

Original Article

Everolimus following 5-aza-2-deoxycytidine is a promising therapy in paclitaxel-resistant clear cell carcinoma of the ovary

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Abstract: Our previous study showed that 5-aza-2-deoxycytidine (5-aza-dC) could inhibit tumor growth by enhancing the susceptibility of ovarian clear cell carcinoma (OCCC) to paclitaxel through decreasing AKT/mTOR expressions. The objective of the study is to evaluate the antitumor efficacy of everolimus (RAD001) and 5-aza-2-deoxycytidine (5-aza-dC) by targeting AKT/mTOR and EZH2 in OCCC. Paclitaxel-sensitive and resistant OCCC cell lines were established. *In vitro* proliferative and apoptotic assays and flow cytometry were performed. The expressions of EZH2, PIK3IP1, phospho-AKT, phospho-mTOR and phospho-Rictor in the OCCC cell lines were evaluated by Western blotting. *In vivo* animal experiments with RAD001 and 5-aza-dC were performed. RAD001 alone showed significant *in vitro* antitumor activity and inhibited *in vivo* tumor growth in paclitaxel-sensitive and resistant OCCC cells. In addition, 5-aza-dC enhanced the antitumor effects when combined with paclitaxel or RAD001 in both paclitaxel-sensitive and resistant tumors. Activation of phospho-AKT ser473 and PIK3IP1 was observed in RAD001-treated paclitaxel-sensitive and resistant OCCC cells. In contrast, inhibition of phospho-AKT ser473 and EZH2 was observed with RAD001 following 5-aza-dC treatment of paclitaxel-sensitive and resistant OCCC cells. Furthermore, RAD001 following 5-aza-dC enhanced apoptosis of paclitaxel-sensitive and resistant OCCC cells. RAD001 following 5-aza-dC may be a promising treatment strategy for the treatment of both chemo-sensitive and resistant OCCC. Further clinical studies are warranted.

Keywords: Ovarian clear cell carcinoma, everolimus, 5-aza-2-deoxycytidine, AKT/mTOR

Introduction

Ovarian carcinoma is the fourth most common cause of cancer deaths among women in the United States [1]. Cytoreductive surgery followed by platinum-based chemotherapy is the standard initial treatment, and has improved the survival of patients with ovarian cancer [2]. Ovarian clear cell carcinoma (OCCC) accounts for 12% of patients with ovarian cancer, and it is the second leading cause of death from ovarian cancer [3]. It is the second most common subtype in North America, and the overall incidence is higher in Taiwan and Japan [1, 4, 5]. A combination of paclitaxel and platinum is currently the gold standard treatment regimen for

ovarian cancer [6], and it is used to treat all subtypes of ovarian neoplasms including OCCC. Compared to ovarian serous carcinoma, OCCC is relatively resistant to platinum and taxane-based chemotherapy, and this chemo-resistance is associated with poor prognosis [4, 5, 7, 8]. With regards to second-line or salvage therapy, the response rate for recurrent or refractory OCCC is extremely low compared to other histologic tumors, and even in patients with platinum-sensitive OCCC, the reported response rate is lower than 10% [9]. Therefore, in order to improve the survival of patients with OCCC, the development of novel strategies for both first-line and salvage treatment of recurrent disease are urgently needed. We recently

reported that methylation of HIN-1 promoter is a novel epigenetic biomarker associated with poor outcomes in patients with OCCC, and that the ectopic expression of the HIN-1 gene increased sensitivity to paclitaxel partly through the Akt pathway [10]. Akt targets several substrates and promotes protein synthesis and cell growth through phosphorylation of the negative regulator of mTOR, the TSC1/TSC2 complex [11]. mTORC1 activation has been reported to positively regulate mRNA translation initiation through its downstream effectors p70S6K and 4EBP1 [12]. In addition, mTORC1 is more frequently activated in clear cell carcinoma than in the more common subtype serous carcinoma (86.6% vs. 50%). It has also been suggested that patients with OCCC are more responsive to mTORC1-targeted therapy [13]. Although rapalogs have demonstrated significant inhibitory effects on a variety of human malignancies, their mechanism may not be sufficient to achieve a broad and robust anticancer effect due to their inability to inhibit mTORC2 activity [14]. The clinical benefits of rapalogs were not approved in many kinds of malignancies. For example, a phase III clinical study reported that patients with renal cell carcinoma treated with everolimus experienced a median progression free interval of only 4 months [15].

We recently reported that demethylating agents can restore HIN-1 expression and decrease the expressions of phospho-Akt at ser473 and Thr308 and phospho-mTOR in paclitaxel-resistant OCCC cells through the HIN-1-AKT-mTOR signaling pathway to inhibit tumor growth [16]. In addition, a recent report demonstrated that *PIK3IP1*, a direct target of ARID1A and EZH2, is upregulated by EZH2 inhibition and contributes to synthetic lethality by inhibiting PI3K-AKT signaling, and that pharmacological inhibition of EZH2 represents a novel treatment strategy for cancers involving *ARID1A* mutations in OCCC [17]. Drug combinations are gaining increasing attention in the clinical setting as the most appropriate approach to target tumors and avoid acquired resistance. Activation of PI3K/Akt/mTOR pathway inhibitors in combination with classical anticancer agents has proven highly effective in several experimental systems [18-20]. Therefore, in this study we analyzed the effect of treatment with 5-aza-2-deoxycytidine (5-aza-dC) followed by the mTOR inhibitor everolimus (RAD001). 5-aza-dC exerted

at least an additive effect with RAD001, possibly through the PIK3-AKT-mTOR and EZH2-PIK3IP1 pathway. Both in vitro and in vivo results appeared to be correlated with the molecular changes induced by the drugs individually and in combination. Our results provide evidence that targeting not only AKT and mTOR but also EZH2 and PIK3IP1 with two drugs with different anti-tumor mechanisms could be a new strategy to achieve better antitumor activity. In addition, we investigated the role of AKT/EZH2/PIK3IP1/mTOR signaling in acquired resistance to paclitaxel and RAD001 in OCCC.

Methods

Culture of cell lines

The OCCC cell line ES2 was obtained from the American Type Culture Collection (Manassas, VA). The ES2 cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C, and were grown in McCoy's 5A medium with 10% FBS. Paclitaxel-resistant ES2 cells were developed by continuous paclitaxel exposure as described previously [16]. The paclitaxel-resistant ES2 cell line was named ES2TR160.

Reagents

Paclitaxel was purchased from Genovate Biotechnology (Hsinchu, Taiwan), and RAD001 and 5-aza-dC were purchased from Cell Signaling Technology (Beverly, MA) and Sigma (St. Louis, MI) for the following experiments.

Cytotoxicity by MTT assays

The sensitivities of ES2 and ES2TR160 cells to paclitaxel, RAD001 and 5-aza-dC were assessed by MTT assay (Sigma). Briefly, cells (4,000 cells/well) in a 96-well plate were exposed to concentrations of 1, 10, 10², and 10³ mM of RAD001, paclitaxel, or 5-aza-dC for 72 h at 37°C. The cells exposed to culture medium only served as negative controls. MTT at a final concentration of 0.5 mg/ml was added to the cells and incubated at 37°C for the last 3 h. At the end of incubation, the cultured medium was removed and 200 µl of DMSO (Sigma) was added to dissolve the blue formazan crystals. The optical density was measured at 490 nm using a universal microplate reader Elx800 (Bio-tek Instruments, Winooski, VT). Inhibitory concentration 50 (IC₅₀) values (the concentra-

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tion of drugs that produced a 50% reduction in absorbance) were analyzed and recorded [16].

Cell proliferation by MTT assays

Cell proliferation was also determined by MTT assays as described earlier. Briefly, cells (1×10^4 cells/well in a 6-well plate) were treated with various concentrations of RAD001, paclitaxel, or 5-aza-dC for 1, 3, and 6 days in 5% CO₂ at 37°C. The assays were then evaluated as described earlier.

Annexin V apoptosis assays

Apoptosis-positive cells were analyzed with an FITC Annexin V apoptosis detection kit I (BD Biosciences, NC) according to the manufacturer's protocol with minor modifications. Briefly, ES2 and ES2TR160 cells were seeded on 12-well chamber slides (Ibidi, Martinsried, Germany) at a density of 7500 cells/well for 24 h and then treated with RAD001, paclitaxel, 5-aza-dC, 5-aza-dC and paclitaxel, or 5-aza-dC and RAD001 for a further 24 h. Each well was washed with buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 1 mM CaCl₂) following 15 min of incubation in binding buffer containing annexin V-FITC and propidium iodide at room temperature in the dark. Images of apoptotic cells were then recorded using a fluorescence microscope (Nikon ECLIPSE 80i, NY). Positive annexin V cells were counted, and the percentage of apoptotic cells was defined as the number of positive annexin V cells divided by the total number of cells.

Western blot analysis

The tumor cells were first treated with the agents described earlier for 3 days. They were then lysed in PBS containing 1% Triton X-100 using an ultrasonic cell disruptor. Lysates were separated by SDS-PAGE (12.5%) and transferred to a PVDF membrane. The membrane was blocked in blocking buffer (TBS containing 0.2% Tween 20 and 1% I-block) and incubated with polyclonal Ab including anti-mTOR, p-mTOR, Rictor, p-Rictor, AKT, AKT p-Akt (ser473), EZH2, and PIK3IP1 Abs (Cell Signaling) separately for 1 hour. A purified rabbit anti-human GAPDH polyclonal Ab (Santa Cruz Biotechnology, Inc., Texas) was also applied at the same time to normalize the signals, followed by an alkaline phosphatase-conjugated anti-rabbit Ab (Vector Laboratories, CA). The membrane was washed

and the bound Abs were visualized by developing with NBT/BCIP as chromogenes.

In vivo animal experiments

All of the experiments were approved by the Institutional Animal Care and Use Committee of Cathay General Hospital. Five- to 7-week-old NOD/SCID (NOD.CB17 *Prkdc^{scid}/Jnar1*) mice were purchased from the National Animal Center (Taipei, Taiwan) and maintained in accordance with institutional policies. All of the experiments were approved by the Institutional Animal Care and Use Committee of Cathay General Hospital. The mice (n=36) were inoculated subcutaneously into the bilateral flank with 1×10^6 ES2 (n=18) or 1×10^6 ES2TR160 tumor cells (n=18). Mice treated with either PBS defined as the mock treatment group, or those treated with 0.25 mg/kg 5-aza-dC given subcutaneous inductions for 4 days (day 1-4) after inoculation (day 0), defined as the 5-aza-dC treatment group [21], with 9 mice in each group. Tumor growth was measured using calipers, and volumes were calculated based on the modified ellipsoid formula ($L \times W \times W/2$). On day 8 (tumor >50 mm³), the mice were further treated with either PBS (n=3), or 20 mg/kg ip paclitaxel per week (n=3), or oral 2.5 mg/m² RAD001 twice per week (n=3) in each group [22]. Tumor volume was measured every other day after tumor challenge, and all of the experiments were carried out in duplicate. Tumor weight was measured when the mice were sacrificed.

Statistical analysis

The median IC₅₀ values of RAD001 and paclitaxel were calculated using Sigma Plot 8.0 software (SPSS Inc., Chicago, IL). All numerical data were expressed as the mean \pm SD. Significant differences between two groups were determined using the Student's t-test, and significant differences among more than two groups were determined with one-way ANOVA. A *p* value less than 0.05 was considered to be statistically significant.

Results

In vitro growth-inhibitory effects of RAD001 on paclitaxel-sensitive and resistant OCCC cells

To examine the effect of RAD001 on the proliferation of OCCC cells, MTT assays were per-

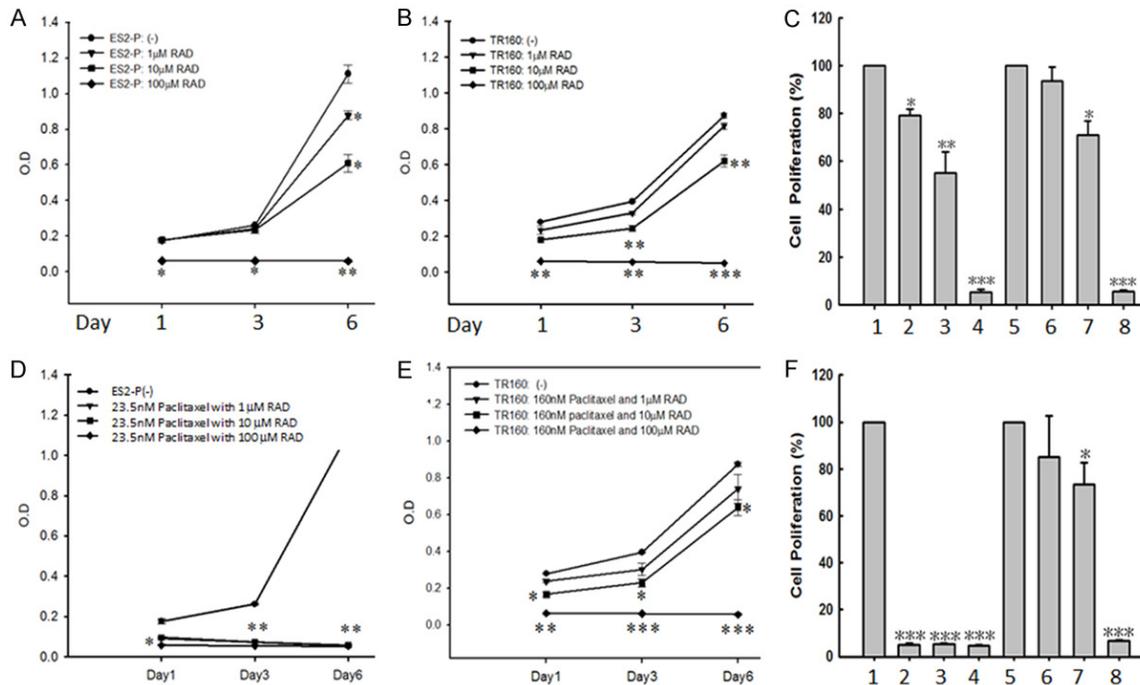


Figure 1. *In vitro* growth-inhibitory effects of RAD001 and/or paclitaxel on paclitaxel-sensitive (ES2) and paclitaxel-resistant (ES2TR160) OCCC cell lines. A. The growth curves of ES2 parental cells treated with 0, 1, 10, and 100 mM RAD001 for 1, 3 and 6 days. B. The growth curves of ES2TR160 cells treated with 0, 1, 10, and 100 mM RAD001 for 1, 3 and 6 days. C. Bar figures of the percentages of growth inhibition of RAD001 on ES2 and ES2TR160 cells. Axis number 1 to 4 in order represent ES2 cells treated with 0, 1, 10, and 100 mM RAD001 for 6 days, respectively; Axis number 5-8 in order represent ES2TR160 cells treated with 0, 1, 10, and 100 mM RAD001 for 6 days, respectively. D. The growth curves of ES2 parental cells treated with 23.5 nM paclitaxel and 1, 10, and 100 mM RAD001 for 1, 3, and 6 days. E. The growth curves of ES2TR160 cells treated with 160 nM paclitaxel and 1, 10, and 100 mM RAD001 for 1, 3, and 6 days. F. Bar figures of the percentages of growth inhibition of RAD001 and paclitaxel on ES2 and ES2TR160 cells. Axis number 1 to 4 in order represent ES2 cells treated with 23.5 nM paclitaxel and 1, 10, and 100 mM RAD001 for 6 days respectively; Axis number 5-8 in order represent ES2TR160 cells treated with 23.5 nM paclitaxel and 1, 10, and 100 mM RAD001 for 6 days, respectively (*represents $P < 0.05$, **represents $P < 0.005$, ***represents $P < 0.001$).

formed on paclitaxel-sensitive ES2 and paclitaxel-resistant ES2TR160 cells. The IC_{50} concentrations of RAD001 in ES2 and ES2TR160 cell lines were 159 mM and 127 mM, respectively. The IC_{50} concentrations of paclitaxel in ES2 and ES2TR160 cell lines were 23.5 nM and 220 nM, respectively. The MTT assays of ES2 (**Figure 1A**) and ES2TR160 (**Figure 1B**) OCCC cells treated with RAD001 for 1, 3 and 6 days showed inhibition of the proliferation of ES2 and ES2TR160 OCCC cells in a dose-dependent manner. The growth-inhibition effect of RAD001 on ES2 and ES2TR160 cell lines was then evaluated. As shown in **Figure 1C**, RAD001 significantly inhibited the growth of ES2 cells by 1 mM ($P < 0.05$, Student's t-test) and ES2TR160 cells by 10 mM ($P < 0.05$, Student's t-test).

The growth-inhibition effect of RAD001 combined with paclitaxel on OCCC cells was further evaluated. A combination of paclitaxel with a low dose of RAD001 (1 mM) inhibit the proliferation of ES2 cells by 93.2% (**Figure 1D** and **1F**). In comparison, a combination of paclitaxel with RAD001 at concentrations of 1 and 100 mM inhibited the proliferation of ES2TR160 cells by 15.3% and 93.4%, respectively (**Figure 1E** and **1F**). These results suggested that RAD001 may be a promising agent for the treatment of both paclitaxel-sensitive and resistant OCCC when combined with paclitaxel.

We next examined whether 5-aza-dC could inhibit the proliferation of ES2 and ES2TR160 cells. The proliferation of ES2 cells was inhibited by 59.0%, 97.5%, and 66.7% when treated with 10 mM of RAD001, 10 mM of 5-aza-dC,

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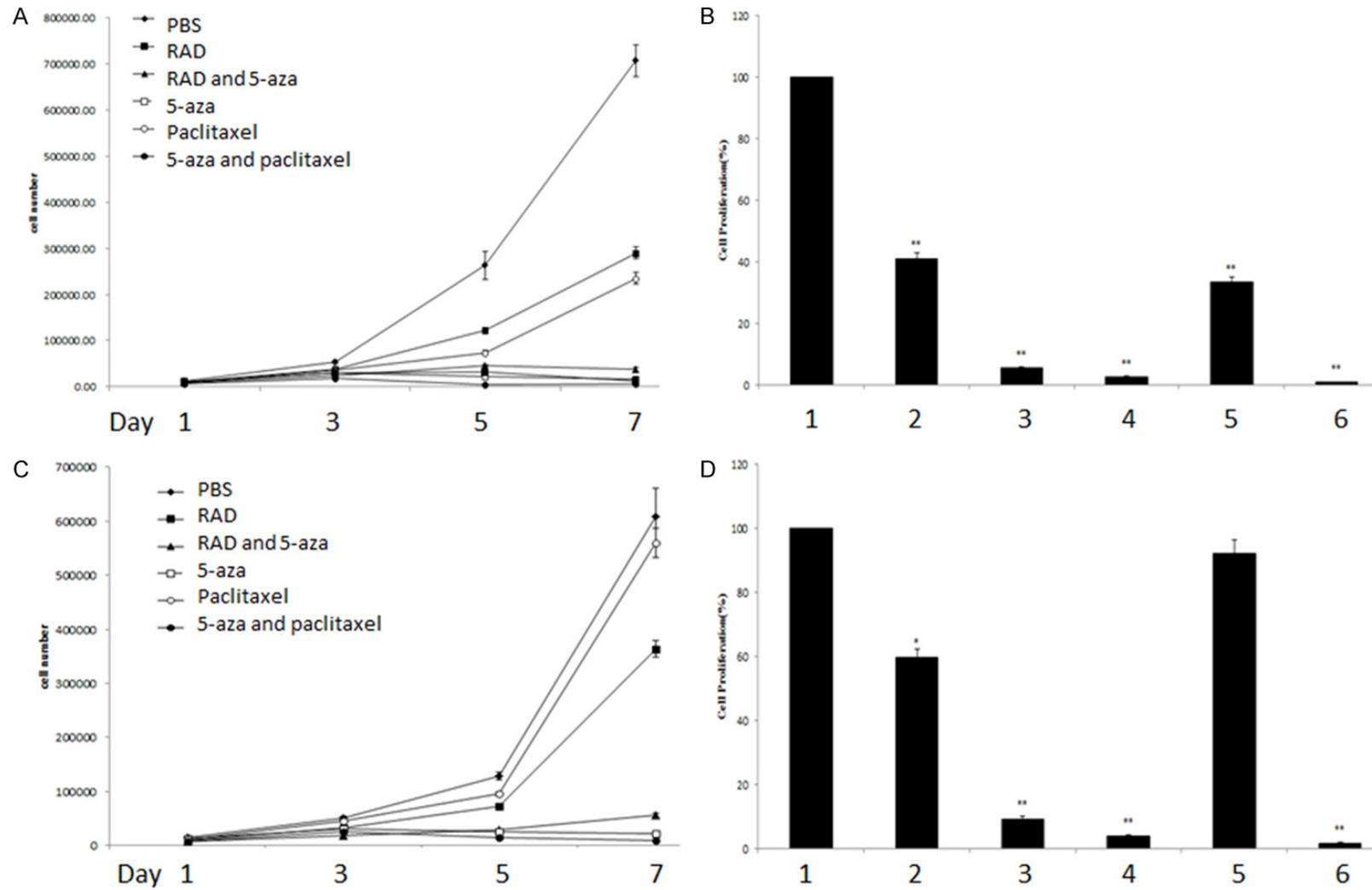


Figure 2. The growth curves and bars of cell proliferation in ES2 and ES2TR160 cells treated with 10 mM 5-aza-dC, RAD001, paclitaxel, or 5-aza-dC followed by paclitaxel or RAD001 at IC₅₀ concentrations. A: The growth curves of ES2 parental cells. B: Bar figures of the percentages of growth inhibition in ES2 parental cells treated with different regimens. (1. PBS, 2. RAD001, 3. RAD001 and 5AZA-dC, 4. 5AZA-dC, 5. Paclitaxel, 6. 5AZA-dC and paclitaxel). C: The growth curves of ES2TR160 cells. D: Bar figures of the percentages of growth inhibition in ES2TR160 cells treated with with different regimens. (1. PBS, 2. RAD001, 3. RAD001 and 5AZA-dC, 4. 5AZA-dC, 5. Paclitaxel, 6. 5AZA-dC and paclitaxel).

and 23.5 nM of paclitaxel alone, respectively ($P < 0.001$, one-way ANOVA) (**Figure 2A** and **2B**). In addition, the inhibition of the proliferation of ES2 cells increased to 94.7% and 99.2% when treated with 5-aza-dC and RAD001, or 5-aza-dC and paclitaxel, respectively ($P < 0.001$, one-way ANOVA) (**Figure 2A** and **2B**). Furthermore, 5-aza-dC significantly inhibited the proliferation of ES2TR160 cells (96.3%) compared with RAD001 (42.2%) or paclitaxel (7.9%) alone (**Figure 2C** and **2D**, $P < 0.001$, one-way ANOVA).

In vivo growth-inhibitory effects of 5-aza-dC and paclitaxel on paclitaxel-sensitive and resistant OCCC cells

To examine the *in vivo* growth-inhibitory effects of 5-aza-dC, the athymic mice were subcutaneously inoculated with 1×10^6 ES2 or ES2TR160 cells. The mice were then treated with either PBS or 0.25 mg/kg 5-aza-dC on days 1-4, as described in the Materials and Methods. When the tumors reached approximately 50 mm³ (on day 8), the mice were randomized into 3 treatment groups, receiving no treatment as control groups, treatment group with ip 20 mg/kg paclitaxel per week without 5-aza-2-dC induction on day 1-4, and ip 20 mg/kg paclitaxel per week with 5-aza-2-dC induction on day 1-4 as described in "Material and Methods". Overall, drug treatment was well tolerated with no apparent toxicity throughout the study. The ES2-bearing mice treated with PBS alone had a similar mean tumor size at day 23 (316.49 mm³) compared to the 5-aza-dC (363.64 mm³, $P = 0.150$) and paclitaxel-treated (265.93 mm³, $P = 0.119$) groups. However, the mice treated with 5-aza-dC and paclitaxel had the lowest tumor volumes (140.36 mm³, $P < 0.001$) compared with the other three groups at day 23 ($P < 0.001$, one-way ANOVA) (**Figure 3A**). In addition, the ES2TR160 tumor-bearing mice that were treated with 5-aza-dC and paclitaxel had significantly smaller tumor volumes (209.77 mm³) than those treated with PBS (372.79 mm³, $P = 0.001$), paclitaxel (293.35 mm³, $P = 0.027$), or 5-aza-dC (308.88 mm³, $P = 0.039$) at day 23 (**Figure 3B**).

We further evaluated the weights of the tumors of the ES2 and ES2TR160-bearing mice in the different groups. The ES2-bearing mice treated with 5-aza-5C and paclitaxel had a lower tumor weight (0.19 ± 0.10 g) than those treated with PBS (0.46 ± 0.05 g) or paclitaxel alone ($0.37 \pm$

0.08 g) ($P < 0.001$, one-way ANOVA). In addition, the ES2TR160-bearing mice treated with 5-aza-5C and paclitaxel had a lower tumor weight (0.26 ± 0.10 g) than those treated with paclitaxel (0.46 ± 0.08 g) or PBS alone (0.65 ± 0.09 g) ($P = 0.001$, one-way ANOVA) (**Figure 3C**). These results indicated that 5-aza-dC followed by paclitaxel had a significant antitumor effect on both paclitaxel-sensitive and resistant OCCC.

In vivo growth-inhibitory effects of RAD001 and 5-aza-dC on paclitaxel-sensitive and resistant OCCC

To further examine the *in vivo* growth-inhibitory effects of RAD001 and 5-aza-dC followed by RAD001, we used a subcutaneous xenograft model in athymic mice inoculated with ES2 or ES2TR160 cells. As shown in **Figure 4A**, the mean ES2 tumor volumes were similar in the mice treated with PBS (316.5 mm³) or 5-aza-dC (363.6 mm³) alone ($P = 0.167$, one-way ANOVA) 23 days after tumor inoculation. However, the mice treated with 5-aza and RAD001 had a significantly smaller volume (176.1 mm³) than those treated with PBS alone (316.49 mm³), paclitaxel (265.93 mm³), and RAD001 (253.97 mm³) ($P = 0.006$, one-way ANOVA) (**Figure 4A**). The ES2TR160 tumor-bearing mice treated with 5-aza-dC and RAD001 also had a significantly smaller mean tumor volume (188.7 mm³) than the other groups after 23 days of tumor inoculation (PBS 333.75 mm³, 5-aza-dC 308.88 mm³, paclitaxel 293.34 mm³, and RAD001 239.54 mm³, $P < 0.001$, one-way ANOVA) (**Figure 4B**). These results indicated that 5-aza-dC combined with RAD001 had potent anti-tumor effects in both of paclitaxel-sensitive and resistant OCCC tumors.

RAD001 combined with 5-aza-dC could inhibit not only the phosphorylation of Akt, mTOR, and Rictor, but also EZH2 signaling pathways

We previously reported that an overexpression of the HIN-1 gene significantly reduced cell growth, induced apoptosis, and increased sensitivity to paclitaxel by reducing the phosphorylation of Akt (Ho et al., 2012). In this study, 5-aza-dC with or without RAD001 enhanced the expression of HIN-1 (**Figure 5A**). In addition, the phosphorylation of mTOR and Rictor (mTORC-2) was decreased in both the ES2 and ES2TR100 groups treated with RAD001 (mTOR

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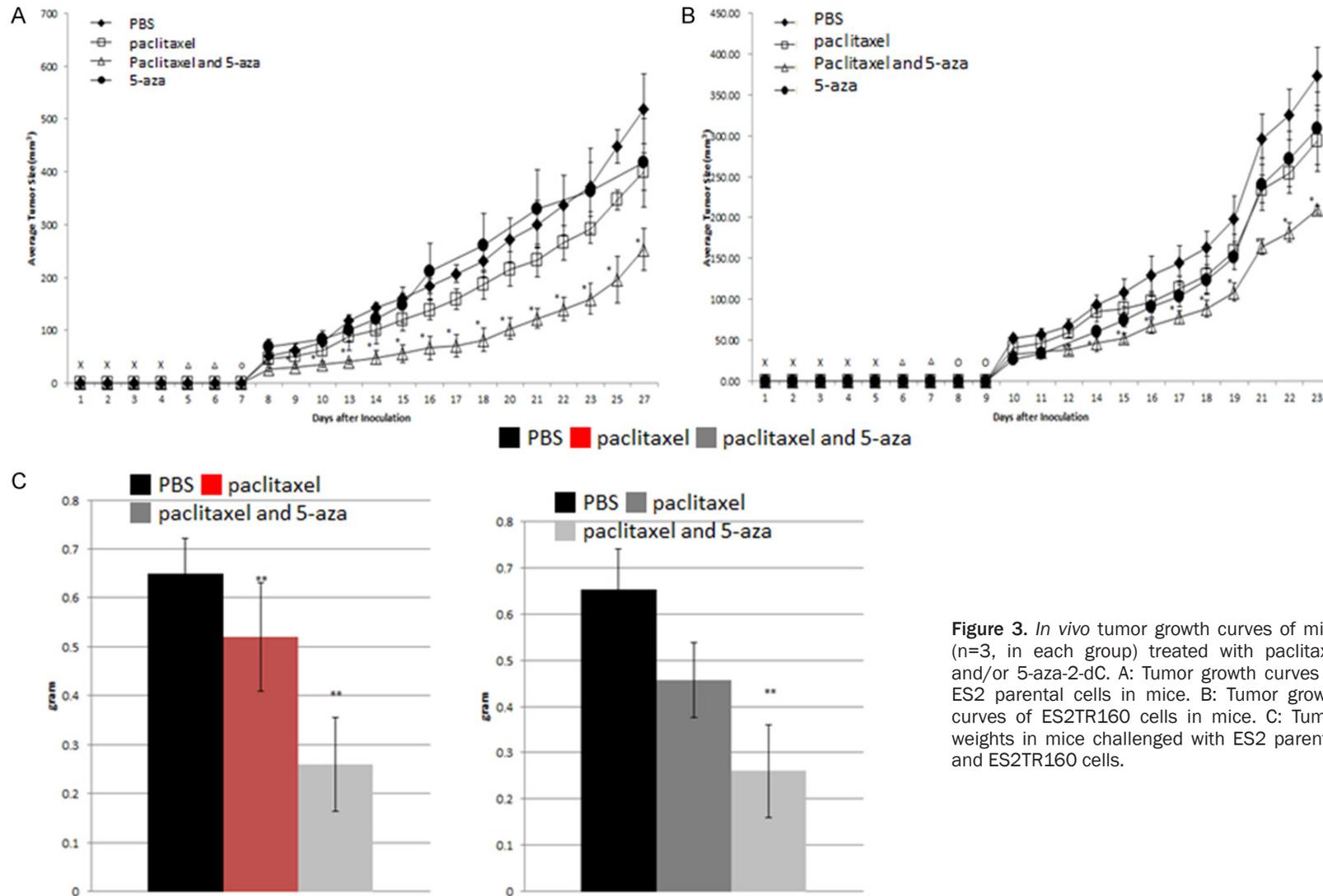


Figure 3. *In vivo* tumor growth curves of mice (n=3, in each group) treated with paclitaxel and/or 5-aza-2-dC. A: Tumor growth curves of ES2 parental cells in mice. B: Tumor growth curves of ES2TR160 cells in mice. C: Tumor weights in mice challenged with ES2 parental and ES2TR160 cells.

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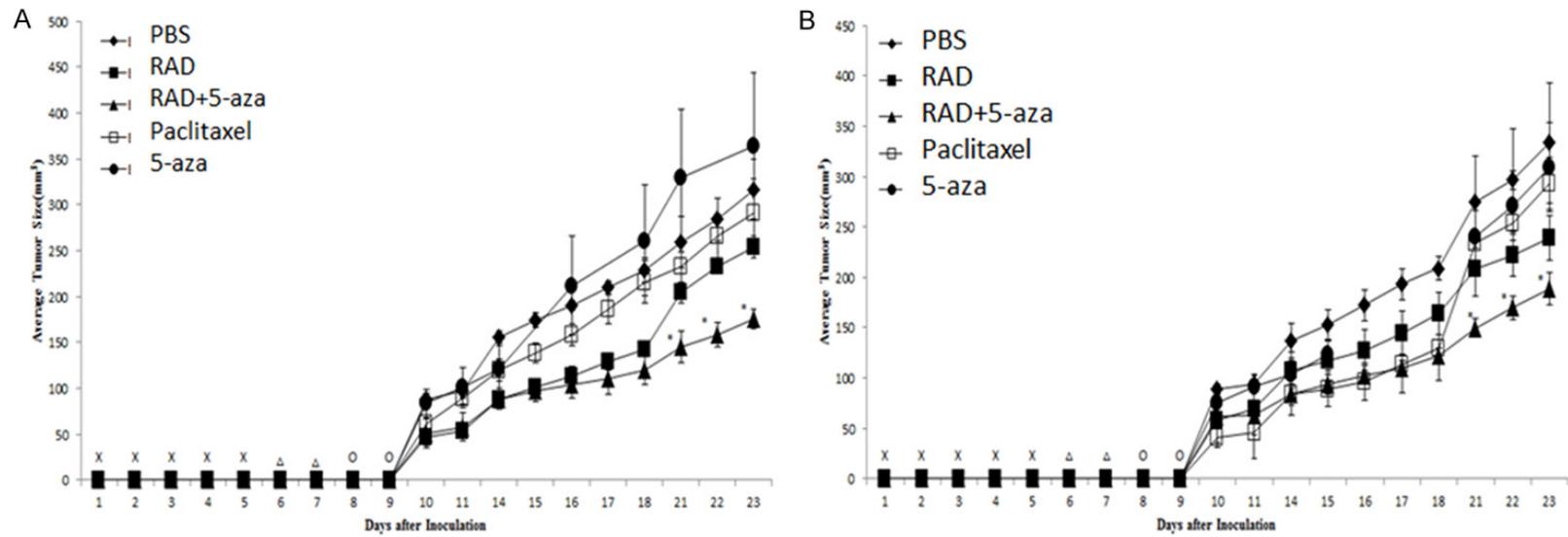


Figure 4. *In vivo* tumor growth curves of mice (n=3, in each group) treated with RAD001 alone or combined with 5-aza-2-dC. A: *In vivo* tumor growth curves of ES2 parental cells. B: *In vivo* tumor growth curves of ES2TR160 cells.

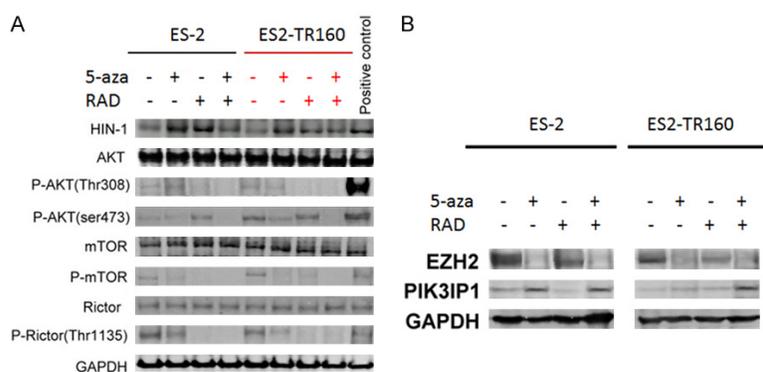


Figure 5. Western blotting analyses of ES2 parental and ES2TR160 cells treated with RAD001 and/or 5-aza-2-dC. A: The expression levels of HIN-1, mTOR, p-mTOR, Rictor, p-Rictor, AKT, p-AKT ser473, and p-AKT ser308. B: The expression levels of EZH2 and PIK3IP1.

inhibitor) alone or RAD001 with 5-aza-dC (**Figure 5A**). These results indicated that inhibition of mTOR enhanced the expression of HIN-1 and downregulated the phosphorylation of mTOR and Rictor.

The phosphorylation of AKT ser473 increased in the ES2 and ES2TR160 cells treated with RAD001 (**Figure 5A**). However, 5-aza-dC inhibited the phosphorylation of AKT (thr308), AKT (ser473), mTOR, and Rictor in both ES2 and ES2TR160 cells. These results indicated that 5-aza-dC combined with RAD001 could reverse paclitaxel-resistance through downregulating the phosphorylation of several signaling pathways.

We further evaluated whether the expression of EZH2 or PI3K3IP1 was influenced by 5-aza-dC or RAD001. As shown in **Figure 5B**, the expression of EZH2 decreased in the ES2 and ES2TR160 cells treated with 5-aza-dC and RAD001, whereas the expression of PIK3IP1 increased (**Figure 5B**). These results indicated that 5-aza-dC combined with RAD001 could regulate the expressions of EZH2 and PIK3IP1 in both the paclitaxel-sensitive and resistant OCCC tumor cells.

We then investigated the apoptosis of OCCC tumor cells treated with different regimens. As shown in **Figure 6A**, the percentage of annexin V-positive ES2 cells treated with 5-aza-dC with RAD001 was higher (96.7±1.0%) than the other groups (P<0.001, one-way ANOVA), whereas there was no significant difference in the percentage of annexin V-positive cells in the ES2

cells treated with paclitaxel alone (31.3±5.0%) or paclitaxel with 5-aza-dC (37.4±4.6%) (P=0.191, one-way ANOVA). In addition, the ES2TR160 cells treated with 5-aza-dC with RAD001 had the highest percentage (91.9±5.0%) of annexin V-positive cells among all of the groups (P<0.001, one-way ANOVA). The percentages of annexin V-positive cells treated with paclitaxel alone (20.6±1.32%) and paclitaxel with 5-aza-dC (17.2±2.9%) were not significantly difference (P=0.447, one-way ANOVA)

(**Figure 6B**). These results indicated that treatment with 5-aza-dC followed by RAD001 could enhance apoptosis in both paclitaxel-sensitive and resistant OCCC tumor cells. However, concurrent treatment with 5-aza-dC and RAD001 did not enhance the apoptosis of both paclitaxel-sensitive and resistant OCCC tumor cells (data not shown).

Discussion

In this study, we evaluated the efficacy of RAD001 in vivo using a subcutaneous xenograft model (**Figure 4**). Treatment with RAD001 decreased the ES2- and ES2TR160-derived tumor burden by 20% and 28%, respectively, compared with PBS. In addition the oral administration of RAD001 was well tolerated by the mice. These findings suggest that RAD001 could have a modest antitumor effect as a single agent for clear cell carcinoma as first- and second-line therapy. Moreover, the paclitaxel-resistant clear cell carcinoma cell lines exhibited increased expressions of phospho-AKT ser473 and thr308 and phospho-mTOR compared to the corresponding paclitaxel-sensitive parental cell lines (**Figure 5A**). The increase in phospho-AKT ser473 expression was associated with increased activation of Rictor. The involvement of AKT in resistance to cisplatin has been reported [23, 24]. Although the inhibition of AKT activity can sensitize human ovarian cancer cells to conventional anticancer agents such as cisplatin [23] and paclitaxel [25], there are concerns that inhibiting AKT could affect certain biologically important cell processes such as glucose metabolism [26]. Thus, we

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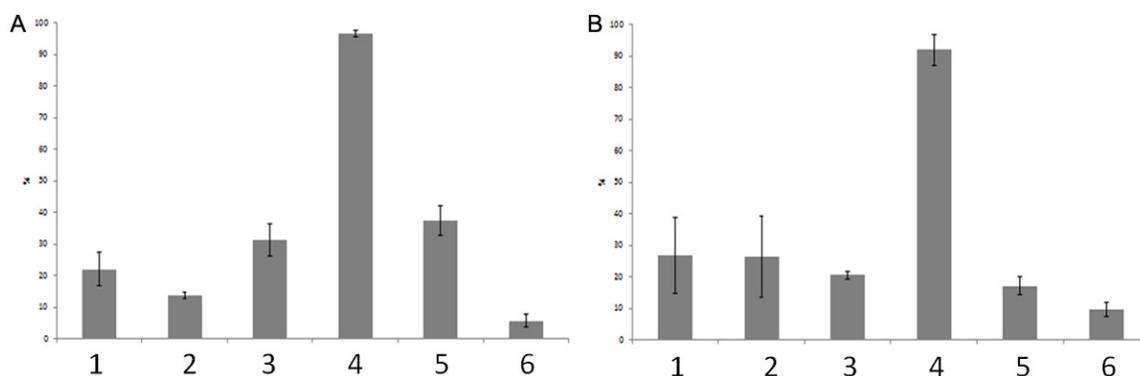


Figure 6. A: Percentages of annexin V-positive ES2 cells treated with different regimens. B: Percentages of annexin V-positive ES2TR160 cells treated with different regimens. Axis number 1 to 6 represents ES2 or ES2TR cells treated with (1) 5AZA-dC (2) RAD001 (3) paclitaxel (4) 5AZA-dC followed by RAD001 (5) 5AZA-dC and paclitaxel (6) no treatment.

used RAD001 to target downstream therapeutic effectors such as mTOR, and the results showed that RAD001 treatment inhibited tumor growth in the paclitaxel-resistant ES2TR160 cells *in vitro*. However, the *in vivo* antitumor effect of RAD001 was not as pronounced in the paclitaxel-resistant cell-derived tumors as in the paclitaxel-sensitive cell-derived tumors (**Figure 4A** and **4B**).

AKT activation has been reported to be a biomarker to predict sensitivity to mTOR inhibitors [19, 27, 28]. However, our results indicate that RAD001 treatment in paclitaxel-resistant OCCC cells may have activated phospho-AKT ser473/mTORC2 signaling, and this may cause cancer cell proliferation. Our results suggest that mTORs inhibition may be only modestly efficacious in the clinical management of paclitaxel-resistant clear cell carcinoma. Recently, Hisamatsu et al. [29] reported that treatment with RAD001 induced mTORC2-mediated AKT activation in both RAD001-sensitive and RAD001-resistant OCCC cells, and that inhibition of mTORC2 during RAD001 treatment enhanced the anti-tumor effect of RAD001 and prevented OCCC cells from acquiring resistance to RAD001. We used phospho-Rictor as an mTORC2 surrogate to represent RAD001-resistant cells, and the expressions of phospho-AKT ser373 and phospho-Rictor were increased in the ES2 and ES2TR160 cells treated with RAD001, which may indicate that anti-mTORC2 drugs may be able to overcome RAD001 resistance. In our previous study, we found that 5-aza-dC

could inhibit phospho-AKT ser373 and phospho-mTOR [16]. In the current study, 5-aza-dC followed by RAD001 treatment suppressed phospho-AKT ser373 and thr308, phospho-mTOR and Rictor activity. Importantly, treatment with 5-aza-dC followed by RAD001 significantly enhanced the efficacy of RAD001 in inhibiting tumor growth in our experimental model. Moreover, the continuous inhibition of phospho-AKT ser473 by 5-aza-dC prevented the OCCC cells from acquiring resistance to RAD001 in the paclitaxel-treated OCCC cells. Of note, RAD001 treatment following 5-aza-dC enhanced apoptosis in paclitaxel-sensitive and resistant OCCC cells and significantly enhanced the therapeutic efficacy of RAD001 in OCCC.

EZH2, the catalytic subunit of polycomb repressive complex 2, silences gene expression through the generation of the lysine 27 trimethylation mark on histone H3 (H3K27Me3) by its catalytic SET domain [30]. EZH2 is often overexpressed in OCCC (Li et al., 2010), and EZH2 was overexpressed in the ES2 and ES2TR160 cells in this study whereas the expression of *PIK3IP1* was decreased. 5-aza-dC treatment followed by RAD001 successfully suppressed EZH2 and increased *PIK3IP1* expression. In mice inoculated subcutaneously with ES2 and ES2TR160 cells, treatment with paclitaxel did not significantly decrease the ES2- and ES2TR160-derived tumor burden with PBS. However, 5-aza-dC followed by paclitaxel significantly decreased the ES2- and ES2TR160-derived tumor burden with PBS (**Figure 3**).

These results suggest that 5-aza-dC can enhance the anti-tumor effect of paclitaxel in both paclitaxel-sensitive and also paclitaxel-resistant OCCC cells. Because the ES2 and ES2TR160 cells used in this study mimic the development of resistance in paclitaxel-treated patients, our results suggest that administering 5-aza-dC followed by RAD001 or paclitaxel may be a promising treatment strategy for patients with OCCC.

OCCC is a distinct subtype of epithelial ovarian cancer, and it is associated with poorer sensitivity to platinum-based chemotherapy and a worse prognosis than more common serous carcinomas both in first-line and recurrent settings [7, 9, 31]. We previously reported that treatment with paclitaxel-platinum-based chemotherapy resulted in better survival than treatment with platinum-based chemotherapy among our patients with advanced pure clear cell carcinoma, especially in those with optimal cytoreduction (40.95 vs. 9.02 months, $P=0.028$) (Ho et al., 2004). However, the response rate to salvage chemotherapy for cisplatin-resistant clear cell carcinoma has been reported to be only 1% [9], indicating the urgent need for new treatment strategies for recurrent clear cell carcinoma of the ovary.

Previous investigations have indicated that mTORC1 is frequently activated in OCCC, and *in vitro* studies have suggested that mTORC1 can be targeted therapeutically [21, 32]. In addition to mTORC1, the role of mTORC2 as a therapeutic target in OCCC has also recently been proposed [29]. There is emerging evidence that mTORC2 may be an important driver for tumorigenesis. For example, it has been reported that the exogenous overexpression of Rictor can promote mTORC2 activity, increase the proliferative and invasive potential of cancer cells [33], and induce malignant tumors in mice [34]. In the current study, we found that Rictor, an essential component of the mTORC2 complex, was highly expressed in paclitaxel-sensitive and paclitaxel-resistant OCCC cells. These results suggest that mTORC2 plays an important role in the development of OCCC, and that it may represent a viable therapeutic target in this disease. The Rictor-mTORC2-AKT feedback loop induced by RAD001 treatment has been reported to be, at least in part, responsible for acquired resistance to RAD001 [29]. In addition,

our results revealed the activation of phosphor-AKT ser473 after RAD001 treatment, which is consistent with previous reports.

Early trials of mTOR inhibitors have shown some clinical benefits in gynecologic malignancies, particularly in endometrial and ovarian cancers. Recent results of combinations of PI3K/mTOR inhibitors and other treatment modalities such as hormonal therapy, chemotherapy, or targeted therapy have been encouraging, demonstrating clinical benefit without significant additional toxicity [35-37]. A more attractive approach would be sequential treatment followed by the inhibition of PI3K by molecular profiling of the resistant tumor [38]. In the current study, we used RAD001 as an mTOR inhibitor, and it showed significant *in vitro* and *in vivo* antitumor activity toward OCCC cells. Furthermore, our results showed that treatment with RAD001 effectively attenuated the phosphorylation of mTOR and Rictor *in vitro* and markedly inhibited the proliferation of OCCC cells. Nevertheless, there are concerns that inhibiting mTOR may trigger a feedback mechanism that activates AKT to potentially promote tumor growth and consequently reduce the antitumor effect of mTOR inhibitors [39-42].

Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer have recently been identified by methylome and expression profiling [43]. However, few potential key drivers of chemo-resistance in OCCC whose expressions can be silenced by DNA methylation have been identified, and further studies to identify therapeutic targets to overcome resistance to various drugs are warranted. Preclinical and clinical studies have strongly supported the use of a combination of regimens, and reported that hypomethylating agents such as 5-aza-dC can restore platinum sensitivity in chemo-resistant ovarian cancer cell lines, xenografts, and patients with ovarian cancer [44-47]. 5-aza-2-deoxycytidine (decitabine) has been shown to inhibit the *ex vivo* tumor growth of OCCC partly through affecting the HIN-1-related AKT-mTOR signaling pathway in both paclitaxel-sensitive and paclitaxel-resistant OCCC [16]. In the current study, 5-aza-dC followed by RAD001 more effectively inhibited ES2- and ES2TR160-derived tumors by up to 45% compared to RAD001 alone.

In conclusion, treatment with 5-aza-dC followed by RAD001 is a promising treatment strategy by enhancing apoptosis of OCCC cells in both first-line chemotherapy and as second-line treatment for recurrent OCCC previously treated with paclitaxel. This combination can also overcome RAD001 chemo-resistance through inhibiting phospho-AKT and phospho-Rictor.

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Disclosure of conflict of interest

None.

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