RAD001 (Everolimus) Delays Tumor Onset and Progression in a Transgenic Mouse Model of Ovarian Cancer

Seiji Mabuchi, Deborah A. Altomare, Denise C. Connolly, Andres Klein-Szanto, Samuel Litwin, Matthew K. Hoelzle, Harvey H. Hensley, Thomas C. Hamilton, and Joseph R. Testa

Abstract

The mammalian target of rapamycin (mTOR) is thought to play a critical role in regulating cell growth, cell cycle progression, and tumorigenesis. Because the AKT-mTOR pathway is frequently hyperactivated in ovarian cancer, we hypothesized that the mTOR inhibitor RAD001 (Everolimus) would inhibit ovarian tumorigenesis in transgenic mice that spontaneously develop ovarian carcinomas. We used TgMISIIR-TAg transgenic mice, which develop bilateral ovarian serous adenocarcinomas accompanied by ascites and peritoneal dissemination. Fifty-eight female TgMISIIR-TAg mice were treated with 5 mg/kg RAD001 or placebo twice weekly from 5 to 20 weeks of age. To monitor tumor development, mice were examined biweekly using magnetic resonance microimaging. In vivo effects of RAD001 on Akt-mTOR signaling, tumor cell proliferation, and blood vessel area were analyzed by immunohistochemistry and Western blot analysis. RAD001 treatment markedly delayed tumor development. Tumor burden was reduced by ≈84%. In addition, ascites formation, together with peritoneal dissemination, was detected in only 21% of RAD001-treated mice compared with 74% in placebo-treated animals. Approximately 30% of RAD001-treated mice developed early ovarian carcinoma confined within the ovary, whereas all placebo-treated mice developed advanced ovarian carcinoma. Treatment with RAD001 diminished the expression of vascular endothelial growth factor in tumor-derived cells and inhibited angiogenesis in vivo. RAD001 also attenuated the expression of matrix metalloproteinase-2 and inhibited the invasiveness of tumor-derived cells. Taken together, these preclinical findings suggest that mTOR inhibition, alone or in combination with other molecularly targeted drugs, could represent a promising chemopreventive strategy in women at high familial risk of ovarian cancer. [Cancer Res 2007;67(6):2408–13]

Introduction

Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States (1). Due to the asymptomatic nature of early disease stages, ≈70% of cases have spread beyond the ovary at diagnosis and the cure rate for these patients is <20%. To improve survival, not only is there an urgent need to develop improved chemotherapeutic agents but also for novel chemoprevention strategies to prevent or delay disease progression. However, development of chemoprevention strategies for ovarian cancer has been delayed due, in part, to the lack of an appropriate experimental model. Recently, a transgenic mouse model of spontaneous epithelial ovarian cancer was developed by expression of SV40 Tag/tag under transcriptional control of the MISIIR promoter (2). Tumors arising in TgMISIIR-TAg mice resemble poorly differentiated ovarian adenocarcinomas in women, frequently accompanied by malignant ascites and peritoneal spreading (2). SV40 Tag binds to and functionally inactivates p53 and Rb (3), which are frequently mutated in human ovarian cancer (4). SV40 Tag binds protein phosphatase PP2A and inhibits its activity, resulting in activation of PI3K-AKT and mitogen-activated protein kinase (MAPK) signaling (3). Notably, AKT is frequently hyperactivated in human ovarian cancer (5). Moreover, expression of activated Akt in ovarian surface epithelial cells is sufficient to induce tumor formation in p53-null mice (6), suggesting that Akt is an important target for chemoprevention. Because elevated Akt activity results in hypersensitivity to mTOR inhibition (7), TgMISIIR-TAg-DR26 mice might serve as a valuable model to assess the chemopreventive potential of the mTOR inhibitor RAD001 (Everolimus). In this report, we show that RAD001 markedly delays tumor onset and progression in a mouse model of ovarian cancer.

Materials and Methods

Reagents. RAD001 and placebo were obtained from Novartis Pharma AG (Basel, Switzerland). Anti–phosphorylated (phospho)-p70S6K, anti–phospho-AKT, anti–phospho–mammalian target of rapamy cin (mTOR), anti–phospho-GSK3, anti–cleaved caspase-3, and anti–matrix metalloproteinase (MMP) 2 antibodies were from Cell Signaling (Beverly, MA). Anti–vascular endothelial growth factor (VEGF), anti–SV40 Tag antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–β-actin was from Sigma (St. Louis, MO). Anti–MMP-2 antibody for immunohistochemistry was from Chemicon (Temecula, CA), and anti–CD31/platelet/endothelial cell adhesion molecule 1 (PECAM-1) from PharMingen (San Diego, CA).

Transgenic mice. The ovarian pathology of female TgMISIIR-TAg founder mice has been described (2). A stably breeding transgenic line, TgMISIIR-TAg-DR26, was established via an affected transgenic male founder. Transgenic female offspring develop bilateral epithelial ovarian carcinoma with complete penetrance, surviving an average of 143 days. Animal experiments were approved by our Institutional Animal Care and Use Committee in accordance with NIH guidelines. Mice were typed for SV40 Tag by isolation of tail genomic DNA as described (2).

Drug preparation. RAD001 was formulated at 2% (w/v) in a micro-emulsion vehicle. For animal studies, RAD001 was diluted in double-distilled water just before administration by gavage. For in vitro analyses, RAD001 was prepared in DMSO.

Chemoprevention study. At 5 weeks of age, female TgMISIIR-TAg-DR26 mice were assigned to two groups receiving either RAD001 (n = 29) or placebo (n = 29). Treatment was given in 0.2 mL containing either 5 mg/kg...
RAD001 or placebo twice weekly. Treatment was continued until 20 weeks of age, when all mice were euthanized. The size of ovaries was measured biweekly, using magnetic resonance microimaging (MRRM). Weights were recorded weekly. At the time of sacrifice, mice were examined for peritoneal dissemination and ascites. Ovarian tumors and peritoneal implants were analyzed histologically. Final ovarian tumor volumes were calculated as follows: \( V = \frac{L \times W \times D \times \pi}{6} \), wherein \( V \) is volume, \( L \) is length, \( W \) is width, and \( D \) is depth.

**Magnetic resonance microimaging.** MRRM was done with a 7-Tesla vertical wide-bore magnet, using a Bruker DRX 300 spectrometer with a microimaging accessory. Mice were anesthetized by exposure by the ear with an isoflurane/oxygen mixture for 10 min, after which the isoflurane concentration was reduced to 1%. Mice received a 0.2-mL gadolinium-diethylentriaminepentaacetic acid injection of 10.1 ml injected at the site of a contrast agent (Berlex, Montville, NJ). Contrast agents should be injected into the peritoneal cavity after scanning is performed.

**Histologic analysis.** Tumors were fixed in 10% neutral buffered formalin for 24 h, and sections were cut at 5-μm thickness. Sections were stained with hematoxylin and eosin (H&E) for histology, but they were not counterstained. Sections were then dehydrated through a graded series of alcohols and cleared in xylene. All sections were then mounted with Permount and viewed with an Axioskop microscope.

**Results**

Because this mouse model expresses SV40 tag in ovarian epithelium (2), resulting tumor cells were expected to have activation of the Akt-mTOR pathway. At 9 to 14 weeks of age, some tumors were still confined within the ovary and strong immunoreactivity for Tag, phospho-Akt, phospho-mTOR, and phospho-p70S6K was detected (Fig. 1A), indicating that Akt-mTOR signaling is activated even in early ovarian lesions. Similar results were observed in more advanced tumors. Thus, TgMISIIR-TAg-DR26 mice were deemed a useful preclinical model to test the efficacy of RAD001 as an agent for early intervention/prevention of ovarian cancer.

To determine if RAD001 suppresses tumorogenesis, TgMISIIR-TAged-DR26 mice were treated with placebo or 5 mg/kg of RAD001 twice weekly. Because no obvious ovarian tumors were identified at 5 to 6 weeks of age in a pilot study of TgMISIIR-TAg-DR26 mice, treatment was started at 5 weeks and continued to 20 weeks of age. Body weight of RAD001-treated mice was ~10% lower than in placebo-treated mice (Supplementary Fig. S1), which was attributed to the large size of tumors in control mice. To rule out toxicity due to treatment with RAD001, a pathologist (A.K.S.) did a histologic assessment of various organs (liver, spleen, pancreas, kidney, small and large intestines, uterus, and ovaries) from seven RAD001-treated mice, and no toxic changes were observed.

Representative MRM images of ovarian tumors at 19 weeks of age and necropsy photos of the same mice at 20 weeks of age are shown in Fig. 1B. Figure 1C depicts time to tumor development based on MRM scans. Although nearly all mice developed ovarian tumors, RAD001 treatment significantly delayed tumor onset and progression. Median time for ovaries to reach a volume of 50 mm³ was 90 days in the placebo group compared with >122 days in RAD001-treated mice (Table 1). Median tumor burden measured at time of euthanasia was 429.2 mm³ in controls versus 26.2 mm³ in RAD001-treated mice; moreover, histologic analysis revealed early ovarian tumors confined within the ovary in 32% of RAD001-treated mice compared with 0% in controls.

Because ascitic fluid volume and size of disseminated tumors correlate with patient prognosis and contribute to patient malnutrition through loss of protein and/or suppressed appetite (11), it is important to control dissemination of tumors and ascites production. Ascites formation and peritoneal dissemination were detected in 73.9% of placebo-treated mice but only 21.4% in RAD001-treated mice (Table 1), demonstrating that RAD001 treatment during the early phase of spontaneous tumorogenesis significantly delays tumor development.

Rapamycin inhibits mTOR's ability to phosphorylate p70S6K and 4E-BP1, thereby inhibiting cell cycle progression of cancer cells (7). To investigate the mechanism by which RAD001 inhibits tumorigenesis in TgMISIIR-TAg-DR26 mouse, we conducted cell proliferation assays using MOVCA5 and MOVCA6 cells. Proliferation was significantly inhibited by treatment with RAD001 in both lines (Fig. 2A). Treatment with 20 nmol/L RAD001 markedly decreased the number of Ki-67-positive cells and induced cleavage of PARP as
well (Supplementary Fig. S2). The apoptotic effect is noteworthy given that rapamycin can induce apoptosis in some settings (7). Immunostaining of tumor sections showed that RAD001 treatment results in a marked decrease in Ki-67–positive cells but only a modest increase in the number with immunoreactivity for cleaved caspase-3 (Supplementary Fig. S3). This suggests that RAD001 inhibits tumorigenesis in this model mainly by inhibiting cell proliferation rather than by inducing apoptosis.

As shown in Fig. 2B, Akt, mTOR, and p70S6K were phosphorylated in MOVCAR cells, indicating activation of Akt-mTOR signaling. Phosphorylation of p70S6K was significantly decreased by RAD001 treatment, indicating inhibited downstream signaling of mTOR. Similarly, ovarian tumors from RAD001-treated mice showed markedly decreased phospho-p70S6K staining (Supplementary Fig. S4). Western blotting and immunohistochemistry revealed that RAD001 does not affect expression of SV40 Tag (Supplementary Fig. S5).

VEGF is known to stimulate tumor angiogenesis and mediate ascites formation (12). Because rapamycin can decrease expression of VEGF in ovarian tumors (13), we tested whether RAD001 affects the expression of VEGF in MOVCAR cells (Fig. 2C). VEGF expression was found to be significantly inhibited by RAD001 in both tested MOVCAR lines.

To examine if RAD001 inhibits angiogenesis in vivo, distribution of the endothelial marker CD31 was assessed by immunohistochemistry (Fig. 3A). Large CD31-immunopositive vessels were observed in tumors from placebo-treated mice, whereas fewer and smaller vessels were observed in tumors from RAD001-treated mice. There was a significant decrease of microvessel area in RAD001-treated tumors compared with control tumors (Fig. 3B). Thus, RAD001’s antitumor effect may be associated, in part, with inhibition of angiogenesis.

MMP-2 expression is an early event in the invasiveness of ovarian carcinoma (14). Elevated MMP-2 expression is associated with tumor invasiveness and metastatic potential, ascites formation, peritoneal dissemination, and poor prognosis in patients with ovarian cancer (14, 15). We found that MMP-2 is expressed in early ovarian tumors from our mice (Fig. 1A). MMP-2 expression was observed in both tested MOVCAR lines, and treatment with
RAD001 significantly attenuated expression of MMP-2 (Fig. 2D) and inhibited cell invasion through Matrigel (Fig. 3C, D). These findings are consistent with the fact that RAD001 inhibits tumor dissemination and ascites production in TgMISIIR-TAg-DR26 mice.

**Discussion**

Our data show that mTOR inhibition markedly delays tumorigenesis in a murine model of ovarian cancer. Although RAD001 treatment did not reduce tumor incidence, it resulted in a remarkable reduction in the number of mice with advanced disease and inhibited ascites formation and peritoneal dissemination. These findings have important implications, because the cure rate of patients with early ovarian cancer is nearly 90% compared with <20% in patients with advanced disease (16).

However, some tumors in RAD001-treated mice escaped the inhibitory effect of RAD001 and became large, indicating the presence of an alternative proliferation pathway. Germane to this, recent work indicates that a combination of rapamycin and a MAPK inhibitor is necessary to inhibit the growth of ovarian tumors that have two redundant proliferation signals (i.e., mTOR and MAPK; ref. 17). In MOVCAR cells, both Akt-mTOR (Fig. 2B) and MAPK pathways (Supplementary Fig. S6) are activated, likely due to the expression of SV40 tag. Therefore, treatment with RAD001 and a MAPK inhibitor might be more efficacious in RAD001-resistant TgMISIIR-TAg-DR26 tumors.

Another possible reason why some tumors escaped the inhibitory effect of RAD001 is that drug treatment may have stimulated a survival pathway. Although RAD001 effectively inhibited phosphorylation of p70S6K, phosphorylation of Akt (as well as mTOR and GSK3) was up-regulated by RAD001 treatment in MOVCAR cells and tumors (Fig. 2B; Supplementary Fig. S4). This is consistent with recent work demonstrating that mTOR inhibition triggers a feedback mechanism that activates Akt, which can then activate other downstream pathways that may promote tumor growth (18). Thus, future studies are planned to evaluate the chemopreventive efficacy of RAD001 combined with a PI3K/Akt inhibitor using TgMISIIR-TAg-DR26 mice.

**Table 1. Effect of RAD001 on tumor development in a mouse model of ovarian cancer**

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<thead>
<tr>
<th>Treatment</th>
<th>No. mice</th>
<th>No. mice with tumors (%)</th>
<th>No. mice with advanced tumors (%)</th>
<th>Tumor volume (mm³)</th>
<th>Median time for tumor to reach 50 mm³ (d)</th>
<th>Incidence of ascites formation (%)</th>
<th>Tumor type</th>
<th>Incidence of ascites formation (%)</th>
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<tr>
<td>Placebo</td>
<td>29</td>
<td>29 (100)</td>
<td>29 (100)</td>
<td>0 (0)</td>
<td>429.2</td>
<td>90</td>
<td>73.9</td>
<td>Advanced 73.90</td>
</tr>
<tr>
<td>RAD001</td>
<td>29</td>
<td>28 (96.6)</td>
<td>19 (67.9)*</td>
<td>9 (32.1)*</td>
<td>26.2*</td>
<td>&gt;122</td>
<td>21.4*</td>
<td>Advanced 31.6*</td>
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*Significantly different from placebo group (P < 0.01).
Chemoprevention will likely require long-term treatment. Thus, a low toxicity profile is required for an effective chemopreventive agent. RAD001 is approved in Europe as an immunosuppressive agent in the solid organ transplantation setting, and there are extensive safety data regarding long-term usage of RAD001 (19). The dosage used in our study seemed to be well tolerated, with no toxic changes evident histologically.

The risk of developing ovarian cancer is 30% to 60% in women with \textit{BRCA1} mutations and 10% to 30% in those with \textit{BRCA2} mutations. Currently, prophylactic oophorectomy is the only way to reduce lifetime risk of ovarian cancer in these women (20). Whereas there is no clearly defined ovarian preneoplastic lesion, phospho-AKT/phospho-mTOR immunostaining is frequently found in inclusion cysts and other histologic lesions in ovaries removed prophylactically from women with hereditary \textit{BRCA1} or \textit{BRCA2} mutations. Collectively, our findings suggest that mTOR inhibition might be a useful strategy to prevent or delay onset of ovarian cancer in women with hereditary ovarian cancer syndrome.

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\textsuperscript{7} Unpublished data.

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**Figure 3.** RAD001 inhibits angiogenesis and invasiveness. \textit{A,} immunohistochemical staining of representative ovarian carcinomas from Tg\textit{MISIIIR-TAg} transgenic mice. Serial sections of an ovarian cancer tissue microarray were stained with anti–CD31/PECAM-1 antibody. Magnification, \(\times50.\) \textit{B,} histogram indicating microvessel area of individual ovarian tumors analyzed by anti-CD31 immunostaining. Significant differences are indicated by asterisks (**, \(P < 0.01).\) \textit{C,} effect of RAD001 on invasiveness of MOVCAR cells. The cells were seeded on Matrigel-coated filters, treated with 20 nmol/L of RAD001, and incubated for 24 h. Magnified views of undersides of filters are shown. \textit{D,} histogram depicting the relative number of cells that penetrated through the Matrigel, with the number of penetrating cells in the vehicle control set arbitrarily at 1.0 (100%). \textit{Columns,} mean from at least three separate experiments; \textit{bars,} SE. Significant differences are indicated by asterisks (**, \(P < 0.01).\)
References

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