Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 253 (2007) 236–248
CANCER Letters
www.elsevier.com/locate/canlet

# An activated mTOR/p70S6K signaling pathway in esophageal squamous cell carcinoma cell lines and inhibition of the pathway by rapamycin and siRNA against mTOR

Guiqin Hou, Lexun Xue *, Zhaoming Lu, Tianli Fan, Fang Tian, Yanli Xue

*Laboratory for Cell Biology, The First Affiliated Hospital, Zhengzhou University, 40 Daxue Road, Zhengzhou, Henan 450052, PR China*

Received 7 December 2006; received in revised form 29 January 2007; accepted 30 January 2007

**Abstract**

mTOR/p70S6K pathway is considered a central regulator in various malignant tumors, but its roles in esophageal squamous cell carcinoma (ESCC), which is a common cause of mortality in China, remain unknown. Here, we identify that the mTOR/p70S6K pathway is activated in ESCC; rapamycin and siRNA against mTOR rapidly inhibited expression of mTOR and the phosphorylation of its major downstream effectors, p70S6K and 4E-BP1, arrested cells in the $G_0/G_1$ phase and induced apoptosis of ESCC cells. The findings may lay a foundation for making further investigations on the mTOR/p70S6K pathway as a potential target for ESCC therapy.


*Keywords:* mTOR; p70S6K; Rapamycin; siRNA; Apoptosis; Esophageal squamous cell carcinoma

## 1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed cancers in developing countries, especially in China [1]. However, the understanding of etiology and mode of carcinogenesis of this disease is still lacking [2]. Although therapy strategies have been improved, the prognosis of patients with ESCC is still poor. Moreover, cells of ESCC are known to develop resistance to chemotherapeutic drugs, thus resulting in a dramatic decrease in the 5-year survival rate for ESCC. Obviously, a better understanding of the molecular mechanisms in carcinogenesis and progression of ESCC helps to improve the prognosis of patients with ESCC.

The mammalian target of rapamycin (mTOR) that is an evolutionarily conserved serine–threonine kinase of a 289-kDa in length belongs to the PIKK [phosphoinositide 3-kinase (PI3K)-related kinase] family [3–5]. mTOR signaling pathway is frequently activated in human cancers [6–9]. In mammals, the two best-characterized targets of mTOR are the ribosomal S6 kinases S6K1 and S6K2, and the eukaryotic initiation factor (eIF4E)-binding protein 1 (4E-BP1). mTOR activation leads to phosphorylations of S6K1/2 and 4E-BP1. The latter releases from the cap-dependent translation initiation factor eIF4E, ultimately resulting in enhanced translation from subset of genes required for cell growth

* Corresponding author. Tel.: +86 371 66658332; fax: +86 371 66997182.
*E-mail addresses:* xuelx@371.net, xuelx@zzu.edu.cn (L. Xue).

0304-3835/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2007.01.026

[10–13]. These two events lead to an increase in ribosomal biogenesis and the selective translation of special mRNA populations [14–19].

mTOR has recently been recognized as an important and attractive therapeutic target for cancer therapy [20,21]. The potential applications of mTOR inhibitors for treating various cancers including renal, prostate, breast, pancreatic, and lung cancers, etc., have been actively studied both preclinically and clinically [22–24]. However, the mTOR/p70S6K signaling pathway in ESCC has not been investigated so far, which impel the authors to investigate the role of mTOR/p70S6K signaling pathway for finding a novel target for the anticancer drugs in ESCC. In the present study, we investigated the activated mTOR and its major downstream members in ESCC cell lines, as well as the changes of mRNA and protein expression levels, cell cycle, and apoptosis in the ESCC cells treated with rapamycin and small interference RNA (siRNA) against mTOR. Here, we present that mTOR/p70S6K signaling pathway is constitutively activated in the ESCC cell lines. Furthermore, rapamycin can specially block mTOR-mediated signaling pathway, and siRNA targeting mTOR cannot only inhibit the pathway, arrest cells to $G_0/G_1$ phase and induce apoptosis, but also increase the sensitivity of EC9706 cells to rapamycin.

## 2. Materials and methods

### 2.1. Cell lines and cell cultures

A poorly differentiated ESCC cell line EC9706 and a well differentiated ESCC cell line Eca109 were provided by the State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Sciences (Beijing, China). Cervical cancer cell line HeLa 229 as control cells was purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Each of the three cell lines was cultured in RPMI 1640 medium(Gibco-BR2, Rockville, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, USA ), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in the presence of 5% $CO_2$, as described in the previous study from our laboratory [25].

### 2.2. Immunocytochemical analysis

The immunoreactivity was determined using the SP kit (Santa Cruz, USA) according to the manufacturer's instructions. Briefly, the three cell lines were plated on several sterile glass slides and incubated at 37 °C for 24 h in presence of 5% $CO_2$. The slides were rinsed three times in phosphate-buffered saline (PBS, pH 7.4) and fixed with 4% formaldehyde at room temperature (RT) for 10 min. After rinsed in PBS and treated with 3% $H_2O_2$ for 10 min, the cells were blocked with 5% normal goat serum for 30 min in a humidified box at RT to eliminate nonspecific binding, and then incubated with anti-human mTOR antibodies (1:100), p-p70s6K and p-4E-BP1, as well as PBS as a control, respectively, at 4 °C overnight. After the slides were rinsed three times in PBS and incubated with corresponding secondary antibodies for 30 min, they were developed with a 0.03% DAB solution. Subsequently, photomicrographs (magnification, 400×) were taken immediately.

### 2.3. Treatment with rapamycin and siRNA against mTOR

EC9706 cells trypsinized were incubated for 24 h and continued to incubate for 6 more hour after deprivation of serum. Subsequently, the serum-starved cells were treated with rapamycin at different concentrations in the medium containing 10% FBS.

EC9706 cells were treated with siRNA against mTOR or universal negative (nonsilencing) control siRNAs (Santa Cruz, USA) according to the manufacturer's instructions. Briefly, transfection reagent (Santa Cruz, USA) was incubated with 0.8 ml of serum-free medium for 10 min, subsequently a mixture of siRNA was added to the medium mentioned above. After incubation at RT for 15 min, the mixture was added to EC9706 cells ($2 \times 10^5$ cells/well) grown in six-well plates for 24 h to 60% confluence in medium without antibiotics at siRNA concentration of 100 nM, and continued to incubate for 6 h. One milliliter of medium containing 20% FBS was added to each well without removing the transfection mixture. After being cultured for 24 h, new medium was added to the cells washed with PBS. Subsequently, the treated cells were collected for further experiments.

### 2.4. Semi-quantitative RT-PCR

Total RNA was prepared from ESCC cell lines with Trizol reagent (Invitrogen, Carlsbad, USA) and reversely transcribed to cDNA using AMV First Strand DNA Synthesis Kit (Biotech Company, Shanghai, China). Briefly, a 1 μg of the isolated RNA was reversely transcribed to cDNA at 37 °C for 1 h in a 20-μl of reaction mixture containing 1 μl AMV reverse transcriptase, 1 μl random hexamer, 4 μl 5× AMV buffer, 1 μl RNase inhibitor (20 U/μl), 2 μl dNTP (10 mM). The PCR amplification mixture (25 μl) consisted of 0.5 μl cDNA mixture, 0.5 U *Taq* DNA polymerase, 2.5 μl of 10× PCR buffer, 2.5 mM dNTP mixture, and 50 pM sense and antisense primers each. Glyceraldehyde-3-3phosphate-dehydrogenase (GAPDH) was used as an internal control. mTOR, p70S6K, and 4E-BP1 were analyzed by PCR and the used oligonucleotide primers included: for mTOR (193 bp), forward primer

5′ CGC TGT CAT CCC TTT ATC G 3′ and reverse primer 5′ ATG CTC AAA CAC CTC CAC C 3′; for p70S6K (188 bp), forward primer 5′ TAC TTC GGG TAC TTG GTA A 3′ and reverse primer 5′ GAT GAA GGG ATG CTT TAC T 3′; for 4E-BP1 (153 bp), forward primer 5′ ACC GGA AAT TCC TGA TGG AG 3′ and reverse primer 5′ CCC GCT TAT CTT CTG GGC TA 3′; and for 570 bp of GAPDH, forward primer 5′ GCA CCG TCA AGG CTG AGA A 3′ and reverse primer 5′ AGG TCC ACC ACT GAC ACG TTG 3′. The PCR conditions were as follows: for initial denaturing at 95 °C for 3 min, followed by 30 PCR cycles with temperatures below: 95 °C for 30 s, for mTOR 54 °C, for p70S6K 49 °C and for 4E-BP1 56 °C for 30 s each, and then 72 °C for 1 min; a final extension at 72 °C for 5 min. The amplified products were subjected to electrophoresis on 1% agarose gels containing 0.2 μg/μl ethidium bromide and visualized under a UV light [4,25].

### 2.5. Western blots

EC9706 cells treated with rapamycin at different concentration of 0, 20, and 50 nM were harvested and lysed for 20 min in cold lysis buffer. After centrifugation at 12,000 rpm for 5 min, the supernatant was harvested as the total cellular protein extracts. The protein concentrations were determined using Bradford method. The total cellular protein extracts were separated on 15% SDS–PAGE for 4E-BP1 and P-4E-BP1, on 8% SDS–PAGE for mTOR, and on 10% SDS–PAGE for p70S6K, p-p70S6K, Akt1/2, and β-actin. Proteins were electrotransferred to supported nitrocellulose membranes (Amersham, Uppsala, Sweden) by a semi-dry transferor. The membranes were blocked in 5% skimmed milk in PBS-T containing 0.05% Tween 20 at RT for 2 h, and then incubated at RT for 2 h with antibodies to mTOR, p70s6K, p-P70s6K, 4E-BP1, p-4E-BP1, AKT1/2, and β-actin diluted in 1% skimmed milk in PBS-T, respectively, followed by incubating with the appropriate HRP-linked secondary antibodies. Finally, the bands of specific proteins on the membranes were developed with DAB solution according to manufacturer's instructions. The membranes were rinsed three times with PBS-T between the incubations described above [4,26].

### 2.6. Cell cycle analysis

Because cell proliferation and growth are controlled by the progression of cell through the well-defined stages of cell cycle, the effects of rapamycin and siRNA against mTOR on the cell cycle progression of EC9706 cells were, respectively, investigated by use of propidium iodide staining and flow cytometry. Becton Dickinson FACScan Flow Cytometer (FACScan, Mountain View, USA) was used for determining DNA contents. Cells treated, respectively, with rapamycin at different concentrations and siRNA against mTOR were fixed in 70% cold ethanol, and then kept at 4 °C until staining. The fixed cells were incubated with RNase (50 μg/ml) for 30 min at 37 °C, and then 5 μl propidium iodide (50 μg/ml) was added to cell suspension and continued to incubate at RT for 30 min in the dark before analysis [26].

### 2.7. Apoptosis assay by flow cytometry

EC9706 cells treated, respectively, with rapamycin and siRNA against mTOR were trypsinized, washed with cold PBS, and resuspended in PBS. Annexin V-FITC (BD Biosciences, USA) at final concentration of 1 μg/ml and 250 ng of propidium iodide were added to a mixture containing 100 μl of cell resuspension and binding buffer (BD Biosciences, USA) each. After cells were vortexed and incubated for 15 min at RT in the dark, 400 μl of binding buffer was added to the mixture for flow cytometric analysis using Becton Dickinson FACScan Flow Cytometer (FACScan, Becton Dickinson, Mountain View, USA).

### 2.8. Cell proliferation assay

WST-8 is better than MTT for analyzing cell proliferation, because it can be reduced to soluble formazan by dehydrogenase in mitochondria and has little toxicity to cells. Cell proliferation was determined using WST-8 dye (Beyotime Inst Biotech, China) according to manufacture's instructions. Briefly, $5 \times 10^3$ cells/well were seeded in a 96-well flat-bottomed plate, grown at 37 °C for 24 h, and then placed in serum-starved conditions for a further 6 h. Subsequently, cells were treated with rapamycin at increasing concentrations in the presence of 10% FBS for 24 or 48 h. After 10 μl WST-8 dye was add to each well, cells were incubated at 37 °C for 2 h and the absorbance was finally determined at 450 nm using a microplate reader.

### 2.9. Statistical analysis

All experiments results were from at least three separate experiments. The data were performed by one-way analysis of variance using SPSS version13.0 (SPSS, Chicago, USA). Summary statistics were expressed as means ± standard deviations, except as otherwise stated. In all statistical analyses, a $P$ value $< 0.05$ was considered statistically significant, and all $P$ values were two-sided.

## 3. Results

### 3.1. mTOR and its downstream targets in the ESCC cell lines

To investigate whether mTOR/p70S6K signaling pathway is activated in the two ESCC cell lines, mRNA and protein expression levels were examined

by immunocytochemistry, Western blots and RT-PCR, respectively. As shown in Fig. 1a, both the ESCC cell lines and HeLa 229 cells have intense immunoreactivity to mTOR and its downstream target p-p70S6K. The results of Western blots showed that there were high expression levels of mTOR and p-p70S6K proteins while the expression of p70S6K protein was weak in the two ESCC cell lines (Fig. 1b and c). There were statistically different levels of proteins of mTOR and p70S6K in the two cell lines, i.e., the protein levels of mTOR ($P < 0.05$) and p-p70S6K ($P < 0.01$) were higher in the poorly differentiated cell line EC9706 than in the well differentiated cell line Eca109 (Fig. 1d). Besides, the results of RT-PCR showed that mTOR signaling pathway was activated in the two ESCC cell lines (Fig. 2a–c). The mRNA levels of mTOR was higher, but the mRNA levels of p70S6K and 4E-BP1 were lower ($P < 0.01$) in the poorly differentiated cell line EC9706 than in the well-differentiated cell line Eca109 (Fig. 2d). Thus, it is likely that the expression levels of mTOR mRNA and protein are used to identify the differentiation degree of ESCC.

### 3.2. Expression changes of mRNAs and proteins in EC9706 cells treated with rapamycin

After cells were treated with rapamycin at different concentrations, the expressions of mRNAs and proteins of mTOR, p70S6K, and 4E-BP1 were measured by RT-PCR and Western blots, respectively. Rapamycin significantly decreased mRNA expression of mTOR ($P < 0.01$) while mRNA expressions of p70S6K and 4E-BP1 were increased ($P < 0.01$) in rapamycin-treated cells in a dose-dependent manner (Fig. 3). The results suggest rapamycin potently inhibits the mRNA level of mTOR and increases the unphosphorylated levels of the two downstream effectors, p70S6K and 4E-BP1.

The results of Western blots revealed that rapamycin inhibited the protein expression of mTOR in a dose-dependent manner ($P < 0.01$) (Fig. 4a and c). Rapamycin significantly blocked the phosphorylation and promoted the unphosphorylation of p70S6K at 6 h after exposure to 20 nM of rapamycin ($P < 0.01$) (Fig. 4b and d). Similarly, the effect of rapamycin on phosphorylation state of 4E-BP1 was detected with antibodies to 4E-BP1 and p-4E-BP1. As shown in Fig. 4, the increase in the intensity

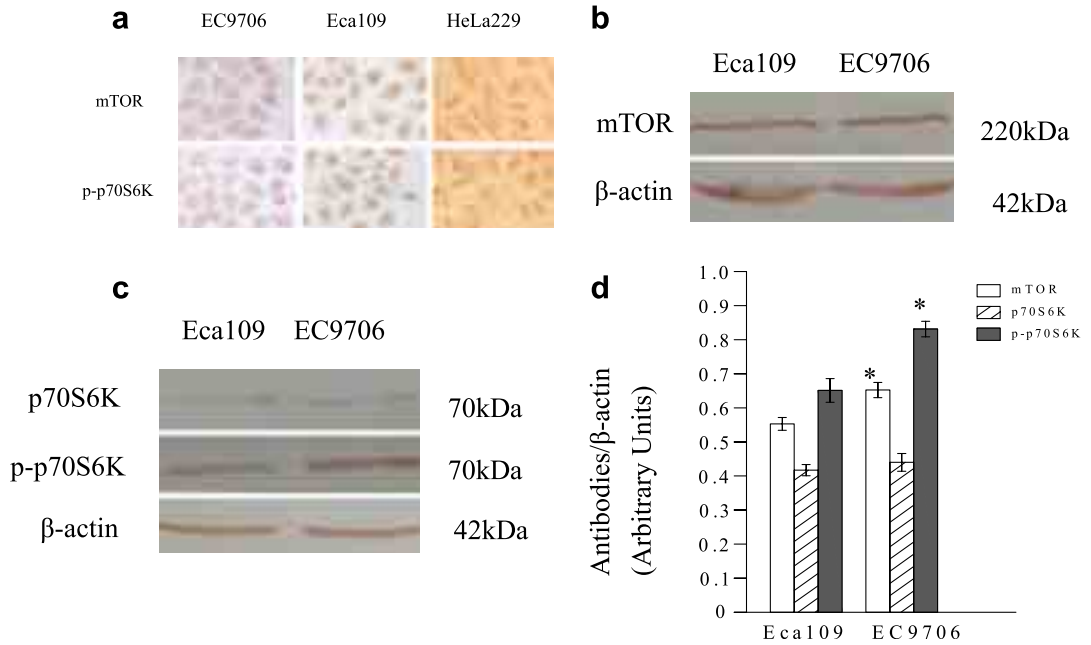


Fig. 1. Expressions of mTOR and p-p70S6K proteins in different cell lines. (a) Expressions and localizations of mTOR and p-p70S6K were investigated immunocytochemically in the nuclei and cytoplasm of both ESCC cell lines ( EC9706 and Eca109) and positive control cell line HeLa 229. (b and c) Western blot analysis was carried out with antibodies to mTOR, p70S6K, p-p70S6K, and β-actin (loading control) in the two ESCC cell lines, respectively. (d) Semi-quantitated values of three independently repeated Western blot experiments were statistically analyzed by densitometry using TotalLab 2.0 software, are expressed as means ± SD. $^*P < 0.05$, compared to those of Eca109 cells.

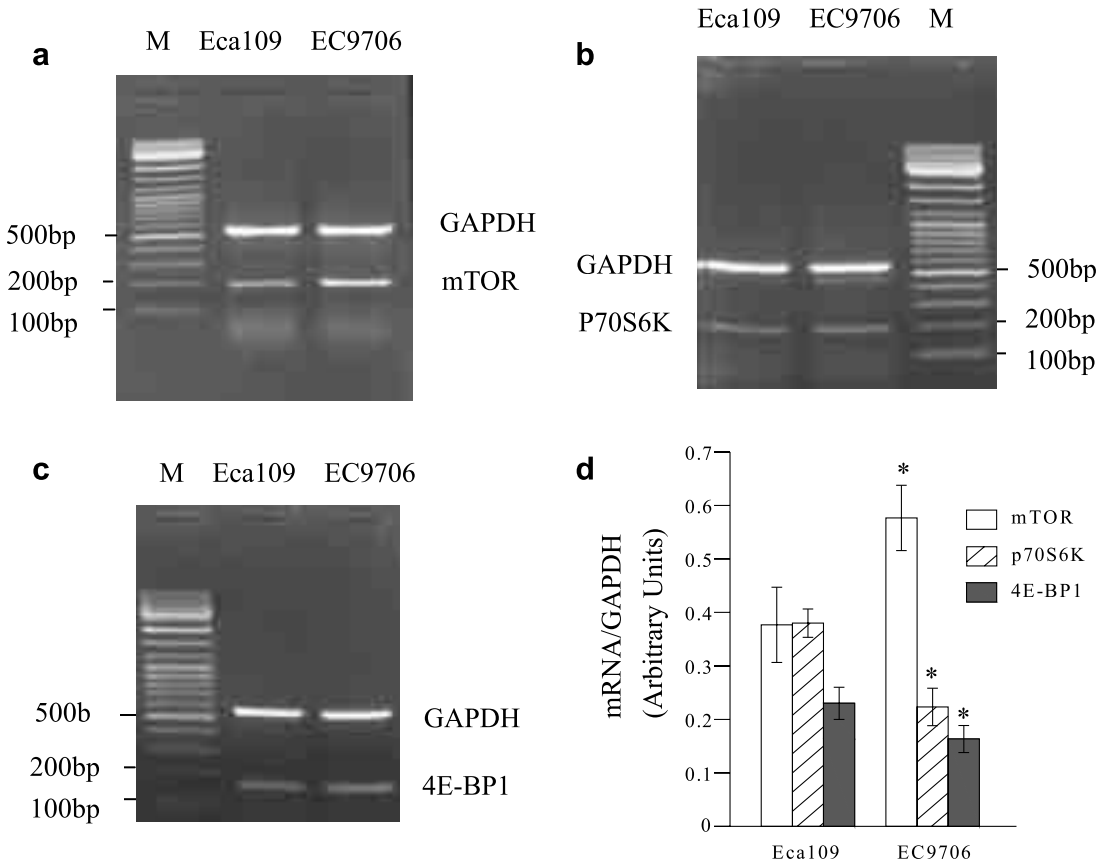


Fig. 2. Determination of mRNA levels of mTOR, p70S6K, and 4EBP1. (a–c) Analysis of mRNA levels of mTOR, p70S6K and 4EBP1 in the two ESCC cell lines by RT-PCR. (d) Semi-quantitated values of mRNA levels of mTOR, p70S6K, and 4EBP1 to GAPDH, respectively. Results from three independently repeated experiments, which were statistically analyzed by densitometry using BandScan 5.0 software, are expressed as means ± SD. $^{*}P < 0.05$, compared to those of Eca109 cells.

of the bands γ and α was due to lower phosphorylated form of 4E-BP1, indicating that rapamycin inhibits the phosphorylation of 4E-BP1 in EC9706 cells ($P < 0.01$). However, rapamycin did not have any effect on Akt ($P > 0.05$), an upstream factor of mTOR (Fig. 4b and g).

### *3.3. Effects of siRNA against mTOR on mTOR/p70S6K pathway in EC9706 cells*

To investigate the best interference effect of siRNA against mTOR, total RNAs of the cells transfected with 4 μl siRNA (10 μM) for different durations (0, 24, 48, 72, and 96 h) were, respectively, extracted, followed by analyzing the mRNA levels using RT-PCR. As shown in Fig. 5a and c, the mRNA level of mTOR was significantly decreased in the cells transfected with siRNA against mTOR compared to that of the cells untransfected and transfected with control siRNA. The mRNA expression of mTOR was the lowest ($P < 0.01$) at 72 h after transfection, by about 20-fold lower than that of untransfected cells, and began to increase at 96 h, indicating that siRNA against mTOR has the biggest inhibitory effect at 72 h after transfection. Also, the mRNA level of p70S6K was increased at 24 h, reaching to the highest expression at 72 h after transfection ($P < 0.01$) (Fig. 5b and d).

According to the results of RT-PCR mentioned above, the total proteins were obtained from EC9706 cells transfected with siRNA against mTOR for 72 h. The changes of the proteins in the mTOR/p70S6K signaling pathway were analyzed using Western blots. Cells untransfected and transfected with control siRNA are as controls. As shown in Fig. 6, in EC9706 cells transfected with siRNA against mTOR, protein level of mTOR was reduced about 3.5-fold at 72 h after transfection in all three separate experiments ($P < 0.01$) (Fig. 6a and c), protein levels of p70S6K and 4E-BP1 were significantly increased but protein levels of p-p70S6K and p-4E-BP1 were significantly decreased ($P < 0.01$) compared to untransfected cells, indicating that siRNA against mTOR inhibits mTOR, p-p70S6K, and p-4E-BP1 but promotes unphosphoryla-

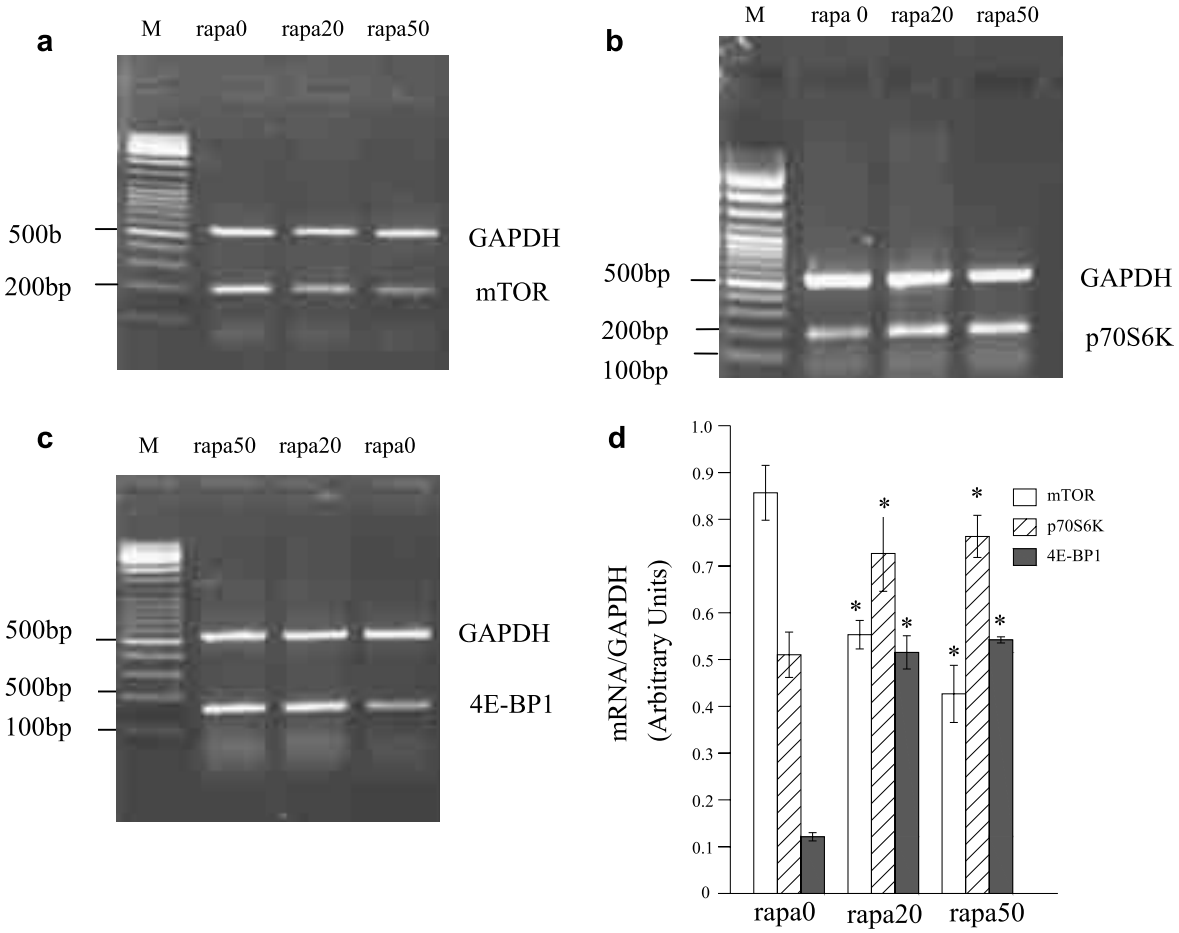


Fig. 3. mRNA levels of mTOR and its downstream effectors in EC9706 cells. (a–c) Analysis of mRNAs of mTOR and its two downstream effectors, p70S6K and 4EBP1, by RT-PCR in cells treated with rapamycin at different concentrations. (d) Semi-quantitative value of mRNA levels of mTOR, p70S6K, and 4EBP1 to GAPDH, respectively. Results from three independently repeated experiments, which were statistically analyzed by densitometry using BandScan 5.0 software, are expressed as means ± SD. $^{*}P < 0.05$, compared to those of EC9706 cells treated without rapamycin.

tion of p70S6K and 4E-BP1 (Fig. 6b, d, f, and g). However, siRNA against mTOR had no effect on the total protein level of Akt ($P > 0.05$) (Fig. 6b and e).

### 3.4. Cell cycle analysis

After cells were treated with rapamycin, the proportion of cells at $G_0/G_1$ phase significantly increased compared to untreated cells ($P < 0.01$). When cells were treated with 20 and 50 nM of rapamycin for 24 h, the proportion of cells at the $G_0/G_1$ phase increased from 63.23% to 72.81% ($P < 0.05$) and to 78.38% ($P < 0.05$), respectively, and there was a significant difference ($P < 0.01$) between low and high concentrations of rapamycin as well, indicating that rapamycin effectively arrests EC9706 cells in the $G_0/G_1$ phage of cell cycle. On the other hand, the proportion of cells at the S phase was decreased ($P < 0.01$), compared to untreated cells (Fig. 7a and c).

To examine if siRNA against mTOR has an impact on cell cycle of ESCC cells, EC9706 cells were transfected with it for 48 h, and untransfected cells and cells transfected with control siRNA were served as controls. To determine if combination of siRNA against mTOR with rapamycin has a collaborative effect, the cells transfected with siRNA against mTOR for 24 h were exposured to 20 nM of rapamycin for a further 24 h. The results of flow cytometry analysis showed that siRNA against mTOR inhibited the cell cycle progression, leading to that the proportion of cells at the $G_0/G_1$ phage was significantly increased from 63.90% to 75.45% ($P < 0.01$). Control siRNA had no effect on cell cycle of EC9706 cells. Besides, cells treated with combination of siRNA against mTOR with rapamycin had higher proportion (79.05%) of cells at the $G_0/G_1$ phase than those only treated with siRNA against mTOR (75.45%) (Fig. 7b and d), and there was a statistically significant difference ($P < 0.05$). The find-

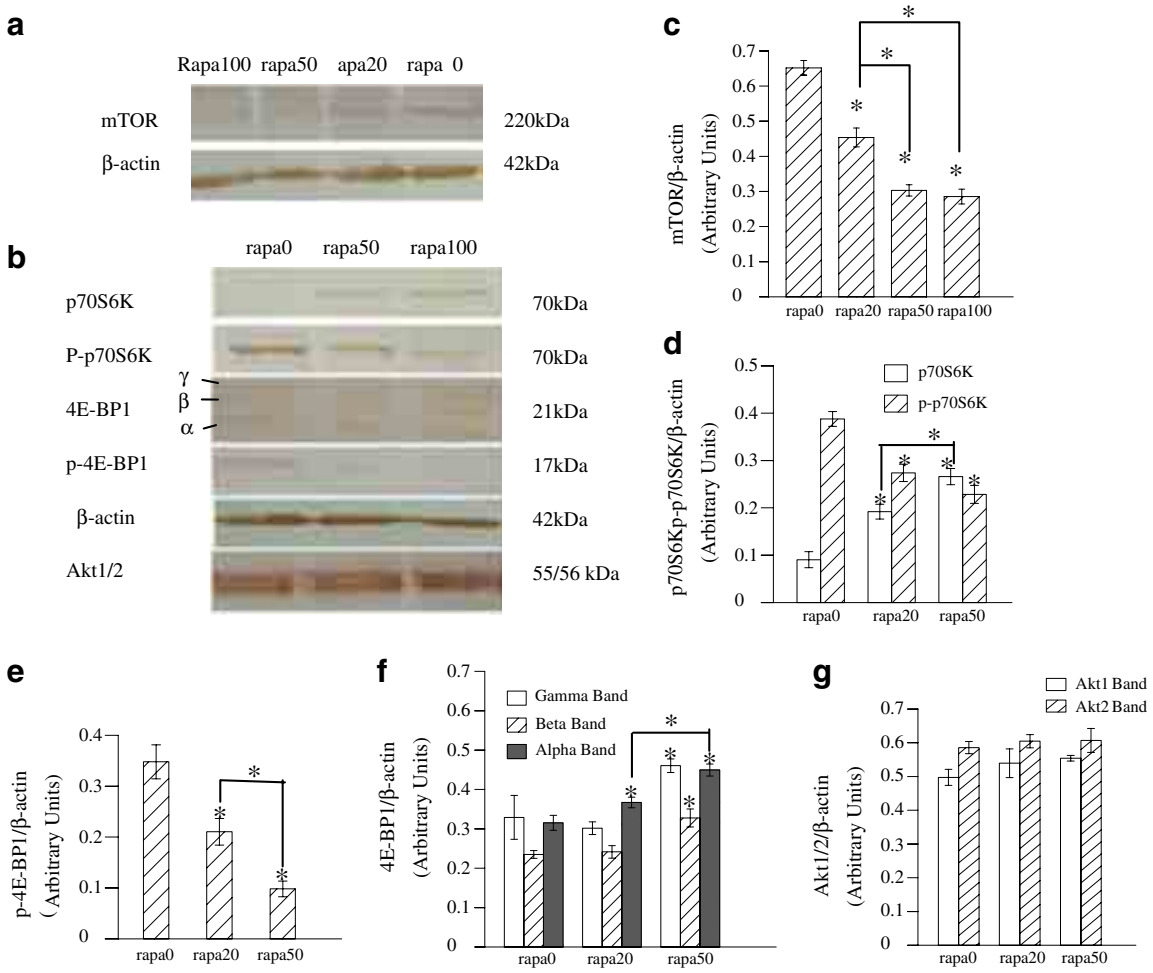


Fig. 4. Effects of rapamycin on mTOR as well as phosphorylation of p70S6K , 4EBP1, and Akt. After serum-starved EC9706 cells were treated with rapamycin at deferent concentrations for 6 h, the cell extracts were subjected to Western blots with the following antibodies. (a) Antibodies to mTOR and β-actin (loading control). (b) Antibodies to p70S6K, p-p70S6K, 4EBP1, p-4EBP1, β-actin, and Akt1/2, respectively. (c–g) Semi-quantitative values of three independently repeated experiments, which were statistically analyzed by densitometry using TotalLab 2.0 software, are expressed as means ± SD. $^{*}P < 0.05$, compared to those of EC9706 cells treated without rapamycin.

ings suggest that the combination of siRNA against mTOR with rapamycin has a collaborative effect on cell cycle phase.

### 3.5. Apoptosis assay

To examine whether rapamycin is able to affect apoptosis of ESCC cells, EC9706 cells were, respectively, treated with 20 and 50 nM of rapamycin for 4 h, followed by assessing apoptosis by means of flow cytometric analysis. Cells treated with apoptotic reagent and untreated cells were, respectively, served as positive and negative controls. The result showed that rapamycin markedly induced EC9706 cell apoptosis ($P < 0.01$) in a dose-dependent manner (Fig. 8a). After exposure to rapamycin at different concentrations of 20 and 50 nM for 4 h, the proportion of positive cells for Annexin V and propidium iodide (region II) (20 nM, 19.11%; 50 nM, 43.54%) was significantly increased ($P < 0.01$), compared to untreated cells (negative control: 4.62%) (Fig. 8c).

Finally, apoptosis was observed in EC9706 cells transfected with siRNA against mTOR for 48 h, and siRNA against mTOR for 44 h combined with 20 nM of rapamycin for a further 4 h. The results revealed that siRNA against mTOR induced apoptosis of EC9706 cells (Fig. 8b). The proportion of cells positive for annexin V and propidium iodide (region II) was markedly increased from 6.11% (control) to 30.4% ($P < 0.01$). The apoptotic proportion of EC9706 cells treated with siRNA against mTOR combined with rapamycin (57.38%) was significantly higher ($P < 0.01$) than that of EC9706 cells treated with siRNA against mTOR alone (30.40%) (Fig. 8d), sug-

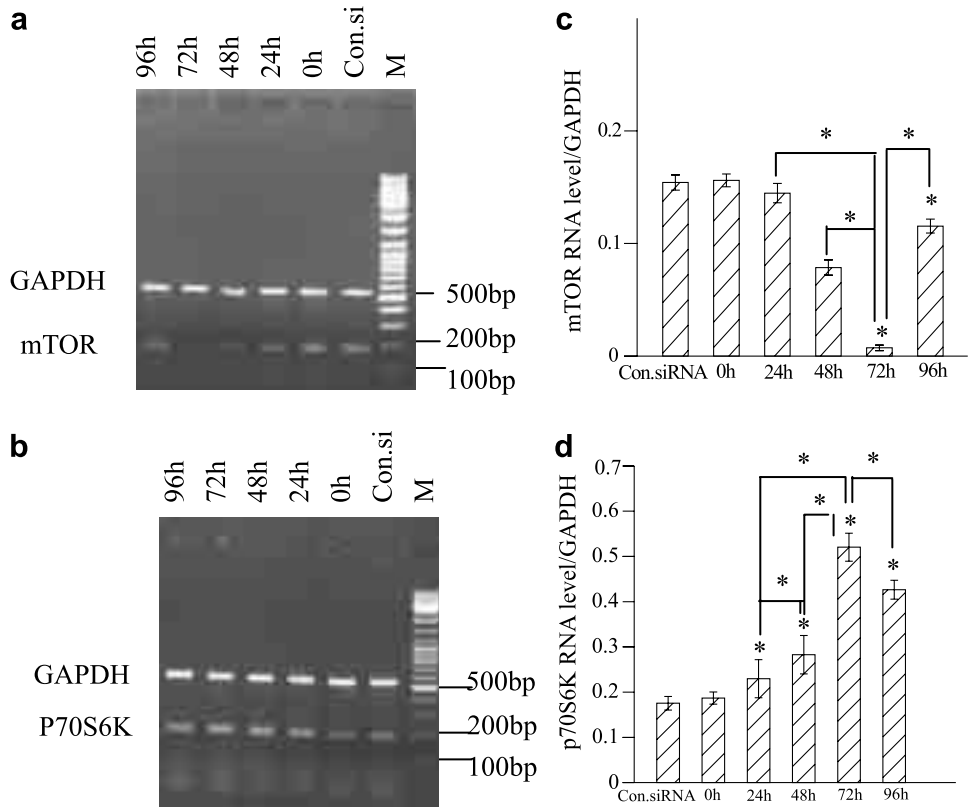


Fig. 5. Determination of mRNAs of mTOR and p70S6K in EC9706 cells interfered with siRNA against mTOR. (a and b) Analysis of mTOR and p70S6K mRNA levels by RT-PCR in cells interfered with siRNA against mTOR. (c and d) Semi-quantitative values of mRNA levels of mTOR and p70S6K to GAPDH, respectively. Results from three independently repeated experiments, which were statistically analyzed by densitometry using BandScan 5.0 software, are expressed as means ± SD. $^{*}P < 0.05$, compared to those of untreated EC9706 cells.

gesting that the combination of siRNA against mTOR with rapamycin has a collaborative effect on apoptosis of EC9706 cells as well.

### 3.6. Effects of rapamycin on ESCC cell proliferation

To determine effects of rapamycin on proliferation of ESCC cells, EC9706 cells were treated with rapamycin at different concentrations (20, 50, 100, 150, and 200 nM) for 24 and 48 h, respectively. As shown in Fig. 9, proliferation of EC9706 cells exposuring to rapamycin became slower compared to that of untreated cells ($P < 0.01$), and inhibitory effects of rapamycin on proliferation of EC9706 cells were in a dose- and time-dependent manner.

## 4. Discussion

Aberrant activation of mTOR has been shown in many cancers including human ovarian, breast and hepatocellular carcinomas, as well as squamous cell carcinomas of the head and neck [6–9]. However, study on mTOR in ESCC has not been reported so far. In the present study, we found that the overexpression of mTOR in the two ESCC cell lines and the expression level of mTOR was higher in poorly differentiated EC9706 cells than in well-differentiated Eca109 cells ($P < 0.05$). On the other hand, high expressions of phosphorylated p70S6K (p-p70S6K) and 4E-BP1 (p-4E-BP1) were detected in the two cell lines. Thus, mTOR/p70S6K signaling pathway is activated in the two ESCC cell lines.

In mammals, mTOR is regulated by a kinase cascade consisting of pI3K, pI3K-dependent kinase l, and Akt [27–29]. The downstream effectors of mTOR include p70S6K and 4E-BP1, which are related to translation, and p-p70S6K has higher activity to promote translation than p70S6K. mTOR can promote the phosphorylation of p70S6K and 4E-BP1, thus stimulating the translation of proteins required for the cell cycle progres-

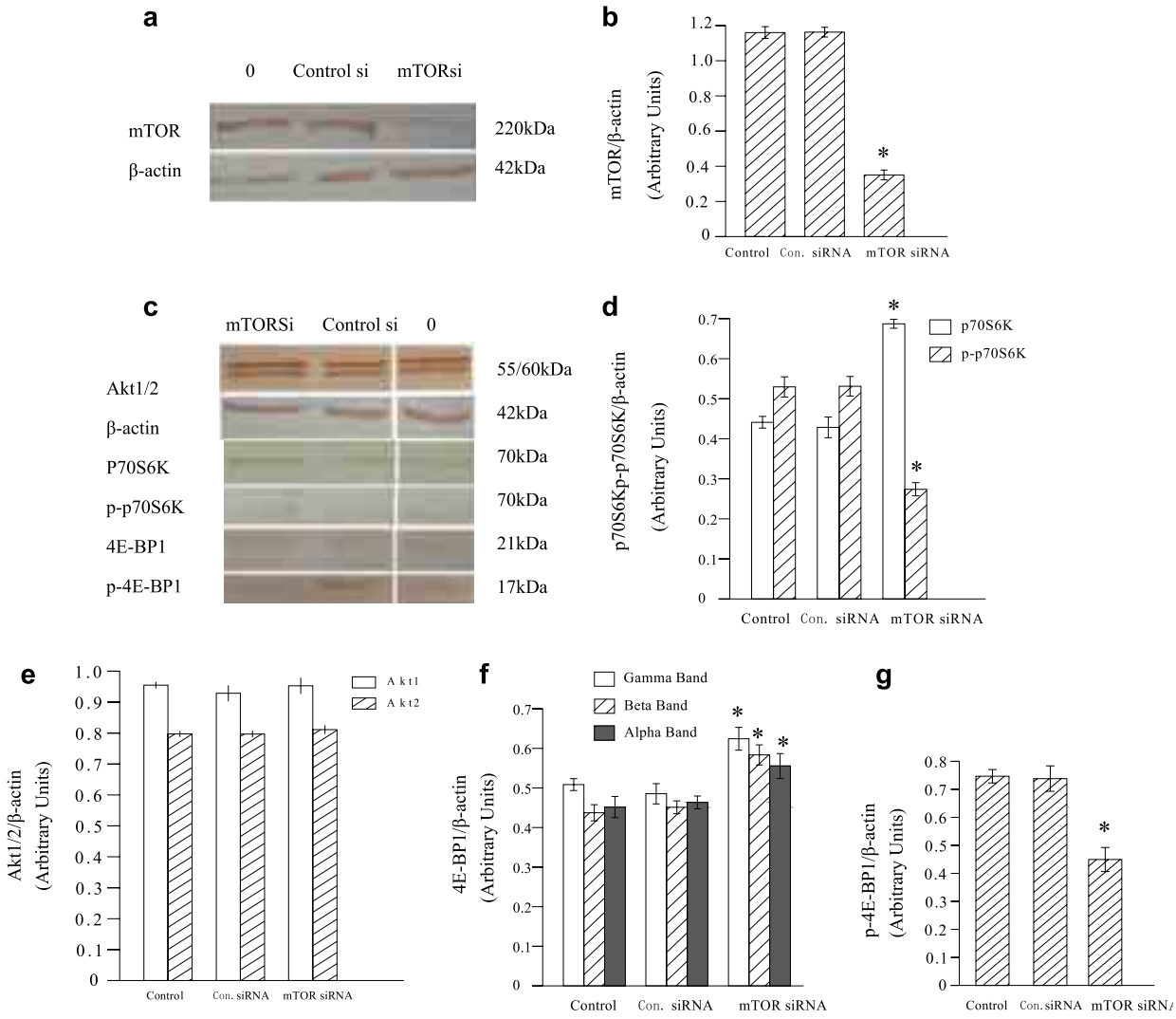


Fig. 6. siRNA against mTOR inhibits protein expressions of mTOR and phosphorylation of p70S6K and 4EBP1 but not Akt. The cell extracts from EC9706 cells interfered with siRNA against mTOR and control siRNA for 72 h were subjected to Western blots with (a). Antibodies to mTOR and β-actin (loading control). (b) Antibodies to Akt1/2, p70S6K, p-p70S6K, 4EBP1, p-4EBP1, and β-actin (loading control), respectively. (c–g) Semi-quantitative values of three independently repeated experiments, which were statistically analyzed by densitometry using TotalLab 2.0 software, are expressed as means ± SD. $^{*}P < 0.05$, compared to those of untreated EC9706 cells.

sion from the $G_1$ to S phase [29–31]. Previous reports have identified that rapamycin can inhibit mTOR and the phosphorylation of p70S6K and 4E-BP1 in many cancers [32–34], but not in ESCC. The results of the present study showed that 20 nM of rapamycin significantly inhibited the phosphorylation of p70S6K and 4E-BP1. Rapamycin markedly arrested ESCC cells to the $G_0/G_1$ phase of cell cycle, resulting in the significantly increased proportion of cells at the $G_0/G_1$ phase. Besides, 50 nM of rapamycin significantly increased the proportion of the ESCC cells positive for annexin V and propidium iodide, resulting in the 10-fold higher apoptosis ratio in rapamycin-treated cells than in untreated cells. However, the expression of Akt was not affected by rapamycin, suggesting that Akt is an upstream factor of mTOR and rapamycin is a special inhibitor of mTOR.

mTOR is a central signal integrator that receives signals arising from growth factors, nutrients, etc., and a central controller of eukaryotic cell growth and proliferation [3,34,35]. It has been demonstrated that the activated mTOR has many functions not only for growth but also for invasion and metastasis

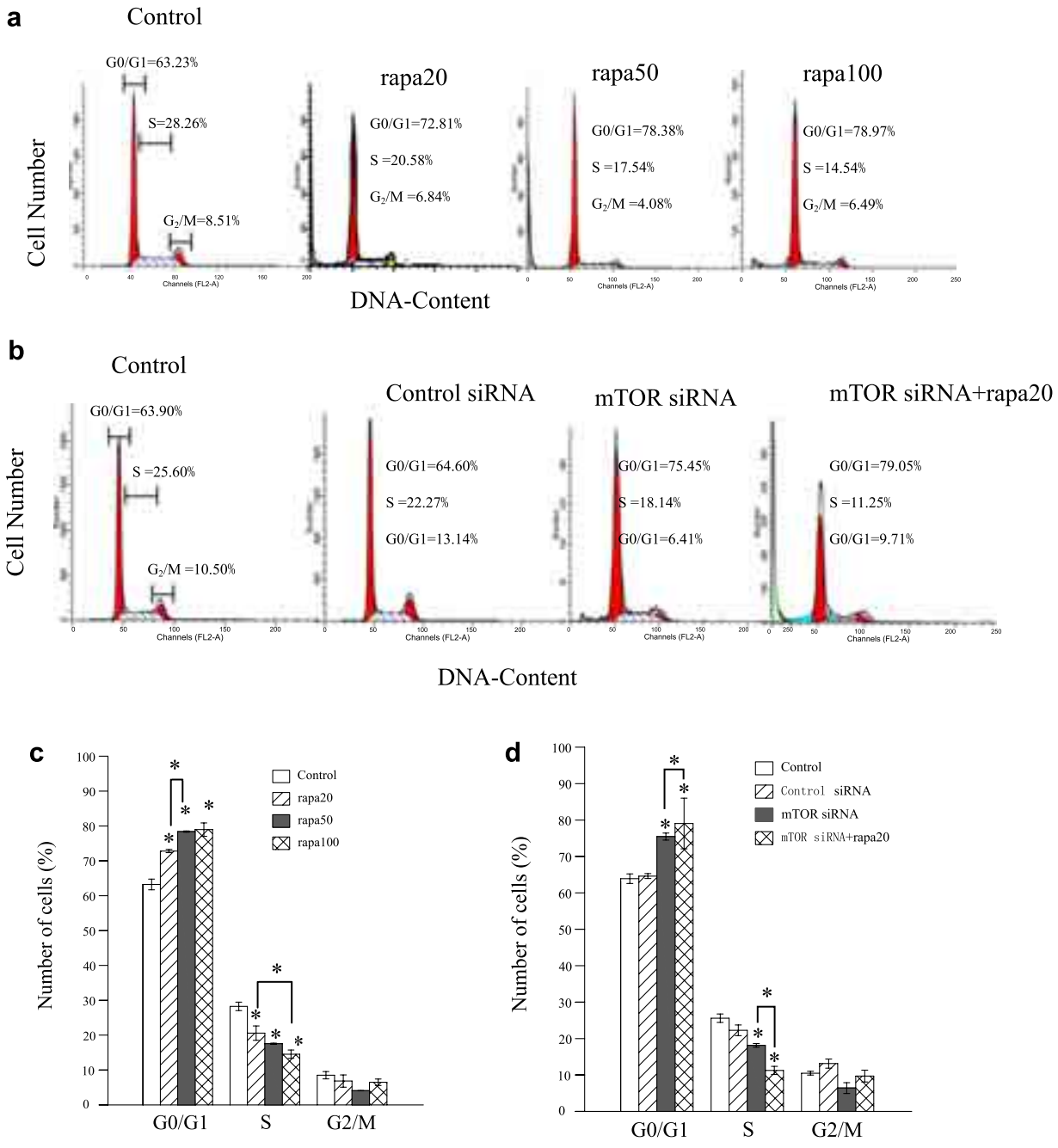


Fig. 7. Rapamycin and siRNA against mTOR arrest EC9706 cells in $G_0/G_1$ phase of the cell cycle. EC9706 cells were seeded in six-well plate containing 10% FBS-RPMI 1640 and grown overnight. (a and c) The cells treated with rapamycin at different concentrations for 24 h. (b and d) The cells transfected with siRNA against mTOR for 48 h and then continuously incubated with or without rapamycin for a further 24 h. Cell cycle was analyzed using the Cellular DNA Flow Cytometry. Untreated cells and cells transfected with control siRNA were respectively served as controls. Results pooled from three independently repeated experiments are expressed as means ± SD. $^*P < 0.05$, compared to untreated cells.

of tumors [36–38]. RNA interfering has become a powerful strategy to understand functions of the genes [39]. In the present study, a chemically synthesized siRNA against mTOR was used to reduce mTOR protein expression in ESCC cells. It was shown that the expression of mTOR was specifically inhibited in EC9706 cells treated with siRNA against mTOR, reaching the lowest value at 72 h. Further-

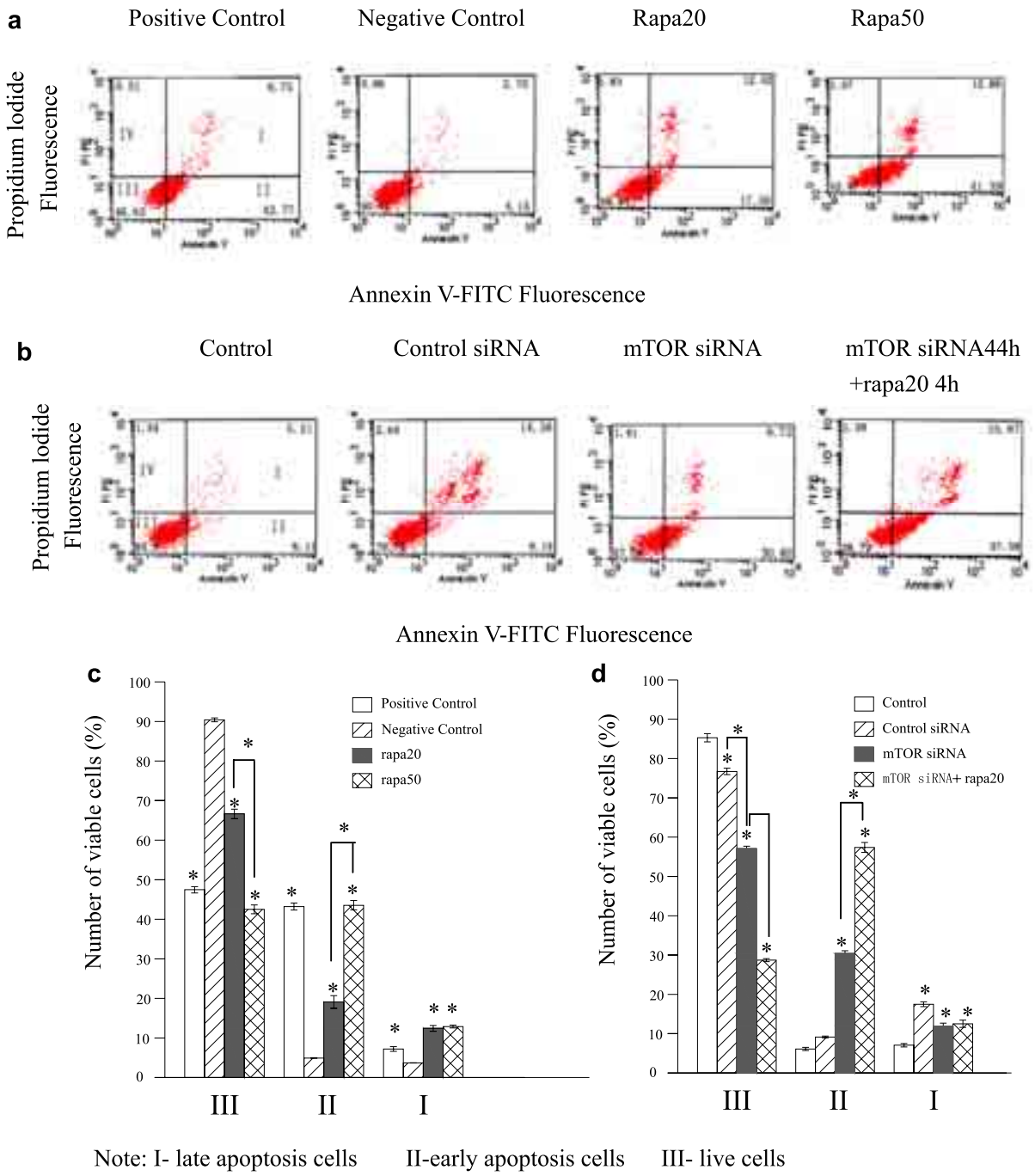


Fig. 8. Induction of apoptosis in EC9706 cells by rapamycin and siRNA against mTOR. EC9706 cells were seeded in six-well plate containing 10% FBS-RPMI 1640 and grown overnight. (a and c) Cells treated with rapamycin at different concentrations or apoptosis reagents for 4 h. (b and d) Cells transfected with siRNA against mTOR for 44 h and then with or without rapamycin for a further 4 h, followed by apoptosis assay using the Annexin V-FITC Apoptosis Detection Kit, as indicated in Section 2. Cells transfected with control siRNA and untreated cells were served as controls. Results pooled from three independent experiments are expressed as means ± SD. $^{*}P < 0.05$, compared to untreated cells.

more, the effect of siRNA against mTOR was similar to rapamycin, i.e., it was able to inhibit phosphorylation of p70S6K and 4E-BP1 and to increase their unphosphorylated form, to arrest EC9706 cells to the $G_0/G_1$ phase of cell cycle, and to induce apoptosis of EC9706 cells. In EC9706 cells treated with siRNA

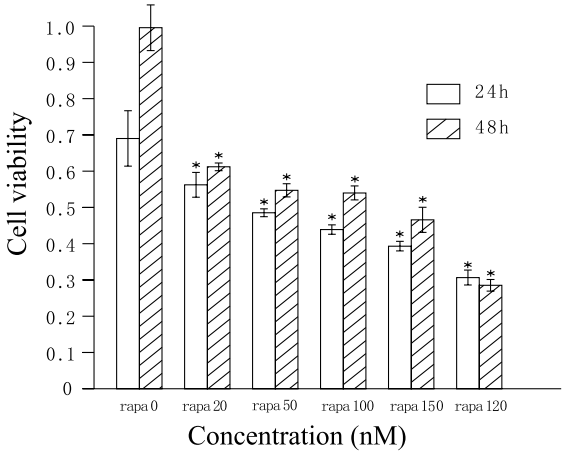


Fig. 9. Effects of rapamycin on proliferation of EC9706 cells. To determine the effects of rapamycin on proliferation of EC9706 cells, the absorbance of cells treated with rapamycin at different concentrations for 24 or 48 h were detected with WST-8 dye. Results pooled from three independent experiments are expressed as means ± SD. $^{*}P < 0.05$, compared to untreated cells.

against mTOR combined with rapamycin, the proportions of cells at $G_0/G_1$ phase and of apoptotic cells were higher than those of cells treated with siRNA against mTOR alone, indicating that the combination of siRNA against mTOR with rapamycin has a synergistic effect on cell cycle and apoptosis of EC9706 cells. Besides, the inhibitory effect of rapamycin on the proliferation of EC9706 cells was demonstrated using WST-8 dye.

In the present study, we identify that the mTOR/p70S6K signaling pathway is activated in ESCC, which might be an important mechanism responsible for the survival and proliferation of ESCC cells. Further studies are underway to investigate the potential use of the mTOR/p70S6K pathway as a specific target for therapy in ESCC and the sensitivity of mTOR inhibitors to ESCC in vivo.

## Acknowledgment

This study was supported by a grant from the "211 Project" (Education Ministry of China, 2002-2).

## References

[1] P. Pisani, D.M. Parkin, F. Bray, J. Forlay, Estimates of the worldwide mortality from 25 cancers in 1990, Int. J. Cancer 83 (1999) 18–29.
[2] R. Montesano, M. Hollstein, P. Hainant, Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review, Int. J. Cancer 69 (1996) 225–235.
[3] N. Hay, N. Sonenberg, Upstream and downstream of mTOR, Genes Dev. 18 (2004) 1926–1945.
[4] S. Han, F.R. Khuri, J. Roman, Fibronectin stimulates non-small cell lung carcinoma cell growth through activation of Akt/mammalian target of rapamycin/S6 kinase and inactivation of LKB1/AMP-activated protein kinase signaling pathway, Cancer Res. 66 (2006) 315–323.
[5] R.J. Shaw, L.C. Cantley, Ras, PI(3)K and mTOR signaling controls tumor cell growth, Nature 441 (2006) 424–430.
[6] G. Xu, W. Zhang, P. Bertramp, X.F. Zheng, H. Mcloeod, Pharmacogenomic profiling of the PI3K/PTEN -AKT/mTOR pathway in common human tumors, Int. J. Oncol. 24 (2004) 893–900.
[7] J.A. van der Hage, L.J. van den Broek, C. Legrand, P.C. Clahsen, C.J. Bosch, E.C. Robanus-Maandaq, et al., Overexpression of p70S6 kinase protein is associated with increased risk of locoregional recurrence in node-negative prememopausal early breast cancer patients, Br. J. Cancer 90 (2004) 1543–1550.
[8] F. Sahin, R. Kannangai, O. Adegbola, J. Wang, G. Su, M. Torbenson, mTOR and p70S6 kinase expression in primary liner Neoplasms, Clin. Cancer Res. 10 (2004) 8421–8425.
[9] P. Amornphimoltham, V. Patel, A. Sodhi, N.G. Nikitakis, J.J. Sauk, E.A. Sausville, et al., Mammalian target of rapamycin, a molecular target in squamous cell carcinomas of the head and neck, Cancer Res. 65 (2005) 9953–9961.
[10] C.S. Beevers, F. Li, L. Liu, S. Huang, Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells, Int. J. Cancer 119 (2006) 757–764.
[11] L. Liu, F. Li, J.A. Cardelli, K.A. Martin, J. Blenis, S. Huang, Rapamycin inhibits cell motility by suppression of mTOR-mediated S6K1 and 4EBP1 pathways, Oncogene 25 (2006) 7029–7040.
[12] T. Sugatani, K.A. Hruska, Akt1/Akt2 and mammalian target of rapamycin/Bim play critical roles in osteoclast differentiation and survival, respectively, whereas Akt is dispensable for cell survival in isolated osteoclast precursors, J. Biol. Chem. 280 (2005) 3583–3589.
[13] D. Morgensztern, H.L. Mcleod, PI3K/Akt/mTOR pathway as a target for cancer therapy, Anticancer Drugs 16 (2005) 797–803.
[14] Q. Meng, C. Xia, J. Fang, Y. Rojanasakul, B.H. Jiang, Role of PI3K and AKT specific isoforms in ovarian cancer cell migration, invasion and proliferation through the p70S6K1 pathway, Cell. Signal. 18 (2006) 2262–2271.
[15] E.K. Rowinsky, Targeting the molecular target of rapamycin (mTOR), Curr. Opin. Oncol. 16 (2004) 564–575.
[16] R.D. Rao, J.C. Buckner, J.N. Sarkaria, Mammalian target of rapamycin (mTOR) inhibitors as anti-cancer agents, Curr. Cancer Drug Targets 4 (2004) 621–635.
[17] H. Carraway, M. Hidalgo, New targets for therapy in breast cancer: mammalian target of rapamycin (mTOR) antagonists, Breast Cancer Res. 6 (2004) 219–224.
[18] D.C. Fingar, J. Blenis, Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression, Oncogene 23 (2004) 3151–3171.
[19] M.A. Bjornsti, P.J. Houghton, The TOR pathway: a target for cancer therapy, Nat. Rev. Cancer 4 (2004) 335–348.

[20] S. Chan, Targeting the mammalian target of rapamycin (mTOR); a new approach to treating cancer, Br. J. Cancer 91 (2004) 1420–1424.
[21] G.V. Georgakis, A. Younes, From Rapa Nui to rapamycin: targeting PI3K/Akt/mTOR for cancer therapy, Anticancer Ther. 6 (2006) 131–140.
[22] L. Dudkin, M.B. Dilling, P.J. Cheshire, F.C. Harwood, M. Hollinqshead, S.G. Arbuck, et al., Biochemical correlates of mTOR inhibition by the rapamycin ester CCI-779 and tumor growth inhibition, Clin. Cancer Res. 7 (2001) 1758–1764.
[23] T. Asano, Y. Yao, J. Zhu, D. Li, J.L. Abbruzzese, S.A. Reddy, The rapamycin anolog CCI-779 is a potent inhibitor of pancreatic cancer cell proliferation, Biochem. Biophys. Res. Commun. 331 (2005) 295–302.
[24] M.B. Atkins, M. Hidalgo, W.M. Stadler, T.F. Loqan, J.P. Dutcher, G.R. Hudes, et al., Randomized Phase II study of multiple does levels of CCI-779, a novel mammalian target of rapamycin kinase inhibitor, in patients with advanced refractory renal cell carcinomas, J. Clin. Oncol. 22 (2004) 909–918.
[25] F. Tian, W.D. Zang, W.H. Hou, H.T. Liu, L.X. Xue, Nuclear factor-kB signaling pathway constitutively activated in esophageal squamous cell carcinoma cell lines and inhibition of growth of cells by small interfering RNA, Acta Biochim. Biophys. Sin. 38 (2006) 318–326.
[26] H. Momota, E. Nerio, E.C. Holland, Perifosine inhibits multiple signaling pathways in glial progenitors and cooperates with temozolomide to arrest cell proliferation in gliomas in vivo, Cancer Res. 65 (2005) 7429–7435.
[27] K.E. O'Reilly, F. Rojo, Q.B. She, D. Solit, G.B. Mills, D. Smith, et al., mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt, Cancer Res. 66 (2006) 1500–1508.
[28] L.A. deGraffenried, L. Fulcher, W.E. Friedrichs, V. Grunwald, R.B. Ray, M. Hidalqo, Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of PI3 kinase/Akt pathway, Ann. Oncol. 15 (2004) 1510–1516.
[29] Z. Feng, H. Zhang, A.J. Levine, S. Jin, The coordinate regulation of the p53 and mTOR pathway in cells, Proc. Natl. Acad. Sci. USA 102 (2005) 8204–8209.
[30] D.C. Fingar, C.J. Richardson, A.R. Tee, L. Cheathan, C. Tsou, J. Blenis, mTOR controls cell cycle progression through its cell growth effector S6K1 and 4EBP1/eukaryotic translation initiation factor 4E, Mol. Cell Biol. 24 (2004) 200–216.
[31] A. Hahn-Windgassen, V. Nogueira, C.C. Chen, J.E. Skeen, N. Sonenberq, N. Hay, Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity, J. Biol. Chem. 280 (2005) 32081–32089.
[32] P. Smolewski, Recent developments in targeting the mammalian target of rapamycin (mTOR) kinase pathway, Anticancer Drugs 17 (2006) 487–494.
[33] L. Hu, J. Hofmann, Y. Lu, G.B. Mills, R.B. Jaffe, Inhibition of phosphatidylinositol 3′-kinase increases efficacy of paclitaxel in vitro and in vivo ovarian cancer models, Cancer Res. 62 (2002) 1087–1092.
[34] J.E. Kim, J. Chen, Cytoplasmic-nuclear shuttling of FKBP12–rapamycin-associated protein is involved in rapamycin-sensitive signaling and translation initiation, Proc. Natl. Acad. Sci. USA 97 (2000) 14340–14345.
[35] D.E. Martin, M.N. Hall, The expanding TOR signaling network, Curr. Opin. Cell Biol. 17 (2005) 158–166.
[36] S.Y. Sun, L.M. Rosenberg, X. Wang, Z. Zhou, P. Yue, F.R. Khuri, Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition, Cancer Res. 65 (2005) 7052–7058.
[37] O. Treeck, B. Wackwitz, U. Haus, O. Ortmann, Effects of a combined treatment with mTOR inhibitor RAD001 and tamoxifen in vitro on growth and apoptosis of human cancer cells, Gyn. Oncol. 102 (2006) 292–299.
[38] L.A. deGraffenried, W.E. Friedrichs, D.H. Russel, E.J. Donzis, A.K. Middleton, J.M. Silva, et al., Inhibition of mTOR activity restores tamoxifen response in brease cancer cells with aberrant Akt activity, Clin. Cancer Res. 10 (2004) 8059–8067.
[39] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.