

mTOR Is a Promising Therapeutic Target Both in Cisplatin-Sensitive and Cisplatin-Resistant Clear Cell Carcinoma of the Ovary

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Abstract Purpose: Mammalian target of rapamycin (mTOR) plays a central role in cell proliferation and is regarded as a promising target in cancer therapy, including for ovarian cancer. This study aimed to examine the role of mTOR as a therapeutic target in clear cell carcinoma of the ovary, which is regarded as an aggressive, chemoresistant histologic subtype.

Experimental Design: Using tissue microarrays of 98 primary ovarian cancers (52 clear cell carcinomas and 46 serous adenocarcinomas), the expression of phospho-mTOR was assessed by immunohistochemistry. Then, the growth-inhibitory effect of mTOR inhibition by RAD001 (everolimus) was examined using two pairs of cisplatin-sensitive parental (RMG1 and KOC7C) and cisplatin-resistant human clear cell carcinoma cell lines (RMG1-CR and KOC7C-CR) both *in vitro* and *in vivo*.

Results: Immunohistochemical analysis showed that mTOR was more frequently activated in clear cell carcinomas than in serous adenocarcinomas (86.6% versus 50%). Treatment with RAD001 markedly inhibited the growth of both RMG1 and KOC7C cells both *in vitro* and *in vivo*. Increased expression of phospho-mTOR was observed in cisplatin-resistant RMG1-CR and KOC7C-CR cells, compared with the respective parental cells. This increased expression of phospho-mTOR in cisplatin-resistant cells was associated with increased activation of AKT. RMG1-CR and KOC7C-CR cells showed greater sensitivity to RAD001 than did parental RMG1 and KOC7C cells, respectively, *in vitro* and *in vivo*.

Conclusion: mTOR is frequently activated in clear cell carcinoma and can be a promising therapeutic target in the management of clear cell carcinoma. Moreover, mTOR inhibition by RAD001 may be efficacious as a second-line treatment of recurrent disease in patients previously treated with cisplatin. (Clin Cancer Res 2009;15(17):5404–13)

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Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States, with >21,000 new cases each year and an estimated 15,520 deaths in 2008 (1). Cytoreductive surgery followed by platinum-based chemotherapy usually combined with paclitaxel is the standard initial treatment and has improved survival in patients with epithelial ovarian cancer (2). However, there still exists many clinical problems in the treatment of epithelial ovarian cancer. One of the most important problems that needs to be resolved is the management of clear cell carcinoma of the ovary, which was first recognized by the WHO as a distinct histologic subtype in 1973 (3). The precise incidence of clear cell carcinoma is unknown, but it is reported to be 3.7% to 12.1% of all histologic subtypes among epithelial ovarian cancer (4).

There have been two major clinical problems in the clinical management of clear cell carcinoma. First is its poor sensitivity to first-line platinum-based chemotherapy and the association with a worse prognosis than the more common serous adenocarcinomas. In the setting of front-line chemotherapy, the response rate to conventional platinum-based chemotherapy, platinum agent alone or in combination with cyclophosphamide and

Translational Relevance

Clear cell carcinoma of the ovary is a distinctive subtype of epithelial ovarian cancer associated with a poorer sensitivity to platinum-based chemotherapy and a worse prognosis than the more common serous adenocarcinoma. To improve survival, the development of new treatment strategies that target clear cell carcinoma more effectively is necessary. Our results show that mammalian target of rapamycin (mTOR) is more frequently activated in clear cell carcinomas than in serous adenocarcinomas. Our data have relevance for the design of future clinical studies of first-line treatment for patients with clear cell carcinoma of the ovary. Moreover, the finding of increased expression of phospho-mTOR and greater sensitivity to RAD001 in cisplatin-resistant clear cell carcinoma cells than in cisplatin-sensitive cells suggests a novel treatment option for patients with recurrent disease after cisplatin-based first-line chemotherapy.

adriamycin, was reported to be only 11% in clear cell carcinoma. In contrast, patients with serous adenocarcinoma had a response rate of 72% (5). The response to carboplatin-paclitaxel, a current standard regimen, was also reported to be relatively low, ranging from 22% to 56% (6–8). When analyzed by clinical stage, worse clinical outcome in patients with clear cell carcinoma has been more evident in advanced (stage III-IV) than in early-stage disease (stage I-II). In a retrospective analysis (5), a statistically significant difference in overall survival between clear cell carcinoma and serous adenocarcinoma was observed in patients with stage III disease (12.7 months versus 26.8 months, respectively; $P = 0.0015$). However, the difference was not significant in stage I-II disease (31.8 months versus 42.3 months, respectively; $P = 0.2761$). Similar results were reported by several groups of investigators (9–11). A more recent retrospective review of six randomized phase III clinical trials also showed that patients with stage III clear cell carcinoma treated with carboplatin-paclitaxel had a shorter survival compared with those with other histologic subtypes of epithelial ovarian cancer (12).

The second important clinical problem in the management of clear cell carcinoma is the lack of effective chemotherapy for recurrent clear cell carcinomas after front-line treatment with platinum-based chemotherapy. A recent report showed that the response rate for various regimens in the setting of second-line chemotherapy for recurrent clear cell carcinoma was only 1% (13). Therefore, to improve survival of patients with clear cell carcinoma, a better understanding of the mechanism of platinum-resistance and the identification of effective treatment strategies especially for both advanced and recurrent disease are needed.

The sensitivity of cancer cells to chemotherapeutic drug-induced apoptosis depends on the balance between pro-apoptotic and antiapoptotic signals. Therefore, inhibition of antiapoptotic signals, such as those mediated by the AKT pathway, has been proposed as a promising strategy to enhance the efficacy of conventional chemotherapeutic agents (14). Among the numerous AKT substrates, mammalian target of rapamycin

(mTOR) is thought to be one of the major targets of relevance to cancer therapy (15, 16). mTOR phosphorylates p70 S6 kinase (p70S6K) and the 4E-BP1 translational repressor, leading to translation of proteins required for cell proliferation (17). It has been reported that AKT-mTOR signaling is frequently activated in epithelial ovarian cancer (18). Recently, an orally bioavailable derivative of rapamycin, everolimus (RAD001), has been shown to inhibit the proliferation of ovarian cancer cells and enhance sensitivity to cisplatin *in vitro* and *in vivo* (19–22). However, no reports have addressed the impact of mTOR inhibitors on ovarian cancer cells that have acquired resistance after the exposure to platinum agents. Moreover, because most tumor specimens and tumor-derived cell lines used in these investigations have been ovarian serous adenocarcinomas (19–21), the role of mTOR in clear cell carcinoma remains largely unknown.

It has been reported that loss of PTEN expression is common in clear cell carcinoma of the ovary (23). It also has been reported that ovarian endometriosis, from which clear cell carcinoma is thought to arise, is characterized by hyperactivation of the AKT-mTOR pathway (24). Because it is well known that loss of PTEN expression and consequent activation of AKT signaling result in hypersensitivity to mTOR inhibition (20, 25, 26), clear cell carcinoma may be a good candidate for therapy with a mTOR inhibitor.

In the current investigation, we examined the activation status of mTOR both in early-stage and advanced-stage clear cell carcinoma, and we determined whether RAD001 has antineoplastic efficacy in both *in vitro* and *in vivo* models of clear cell carcinoma. Moreover, we investigated the role of AKT/mTOR signaling in the acquired resistance to cisplatin in clear cell carcinoma cells.

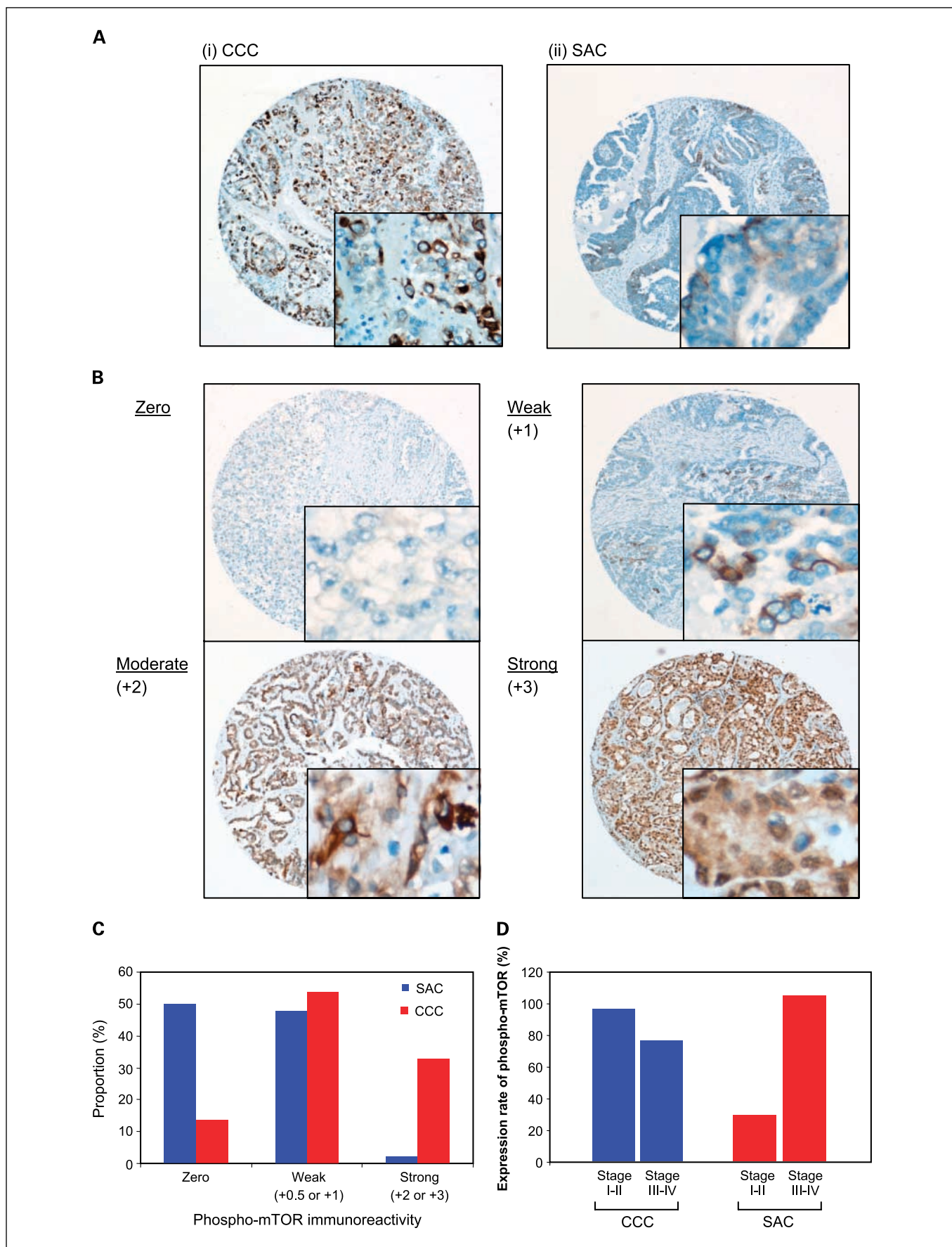
Materials and Methods

Reagents/Antibodies. RAD001 was obtained from Novartis Pharma AG. Enhanced chemiluminescence Western blotting detection reagents were from Perkin Elmer. Antibodies recognizing p70S6K, phospho-p70S6K (Thr389), mTOR, phospho-mTOR (Ser2448), AKT, phospho-AKT (Ser473), poly ADP ribose polymerase (PARP), LC3B, and β -actin were obtained from Cell Signaling Technology. The Cell Titer 96-well proliferation assay kit was obtained from Promega. Cisplatin was purchased from Sigma.

Drug preparation. RAD001 was formulated at 2% (w/v) in a micro-emulsion vehicle (Novartis Pharma AG). RAD001 was prepared according to the manufacturer's protocols. Thus, for animal studies, RAD001 was diluted to the appropriate concentration in double-distilled water just before administration by gavage. For *in vitro* analyses, RAD001 was prepared in DMSO before addition to cell cultures.

Clinical samples. All surgical specimens were collected and archived according to protocols approved by the institutional review boards of the parent institutions. Appropriate informed consent was obtained from each patient. The tumors included 46 serous adenocarcinomas and 52 clear cell carcinomas. Based on International Federation of Gynecology and Obstetrics (FIGO) criteria, 22 serous adenocarcinomas were stage I-II tumors and 24 were stage III-IV tumors. Among the clear cell carcinomas, 27 were stage I-II tumors and 25 were stage III-IV tumors.

Immunohistochemistry. Tumor samples were fixed in 10% neutral buffered formalin (10% formaldehyde, phosphate-buffered) overnight and then embedded in paraffin. In all patients, the diagnosis was based on a light microscopy examination using conventional H&E stain. Ovarian cancer tissue microarrays consisting of two cores from



each tumor sample were prepared by the Tumor Bank Facility at Fox Chase Cancer Center, as described previously (18, 19). Tissue sections were cut at 4 μm , mounted on slides, and processed for either H&E or immunohistochemical staining. For immunohistochemical studies, sections were incubated with the primary antibody, followed by the appropriate peroxidase-conjugated secondary antibody, as reported previously (19). The primary antibody used was anti-phospho-mTOR (Ser 2448) at 1:50 dilution. Negative controls were incubated with primary antibody preabsorbed with blocking peptide (Cell Signaling Technology). Surrounding nonneoplastic stroma served as an internal negative control for each slide. The slides were scored semiquantitatively by a pathologist who was blinded to the clinical outcome. A score of 0 indicated no staining, +0.5 was weak focal staining (<10% of the cells were stained), +1 was indicative of focal staining (10-50% of the cells were stained), +2 indicated clearly positive staining (>50% of the cells were stained), and a score of +3 was intensely positive, as described in detail elsewhere (18). The slides were examined under a bright field microscope. Tumors with staining of +2 or +3 were grouped as the strong-staining group, whereas tumors with staining of +0.5 or +1 were grouped as a weak-staining group. When the two cores from the same tumor sample showed different positivity results, the lower score was considered valid.

Cell culture. Human ovarian clear cell carcinoma cell lines RMG1, RMG2, KOC7C, and HAC2 were kindly provided by Dr. H. Itamochi (Tottori University, Tottori, Japan). These cells were cultured in phenol red free DMEM (Ham's F-12, Gibco Ltd) with 10% fetal bovine serum, as reported previously (27-29).

Establishment of cisplatin-resistant cell lines. Cisplatin-resistant sublines from RMG1 and KOC7C were developed in our laboratory by continuous exposure to cisplatin, as described previously (30). Briefly, cells of both lines were exposed to stepwise increases in cisplatin concentrations. Initial cisplatin exposure was at a concentration of 10 nmol/L. After the cells had regained their exponential growth rate, the cisplatin concentration was doubled and then the procedure was repeated until selection at 10 $\mu\text{mol/L}$ was attained. The resulting cisplatin-resistant sublines, called RMG1-CR and KOC7C-CR, were subcultured weekly and treated monthly with 10 $\mu\text{mol/L}$ cisplatin to maintain a high level of chemoresistance.

Cell proliferation assay. A MTS assay was used to analyze the effect of RAD001 on cell viability as described (31). Cells were cultured overnight in 96-well plates (1×10^4 cells/well). Cell viability was assessed after addition of RAD001 and/or cisplatin at the indicated concentrations for 48 h. The number of surviving cells was assessed by determination of the $A_{490\text{ nm}}$ of the dissolved formazan product after addition of MTS for 1 h as described by the manufacturer (Promega). Cell viability is expressed as follows: $A_{\text{exp group}}/A_{\text{control}} \times 100$.

Western blot analysis. Cells were treated with either DMSO (vehicle) or 10 nmol/L RAD001 for 6 h. Cells were washed twice with ice-cold PBS and lysed in lysis buffer [20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na_3VO_4 , 1 mmol/L β -glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ leupeptin, and 1% Triton X-100] for 10 min at 4°C. Lysates were centrifuged at $12,000 \times g$ at 4°C for 15 min, and protein concentrations of the supernatants were determined using Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was done in 5% nonfat milk in 1 \times Tris-buffered saline. Western blot analyses were done with various specific primary antibodies. Immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-

mouse immunoglobulin by using the enhanced chemiluminescence Western blotting system (Perkin Elmer).

Cell cycle analysis. Cells were incubated with or without 20 nmol/L RAD001 for 2 d. After the cells were washed with PBS, they were fixed with 75% ethanol overnight at 4°C. The cells were then washed twice with PBS and stained with propidium iodide (50 $\mu\text{g/mL}$) in the presence of RNase A (100 $\mu\text{g/mL}$; Roth) for 20 min at 4°C. Cell cycle distribution was determined by analyzing 10,000 cells using a FACScan flow cytometer and Cell Quest software (Becton Dickinson).

Immunofluorescence microscopy. Cells were incubated with or without 20 nmol/L RAD001 for 2 d. Cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 10 min, and then blocked and incubated with anti-LC3B antibody overnight at 4°C. After washing with PBS, the coverslips were incubated with FITC-conjugated secondary antibody (Alexab Fluor 488 anti-rabbit IgG, Molecular Probes) for 1 h, followed by 10 min of incubation with 4',6-diamidino-2-phenylindole. Slides were washed with PBS and mounted with Vectashield hardest mounting medium (Vector Laboratories). Images were acquired with a fluorescence microscope and processed using Photoshop software.

S.c. xenograft model. All procedures involving animals and their care were approved by the Institutional Animal Care and Usage Committee of Osaka University, in accordance with institutional and NIH guidelines. Five- to seven-week-old nude mice ($n = 40$) were inoculated s.c. into the right flank either with 5×10^6 RMG1, RMG1-CR, KOC7C, or KOC7C-CR cells in 200 μL of PBS, with 10 mice in each group. When tumors reached about 50 mm^3 , the mice were assigned into two treatment groups, with 10 mice in each group. The first group was treated with placebo twice a week. The second group was treated with RAD001 (2.5 mg/kg) twice a week. RAD001 was administered intragastrically using an animal-feeding needle. Body weight was measured weekly. Caliper measurements of the longest perpendicular tumor diameters were done every week to estimate tumor volume using the following formula: $V = L \times W \times D \times \pi/6$, where V is the volume, L is the length, W is the width, and D is the depth.

Statistical analysis. Cell proliferation was analyzed by Wilcoxon exact test. Tumor volume of RAD001-treated mice was compared with that of placebo-treated mice and analyzed by Wilcoxon exact test. Immunoreactivity was analyzed using Fisher's exact test. $P < 0.05$ was considered significant.

Results

Difference in phospho-mTOR expression between clear cell carcinomas and serous adenocarcinomas. Immunohistochemical analysis of ovarian cancer tissue microarrays for phospho-mTOR expression was done using 52 clear cell carcinomas of the ovary and 46 ovarian serous adenocarcinomas as described in Materials and Methods. Representative photographs of clear cell carcinoma and serous adenocarcinoma are shown in Fig. 1A. Phospho-mTOR immunoreactivity was scored semiquantitatively (Fig. 1B), and stronger immunoreactivity for phospho-mTOR was observed in clear cell carcinomas than in serous adenocarcinomas. Among the 46 serous adenocarcinomas, 23 (50%) showed negative staining, 7 (15.2%) were scored as +0.5, 15 (32.6%) were +1, and 1 (2.1%) was scored as +2. In contrast, among the 52 clear cell carcinomas, 7 tumors (13.4%) had negative staining; 5 (9.6%) were scored as +0.5, 23 (44.2%) as +1, 15 (28.8%) as +2, and 2 (3.8%) as +3. The

Fig. 1. mTOR is frequently activated in ovarian clear cell carcinomas. Ovarian cancer tissue microarrays were stained with anti-phospho-mTOR antibody. A, representative photographs of ovarian tissue microarray cores of (i) clear cell carcinoma (CCC) and (ii) serous adenocarcinoma (SAC). Magnifications, $\times 100$, and $\times 400$ (inset). B, phospho-mTOR immunoreactivity was scored semiquantitatively from zero to strong, as described in Materials and Methods. Representative photos of weak, moderate, and strong staining are shown. Magnifications, $\times 100$, and $\times 400$ (inset). C, histogram indicating immunostaining profile. Proportion indicates proportion of tumors examined. Phospho-mTOR expression was more frequent and stronger in intensity in clear cell carcinomas than in serous adenocarcinomas. D, histogram indicating the expression of phospho-mTOR according to clinical stage.

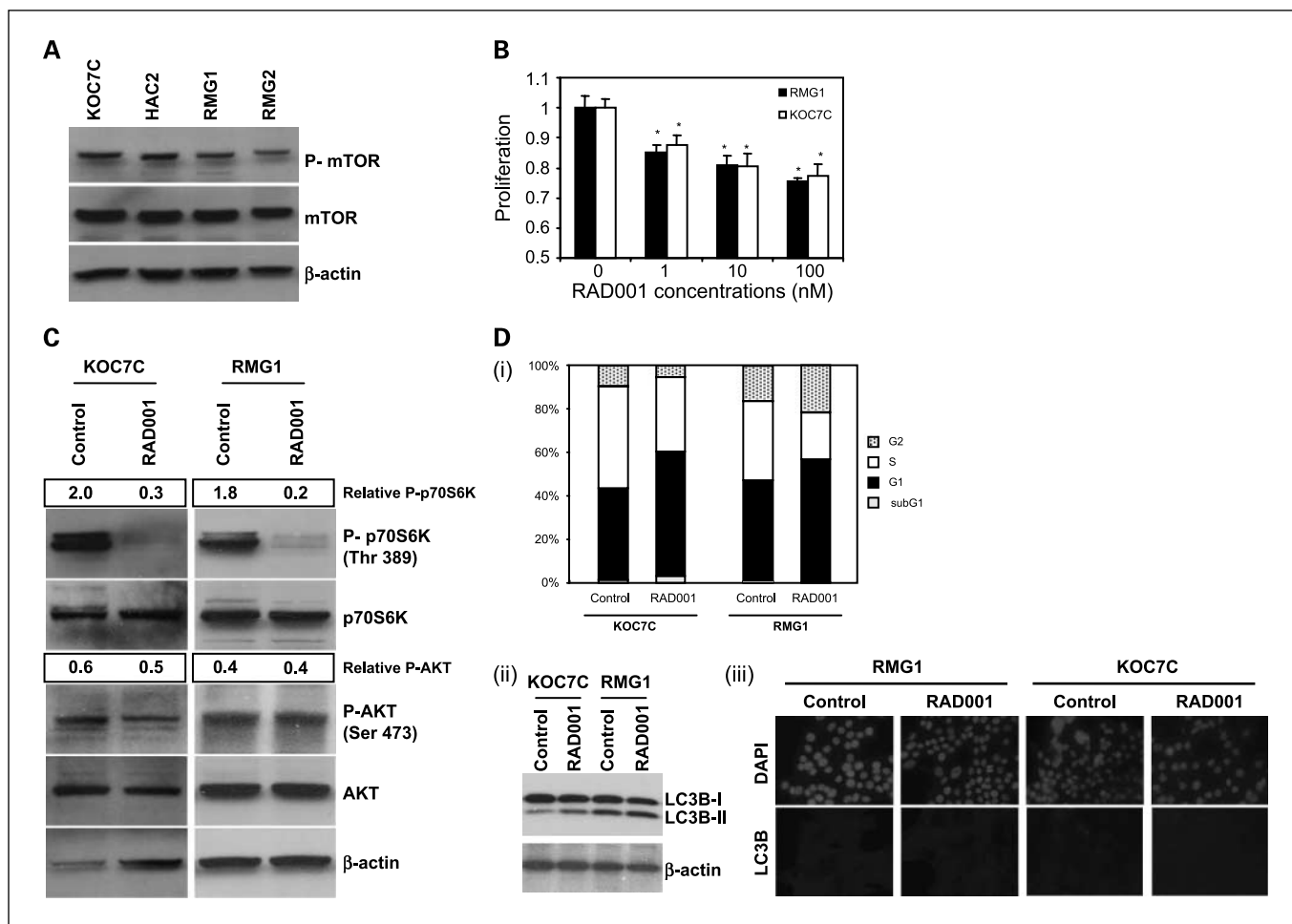


Fig. 2. RAD001 inhibits mTOR activity and proliferation of cisplatin-sensitive clear cell carcinoma cells. *A*, analysis of phospho-mTOR status in four ovarian clear cell carcinoma cell lines. Clear cell carcinoma cells were serum-starved overnight, after which the expression of phospho-mTOR was determined by Western blot analysis. Actin expression was used as a loading control. *B*, sensitivity of clear cell carcinoma cells to RAD001. Clear cell carcinoma cells were treated with the indicated concentrations of RAD001 in the presence of 5% fetal bovine serum (FBS) for 72 h. Cell viability was assessed by MTS assay. Points, mean; bars, SD (*, significantly different from control; $P < 0.01$). *C*, RAD001 attenuates the phosphorylation of p70S6K *in vitro*. KOC7C and RMG1 cells were treated with 10 nmol/L of RAD001 for 6 h in the presence of 5% FBS. Cells were harvested, and equivalent amounts (30 μ g) of protein were subjected to SDS-PAGE and blotted with anti-phospho-p70S6K (Thr³⁸⁹), anti-p70S6K, anti-phospho-AKT (Ser⁴⁷³), anti-AKT, or anti- β -actin antibodies. Blots were scanned and quantified by densitometry. The density of phospho-p70S6K or phospho-AKT was normalized to the corresponding density of bands for total p70S6K or AKT, respectively, and the results are depicted as "Relative P-AKT" or "Relative P-TOR" values. *D*, (i), RAD001 induced an increase in the percentage of cells in G₁ phase. RMG1 and KOC7C cells were treated with or without 10 nmol/L RAD001 for 48 h. Cell cycle analyses were done with a FACScan as described in the Materials and Methods. (ii, iii), RAD001 does not induce autophagy. KOC7C and RMG1 cells were treated with 10 nmol/L of RAD001 for 48 h in the presence of 5% FBS. (ii), cells were harvested, and equivalent amounts (30 μ g) of protein were subjected to SDS-PAGE and blotted with anti-LC3B or anti- β -actin antibodies. (iii), cells were fixed, stained with anti-LC3B antibodies, and viewed with a fluorescence microscope.

frequency of strong phospho-mTOR immunoreactivity was significantly higher, and the frequency of tumors with no immunoreactivity was significantly lower in clear cell carcinomas than in serous adenocarcinomas (Fig. 1C). These results indicate that clear cell carcinomas may be more strongly dependent on mTOR for tumor progression than are serous adenocarcinomas.

When analyzed by clinical stage, phospho-mTOR expression was observed in 76% of advanced-stage clear cell carcinomas and in 96% of early-stage clear cell carcinomas (Fig. 1D). Thus, most patients with clear cell carcinoma may be candidates for therapy with a mTOR inhibitor. In contrast, in serous adenocarcinomas, phospho-mTOR expression was uncommon in early-stage tumors, although it was significantly increased in advanced-stage tumors. Therefore, in serous adenocarcinomas, mTOR inhibition may be a therapeutic option only in advanced-stage disease.

Collectively, these results indicate that pharmacologic inhibition of mTOR may be a promising therapeutic strategy in the management of clear cell carcinomas, both in early-stage and in advanced-stage disease.

In vitro growth-inhibitory effect of RAD001 on cisplatin-sensitive clear cell carcinoma cell lines. Given the frequent mTOR activation found in human clear cell carcinoma tumor specimens (Fig. 1), we evaluated the expression of phospho-mTOR in four human clear cell carcinoma cell lines by Western blotting. As shown in Fig. 2A, under serum-starvation conditions, mTOR was phosphorylated in all clear cell carcinoma cell lines tested, which is consistent with immunohistochemical results observed with tumor samples. We next examined the efficacy of mTOR pathway inhibition by RAD001 on the proliferation of clear cell carcinoma cells *in vitro*. For this purpose, we did a MTS assay using two of these clear cell carcinoma cell lines with activated AKT/

mTOR signaling. As shown in Fig. 2B, RAD001 inhibited the proliferation of RMG1 and KOC7C cells *in vitro*, with ~25% inhibition at the highest drug concentration tested (Fig. 2B).

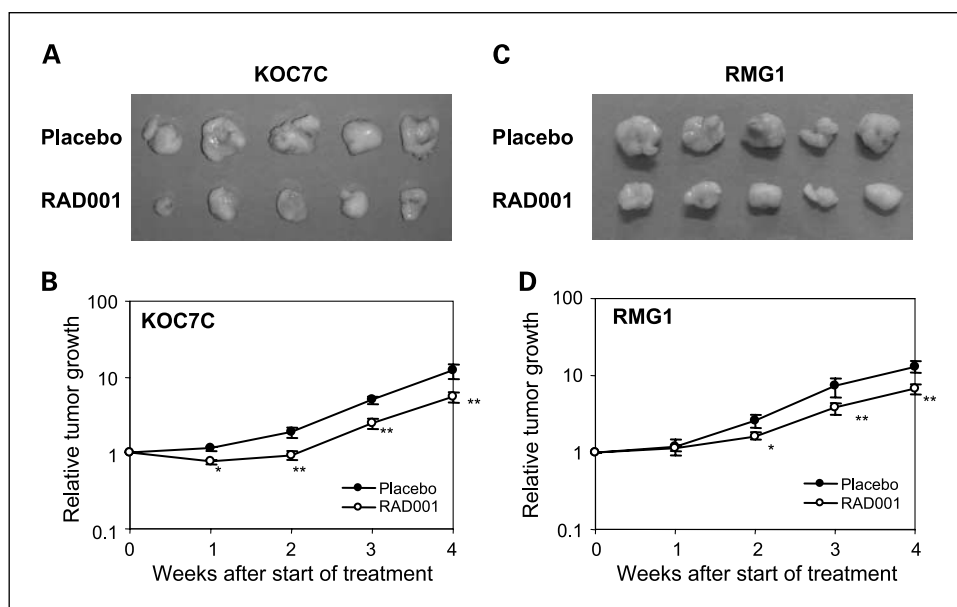
RAD001 attenuates phosphorylation of p70S6K in vitro. To determine if the antiproliferative effects of RAD001 result from inhibition of mTOR signaling, we examined the effect of RAD001 on the phosphorylation of downstream p70S6K in RMG1 and KOC7C cells. As shown in Fig. 2C, AKT, mTOR, and p70S6K were phosphorylated in both cell lines, indicative of the hyperactivation of the AKT/mTOR pathway. As expected, phosphorylation of the downstream effector p70S6K was significantly decreased in both cell lines by treatment with RAD001, indicating that RAD001 effectively inhibits mTOR signaling in clear cell carcinoma cells. Although previous studies have shown that mTOR inhibition is associated with a feedback activation of AKT that may result in resistance to mTOR inhibition (32–35), no significant increase in the phosphorylation of AKT was observed in response to RAD001 in these clear cell carcinoma cell lines. Rapamycin and its derivatives are generally regarded as having cytostatic effects; in some tumor cells, however, these agents have also been reported to induce apoptosis (36). To determine the mechanism by which RAD001 inhibits cell proliferation, we first examined the effect of RAD001 on cell cycle progression by flow cytometry. As shown in Fig. 2D (i), the percentage of cells in G₁ phase was significantly increased in both RMG1 and KOC7C cells after 2-day treatment with 10 nmol/L RAD001. In both cell lines, the percentage of apoptotic cells in the sub-G₁ peak did not change after treatment with RAD001. Moreover, as shown in Fig. 4B, treatment with 10 nmol/L RAD001 did not induce cleavage of PARP in these cells. We also examined whether treatment with RAD001 induces autophagic cell death in clear cell carcinoma cells. It has been reported that LC3B-I is converted to LC3B-II during autophagy (37). As shown in Fig. 2D (ii), however, the conversion of LC3B-I to the lower migrating form LC3B-II was not induced in response to treatment with RAD001 in RMG1 or KOC7C cells. Furthermore, as shown in Fig. 2D (iii), treatment with 10 nmol/L RAD001 did not induce punctate staining for

LC3B, an indicator of autophagy associated with the concentration of LC3 in autophagosomal vacuoles (37). Collectively, these results suggest that RAD001 most likely affects clear cell carcinoma cells by inducing cell cycle arrest (17).

Effect of RAD001 on the growth of ovarian clear cell carcinoma. To further examine the *in vivo* growth-inhibitory effect of RAD001, we employed a s.c. xenograft model in which athymic mice were inoculated s.c. with RMG1 or KOC7C cells. When tumors reached ~50 mm³, the mice were randomized into two treatment groups receiving placebo or RAD001, as described in Materials and Methods. Drug treatment was well tolerated, with no apparent toxicity throughout the study. Tumor volume was measured weekly after the start of treatments (Fig. 3B and D). The appearance of tumors 4 weeks from the first day of treatment is also shown in Fig. 3A and C. Histologically, these s.c. tumors were clear cell carcinomas (data not shown). Mean RMG1-derived tumor burden in mice treated with RAD001 was 332.5 mm³ compared with 652.5 mm³ in placebo-treated mice, and mean KOC7C-derived tumor burden in animals treated with RAD001 was 276 mm³ compared with 605.5 mm³ in placebo-treated mice. Overall, treatment with RAD001 decreased RMG1-derived and KOC7C-derived tumor burden by 49% and 55%, respectively, compared with placebo. These results indicate that RAD001 has significant antitumor effects as a single agent in clear cell carcinoma.

Increased mTOR activation and the sensitivity to RAD001 in cisplatin-resistant cell lines. Cisplatin resistance is regarded as a major clinical problem in the management of clear cell carcinoma of the ovary (10, 11). It has been previously reported that AKT is involved in the resistance of ovarian serous adenocarcinoma cells to cisplatin (38, 39). To examine whether AKT/mTOR signaling is involved in cisplatin resistance in clear cell carcinoma, we established cisplatin-resistant sublines from RMG1 and KOC7C cells, as described in Materials and Methods. To examine whether these sublines had acquired resistance to cisplatin, we first evaluated the sensitivity of these cell lines to cisplatin by MTS assay. As shown in Fig. 4A, clear differential

Fig. 3. Effect of RAD001 on the growth of cisplatin-sensitive clear cell carcinoma-derived tumor cells *in vivo*. Athymic nude mice were inoculated s.c. with KOC7C cells or RMG1 cells. When the tumors reached an average size of ~50 mm³, mice were treated with placebo or 2.5 mg/kg RAD001 twice a week for 4 wk. A and C, appearance of s.c. tumors. B and D, graphs depicting weekly tumor volumes (mm³) for each treatment group. Points, mean; bars, SD; *, *P* < 0.05, significantly different from placebo-treated mice; **, *P* < 0.01, significantly different from placebo-treated mice.



sensitivity to cisplatin was observed between cisplatin-sensitive parental and respective cisplatin-resistant sublines. We next examined cisplatin-induced apoptosis in these cell lines. Treatment with cisplatin induced cleavage of PARP in parental cells, but not in cisplatin-resistant sublines (Fig. 4B). Using these cell lines, we investigated the activity of AKT/mTOR in both cisplatin-resistant sublines and parental chemosensitive cells by Western blotting. As shown in Fig. 4C, higher phospho-AKT and phospho-mTOR expression was observed in both chemoresistant cell lines compared with their respective parental cell lines. Increased activation of AKT/mTOR signaling was also observed in another cisplatin-resistant subline, HAC2-CR, which was established from parental HAC2 cells (data not shown). The increased phosphorylation of AKT and mTOR was inhibited by treatment with a phosphoinositide 3-kinase inhibitor, LY294002 (data not shown).

Because it is well known that loss of PTEN expression and consequent activation of AKT result in hypersensitivity to mTOR inhibition (20, 25), we considered chemoresistant sublines to be good candidates for treatment with RAD001. Thus, we next examined the inhibitory effect of RAD001 on chemoresistant and parental chemosensitive clear cell carcinoma cell lines by MTS assay (Fig. 4D). A clear differential effect was shown depending on the cell sensitivity to cisplatin. Cisplatin-resistant RMG1-CR and KOC7C-CR cells are significantly more sensitive to RAD001 than are their respective parental cell lines RMG1 and KOC7C. We also confirmed that treatment with RAD001 effectively inhibited the phosphorylation of p70S6K *in vitro*, without inducing negative feedback activation of AKT (data not shown). Moreover, using RMG1-CR and KOC7C-CR cells, we next determined whether the treatment with RAD001 enhances the efficacy of cisplatin. As shown in Fig. 4E, in the presence of 10 nmol/L RAD001, the ability of cisplatin (0-10 μ mol/L) to inhibit cell proliferation was not enhanced in these cisplatin-resistant cell lines. These results suggest that RAD001 may have efficacy as a single agent for cisplatin-resistant clear cell carcinomas.

Effect of RAD001 on the cisplatin-resistant clear cell carcinoma *in vivo*. To further examine the *in vivo* effect of RAD001 on cisplatin-resistant sublines, athymic mice were inoculated s.c. with RMG1-CR or KOC7C-CR cells, and were randomized into two treatment groups receiving placebo or RAD001, as described in Materials and Methods. The appearance of the tumors 4 weeks from the first day of treatment is shown in Fig. 5A, C. Moreover, corresponding graphs depicting diminished tumor volumes for RAD001-treated mice relative to placebo-treated mice are presented in Fig. 5B and D. Mean RMG1-CR-derived tumor burden in mice treated with RAD001 was 163 mm³ compared with 553 mm³ in placebo-treated mice, and mean KOC7C-CR-derived tumor burden in animals treated with RAD001 was 218.5 mm³ compared with 710 mm³ in placebo-treated mice. Treatment with RAD001 decreased RMG1-CR-derived tumor burden by 72% compared with only 49% reduction in RMG1-derived tumors (Fig. 3C and D). Similar results were obtained in mice inoculated with KOC7C-CR cells. Treatment with RAD001 decreased KOC7C-CR-derived tumor burden by 69% compared with a 55% reduction in RAD001-treated KOC7C-derived tumors (Fig. 3A and B). Collectively, these *in vitro* and *in vivo* data suggest that the antitumor effect of RAD001

is greater in cisplatin-resistant clear cell carcinoma than in cisplatin-sensitive clear cell carcinoma.

Discussion

Despite recent developments in platinum-based combination chemotherapy, patients with clear cell carcinoma of the ovary, especially in advanced-stage or recurrent disease, have a worse progression-free survival and overall survival when compared with patients with a serous histology (5-11). Therefore, to improve survival, new strategies are necessary to more effectively treat clear cell carcinoma.

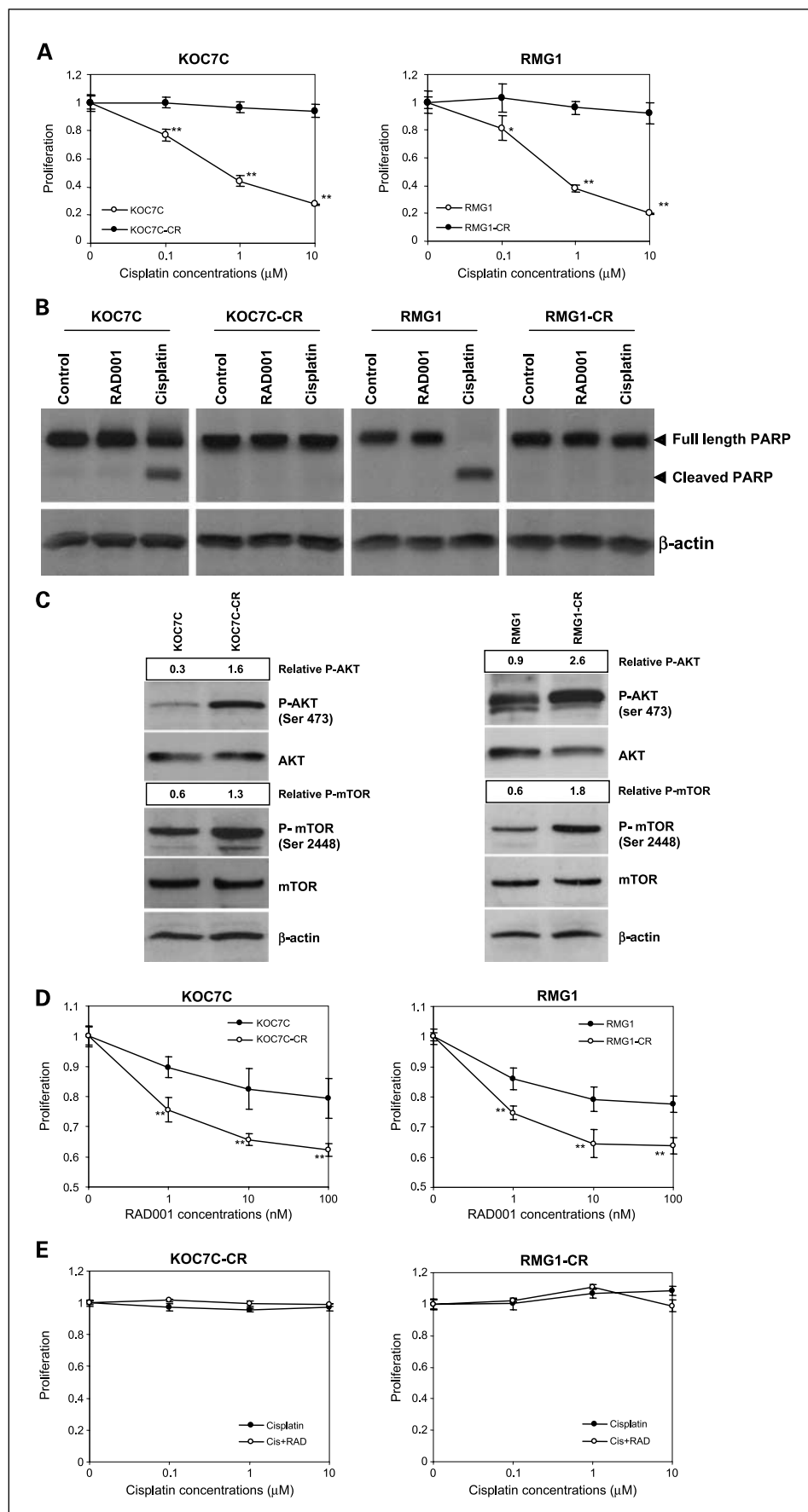
In the present study, we observed activation of mTOR in 86.6% of clear cell carcinoma of the ovary (Fig. 1). Importantly, the frequency of strong phospho-mTOR immunoreactivity in clear cell carcinomas was significantly higher than that found in serous adenocarcinomas, indicating that clear cell carcinomas are more strongly dependent on mTOR signaling for tumor progression than are serous adenocarcinomas. In addition, mTOR was frequently activated in both stage III-IV clear cell carcinomas (76%) and stage I-II clear cell carcinomas (96%). Therefore, mTOR seems to be a promising target for the treatment of patients with both early- and advanced-stage clear cell carcinoma. In contrast, phospho-mTOR expression was uncommon in early-stage serous adenocarcinomas but was significantly increased in advanced-stage serous adenocarcinomas. The very high frequency of mTOR activation observed in early-stage clear cell carcinomas suggests that hyperactivation of mTOR kinase is an early event in the development of clear cell carcinomas. This is noteworthy in light of the fact that activated AKT/mTOR signaling has been reported in ovarian endometriosis, from which clear cell carcinoma is thought to arise (24). We have recently shown that the mTOR inhibitor RAD001 markedly inhibited tumor onset and progression in a transgenic mouse model of ovarian cancer that develops ovarian serous adenocarcinomas with activated AKT/mTOR signaling. Thus, mTOR might be a reasonable target for the chemoprevention of clear cell carcinoma in patients with ovarian endometriosis.

Our data show that treatment with RAD001 effectively attenuates the phosphorylation of p70S6K *in vitro* and markedly inhibits the proliferation of ovarian clear cell carcinoma cells. There exists a concern in inhibiting mTOR, in that mTOR inhibition may trigger a feedback mechanism that activates AKT to potentially promote tumor growth and may consequently reduce the antitumor effect of mTOR inhibitors (32-35). Although such a feedback has been observed in several cancer cell types including breast cancer (32), rhabdomyosarcoma (33), non-small cell lung cancer (34), and multiple myeloma (35), treatment with RAD001 in the current study did not induce activation of AKT in ovarian clear cell carcinoma cells (Fig. 2D).

We also evaluated the efficacy of RAD001 *in vivo*, employing s.c. xenograft models (Fig. 3). In mice inoculated s.c. with RMG1 or KOC7C cells, treatment with RAD001 significantly inhibited tumor growth. Moreover, orally administered RAD001 in our treatment schedule was well tolerated. Taken together, these findings indicate that RAD001 could have significant antitumor effects as a single agent for clear cell carcinoma in a setting of front-line therapy.

An additional important finding in our study is the antitumor activity of RAD001 in cisplatin-resistant clear cell carcinoma. In general, patients with platinum-resistant recurrent epithelial

Fig. 4. Phospho-mTOR expression and its role as a therapeutic target in cisplatin-resistant clear cell carcinoma cells. **A and B,** establishment of cisplatin-resistant variant cell lines. Cisplatin-resistant sublines were established as described in Materials and Methods. **A,** cisplatin-sensitive parental (KOC7C and RMG1) and cisplatin-resistant variant (KOC7C-CR and RMG1-CR) cells were treated with the indicated concentrations of cisplatin in the presence of 5% fetal bovine serum for 72 h. Cell viability was assessed by MTS assay. Points, mean; bars, SD (*, $P < 0.05$; **, $P < 0.01$). **B,** effect of cisplatin on the cleavage of PARP in cisplatin-sensitive parental and cisplatin-resistant variant cell lines. KOC7C, KOC7C-CR, RMG1, and RMG1-CR treated with 10 $\mu\text{mol/L}$ cisplatin or 10 nmol/L RAD001 for 24 h. Cells were harvested, and then lysates were subjected to Western blotting using anti-PARP or anti- β -actin antibody. **C,** activation of AKT/mTOR signaling in cisplatin-sensitive parental and cisplatin-resistant variant cells *in vitro*. KOC7C, KOC7C-CR, RMG1, and RMG1-CR cells were serum-starved overnight. Cells were harvested, and equivalent amounts (30 μg) of protein were subjected to SDS-PAGE and blotted with anti-phospho-mTOR (Ser²⁴⁴⁸), anti-mTOR, anti-phospho-AKT (Ser⁴⁷³), anti-AKT, or anti- β -actin antibodies. AKT, phosphor-AKT, and β -actin were from the same membrane and 8% gel; mTOR and phosphor-mTOR were from a separate 6% gel. Blots were scanned and quantified by densitometry. The density of phospho-AKT or phospho-mTOR bands was normalized to the corresponding density of total AKT or mTOR bands, respectively, and the results are shown as "Relative P-AKT" or "Relative P-TOR" values. **D,** enhanced sensitivity to RAD001 in cisplatin-resistant clear cell carcinoma cells *in vitro*. Cisplatin-sensitive parental (KOC7C and RMG1) and cisplatin-resistant variant (KOC7C-CR and RMG1-CR) cells were treated with the indicated concentrations of RAD001 in the presence of 5% FBS for 72 h. Cell viability was assessed by MTS assay. Points, mean; bars, SD (**, $P < 0.01$). **E,** cisplatin-resistant variant (KOC7C-CR and RMG1-CR) cells were treated with the indicated concentrations of cisplatin in the presence or absence of 10 nmol/L RAD001 for 72 h. Cell viability was assessed by MTS assay.



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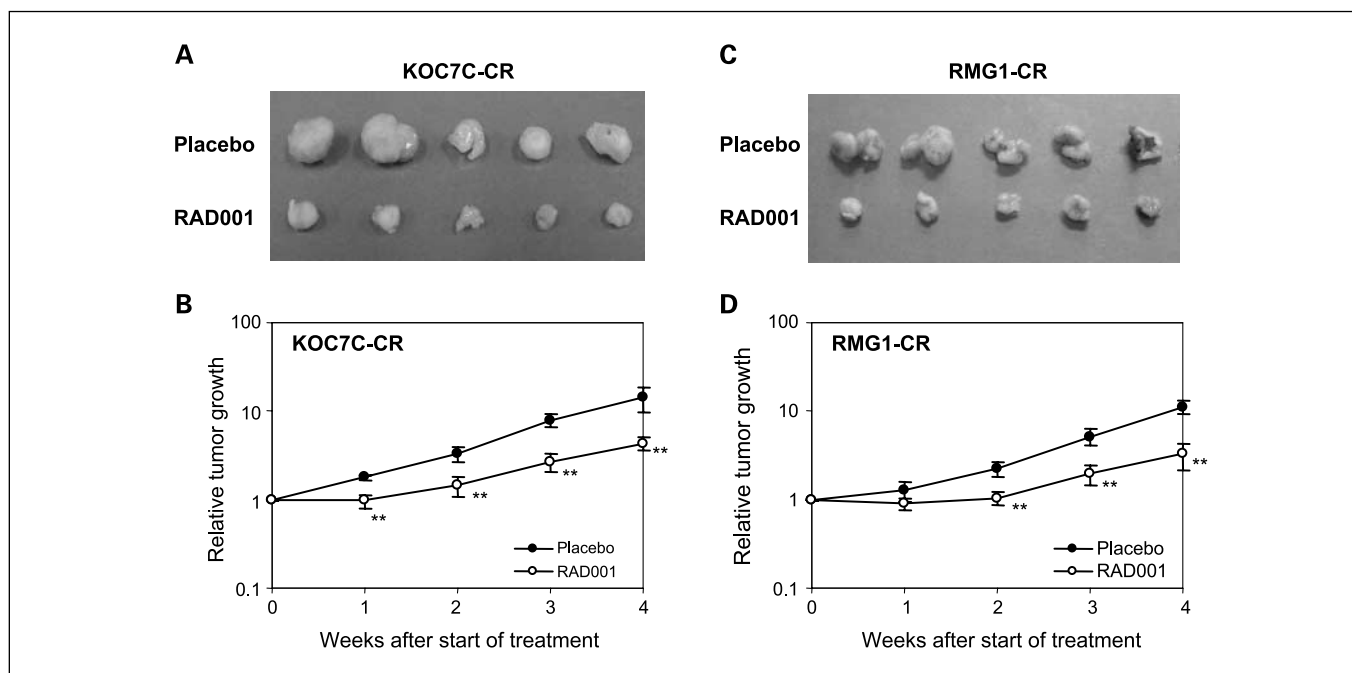


Fig. 5. Effect of RAD001 on the growth of cisplatin-resistant clear cell carcinoma–derived tumors *in vivo*. Athymic nude mice were inoculated s.c. with KOC7C-CR cells or RMG1-CR cells. When the tumors reached an average size of $\sim 50 \text{ mm}^3$, the mice were treated with placebo or 2.5 mg/kg RAD001 twice a week for 4 wk. **A** and **C**, appearance of s.c. tumors. **B** and **D**, graphs depicting weekly tumor volumes (mm^3) for each treatment group. Points, mean; bars, SD; **, $P < 0.01$, significantly different from placebo-treated mice.

ovarian cancer have been treated with antineoplastic agents that do not exhibit cross-resistance with platinum agents. However, these patients have dismal prognosis, with overall response rate ranging from 9% to 33% (40). Unfortunately, the prognosis of patients with cisplatin-resistant clear cell carcinomas is even worse. For example, in one study, the response rate for salvage chemotherapy for cisplatin-resistant clear cell carcinoma was only 1% (13), indicative of the urgent need of new treatment strategies for recurrent clear cell carcinoma of the ovary.

In this study, we found that cisplatin-resistant clear cell carcinoma cell lines exhibit enhanced phospho-mTOR expression compared with the corresponding cisplatin-sensitive parental cell lines (Fig. 4B). The increased phospho-mTOR expression was associated with increased activation of AKT. The involvement of AKT in the resistance to cisplatin has been reported previously (38, 41). Although we and others have previously reported that inhibition of AKT activity sensitizes human ovarian cancer cells to conventional anticancer agents such as cisplatin (38) and paclitaxel (42), there are concerns associated with inhibiting AKT, because AKT also mediates certain biologically important cell processes such as glucose metabolism (43). Thus, a safer approach may be to target downstream therapeutic effectors such as mTOR. Interestingly, our cisplatin-resistant clear cell carcinoma cells showed significantly higher sensitivity to RAD001 *in vitro*, compared with the respective cisplatin-sensitive parental cell lines. Furthermore, the *in vivo* antitumor effect of RAD001 was also greater in cisplatin-resistant cell-derived tumors than in cisplatin-sensitive cell-derived tumors (Fig. 5). It has been previously reported that AKT activation may be a biomarker to predict the sensitivity to mTOR inhibitors (20, 25, 26). Although AKT activation is not the sole determinant of sensitivity to mTOR inhibition (26), our results

indicate that enhanced sensitivity to mTOR inhibitors in cisplatin-resistant clear cell carcinoma cells is associated with, at least in part, the activation of AKT/mTOR signaling. Because the RMG1-CR and KOC7C-CR cells used in this study mimic the clinical situation of resistance development in cisplatin-treated patients, our results may suggest that a mTOR inhibitor might have efficacy for the clinical management of cisplatin-resistant clear cell carcinomas.

We should note, however, that a potential limitation of our experimental design is the use of a s.c. xenograft model. Peritoneal dissemination is the main process involved in the progression in human ovarian cancer. Thus, i.p. injection of cancer cells would more accurately model advanced disease. Therefore, further investigation using an i.p. model or a genetically engineered mouse model of ovarian cancer would be helpful.

Our results indicate that RAD001 is a promising agent for the treatment of clear cell carcinoma of the ovary both as a front-line treatment and as a salvage treatment for recurrence after platinum-based chemotherapy. A recent phase III study showed that RAD001 had significant activity in some patients with advanced renal cell carcinoma (44). For patients with recurrent ovarian cancer, the Southwest Oncology Group will soon initiate a randomized phase II trial of carboplatin and paclitaxel with or without everolimus in patients with ovarian cancer in first relapse. We believe that our data support the scientific justification for this and future clinical trials with RAD001 in patients with clear cell carcinoma of the ovary, a chemoresistant histologic subtype characterized by frequent hyperactivation of mTOR pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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