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

ZHI GANG WANG[1,2], TAKUYA FUKAZAWA[2], TOSHIO NISHIKAWA[2], NOBUYUKI WATANABE[2], KAZUFUMI SAKURAMA[2], TAKAYUKI MOTOKI[2], SHINJI HATAKEYAMA[3], OSAMU OMORI[3], TOSHIAKI OHARA[2], SHUNSUKE TANABE[2], YASUHIRO FUJIWARA[2], MUNENORI TAKAOKA[2], YASUHIRO SHIRAKAWA[2], TOMOKI YAMATSUJI[2], NORIAKI TANAKA[2] and YOSHIO NAOMOTO[2]

[1]College of Life Science, Inner Mongolia University, The Key Laboratory of Mammal Reproductive Biology and Biotechnology, Ministry of Education, Hohhot 010021, P.R. China; [2]Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Graduate School of Medicine and Dentistry, Okayama University, 2-5-1 Shikatacho, Okayama 700-8558; [3]Novarutis Pharma, Tsukuba, Japan

Received October 13, 2009; Accepted January 15, 2010

DOI: 10.3892/or_00000747

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.

Correspondence to: Dr Yoshio Naomoto, Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan
E-mail: ynaomoto@md.okayama-u.ac.jp

Key words: RAD001 (everolimus), mTOR signaling, cell growth, esophageal cancer cells

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,

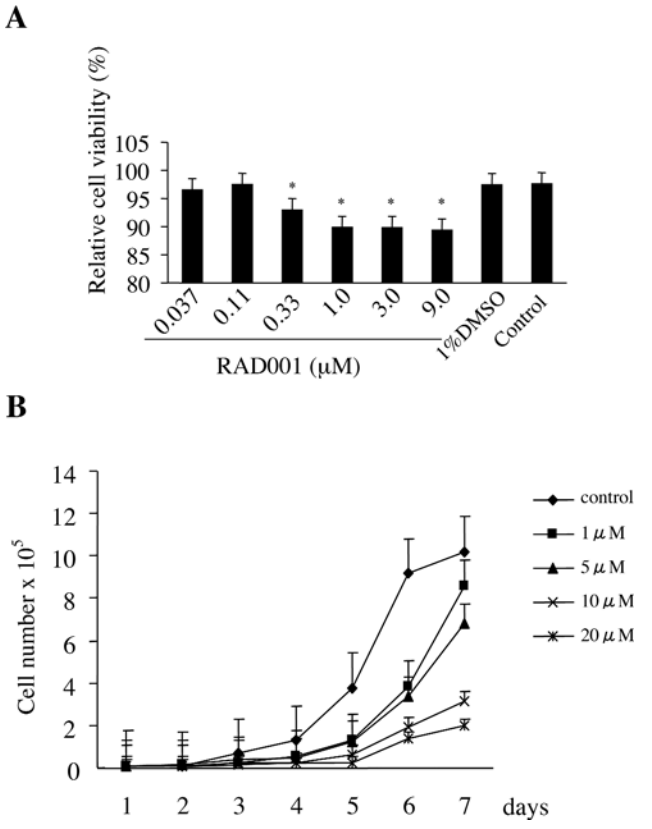

Figure 1. RAD001 suppresses proliferation of SEG-1 esophageal cancer cells. (A) The effects of RAD001 and its menstruum DMSO on cell proliferation of SEG-1 esophageal cancer cells were examined by trypan blue exclusion assay. The survival rate of cells treated with RAD001 over 0.33 μM was significant 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.

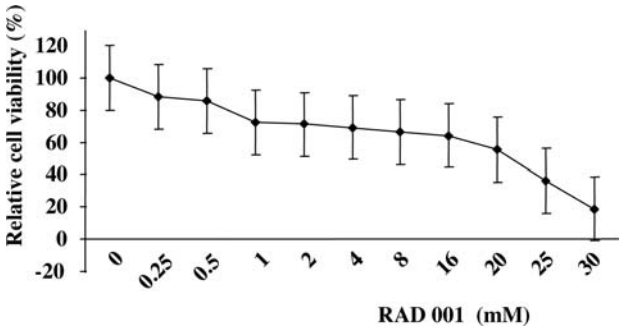

Figure 2. Inhibition curve of RAD001 on SEG-1 cell growth. SEG-1 esophageal cancer cells were treated with different concentration of RAD001 (0.25-30 μM) for 48 h and were determined the susceptibility to RAD001 by MTT assay. RAD001 induced a dose-dependent decrease in cell proliferation.

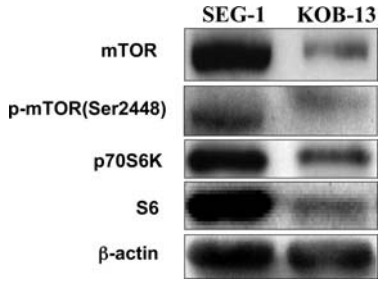

Figure 3. Expressions of mTOR, p70S6K and S6 were higher in SEG-1 esophageal cancer cells than in KOB-13 normal esophageal epithelial cells. Expressions of mTOR, p70S6K and S6 were detected by Western blotting. mTOR, p70S6K and S6 were overexpressed in SEG-1 esophageal cancer cells compared with those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

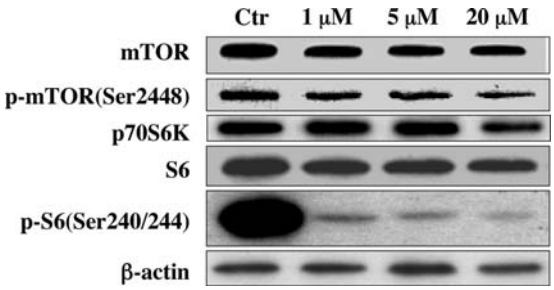

Figure 4. Activations of mTOR and its downstream molecules were inhibited by RAD001 in SEG-1 esophageal cancer cells. Expression of mTOR, p70S6K, and S6 and phosphorylation of phospho-mTOR (Ser2448), and phosphor-S6 (Ser240/244) status were examined by Western blotting at 48 h after the addition of RAD001.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

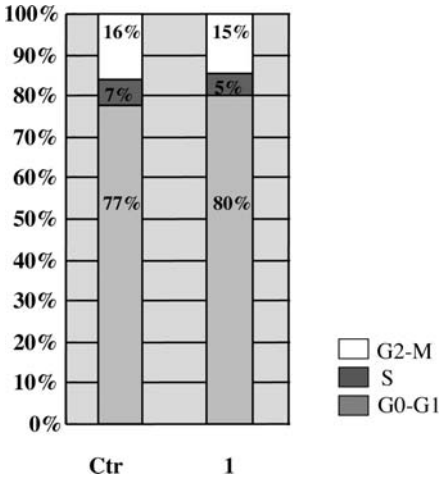

Figure 5. RAD001 induces G1/S cell cycle arrest in SEG-1 esophageal cancer cells. RAD001 treatment kept for 48 h and cell cycle analysis was performed by flow cytometry. RAD001 induces G1/S cell cycle arrest in SEG-1 esophageal cancer cells. (Ctr), Control without RAD001; (1), treatment with 5 μM RAD001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

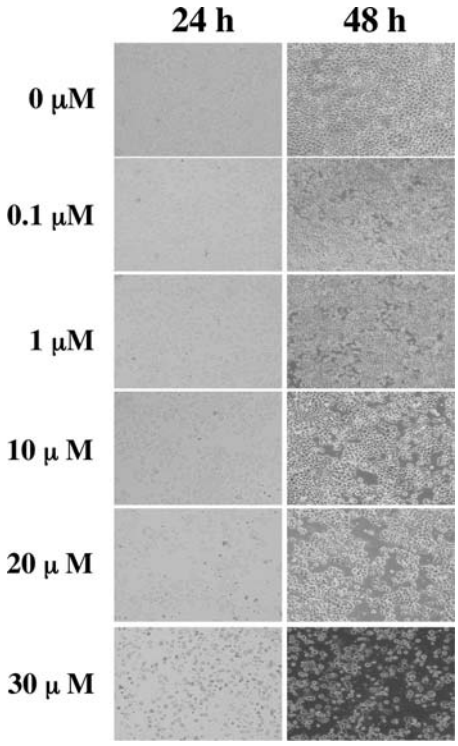

Figure 6. RAD001 induces morphological damage of SEG-1 esophageal cancer cells. SEG-1 cells were treated with indicated concentrations of RAD001 and microscopic images were taken at 24 h (A) and at 48 h (B). Morphological change was observed in 24 h and cell density was obviously decreased by the treatment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

showed that there was a relationship between the disorganization 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.

Acknowledgments

We thank Mr. Tanida for technical assistance. Grant support: The Ministry of Education, Science, and Culture, Japan.

References

1. Lambert R and Hainaut P: Esophageal cancer: cases and causes (Part I). Endoscopy 39: 550-555, 2007.
2. Lambert R and Hainaut P: Esophageal cancer: the precursors (Part II). Endoscopy 39: 659-664, 2007.
3. Wu X, Chen VW, Andrews PA, Ruiz B and Correa P: Incidence of esophageal and gastric cancer among Hispanics, non-Hispanic whites and non-Hispanic blacks in the United States: subsite and histology differences. Cancer Causes Control 18: 585-593, 2007.
4. Wu X, Chen VW, Ruiz B, Andrews P, Su LJ and Correa P: Incidence of esophageal and gastric carcinomas among American Asians/Pacific Islanders, Whites, and Blacks: subsite and histology differences. Cancer 106: 683-692, 2006.
5. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics, 2007. CA Cancer J Clin 57: 43-66, 2007.
6. Bosetti C, Levi F, Ferlay J, Garavello W, Lucchini F, Bertuccio P, Negri E and Vecchia CL: Trends in oesophageal cancer incidence and mortality in Europe. Int J Cancer 122: 1118-1129, 2008.
7. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 55: 74-108, 2005.
8. Wan X and Helman LJ: The biology behind mTOR inhibition in sarcoma. Oncologist 12: 1007-1018, 2007.
9. Hou G, Xue L, Lu Z, Fan T, Tian F and Xue Y: An activated mTOR/p70S6K signaling pathway in esophageal squamous cell carcinoma cell lines and inhibition of the pathway by rapamycin and siRNA against mTOR. Cancer Lett 253: 236-248, 2007.
10. Tsang CK, Qi H, Liu LF and Zheng XFS: Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug Discov Today 12: 112-124, 2007.
11. Vega F, Medeiros LJ, Leventaki V, Atwell C, Cho-Vega JH, Tian L, Claret FX and Rassidakis GZ: Activation of mammalian target of rapamycin signaling pathway contributes to tumor cell survival in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma. Cancer Res 66: 6589-6597, 2006.
12. Sahin F, Kannangai R, Adegbola O, Wang J, Su G and Torbenson M: mTOR and p70 S6 kinase expression in primary liver neoplasms. Clin Cancer Res 10: 8421-8425, 2004.
13. Jiang H, Coleman J, Miskimins R and Miskimins WK: Expression of constitutively active 4EBP-I enhances p27Kip I expression and inhibits proliferation of MCF7 breast cancer cells. Cancer Cell Int 3: 2, 2003.
14. Mabuchi S, Altomare DA, Connolly DC, Klein-Szanto A, Litwin S, Hoelzle MK, Hensley HH, Hamilton TC and Testa JR: RAD001 (Everolimus) delays tumor onset and progression in a transgenic mouse model of ovarian cancer. Cancer Res 67: 2408-2413, 2007.
15. Haritunians T, Mori A, O'Kelly J, Luong QT, Giles FJ and Koeffler HP: Antiproliferative activity of RAD001 (everolimus) as a single agent and combined with other agents in mantle cell lymphoma. Leukemia 21: 333-339, 2007.
16. Vignot S, Faivre S, Aguirre D and Raymond E: mTOR-targeted therapy of cancer with rapamycin derivatives. Ann Oncol 16: 525-537, 2005.
17. Boulay A, Zumstein-Mecker S, Stephan C, Beuvink I, Zilbermann F, Haller R, Tobler S, Heusser C, O'Reilly T, Stolz B, Marti A, Thomas G and Lane HA: Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. Cancer Res 64: 252-261, 2004.
18. Jundt F, Raetzel N, Muller C, Calkhoven CF, Kley K, Mathas S, Lietz A and Dorken B: A rapamycin derivative (everolimus) controls proliferation though down-regulation of truncated CCAAT enhancer binding protein and NF-κB activity in Hodgkin and anaplastic large cell lymphomas. Blood 106: 1801-1807, 2005.
19. Zitzmann K, De Toni EN, Brand S, Goke B, Meinecke J, Spottl G, Meyer HHD and Auernhammer CJ: The novel mTOR inhibitor RAD001 (everolimus) induces antiproliferative effects in human pancreatic neuroendocrine tumor cells. Neuroendocrinology 85: 54-60, 2007.
20. Wanner K, Hipp S, Oelsner M, Ringshausen I, Bogner C, Peschel C and Decker T: Mammalian target of rapamycin inhibition induces cell cycle arrest in diffuse large B cell lymphoma (DLBCL) cells and sensitizes DLBCL cells to rituximab. Br J Haematol 134: 474-484, 2006.

21. Ferri N, Granata A, Pirola C, Torti F, Pfister PJ, Dorent R and Corsini A: Fluvastatin synergistically improves the antiproliferative effect of everolimus on rat smooth muscle cells by altering p27Kip1/cyclin E expression. Mol Pharmacol 74: 144-153, 2008.
22. Beuvink I, Boulay A, Fumagalli S, Zilbermann F, Ruetz S, O'Reilly T, Natt F, Hall J, Lane HA and Thomas G: The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell 120: 747-759, 2005.
23. Sarkar FH and Li Y: Targeting multiple signal pathways by chemopreventive agents for cancer prevention and therapy. Acta Pharmacol Sin 28: 1305-1315, 2007.
24. Boulay A, Rudloff J, Ye J, Zumstein-Mecker S, O'Reilly T, Evans DB, Chen S and Lane HA: Dual inhibition of mTOR and estrogen receptor signaling in vitro induces cell death in models of breast cancer. Clin Cancer Res 11: 5319-5328, 2005.
25. Wu L, Birle DC and Tannock IF: Effects of the mammalian target of rapamycin inhibitor CCI-779 used alone or with chemotherapy on human prostate cancer cells and xenografts. Cancer Res 65: 2825-2831, 2005.
26. Panwalkar A, Verstovsek S and Giles FJ: Mammalian target of rapamycin inhibition as therapy for hematologic malignancies. Cancer 100: 657-666, 2004.
27. Albert JM, Kim KW, Cao C and Lu B: Targeting the Akt/mammalian target of rapamycin pathway for radiosensitization of breast cancer. Mol Cancer Ther 5: 1183-1189, 2006.
28. Cao C, Subhawong T, Albert JM, Kim KW Geng L, Sekhar KR, Gi YJ and Lu B: Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. Cancer Res 66: 10040-10047, 2006.
29. Mabuchi S, Altomare DA, Cheung M, Zhang L, Poulikakos PI, Hensley HH, Schilder RJ, Ozols RF and Testa JR: RAD001 inhibits human cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an cancer model. Clin Cancer Res 13: 4261-4270, 2007.
30. Goudar RK, Shi Q, Hielmeland MD, Wikstrand CJ, Reese ED, Conrad CA, Traxler P, Lane HA, Reardon DA, Cavenee WK, Wang XF, Bigner DD, Friedman HS and Rich JN: Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition. Mol Cancer Ther 4: 101-112, 2005.
31. Homicsko K, Lukashev A and Iggo RD: RAD001 (Everolimus) improves the efficacy of replicating adenoviruses that target colon cancer. Cancer Res 65: 6882-6890, 2005.
32. Yee KWL, Zeng Z, Konopleva M, Verstovsek S, Ravandi F, Ferrajoli A, Thomas D, Wierda W, Apostolidou E, Albitar M, O'Brien S, Andreeff M and Giles FJ: PhaseI/II study of the Mammalian target of rapamycin inhibitor everolimus (RAD001) in patients with relapsed or refractory hematologic malignancies. Clin Cancer Res 12: 5165-5173, 2006.
33. Schmelzle T and Hall MN: TOR, a central controller of cell growth. Cell 103: 253-262, 2000.
34. Wang ZG, Wu YJ and Shorgen: The mTOR signaling pathway and the regulation of cell growth. (in Chinese) Acta Biophysica Sinaca 22: 333-342, 2007.
35. Albanell J, Dalmases A, Rovira A and Rojo F: mTOR signaling in human cancer. Clin Transl Oncol 9: 484-493, 2007.
36. Hidalgo M and Rowinsky EK: The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene 19: 6680-6686, 2000.
37. Wullschleger S, Loewith R and Hall MN: TOR signaling in growth and metabolism. Cell 124: 471-484, 2006.
38. Shaw RJ and Cantley LC: Ras, PI3K and mTOR signaling controls tumour cell growth. Nature 441: 424-430, 2006.
39. Shah OJ and Hunter T: Turnover of the active fraction of IRS1 involves raptor-mTOR and S6K1-dependent serine phosphorylation in cell culture models of tuberous sclerosis. Mol Cell Biol 26: 6425-6434, 2006.
40. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J and Rosen N: mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 66: 1500-1508, 2006.
41. Wan X, Harkavy B, Shen N, Grohar P and Helman LJ: Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene 26: 1932-1940, 2007.
42. Shi Y, Yan H, Frost P, Gera J and Lichtenstein A: Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like groth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade. Mol Cancer Ther 4: 1533-1540, 2005.
43. Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H and Khuri FR: Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res 65: 7052-7058, 2005.
44. Failly M, Korur S, Egler V, Boulay JL, Lino MM, Imber R and Merlo A: Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells. Mol Cancer Ther 6: 773-781, 2007.
45. Ikezoe T, Nishioka C, Tasaka T, Yang Y, Komatsu N, Togitani K, Koeffler HP and Taguchi H: The antitumor effects of sunitinib (formerly SU11248) against a variety of human hematologic malignancies: enhancement of growth inhibition via inhibition of mammalian target of rapamycin signaling. Mol Cancer Ther 5: 2522-2530, 2006.
46. Kim KW, Mutter RW, Cao C, Albert JM, Freeman MF, Hallahan DE and Lu B: Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. J Bio Chem 281: 36883-36890, 2006.
47. Bai X, Ma D, Liu A, Shen X, Wang QJ, Liu Y and Jiang Y: Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. Science 318: 977-980, 2007.
48. Proud CG: mTOR, unleashed. Science 318: 926-927, 2007.