SPANDIDOS PUBLICATIONS

RAD001 offers a therapeutic intervention through inhibition of mTOR as a potential strategy for esophageal cancer

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Abstract. Esophageal cancer is one of the most frequently occurring cancers in the world. Targeting therapy strategy of cancer with specific inhibitors is developing and has showed promising antitumor efficacy. It is known that mTOR is an important controller of cell growth. RAD001 (everolimus) is a specific inhibitor of mTOR that can block the mTOR signaling pathway. The purposes of this study was to explore the inhibitory effects of RAD001 on mTOR signaling and the mechanism of cell growth suppression by RAD001. We examined both the expression of mTOR, p70S6K and S6 in SEG-1 esophageal cancer cells and KOB-13 normal esophageal epithelial cells and the efficacy of RAD001 against SEG-1 esophageal cancer cells. mTOR, p70S6K and S6 were overexpressed in SEG-1 esophageal cancer cells compared with KOB-13 normal esophageal epithelial cells. SEG-1 esophageal cancer cells were sensitive to RAD001. The survival rate of the cells treated with RAD001 over 0.33 μM was significantly different compared with that of control ($P<0.01$). RAD001 inhibited the phosphorylation of mTOR (Ser2448) and S6 (Ser240/244) in different grades and the expressions of mTOR, p70S6K and S6. As a result, RAD001 induced a dose-dependent decrease in cell proliferation, G1/S arrest and damage of cell shape. Taken together, these data showed that RAD001 can inhibit mTOR signaling and proliferation in SEG-1 esophageal cancer cells *in vitro*. It offers a therapeutic intervention through inhibition of mTOR as a potential strategy for esophageal cancer.

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Introduction

Esophageal cancer is one of the most frequently occurring cancers in the world. The incidences of esophageal cancer differ greatly among different regions and countries, depending on race, eating habits and environments. The incidence is 3-4 times higher in men than in women, and it is an important cause of death, with about 462,000 newly occurring cases and 38,600 deaths per year, the death to incidence ratio reaching 0.8 (1-4). The incidence of esophageal cancer has increased rapidly in the United States over the last three decades (5). It also occurred with high incidence and mortality in Europe (6) and in Southern and Northern temperate zones (1), especially in China (7). New therapy strategy is needed because of the poor prognosis of patients with esophageal cancer. Targeting therapy strategy of cancer with specific inhibitors is developing and has showed promising antitumor efficacy. Increasing knowledge of the signal transduction pathways for growth factors has led to speculation that they could offer novel targets for cancer therapy.

The mammalian target of rapamycin (mTOR) molecular weight M_r 289,000, also named FKBP-rapamycin associated protein (FRAP), is an evolutionarily conserved protein kinase that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family and functions as a serine/threonine kinase. mTOR can integrate and converge a wide range of signals, including intracellular and extracellular nutrients, growth factors and stress conditions, thereby regulating cell growth through the downstream effectors 4EBP1 and p70S6K (S6K1). Thus, mTOR acts as a central regulator of cell growth and cell cycle to mediate the underlying biological processes. The mTOR signaling pathway is abnormally activated in many human cancers, leading to the pathway activation (8-11). S6K1 and elF-4E, downstream targets of mTOR, are known to be activated and overexpressed in

many cancers, and have been found to be associated with the transformation process and oncogenesis (12,13). As a result, the mTOR pathway is considered to be an important target for cancer drug development.

Rapamycin (Sirolimus) is a macrolide antibiotic produced by *Streptomyces hygroscopicus*, which binds FKBP-12 (FK506 binding protein). The rapamycin-FKBP12 complex can inhibit mTOR activity. Rapamycin is the founding member of the family of mTOR inhibitors. The most notable of the rapamycin analogues currently in clinical trials as anticancer agents include RAD001 (everolimus), CCI-779 (temsirolimus), and AP23573. These drugs are highly specific inhibitors of mTOR, and differ only slightly in chemical structure with superior chemical stability and pharmaceutical properties. RAD001, 40-O-(2-hydroxyethyl)-rapamycin, or everolimus, can be administrated orally. RAD001 exhibits the anti-tumor effect *in vitro* and *in vivo* (14-16), and demonstrates a dose-dependent anti-tumor activity in some carcinoma models (17-19). RAD001 induced the G1/S cell cycle arrest and apoptosis (20-22). Now, RAD001 is currently under-going evaluation studies in phase II as an anti-tumor agent.

In this study, we treated SEG-1 esophageal cancer cells with RAD001 to show mTOR signaling has an important role in esophageal cancer cell growth regulation. Our data showed the inhibitive effects of RAD001 on mTOR signaling and esophageal cancer cell growth. It offers a therapeutic intervention through inhibition of mTOR as a potential strategy for esophageal cancer.

Materials and methods

Cell lines and culture conditions. The human esophageal cancer cells SEG-1 kindly provided from Dr David Beer in University of Michigan were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. The KOB-13 normal esophageal epithelial cells were maintained in KSFM supplemented with 10% heat-inactivated fetal bovine serum, EGF and BPE. All cell lines were cultured in 10% CO_2 at 37˚C.

Reagents. RAD001 (everolimus), an orally bioavailable derivative of rapamycin, was synthesized by Novartis Pharma AG (Basel, Switzerland) and dissolved in DMSO (Sigma Chemical Corp., St. Louis, MO). The concentration of DMSO in the final solution did not exceed 1% (v/v).

Trypan blue exclusion assay of cell proliferation. The anti-proliferative activity of RAD001 on SEG-1 esophageal cancer cells growing in culture was determined using a trypan blue exclusion assay. SEG-1 cells were seeded directly in 24-well culture plates at a density of $1x10^4$ per well 24 h before drug treatment. i) For sensitivity experiments, subconfluent cells were treated with 0.037, 0.11, 0.33, 1.0, 3.0, 9.0 μM RAD001 and 1% DMSO (v/v) for 48 h, and then cell cultures were harvested with trypsin and stained with trypan blue. Cell number was counted using a hematocytometer; ii) for growth curve experiments, subconfluent cells were treated with different concentrations of RAD001 (1, 5, 10 and 20 μM) for 48 h, and then cell cultures were harvested with trypsin on the indicated day after treatment and stained with trypan blue. Cell number was counted using a hematocytometer.

MTT assay and IC_{50} calculation. Exponentially growing cells were seeded in 96-well plates at a density of $4x10^3$ cells per well 24 h before drug treatment. Then cells were incubated with RAD001 at various concentrations (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 30 μM) for 48 h. The medium with RAD001 was absorbed and fresh medium was added. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (20 μl) (MTT, 5 g/l; Nacalai tesque, Inc., Japan) was added to each well and incubated for 4 h at 37˚C. The solution was absorbed and the formazan product was dissolved by adding 100 μl of 0.04 M 2-propanol·HCl to each well and incubated for 10 min at 37˚C. The MTT absorbance value was detected at 540/620 nm with a spectrophotometer set (Thermo, Multiskan SX 353, USA). IC_{50} was calculated by Logit model based on the data.

Cell cycle analysis by flow cytometry. For cell cycle analysis, SEG-1 esophageal cancer cells were plated in 6-well tissue culture plates at a density of $3x10^5$ cells per well and incubated for 24 h at 37˚C. Subconfluent cells were treated with 5 μM RAD001 for 48 h, and then harvested. Cells were washed with cold PBS, stained with 50 mg/l propidium iodide (PI). DNA content was analyzed by flow cytometry (FACS Calibur, Becton-Dickinson Co., USA).

Cell shape assay. SEG-1 esophageal cancer cells were seeded directly in 6-well culture plates at a density of $3x10^5$ per well 24 h before drug treatment. Cells were then treated with various concentrations of RAD001 (0.1, 1, 10, 20 and 30 μM) for 24 and 48 h. Cells were imaged with a digital camera mounted to a light microscope (Olympus DP-70).

Western blot analysis. SEG-1 esophageal cancer cells were plated onto 6-well plates at a density of $3x10^5$ per well and then incubated for 24 h in medium. On the following day, cells were treated with different concentrations of RAD001 (1, 5 and 20 μM) for 48 h. Cell cultures were collected after treatment with trypsin and washed three times with cold PBS. Cells then were dissolved in a cell lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 100 g/l Glycerol, 50 g/l Triton X-100, 2 g/l Na_2VO_4, and 4 g/l EDTA. Adding 10 μl PMSF (0.1 M) and 10 μl aprotinin (10 g/l) to each 1 ml lysis buffer just before use. It was put on ice for 15 min and centrifuged at 15,000 r/m at 4˚C for 20 min. The concentrations of protein lysates were measured by the Bio-Rad protein determination method (Bio-Rad laboratories, Hercules, CA). Equal amounts (40 μg) of protein were electrophoresed in 12 or 8% (w/v) SDS poly-acrylamide gels. Proteins were then transferred to Hybond-polyvinylidene difluoride transfer membranes (Amersham, Arlington Heights, IL) and incubated with the primary antibodies overnight at 4˚C, followed by incubation with peroxidase-linked secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence (ECL) (Amersham) was used for signal detection. The primary antibodies were mTOR (Epitomic, Inc.), phosphor-mTOR (Ser2448), S6,

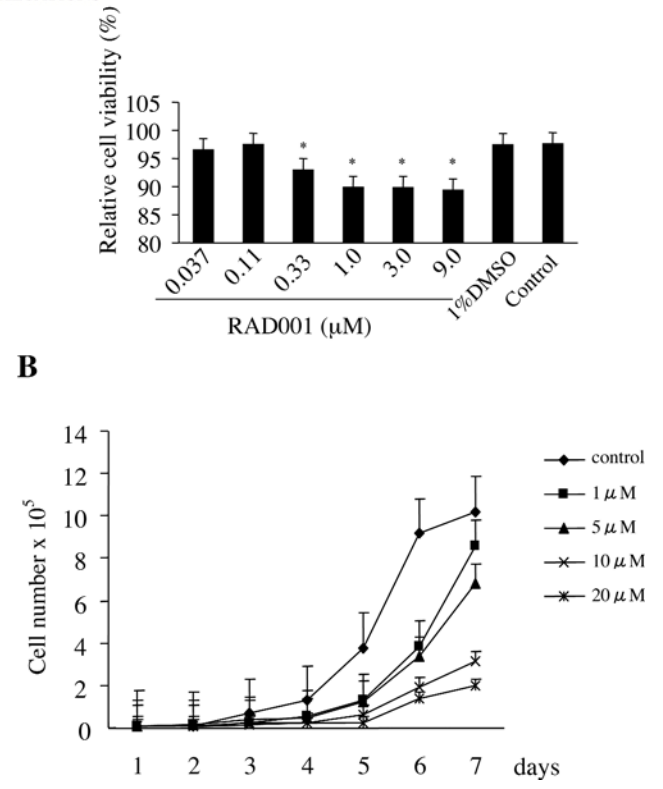

Figure 1. RAD001 suppresses proliferation of SEG-1 esophageal cancer cells. (A) The effects of RAD001 and its menstruum DMSO on cell proliferation of SEG-1 esophageal cancer cells were examined by trypan blue exclusion assay. The survival rate of cells treated with RAD001 over 0.33 μM was significant different compared with that of 1% DMSO and control. (B) RAD001 treatment started at day 1 and cell number in each condition was counted every 24 h until day 7. Each plot mark represents the following: diamond (♦), control (DMSO only); rectangle (■), 1 μM of RAD001; triangle (▲), 5 μM of RAD001; fork (×), 10 μM of RAD001; star (*), 20 μM of RAD001.

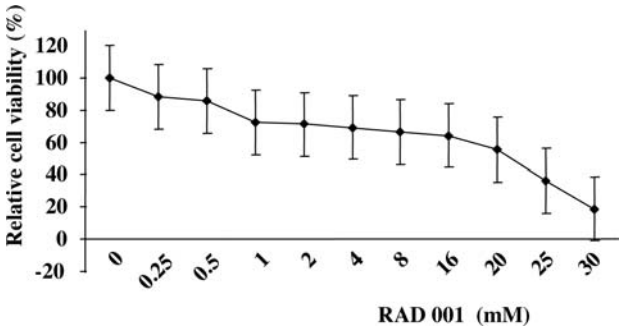

Figure 2. Inhibition curve of RAD001 on SEG-1 cell growth. SEG-1 esophageal cancer cells were treated with different concentration of RAD001 (0.25-30 μM) for 48 h and were determined the susceptibility to RAD001 by MTT assay. RAD001 induced a dose-dependent decrease in cell proliferation.

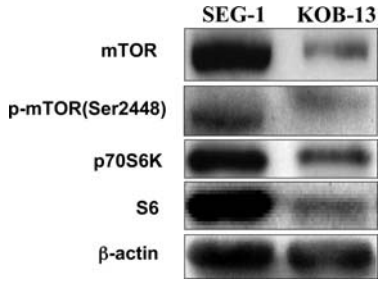

Figure 3. Expressions of mTOR, p70S6K and S6 were higher in SEG-1 esophageal cancer cells than in KOB-13 normal esophageal epithelial cells. Expressions of mTOR, p70S6K and S6 were detected by Western blotting. mTOR, p70S6K and S6 were overexpressed in SEG-1 esophageal cancer cells compared with those in KOB-13 normal esophageal epithelial cells.

phosphor-S6 (Ser240/244) (Cell Signaling Technology, Inc.), p70S6K (Santa Cruz) and ß-actin (Sigma Aldrich).

Statistical analysis. Descriptive statistics were generated for all quantitative data with presentation of mean ± SD. Proliferation of the cells exposed to the drugs was compared to the negative control. Statistical significance was defined as $^{*}p<0.05$.

Results and Discussion

RAD001 inhibits proliferation of SEG-1 esophageal cancer cells. The effects of RAD001 on cell proliferation of SEG-1 esophageal cancer cells were examined by trypan blue exclusion assay. SEG-1 esophageal cancer cells were sensitive to RAD001 and the survival rate of the cells treated with RAD001 over 0.33 μM was significantly suppressed compared with that of control. There was no significant difference between the cells treated with 1% DMSO and control (Fig. 1A). As shown in Fig. 1B, the growth curve demonstrated that of 10 and 20 μM of RAD001 clearly suppressed the SEG-1 cell growth from 6 days to 7 days after treatment. Next, to determine the inhibitory effect of RAD001 on cell growth and optimize its concentration for further experiments, inhibition concentration 50% (IC_{50}) was measured to examine cytostatic effect of RAD001 in esophageal cancer cells. SEG-1 esophageal cancer cells were treated with different concentrations of RAD001 (0.25-30 μM) for 48 h and the susceptibility to RAD001 was determined by MTT assay. The IC_{50} of RAD001 on SEG-1 esophageal cancer cells was 12.04 μM (Fig. 2).

The mTOR, p70S6K and S6 were overexpressed in SEG-1 esophageal cancer cells. To analyze the expression status of mTOR and its downstream target p70S6K and S6 in SEG-1 esophageal cancer cells, we performed Western blot analysis. As shown in Fig. 3, the expression of mTOR, p70S6K and S6 were stronger in SEG-1 esophageal cancer cells than in KOB-13 normal esophageal epithelial cells (Fig. 3).

RAD001 inhibits activation of mTOR and its downstream targets p70S6K and S6. In order to explore the mechanism of RAD001 inhibition in SEG-1 esophageal cancer cells, we investigated the activities of five kinds of molecules related to the mTOR signaling pathway by Western blotting. They were: mTOR and phospho-mTOR (Ser2448), downstream target p70S6K, S6 and phospho-S6 (Ser240/244). As shown

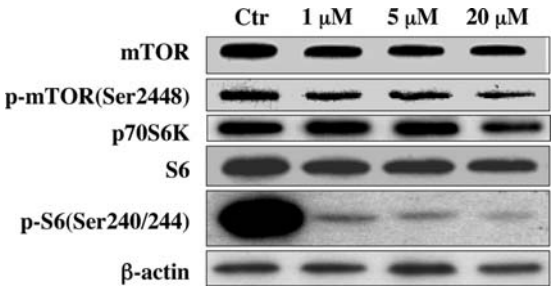

Figure 4. Activations of mTOR and its downstream molecules were inhibited by RAD001 in SEG-1 esophageal cancer cells. Expression of mTOR, p70S6K, and S6 and phosphorylation of phospho-mTOR (Ser2448), and phosphor-S6 (Ser240/244) status were examined by Western blotting at 48 h after the addition of RAD001. ß-actin served as an internal control.

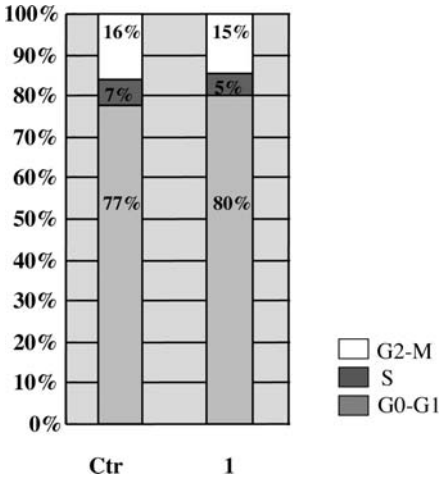

Figure 5. RAD001 induces G1/S cell cycle arrest in SEG-1 esophageal cancer cells. RAD001 treatment kept for 48 h and cell cycle analysis was performed by flow cytometry. RAD001 induces G1/S cell cycle arrest in SEG-1 esophageal cancer cells. (Ctr), Control without RAD001; (1), treatment with 5 μM RAD001 for 48 h.

in Fig. 4, RAD001 inhibited the phosphorylation of mTOR and S6 and slightly suppressed the expressions of mTOR, p70S6K and S6 in SEG-1 cells 48 h after treatment.

RAD001 induced G1/S cell cycle arrest in SEG-1 esophageal cancer cells. Next, to analyze the cell cycle after RAD001 treatment, SEG-1 esophageal cancer cells were incubated with RAD001 (5 μM for 48 h) and FACS analysis was performed. An inhibition of cell cycle progression occurred as a result of RAD001 treatment, as demonstrated by a decreased proportion of cells in the S phase (Fig. 5). These results showed that RAD001 induces G1/S cell cycle arrest in SEG-1 esophageal cancer cells.

Morphological change of SEG-1 esophageal cancer cells after RAD001 treatment. mTOR acts as a central regulator for cell growth. In order to examine the lethal effect in SEG-1 esophageal cancer cells, the cells were treated with different concentrations of RAD001 (0.1-30 μM) for 24 and 48 h. Cell death was clearly observed 48 h after 20 and 30 μM RAD001 treatments in SEG-1 esophageal cancer cells (Fig. 6).

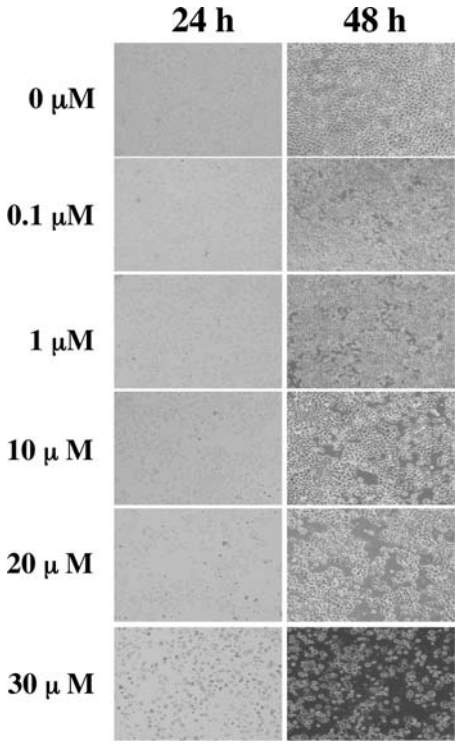

Figure 6. RAD001 induces morphological damage of SEG-1 esophageal cancer cells. SEG-1 cells were treated with indicated concentrations of RAD001 and microscopic images were taken at 24 h (A) and at 48 h (B). Morphological change was observed in 24 h and cell density was obviously decreased by the treatment with the mTOR inhibitor in a dose-dependent fashion.

Conventional therapeutic strategies of tumors include surgery followed by radiation and chemotherapy. In recent years, preventive and therapeutic strategies for targeting the key molecules involved in the signaling transduction pathways of the cell proliferation, migration, and the invasion of tumors have made great progress (8,23-27). Many specific inhibitors have been discovered and are beginning to be used in clinic trials against cancer. RAD001 (everolimus), an orally bioavailable analog of rapamycin, has demonstrated potent antiproliferation effects against a variety of human tumor-derived cell lines grown either *in vitro* or as tumors in animal models (28-31). RAD001 displayed a good safety profile in clinical trails (16,32).

The inhibition of RAD001 on mTOR can affect the phosphorylation of the downstream molecules p70S6K and S6 (9,17). We found that the expression of mTOR, p70S6K and S6 were inhibited and the phosphorylation of mTOR and S6 were inhibited when SEG-1 esophageal cancer cells were treated with RAD001. Furthermore, it displayed a dose dependent effect. These data provided direct evidences that mTOR signaling plays a role in the expression of these genes. The data showed a mechanism of inhibitive effect of RAD001 on SEG-1 esophageal cancer cell proliferation.

mTOR, as a Ser/Thr kinase, belongs to the phosphatidylinositol kinase-related kinase super family, and is the focus of research on signal transduction and cell proliferation. It is believed that mTOR regulates the basic biological process as a central regulator of cell growth (33,34). Studies on the constitution of the mTOR pathway

that there was a relationship between the dysregulation of the mTOR pathway and tumors (35,36). The mTOR signal, together with the PI3K signal, controlled the cell proliferation of tumors. Three recent studies reviewed the research results on mTOR and the occurrence of tumors, therapy, and the design of anti-tumor drugs (8,37,38). We tested the expression of mTOR in SEG-1 esophageal cancer cells and KOB-13 normal esophageal epithelial cells by means of Western blotting. The results showed that expression of mTOR was stronger than that of KOB-13 normal esophageal epithelial cells. At the same time, p70S6K and S6 were also overexpressed, showing the accordance of the expression of mTOR signaling and tumor growth. RAD001, the specific inhibitor of mTOR, can affect the phosphorylation of mTOR (Ser2448) and can greatly inhibit the expression and phosphorylation of S6 with a dose-dependent effect in SEG-1 esophageal cancer cells. These data showed the importance of the mTOR signal pathway in the regulation of cell proliferation.

Recent studies showed that the mTOR/S6K signals exhibited a feedback down-regulation to IRS, which constitutes a feedback regulation cycle of the PI3K/Akt/mTOR/S6K signal pathway (39). The inhibition of rapamycin on mTOR induced the activation of its upstream receptor Akt and exhibited an enhanced resistance to inhibitor of mTOR, which had been confirmed both in the cancer cell lines and in patient tumors treated with RAD001 (8,40-43). There are many reports showing the effectiveness of the combinatorial therapy, in which mTOR inhibitor was combined with other kinase inhibitors, or chemotherapy and radiation treatment (24,28-30,44-46). The results suggested that the combinatorial anticancer therapy strategy was necessary when using the mTOR inhibitors as anti-tumor drugs.

Although rapamycin and its analogs are well developed as anticancer drugs, to find and design new inhibitors of mTOR signaling is needed. The further understanding of the interaction among the molecules in mTOR complex and the molecules in mTOR signal pathway will present a good opportunity for designing and developing specific inhibitors on the pathway. Targeting proteins of the mTOR signal pathway can provide more effective and alternative approaches for developing new drugs. For example, recent results showed that FKBP38 was a key regulator of mTOR. There are no stimulating signals to cell growth produced when FKBP38 binds to mTOR, while the growth signal is released when FKBP38 binds to Rheb (47,48). The discovery provides more targets for studying the inhibitors of mTOR pathway and a new pathway for targeting therapy of cancer.

In conclusion, our results showed the importance of the mTOR signaling pathway in the regulation of esophageal cancer cell proliferation. RAD001 had inhibitory effect in SEG-1 esophageal cancer cells through inhibition of the activity of mTOR and its downstream molecule S6. Thus, we provided a therapeutic intervention through inhibition of mTOR as a potential strategy for esophageal cancer.

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