TP53 signaling have been considered as candidate molecular targets in human cancers [4]. Catalytic subunit alpha of the PI3K (p110α) is a class I PI3K catalytic subunit, encoded by *PIK3CA* gene [5]. *PIK3CA* is frequently mutated or amplified in various types of cancers [6,7]. Especially, the ratio of *PIK3CA* mutation and amplification in CCOC is 45–55% and 18–33%, respectively [8–12]. Therefore, PI3K-AKT signaling pathway is broadly activated in CCOC. The activation of the PI3K pathway is involved in enhanced cellular motility, cell survival, and resistance to chemotherapies [13], and is anticipated as a therapeutic target of in CCOC [14,15].

TP53 is the most frequently mutated gene in human cancer, and induces expression of various target genes, involved in cell cycle arrest (G1 arrest or G2/M arrest), apoptosis induction, DNA repair, and senescence [4]. There is a crosstalk between the PI3K pathway and TP53 signaling. Murine double minute 2 (MDM2) is known to be one of the AKT effectors. AKT phosphorylates MDM2 (Ser-166, Ser-186 and Ser-188), which enhances Mdm2-mediated ubiquitination and degradation of p53 [16–18]. We previously reported that *MDM2* expression is higher in CCOC compared with that in the other histological subtypes, and that high expression of *MDM2* is a poor prognostic factor in CCOC[19]. We also found that RG7112 (an MDM2 inhibitor, which inhibits the binding between MDM2 and p53) significantly suppressed cell viability in CCOC cell lines with wild-type TP53 [19]. In addition, a dual inhibitor of pan-PI3K and mTOR, DS-7423, decreased the phosphorylation level of MDM2, and induced TP53-mediated apoptosis in TP53 wild-type CCOC cells [14]. These results imply the potential of the combination therapy targeting the PI3K signaling and MDM2- p53 binding in CCOC.

In this study, we aimed to clarify the correlation between expression levels of PIK3CA and MDM2 and prognostic outcomes in CCOC, and to evaluate the efficacy of the combination therapy of the PI3K/mTOR inhibitor (DS-7423) and the MDM2 inhibitor (RG7112) against CCOC cells with wild-type TP53.

2. Materials and methods

2.1. Tumor samples and microarray

We previously performed microarray analysis using CCOC samples (accession number: GSE65986) [20]. Briefly, surgical samples were obtained from the University of Tokyo Hospital and Saitama Medical University International Medical Center with the approval of institutional ethical committee. All 75 patients with CCOC gave written informed consent prior to surgery, and high carcinoma cell content was confirmed (>50%) by a pathologist. RNA was extracted from these 75 tumor specimens and hybridized as described previously [20]. Of these, 4 cases were excluded from the analysis because of insufficient information. Clinico-pathological characteristics of 71 patients were listed in Supplementary Table 1.

2.2. Cell lines and compounds

Three CCOC cell lines, OVISE, OVTOKO and RMG-I were purchased from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Osaka, Japan), and JHOC-7 was purchased from RIKEN Cell Bank (Ibaraki, Tsukuba, Japan). All cell lines were authenticated by STR analysis. RG-7112 (MDM2 inhibitor) was purchased from MedChem Express (Monmouth Junction, NJ, USA) and APEXBIO (Houston, TX, USA). DS-7423 {a dual inhibitor of pan-PI3K (P110alpha, beta, gamma and delta) and mTOR} was provided by the Daiichi-Sankyo Company, Ltd. (Tokyo, Japan) (Supplementary Fig. 1) [14].

2.3. MTT assay

MTT assay was performed using Cell Counting Kit-8 with the tetrazolium salt WST-8 (Dojindo; Minatoku, Tokyo, Japan). Cells were seeded on the medium in 96-well plates at densities of 2000 cells/well for 72 h. Proliferation was quantified by monitoring the changes of absorbance using a microplate reader (Bio Tek, Winooski, VT, USA). Each data point was normalized using the values of control wells (with medium and reagents alone) in each experiment.

2.4. Immunoblotting

The cell lines were treated with each compound for 6 h and were lysed as described previously [21]. Protein separated by SDS-PAGE and transferred into membranes were probed with antibodies specific for MDM2, p21, p53 (Santa Cruz; Dallas, TX, USA), phosphorylated p53 (Ser46), phosphorylated p53 (Ser15), cleaved PARP, PUMA, S6, phosphorylated S6 (Ser240/244), Akt, phosphorylated Akt (Ser473) (Cell Signaling Technology; Danvers, MA, USA), survivin (Novus Biologicals; Littleton, CO, USA), and beta-actin (Sigma-Aldrich; St. Louis, MO, USA).

2.5. Cell cycle analysis

Cells (5×10 [5]) were seeded in 60-mm dishes with each compound for 72 h and collected by trypsinization. The cells were washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol and kept overnight at 4 °C. The fixed cells were washed in PBS, resuspended in PBS containing 0.25 mg/mL RNase A (Sigma-Aldrich) for 30 min at 37 °C, incubated with 50 μg/mL propidium iodide (Sigma-Aldrich) at 4 °C for 30 min in the dark, and analyzed on FACS Calibur HG (Becton, Dickinson and Company). Data were analyzed in CELL Quest pro ver. 3.1. (Becton, Dickinson and Company). The experiment was repeated three times.

2.6. Detection of apoptosis

Cells (5×10 [5]) were seeded in 60-mm dishes with each compound for 72 h. After collection by trypsinization, the cells were washed and incubated with annexin V-fluorescein isothiocynate and propidium iodide according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences), then analyzed on FACS Calibur HG. The experiment was repeated three times.

2.7. Tumor xenografts in nude mice

Specific pathogen-free female nude mice (BALB/cAJc1-nu/nu) were purchased from CLEA Japan, Inc. (Meguro, Tokyo, Japan). Nude mice bearing OVISE or RMG-I tumor xenografts were established as described previously [17]. The mice were randomly assigned into four groups of 5–6 mice and received an oral daily gavage of compound for 3 weeks. Three times weekly tumor growth was measured and volume was calculated according to the formula ([major axis] × [minor

axis]^2)/2. Toxicity was assessed based on the weight change of treated mice. After sacrifice, each tumor was resected and partially frozen for the extraction of DNA or RNA. The other piece was fixed in 4% paraformaldehyde for immunoblotting analysis. Our animal experiment was conducted under the approval of institutional animal care and use committee in University of Tokyo (#Med-H18–102).

2.8. Immunohistochemistry

Isolated tumor fragments were fixed and stained using CD31 antibody as described previously [19]. The number of micro-vessels was counted at 400× in four random fields in each tumor.

2.9. Immunofluorescence of 5-Bromo-2-deoxyuridine (BrdU) staining

Undiluted BrdU labeling reagent (Roche; Switzerland) was intraperitoneally injected into mice. After 2 h, mice were sacrificed, and the number of BrdU-positive cells was counted at 400× in four random fields in each tumor.

2.10. TUNEL staining

The frozen tissue sections were fixed with 4% paraformaldehyde for 15 min at room temperature. After an endogenous peroxidase block in H_2O_2 methanol, the sections were incubated on ice for 5 min with permeabillisation buffer (TaKaRa/MK505), then treated with TdT Enzyme (TaKaRa/MK502) and Labeling Safe Buffer (TaKaRa/MK502) for 60 min at 37 °C, followed by the administration of anti FITC HRP conjugate (TaKaRa/MK503) for 30 min at 37 °C. Finally, tumors were stained with 3, 3-diaminobenzidine (DAKO) and hematoxylin (Wako).

2.11. Statistical analysis

Survival impact of mRNA expression of *PIK3CA* and *MDM2* was evaluated using Kaplan-Meier method and log-rank test. The statistical analysis *in vitro* and *in vivo* was conducted using *t*-test. The definition of synergism was calculated by using the Chou–Talalay method. Combination index (CI) of Chou-Talalay defines Synergism (CI < 1), Additive Effect (CI = 1) and Antagonism (CI > 1). Statistical analyses were performed using JMP v11 (SAS, Cary, NC) and GraphPad Prism 6 (La Jolla, CA). In all these tests, differences were considered to be significant at $P < 0.05$. We also compared 4 groups (vehicle, DS-7423, RG-7112, and DS-7423 + RG-7112) in the xenograft studies using two-way analysis of variance (ANOVA).

3. Results

3.1. Exclusive mRNA expression levels and survival impact of *PIK3CA* and *MDM2*

We first evaluated mRNA expression levels of *PIK3CA* and *MDM2* in 71 clear cell carcinomas by expression array. The expression level

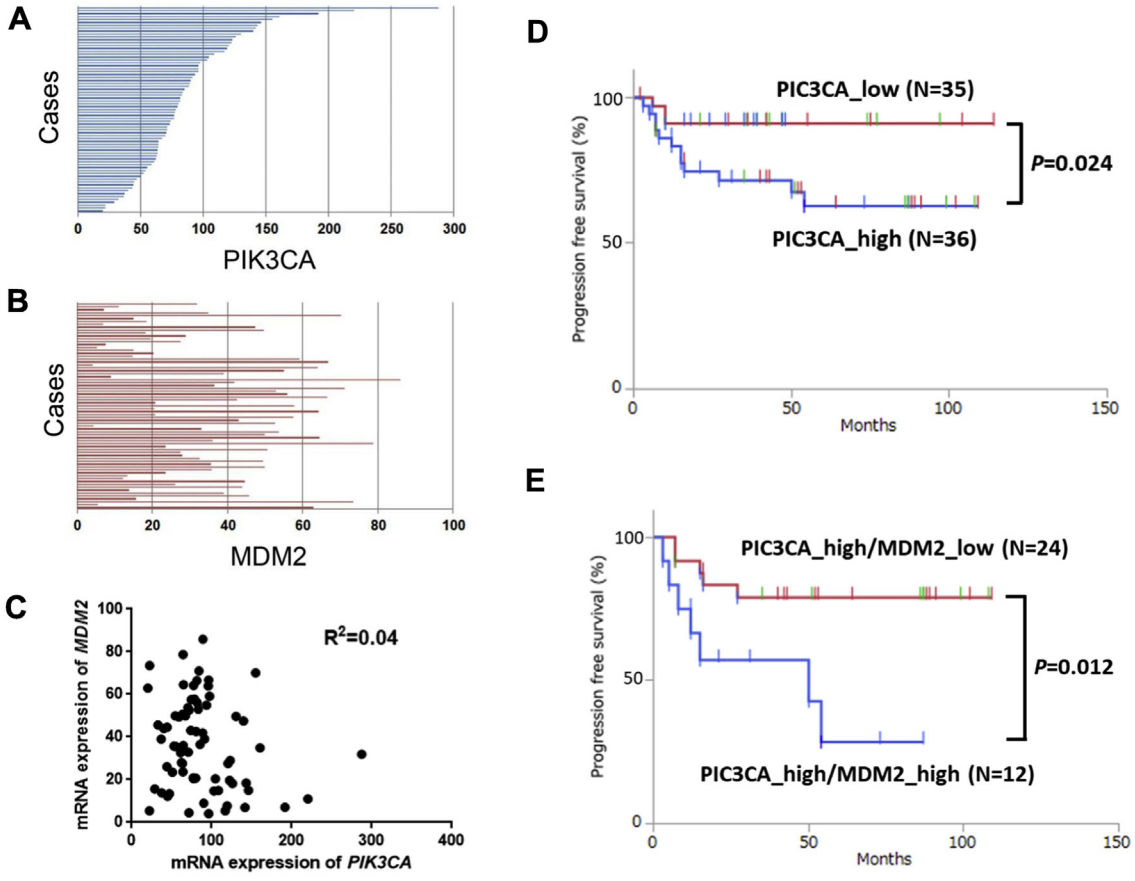

Fig. 1. mRNA expression levels of *PIK3CA* and *MDM2* status, and their prognostic significance in CCOC
(A, B) mRNA expression levels of *PIK3CA* (left) and *MDM2* (right), obtained from microarray dataset.
(C) Correlation of mRNA expression levels between *PIK3CA* and *MDM2*
(D, E) Prognostic significance of expression levels of *PIK3CA* and *MDM2* in CCOC, evaluated by Kaplan-Meier method and log-rank test. High expression of *PIK3CA* was defined as more than median value. High expression of *MDM2* was defined as >2-fold of the median value of the 17 normal tissues, as described previously [19]. Progression free survival was compared between the two groups: *PIK3CA*-high and *PIK3CA*-low in (D), and *PIK3CA*-high/*MDM2*-high and *PIK3CA*-high/*MDM2*-low in (E).

Table 1
Univariate/Multivariate analysis in CCC by age, stage, and *MDM2&PIK3CA* expression.

Characteristics		Univariate			Multivariate		
		HR	95%CI	*P*-value	HR	95%CI	P-value
Progression free survival							
Age	≥50 <50 (ref)	1.69	0.63–5.86	0.32	0.97	0.33–3.54	0.96
Stage	III/IV I/II (ref)	5.66	2.41–13.29	0.0001	6.42	2.48–16.87	0.0002
MDM2&PIK3CA	High Low (ref)[a]	5.94	2.089–14.94	0.0017	6.11	2.08–16.11	0.0019
Overall survival							
Age	≥50 <50 (ref)	1.67	0.54–7.25	0.40	0.52	0.13–2.55	0.39
Stage	III/IV I/II (ref)	8.84	3.34–25.78	<0.0001	13.68	4.30–51.57	<0.0001
MDM2&PIK3CA	High Low (ref)[a]	3.98	1.10–11.68	0.037	4.29	1.14–13.60	0.033

[a] Low: low expression of either MDM2 or PIK3CA or both genes.

of *MDM2* was independent from that of *PIK3CA* ($R^2 = 0.04$; Fig. 1A–C), although MDM2 is phosphorylated *via* activation of the PI3K pathway. High mRNA expression of *PIK3CA* (n = 36) was significantly associated with shorter progression free survival (PFS) ($P = 0.024$, Fig. 1D). As high expression of *MDM2* was also associated with shorter PFS[19], we compared the prognosis between *MDM2*-high (n = 12) and *MDM2*-low (n = 24) in the *PIK3CA*-high CCOCs. PFS of the *MDM2*-high was significantly shorter than that of the *MDM2*-low in this setting ($P = 0.012$, Fig. 1E). There was a trend towards shorter overall survival (OS) in cases with high mRNA expression levels of *PIK3CA* (n = 36) ($P = 0.067$, Supplementary Fig. 2A). OS was significantly shorter in cases for which the mRNA expression levels of both *MDM2* and *PIK3CA* were high ($P = 0.004$, Supplementary Fig. 2B). CCOCs with high expression of both *PIK3CA* and *MDM2* (n = 12) showed significant poor PFS ($p = 0.0017$) and overall survival (OS) ($p = 0.037$), compared with the remaining CCOCs (n = 59) by univariate analysis (Table 1). Multivariate analysis showed that high expression of both *PIK3CA* and *MDM2* is a prognostic factor, independent from stage and age (PFS; $P = 0.0019$, 95%CI; 2.08–16.11, OS; $P = 0.033$, 95%CI; 1.14–13.6) (Table 1). These results highlighted the importance of activity of both MDM2 and PIK3CA in CCOC.

3.2. In vitro evaluation of MDM2 and PI3K inhibition against CCOC without TP53 mutations

We evaluated anti-proliferative effect of dual inhibition of PI3K/mTOR (by DS-7423) and MDM2 (by RG7112), using four CCOC cell lines without *TP53* mutations. The maximal inhibitory concentration (IC_{50}) values of either DS-7423 or RG7112 alone were summarized in Table 2. Addition of DS-7423 (0,0625, 2.5, 10, 40, 200, 800, or 2500 nM) to RG7112 (0.625 uM or 1.25 uM) significantly reduced cell viability in 4 CCOC cell lines (Fig. 2A, Supplementary Fig. 3A). The combination index by Chou–Talalay method were <1.0 in all these 4 cell lines (0.571–0.725) (Table 2), indicating the synergistic effect of these two inhibitors.

We confirmed the expression levels of related molecules in examined using OVISE and OVTOKO (Fig. 2B, Supplementary Fig. 3B). The levels of MDM2, p53 and phospho- p53 (Ser-15 and Ser-46) were increased by RG7112, whereas the levels of phospho-AKT and phospho-S6 were decreased by DS-7423. The combination of DS-7423 (156 nM) and RG7112 (2.5 or 5 uM) induced the expression of pro-apoptotic proteins, such as PUMA and cleaved PARP (Fig. 2B). The expression of p21 was induced by RG7112. The combination increased the ratio of sub-G1 fraction in OVISE and RMG-I cells at MTT assay (from 1.6% to 50.6% and from 3.7% to 26.4%, respectively) (Fig. 2C). Furthermore, the combination significantly increased the number of apoptotic cells by annexin V staining (Fig. 2D). These results were also confirmed in the other 2 cell lines (Supplementary Fig. 3C and 3D).

3.3. Antitumor activity of MDM2 and PI3K inhibition in vivo

The *in vivo* antitumor activity of the combination was examined by using mice xenograft models of OVISE and RMG-I cells. DS-7423 (3 mg/kg) and RG7112 (50 mg/kg) were orally administered daily for three weeks. Although a single agent of either DS-7423 or RG7112 by itself significantly decreased the tumor volume compared with a control (vehicle), the combination of these 2 agents more robustly reduced the tumor volume and tumor weight without causing body weight loss in both OVISE and RMG-I cells (Fig. 3A–F). MDM2, p53, and p21 were upregulated in tumors treated with the combination of DS-7423 and RG7112 (Supplementary Fig. 4A and 4B). As DS-7423 dephosphorylates MDM2, which results in the stabilization of p53 [14], the combination of RG7112 and DS-7423 may contribute to a more robust induction of p53.

3.4. Reduction of tumor vascularity and induction of apoptosis by the dual inhibition of MDM2 and PI3K/mTOR

We previously reported that the amount of microvessels was significantly reduced by the treatment of RG7112 *in vivo* [19]. We performed immunohistochemistry of CD31 to evaluate the effect on tumor vascularity in the xenograft mice by the treatment of the dual inhibition of DS-7423 and RG7112. This combination treatment significantly reduced the number of CD31 positive cells, compared with either single agent alone (Fig. 4A and Supplementary Table 3). Cell proliferative activity was evaluated by BrdU staining. The ratio of BrdU positive cells was also significantly decreased in the xenograft tumors treated with DS-7423 and RG7112, compared with those treated with vehicle and either single agent alone (Fig. 4B and Supplementary Table 4). Finally, the number of apoptotic cells was evaluated by TUNEL staining. The ratio of TUNEL stained cells was significantly higher in tumors

Table 2
IC50 values and combination Index (CI) of RG7112 and DS-7423 in CCC cell lines.

	TP53	DS-7423 (nm)	RG-7112 (μm)	CI
OVISE	Wild type	100	1	0.725
RMG-I	Wild type	80	1.1	0.576
OVTOKO	Wild type	40	2	0.725
JHOC-7	Wild type	100	2.2	0.571

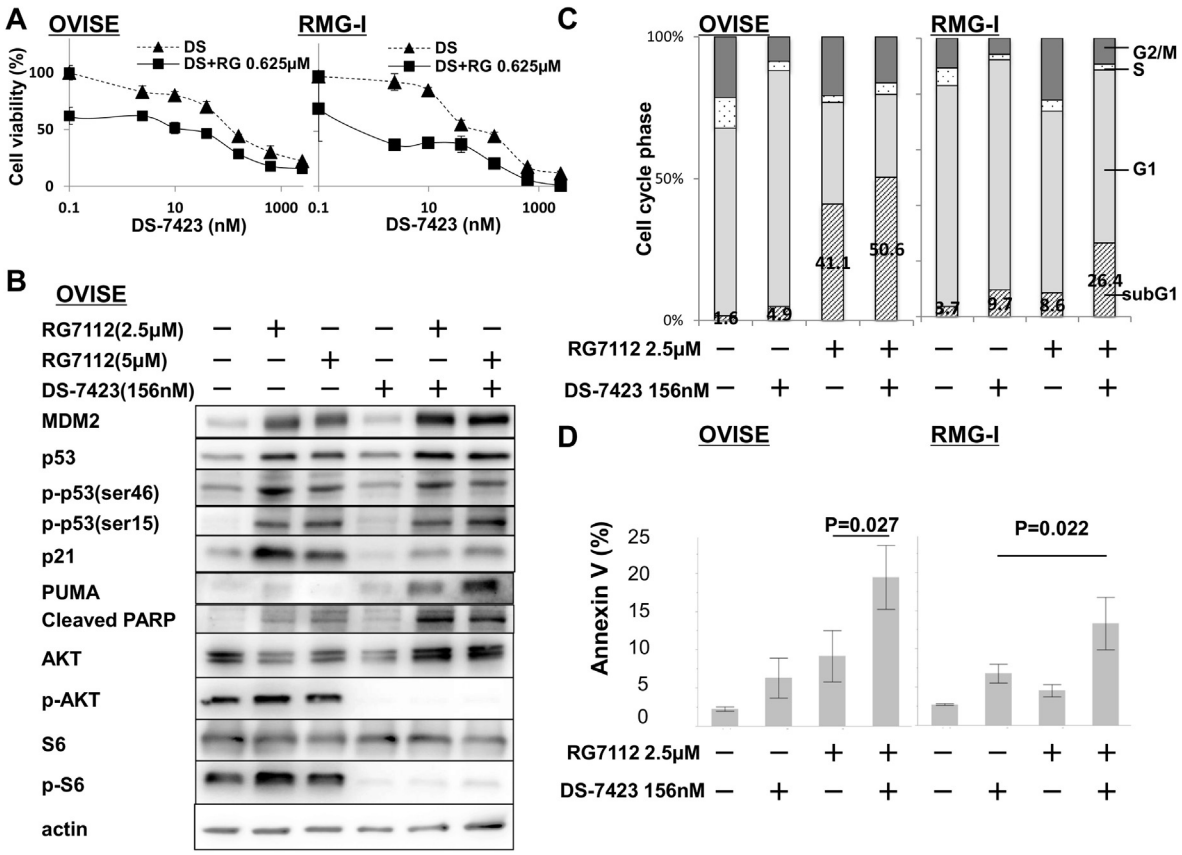

Fig. 2. Synergic effect of DS-7423 in combination with RG7112, *in vitro*
(A-D) *in vitro* effect of DS-7423 in combination with RG7112 using OVISE (and RMG-I) cell lines was examined about (A) cell viability, (B) expression of p53 and PI3K target proteins (C) cell cycle, and (D) apoptosis. *t*-test was used in (D).

treated with the combination of DS-7423 and RG7112, compared with vehicle or either single agent alone (Fig. 4C and Supplementary Table 5).

4. Discussion

In this study, we focused on CCOC and identified that (i) coexistence of high mRNA expression of *PIK3CA* and *MDM2* served as a significant and independent poor prognostic factor, (ii) dual inhibition of the PI3K pathway and MDM2 showed anti-tumor effect with induction of apoptotic cell death *in vitro,* and (iii) the dual inhibition showed *in vivo* anti-tumor effect with reduction of microvessels and cell proliferation, as well as induction of apoptotic cell death.

The PI3K signaling pathway plays key roles in various physiological and cellular processes, including cell proliferation, growth, survival, migration and metabolism [22]. However, the prognostic impact of the *PIK3CA* alterations is still controversial. *PIK3CA* amplification was shown as a poor prognostic factor in gastric, breast, esophageal, head and neck cancer and liposarcoma [23–28]. In CCOC, *PIK3CA* mutation and/or its overexpression by immunohistochemistry were suggested as a favorable prognostic factor in two studies [10,29], which was not compatible with our findings. The discrepancy may be caused by the method of evaluation (mRNA or protein expression, mutation or overexpression) and the cut-off criteria. The PI3K pathway can be activated by various genetic alterations, including *PIK3CA* mutation, *PIK3CA* amplification or any other alterations in this pathway (such as *PTEN* mutation and overexpression of receptor tyrosine kinases) [30]. Therefore, the prognostic impact in this pathway may vary according to the types of alterations (mutation or amplification) and cancers. Taken together with our findings and previous studies in other cancers, over-expression of *PIK3CA* may contribute to the tumor aggressiveness in multiple tumors. Further study is warranted to clarify the key prognostic factors in this pathway.

The PI3K activation enhances Mdm2-mediated ubiquitination and degradation of p53 [31,32], however, no correlation was found between the mRNA expression level of *PIK3CA* and that of *MDM2* in CCOC. This may be explained by several reasons; (i) AKT activates MDM2 mainly *via* its phosphorylation, not *via* its overexpression, (ii) the other PI3K pathway inputs (such as *PIK3CA* mutation) are common in CCOC, and (iii) MDM2 is also regulated by the PI3K independent signaling, such as DNA-damage activate kinases and ribosomal stress [33,34]. Therefore, co-targeting the PI3K pathway and MDM2 may be broadly effective to CCOC with wild-type TP53. Considering the prognostic impact, co-targeting these two signaling may be more beneficial to CCOC patients with co-expression of high *PIK3CA* and *MDM2*.

We previously reported that either PI3K/mTOR inhibition by DS-7423 or MDM2 inhibition by RG7112 significantly showed anti-tumor effect in CCOC cell lines [14,19]. However, the effect of either single inhibition alone was still modest with limited fraction of apoptotic cell death [14,19]. One possible reason of the limitation of a single MDM2 inhibitor may be explained by the activation of the PI3K pathway. An MDM2 inhibitor, Nutlin-3, was shown to induce apoptotic cell death in leukemia cells with wild-type *PTEN*, but not in those with *PTEN* mutations [35]. Taken together with our previous findings, the PI3K pathway may be a key cell survival signaling against the TP53 wild-type cancer cells. Indeed, the apoptotic cell death was synergistically induced by the

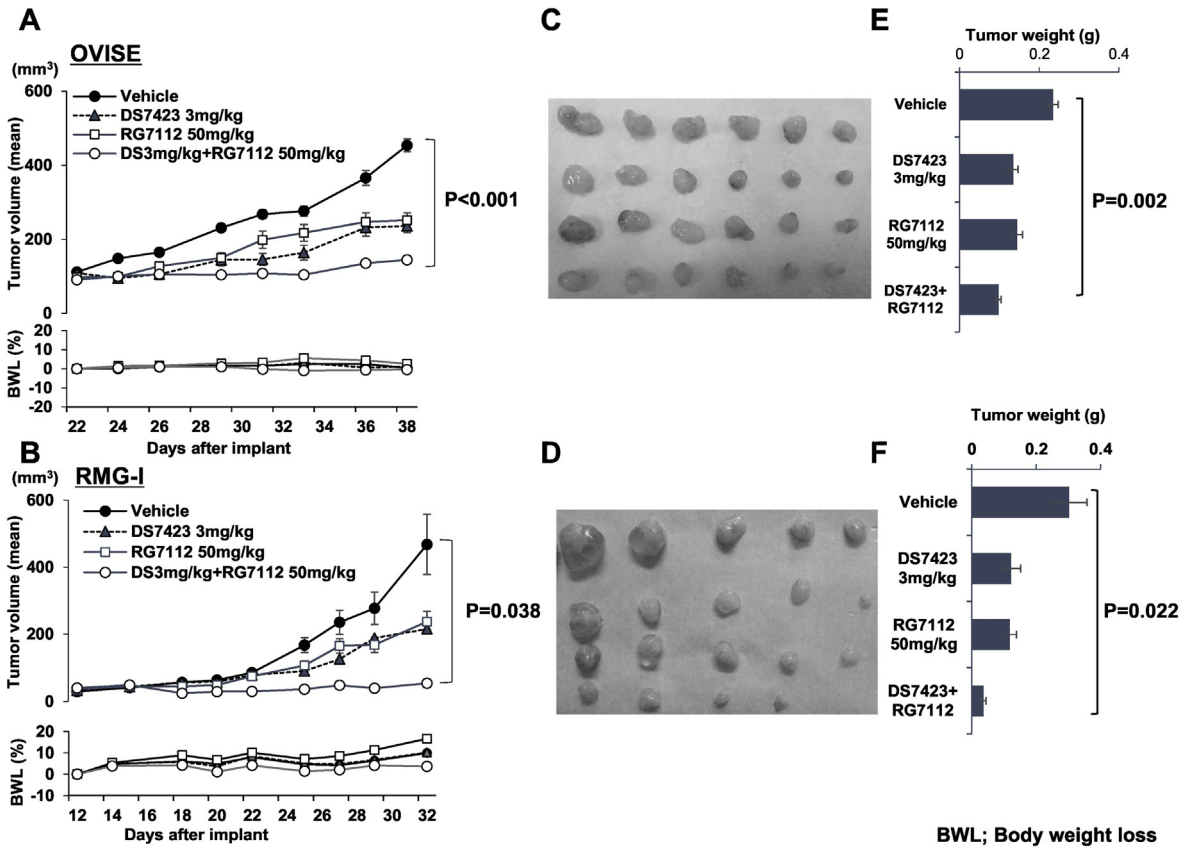

Fig. 3. Synergic effect of DS-7423 in combination with RG7112, *in vivo*
(A, B) Monitoring of tumor size during the treatment of DS-7423, RG7112 and combination of these 2 agents. (C, D) Pictures of tumors after the termination of treatment for 21 days. (E, F) Comparison of tumor weight by *t*-test. The experiments were performed in OVISE (A, C, E) and RMG-I (B, D, F) cell lines.

combination of DS-7423 and RG7112 in our *in vitro* and *in vivo* study. In addition to the cytotoxic effect, cytostatic anti-tumor activities were also identified by this combination treatment. The combination suppressed both proliferation and tumor vascularity *in vivo*.

We previously reported that RG7112 suppresses the hypoxia-induced expression of HIF-1alpha in CCOC cells with wild type TP53 [19]. These data suggested that RG7112 might inhibit tumor vascularization by the stabilization of TP53, which suppresses HIF-1alpha. Indeed, a significant correlation between the number of CD31 positive cells and RG7112 treatment was observed in our study. AKT and mTOR are involved in cell proliferation by phosphorylating various effectors (such as GSK3beta, 4E-BP1 and S6K), and mTOR activates angiogenic signaling by induction of HIF-1alpha and VEGF [36,37]. Therefore, dual inhibition of the PI3K/mTOR pathway and MDM2 may augment various types of anti-tumor activities in cancer cells with wild-type TP53. One of the biggest concerns combining these two small molecule agents is toxicity; however, the combination of DS-7423 and RG7112 showed no significant loss of body weight in mice in this study.

Our study has several limitations. Neither dual PI3K/mTOR inhibitor nor MDM2 inhibitor has been clinically approved in human cancers even as a single agent, although mTOR inhibitors (such as everolimus and temsirolimus) and several PI3K-isoform selective inhibitors (idelalisib, copanlisib, and duvelisib) have been approved in certain human malignancies [38–40]. Therefore, the types of inhibitors targeting the PI3K/mTOR pathway should be further explored in the combination therapy with an MDM2 inhibitor. In addition, the anti-tumor activity of this combination treatment has not been tested in other cancer types. The biomarker(s) for the co-targeting treatment should be further elucidated.

In conclusion, we identified the poor prognostic impact of coexistent high mRNA expression of *PIK3CA* and *MDM2*, and showed the synergistic anti-tumor effect by the combination therapy targeting the PI3K pathway and MDM2 in CCOC. Our findings highlight these two signaling pathways as potent molecular targets in CCOC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.08.028.

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Declaration of competing interest

Drs. Takahiko Seki, Mana Hatanaka and Yasuhide Hirota are employees of Daiichi Sankyo Co. Ltd. DS-7423 is in clinical

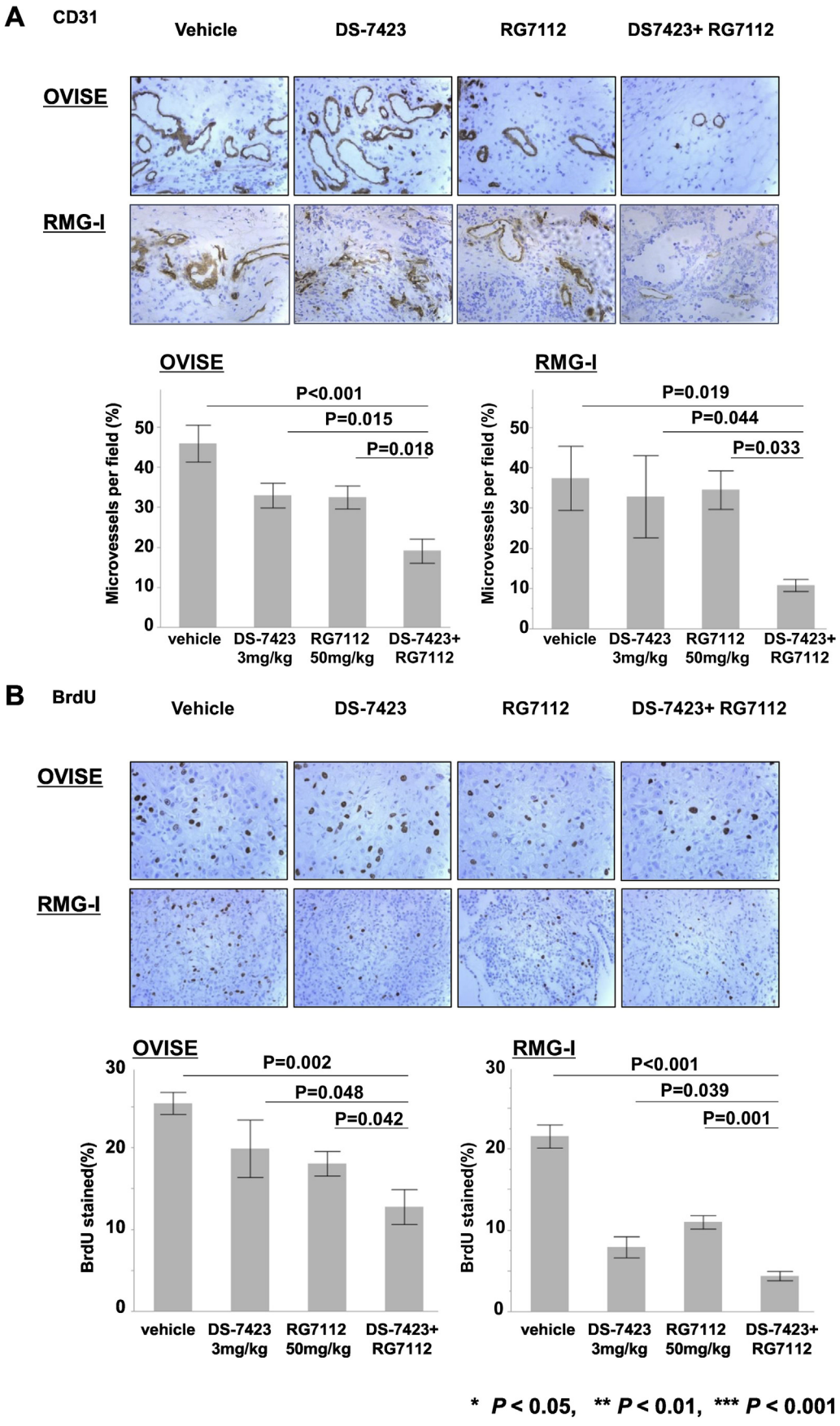

Fig. 4. Tumor vascularity, cell proliferation and apoptosis, evaluated by immunohistochemistry after treatment of DS-7423 and/or RG7112 in mice, inoculated with OVISE or RMG-I cells. Immunohistochemistry of (A) CD31 (B) BrdU and (C) TUNEL to assess the tumor vascularity, cell proliferation and apoptosis, respectively.

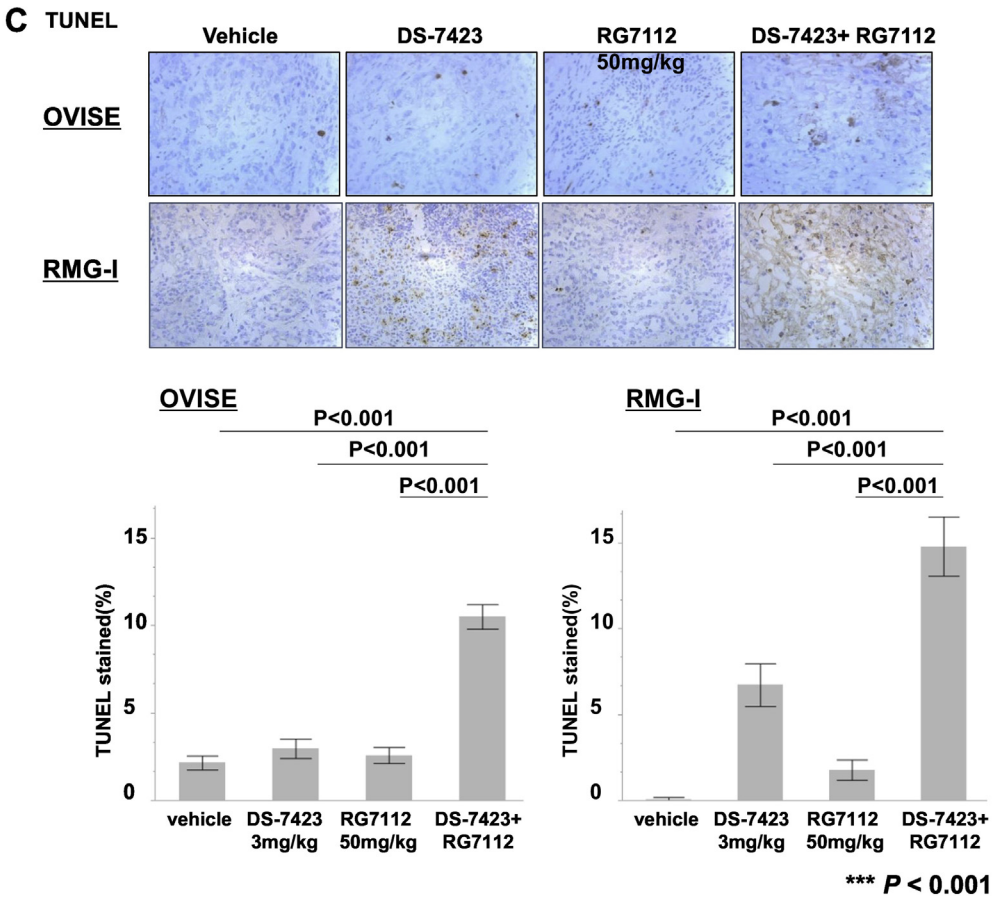

Fig. 4. (*continued*).

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Author contributions

CM, YI and KO conducted main experiments, collected the data, and wrote the manuscript. YU, AN, TK, and HA analyzed the data of expression array in clinical samples. YK, TK, AM, KS, MT and KN conducted experiments and facilitated data analyses. TT, MM, YM, OW-H, KK, KH and KF facilitated clinical data acquisition and interpretation of the data. YO and TF facilitated study conception and study design. All authors provided final approval of the manuscript and agree to the accuracy and integrity of this work.

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