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# Prognostic significance and function of phosphorylated ribosomal protein S6 in esophageal squamous cell carcinoma

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**Ribosomal protein S6 is a key regulator of 40S ribosome biogenesis, and its phosphorylation is closely related to cell growth capacity. However, as a downstream target of S6 kinases, the clinical significance and the roles of S6 and S6 phosphorylation in cell viability and motility of esophageal squamous cell carcinoma remain unclear. Here, we show that high level of phosphorylated-ribosomal protein S6 (p-S6) (immunohistochemistry score $\geq$5) and an increased ratio of p-S6/S6 (immunohistochemistry score $\geq$0.75) were significantly associated with shortened disease-free survival in patients with esophageal squamous cell carcinoma in univariate analysis ($P=0.049$ and $P<0.001$, respectively). After adjusting for age, tumor-nodes-metastasis stage, chemotherapy, and radiation therapy in multivariate analysis, both p-S6 (hazard ratio 2.21, $P=0.005$) and p-S6/S6 (hazard ratio 2.40, $P<0.001$) remained independent adverse prognostic factors. In addition, S6 and S6 kinase 1 knockdown resulted in attenuation of viability by suppressing cyclin D1 expression in esophageal cancer cells. Furthermore, depletion of S6 and S6 kinase 1 resulted in a reduction in esophageal cancer cell migration and invasion. This was paralleled by a reduction in focal adhesion and by suppression of extracellular signal-regulated kinase and c-jun N-terminal kinase phosphorylation, which control cell motility. Collectively, these findings suggest that p-S6 and the ratio of p-S6/S6 are closely relevant to tumor progression and have prognostic significance in esophageal squamous cell carcinoma.**
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**Keywords:** esophagus squamous cell carcinoma; phosphorylation; S6

Esophageal squamous cell carcinoma is one of the most aggressive malignant tumors of the gastrointestinal tract, and has a high mortality rate.[1] The prognosis of esophagus squamous cell carcinoma is poor due to a high incidence of metastasis to lymph nodes, liver, and lung.[1] Despite the development of multimodal therapies including surgery, chemotherapy, radiotherapy, and chemo-radiotherapy, the 5-year survival rate of patients remains in the range of 10 to 25%,[1] underscoring the need for identifying the signaling pathways and downstream targets that lead to esophageal cancer progression and metastasis.[2,3]

Recently, the mammalian target of rapamycin (mTOR)—S6 kinase (S6K1) signaling pathway—has emerged as a critical regulator of cellular metabolism, proliferation, and survival in response to growth factor and nutrient availability.[4–7] Upon growth factor stimulation, mTOR-dependent activation of S6K1 induces phosphorylation of S6, the 40S ribosomal protein at Ser235, Ser236, Ser240, Ser244, and Ser247.[8,9] The importance of S6 phosphorylation is underscored by the finding that mouse embryonic fibroblasts from S6 knock-in mice in which all phosphorylatable serine residues in S6 are substituted by alanines exhibit small cell size,[10] indicating the potential role of S6 phosphorylation in regulating cell growth capacity. In line with this, the regulation of S6 phosphorylation and S6K activity is frequently altered in tumors such as lym-

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phangioleiomyomatosis and renal carcinoma.[11–13] S6 phosphorylation and S6K1 activity are also markedly upregulated in tumors that carry mutations in the tuberous sclerosis complex 1/2 (TSC1/2).[13] Moreover, the antiproliferative efficacy of mTOR inhibitors that inhibit S6 phosphorylation is currently being evaluated as a treatment for human malignancies in several clinical trials,[12,14] suggesting the potential relevance of S6 phosphorylation in the treatment of esophageal cancer.

Although mTOR signaling, which occurs upstream of S6 phosphorylation, is considered to be a potential target in esophagus squamous cell carcinoma *in vitro*,[15] the prognostic significance of S6 phosphorylation and its role in esophageal cancer progression remain unclear. Here, we assessed the clinical relevance of S6 phosphorylation and its prognostic impact on the survival of patients with esophagus squamous cell carcinoma by measuring the levels of p-S6, and the ratio of p-S6 to total S6 (p-S6/S6) in tissues of esophagus squamous cell carcinoma and by evaluating the relationship of these parameters with clinical outcome. We further examined the functional role of S6 in esophageal cancer cell viability and motility, which are critical for migration and invasion.

## Materials and methods

### Patients, Tissue Samples, and Reagents

The clinical features of the patients investigated in this study are reported in Table 1. Informed consent for the use of surgical specimens for research purposes was obtained from all patients. The data were procured from surgical pathology files kept at the Department of Pathology in Samsung Medical Center, Seoul, Korea and included 169 cases of esophagus squamous cell carcinoma and 35 cases of non-tumor esophageal tissues. The tissue specimens were obtained from archival paraffin blocks of samples from patients with esophagus squamous cell carcinoma who underwent esophagectomy at the Samsung Medical Center between January 1996 and December 2007. All eligible, consecutive cases during this time period were identified for this study. Pathologic features of specimens were classified based on the seventh edition of the tumor-nodes-metastasis (TNM) classification (Union for International Cancer Control (UICC)). All archival materials were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin.

**Table 1** Patients characteristics

| *Characteristics* | *Case no. (n = 169)* | *%* |
|---|---|---|
| *Age* | | |
| ≥65 years | 131 | 78 |
| <65 years | 38 | 22 |
| *Sex* | | |
| Male | 163 | 96 |
| Female | 6 | 4 |
| *Tumor size* | | |
| ≥4.0 cm | 95 | 56 |
| <4.0 cm | 74 | 44 |
| *Differentiation* | | |
| W/D | 40 | 24 |
| M/D | 89 | 52 |
| P/D | 40 | 24 |
| *TNM stage* | | |
| I | 27 | 16 |
| II | 58 | 34 |
| III | 64 | 38 |
| IV | 20 | 12 |
| *Tumor invasion* | | |
| T1 | 34 | 20 |
| T2 | 30 | 18 |
| T3 | 96 | 57 |
| T4 | 9 | 5 |
| *LN metastasis* | | |
| Negative | 68 | 40 |
| Positive | 101 | 60 |
| *Distant metastasis* | | |
| Absent | 147 | 87 |
| Positive | 22 | 13 |

### Immunohistochemical Staining Procedure

Immunostaining was performed using S6 (54D2) or p-S6 (Ser 240/244, Cat. 2215) specific antibodies from Cell Signaling Technology, MA, USA. Tissue sections were deparaffinized with xylene, hydrated, and immersed in peroxidase-blocking solution (Dako REAL Peroxidase-Blocking Solution, Dako, CA, USA) for 10 min. The sections were then microwaved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 9.5) for 20 min at 98°, and then incubated with the primary antibodies (1:100 dilution) followed by rinsing with washing buffer (0.1% Tween 20 in distilled water). Sections were further incubated with DAKO REAL EnVision/HRP, Rabbit/Mouse (Envision) detection reagent. After rinsing, the chromogen was developed. The slides were then counterstained with Meyer's hematoxylin, dehydrated, and mounted with Canada balsam for examination.

### Evaluation of Results of Immunohistochemical Staining

We used the scoring method of Sinicrope *et al*[16] to evaluate both the intensity of immunohistochemical staining and the proportion of cells stained with p-S6 or S6. The staining intensity was scored as follows: 1 (weak), 2 (moderate), or 3 (strong). The proportion of positive cells was classified into one of the five positivity categories: 1 (<5%), 2 (5%–25%), 3 (26%–50%), 4 (51%–75%), or 5 (>75%).

Scores for both parameters (staining intensity and proportion of positive cells) were multiplied to generate an immunohistochemistry score, eg, if the reactivity score was 3 (strong) and the proportion of positive cells was 3 (26%–50%), the final immunohistochemistry score is given by $3 \times 3 = 9$. The immunohistochemistry score of S6 and p-S6 in each sample was determined using this method. Because an immunohistochemistry score higher than 4 indicates moderate or strong immunoreactivity, we classified the cases in which immunohistochemistry score was higher than 4 (immunohistochemistry score: 5–15) as 'high p-S6,' and cases with lower scores (immunohistochemistry score: 1–4) as 'low p-S6.' The p-S6/S6 ratio was then calculated by dividing the p-S6 immunohistochemistry score by the S6 immunohistochemistry score. Each lesion was separately examined and scored by two pathologists (SHK and CKP). The pathologists discussed any cases showing a discrepancy in scores until a consensus was reached.

### Cell Culture and Transfection

Human esophageal squamous carcinoma TE8 and TE10 cell lines were purchased from RIKEN (Saitama, Japan). Cells were cultured in RPMI-1640 (Welgene, Korea) containing 10% FBS (Invitrogen, CA, USA). Cells were transfected with siRNAs using G-fectin (GP-2000) with each siRNA. The siRNAs employed were targeted to mRNAs encoding human S6 (5′-TTGTAAGAAAGCCCTTAAATA-3′) and S6K1 (5′-AAAAGGGGGCTATGGAAAGGTTT-3′). A non-silencing control siRNA (Qiagen, Germany) was also used. Cells were harvested at 48 or 72 h after transfection.

### Western Blotting

Cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were then immunoblotted with antibodies (1:1000 dilution) against S6 (54D2), p-S6 (Ser 240/244, Cat. 2215), p-JNK (Thr183/Tyr185, Cat. 9251), extracellular signal-regulated kinase (ERK) (Cat. 9102), p-ERK (Cat. 9101), p-paxillin (Tyr 118, Cat. 2541) (all from Cell Signaling Technology), and with antibodies against S6K1 (Cat. sc230), c-jun N-terminal kinase (JNK) (Cat. sc6254), cyclin D (Cat. sc397), cdk2 (Cat. sc163), p21 (Cat. sc397), p27 (Cat. sc528), pFAK (Tyr 397, Cat. sc1688), focal adhesion kinase (FAK) (Cat. sc558), and $\beta$-actin (Cat. sc1616) (all from Santa Cruz Biotechnology, CA, USA), and with antibodies against paxillin (BD Biosciences, Cat. BD610619, NJ, USA).

### Cell Viability Assay

Aliquots of a TE8 or TE10 cell suspension were mixed with trypan blue dye and left for 5 min at room temperature. The cells were counted using a hemocytometer and the percentage of live cells was determined.

### Migration and Invasion Assay

*In vitro* Matrigel invasion assays were done using 6.5 mm Costar transwell chambers. The transwell filters were coated with Matrigel (Becton Dickinson, NJ, USA), and cells were seeded onto the Matrigel. After incubation, the filter was removed from the chamber, and cells that had invaded the Matrigel were fixed and stained with hematoxylin and eosin solution. The number of cells attached to the filter was determined by counting under a light microscope. The migration assay was conducted in a similar manner as the invasion assay, except that filters were not coated with Matrigel. Assays were performed at least three times.

### Immunocytochemistry

TE8 or TE10 cells were washed and fixed in 4% formaldehyde/PBS, and permeabilized with 0.2% NP-40/PBS. Cells were blocked with bovine serum albumin and then incubated with anti-paxillin (BD Biosciences) and an anti-mouse rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology) or Alexa 488 phalloidin (Invitrogen) containing 1 $\mu$g/ml Hoechst (Invitrogen), and were mounted with anti-fading mounting medium (Dako North America). Cells were visualized and images were collected using fluorescence microscopy (Axiovert, Zeiss, Germany).

### Statistical Analysis

The association between disease-free survival and protein expression status such as p-S6 and the p-S6/S6 ratio was determined using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazard models were fitted for multivariate analysis to determine the prognostic effect of protein expression and clinicopathologic factors. Results on viability, migration, and invasion were analyzed by one-way or two-way analysis of variance (ANOVA). Significance of differences between means was determined using Bonferroni multiple comparison test. Statistical analysis was performed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). A two-sided significance level of 0.05 was used for all statistical analysis.

## Results

### p-S6 Expression in Non-Tumor Esophagus and Esophageal Squamous Cell Carcinoma

Upon insulin stimulation, mTOR-dependent S6K1 activation induces S6 phosphorylation at Ser 240/244.[9] To assess the status of S6 phosphorylation, we

first validated the specificity of antibodies against S6 and p-S6 (Ser 240/244) and then measured the levels of S6 and p-S6 expression in TE8 esophageal cancer cells. The levels of S6 phosphorylation at Ser 240/244 were increased upon insulin stimulation, whereas such levels were reduced by treatment with rapamycin, an inhibitor of the mTOR pathway, indicating the specificity of antibodies detecting either S6 or p-S6 (Figure 1a). Using these antibodies, we assessed the levels of p-S6 expression by immunohistochemistry in esophageal tissues from 35 non-tumor and 169 esophageal cancer patients. Immunohistochemical analysis revealed that S6 expression was found in almost all non-tumor esophageal and esophagus squamous cell carcinoma tissues (Figure 1b). In contrast, immunoreactive intensity of p-S6 was significantly stronger in esophageal cancer cells compared with non-tumor tissue (Figure 1c). Interestingly, strong immunoreactivity for p-S6 was particularly evident in cancer cells along the invading front at the tip of esophagus squamous cell carcinoma mass (Figure 1d).

### Association of Enhanced p-S6 Expression and High p-S6/S6 Ratio with Poor Prognosis in Patients with Esophagus Squamous Cell Carcinoma

Univariate and unsupervised survival analyses of patients with esophagus squamous cell carcinoma were performed using the Kaplan–Meier method. Patients with high levels of p-S6 (immunohistochemistry score $\geq 5$) had a shorter disease-free survival time than patients with low levels of p-S6 (immunohistochemistry score $<5$, Figure 2a). The level of p-S6 was significantly associated with a reduced disease-free survival rate ($P=0.049$) but not with the overall survival rate ($P=0.266$; Figure 2a and Supplementary Figure S1).

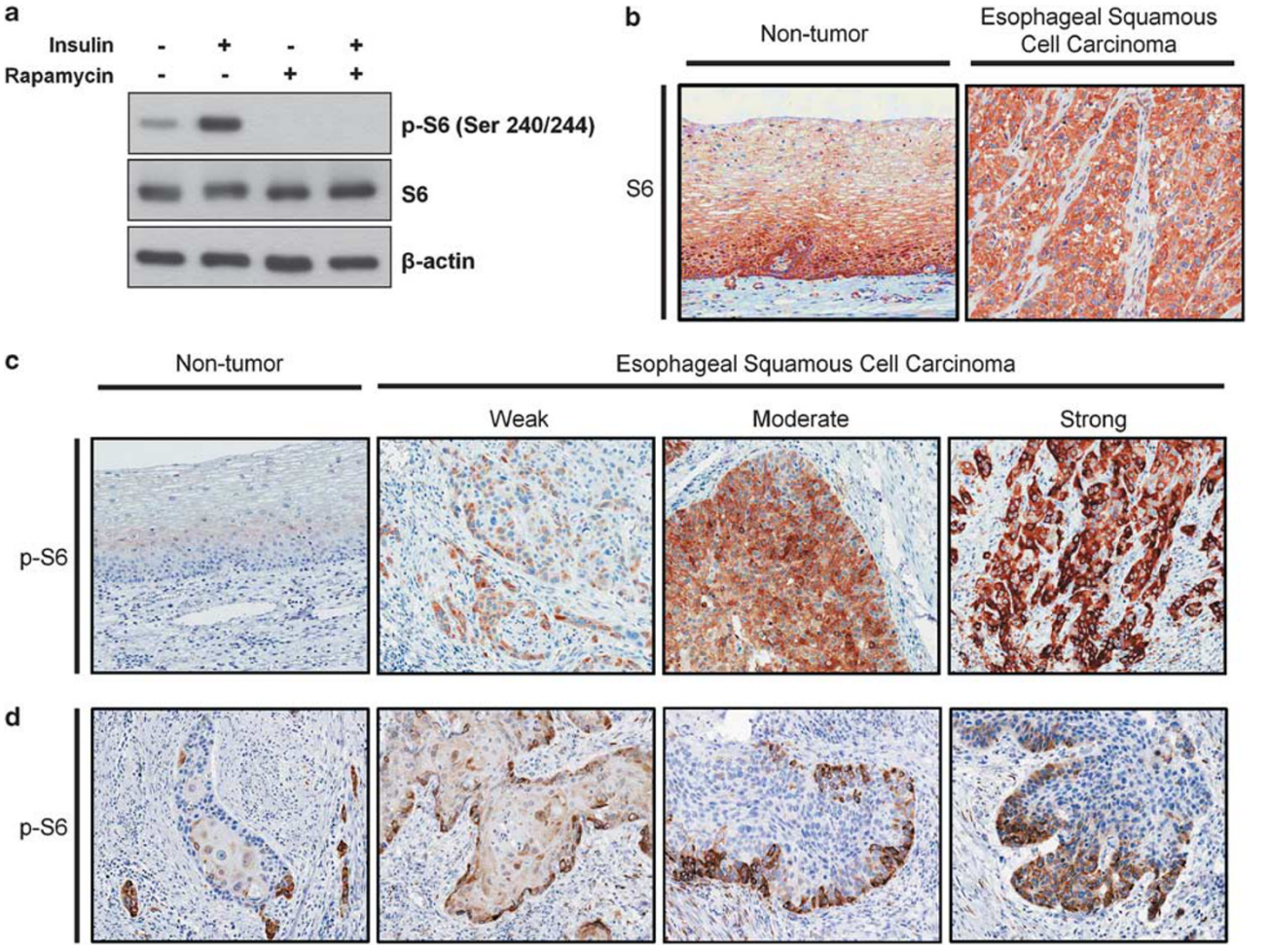


**Figure 1** Immunohistochemical analysis of p-S6 in non-tumor esophageal and esophageal squamous cell carcinoma tissues. (**a**) Expression levels of the indicated proteins were determined in TE8 cells using western blotting. TE8 cells were pre-treated with either vehicle (control) or rapamycin (20 nM) for 30 min and then stimulated with insulin (50 nM) for 30 min. (**b**) Representative S6 immunostaining in non-tumor esophageal or esophagus squamous cell carcinoma tissues. (**c**) Representative p-S6 immunostaining in non-tumor esophageal or esophagus squamous cell carcinoma tissues with weak, moderate, or strong expression. (**d**) Intense immunoreactivity for p-S6 in cancer cells along the invading front at the tip of esophagus squamous cell carcinoma mass. (Original magnification $\times 200$.)

For supervised survival analysis including tumor stage, we divided the patients into stage I + II and stage III + IV, and performed Kaplan–Meier survival analysis separately in each group. A high p-S6 level was a more significant risk factor affecting disease-free survival of early-stage esophagus squamous cell carcinoma patients (I + II, $P = 0.016$) than of late-stage patients (III + IV, $P = 0.080$, Figure 2b and c). These data suggest that p-S6 was more relevant to the survival rate of patients in early stages of esophageal cancer. As immunoreactivity can be influenced by a various confounding factors such as fixation status and staining conditions, we attempted to compensate for these undesirable influences. For this, we stained the same tissues with antibody to S6 and p-S6, and calculated the ratio of p-S6 to S6 (p-S6/S6). We then determined whether the p-S6/S6 ratio was relevant to the clinical outcome of esophagus squamous cell carcinoma. Based on analysis of disease-free survival rate of patients with esophagus squamous cell carcinoma, we found that a high p-S6/S6 ratio was more significantly correlated with unfavorable prognosis ($P < 0.001$) than was the level of p-S6 alone (Figure 2d). Similarly, a high p-S6/S6 ratio was significantly associated with a reduced overall survival rate ($P = 0.044$, Supplementary Figure S1).

### Multivariate Analysis of Prognostic Variables in Patients with Esophagus Squamous Cell Carcinoma

To determine whether p-S6 and the p-S6/S6 ratio were independent prognostic factors in esophagus squamous cell carcinoma, multivariate analyses of p-S6 and the p-S6/S6 ratio for overall and disease-free survival rates of esophageal cancer patients were performed using Cox proportional-hazard regression. In this multivariate survival analysis, age, TNM stage, history of chemotherapy, and radiation therapy, as well as data for p-S6 and the p-S6/S6 ratio, were entered into a Cox proportional-hazard model. Multivariate survival analysis was separately performed according to p-S6 (Table 2) and p-S6/S6 (Table 3). The results showed that p-S6/S6 was a more powerful independent prognostic factor than p-S6. The relative risk of p-S6/S6 in disease-free survival was 2.404 ($P < 0.001$), and the relative risk in overall survival

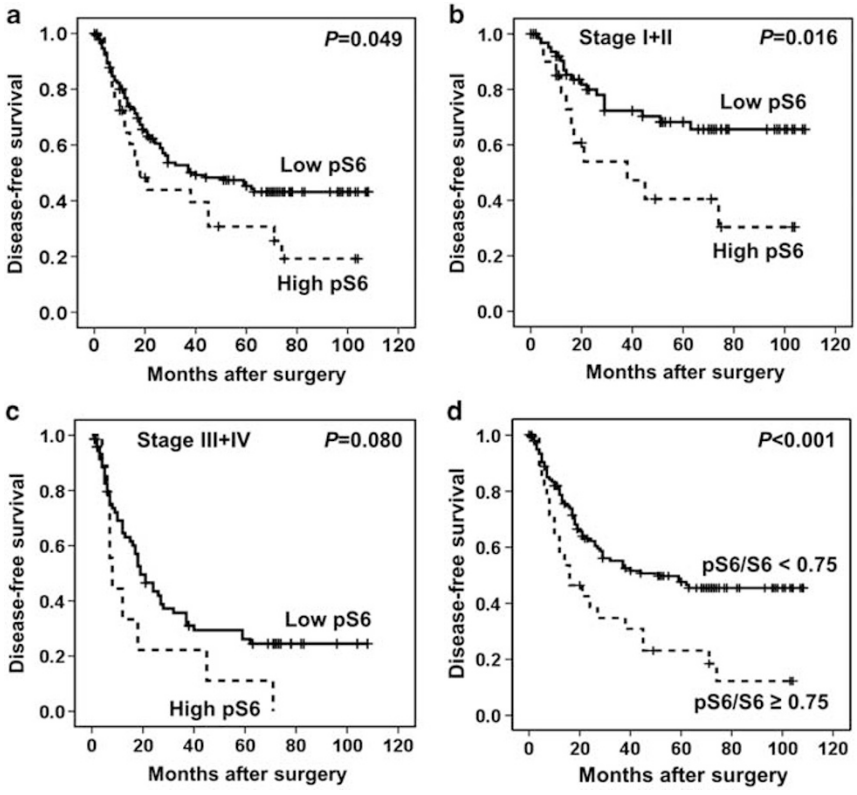


**Figure 2** Kaplan–Meier survival curves for disease-free survival according to the results of p-S6 or p-S6/S6 immunostaining. (**a**) Kaplan–Meier curves illustrating disease-free survival among patients with esophagus squamous cell carcinoma on the basis of p-S6 expression status; low P-S6 (immunohistochemistry score $< 5$, $n = 138$) and high P-S6 (immunohistochemistry score $\geq 5$, $n = 31$). (**b**) Disease-free survival among patients with esophagus squamous cell carcinoma on the basis of p-S6 expression status at stage I + II (low pS6, $n = 64$; high pS6, $n = 21$), or (**c**) at stage III + IV (low pS6, $n = 74$; high pS6, $n = 10$). (**d**) Disease-free survival among patients with esophagus squamous cell carcinoma on the basis of p-S6/S6 ratio (p-S6/S6 $< 0.75$, $n = 140$; p-S6/S6 $\geq 0.75$, $n = 29$). Disease-free survival was significantly worse in patients with high expression of p-S6 or high p-S6/S6 ratio.

**Table 2** Multivariate Cox regression analysis of p-S6 and other covariates for ESCC patients' survival rate

| *Predictors* | *Disease-free survival* | | *Overall survival* | |
|---|---|---|---|---|
| | *HR (95% CI)* | P-*value* | *HR (95% CI)* | P-*value* |
| *Age* | | | | |
| 0–65 | 1.00 | | 1.00 | |
| 65+ | 0.950 (0.6–1.6) | 0.846 | 0.962 (0.5–1.7) | 0.895 |
| *TNM stage* | | | | |
| I | 1.00 | | 1.00 | |
| II | 2.656 (1.0–7.1) | 0.051 | 4.115 (1.2–14.0) | 0.024* |
| III | 6.226 (2.3–16.5) | <0.001* | 10.931 (3.2–37.0) | <0.001* |
| IV | 7.940 (2.9–21.8) | <0.001* | 11.776 (3.3–41.7) | <0.001* |
| *Chemotherapy* | | | | |
| Absent | 1.00 | | 1.00 | |
| Positive | 0.854 (0.5–1.4) | 0.518 | 0.739 (0.4–1.2) | 0.235 |
| *Radiation therapy* | | | | |
| Absent | 1.00 | | 1.00 | |
| Positive | 1.944 (1.3–3.0) | 0.002* | 1.867 (1.2–2.9) | 0.007* |
| *p-S6* | | | | |
| Negative | 1.00 | | 1.00 | |
| Positive | 2.210 (1.3–3.8) | 0.005* | 2.101 (1.2–3.8) | 0.013* |

Abbreviations: CI, confidence interval; HR, hazard ratio.
*$P<0.05$.

**Table 3** Multivariate Cox regression analysis of p-S6/S6 and other covariates for ESCC patients' survival rate

| *Predictors* | *Disease-free survival* | | *Overall survival* | |
|---|---|---|---|---|
| | *HR (95% CI)* | P-*value* | *HR (95% CI)* | P-*value* |
| *Age* | | | | |
| 0–65 | 1.00 | | 1.00 | |
| 65+ | 0.923 (0.5–1.6) | 0.763 | 0.932 (0.5–1.7) | 0.811 |
| *TNM stage* | | | | |
| I | 1.00 | | 1.00 | |
| II | 2.447 (0.9–6. 2) | 0.072 | 3.810 (1.1–12.9) | 0.031* |
| III | 5.233 (2.0–13.5) | 0.001* | 9.240 (2.8–30.4) | <0.001* |
| IV | 6.923 (2.5–18.9) | <0.001* | 10.545 (3.0–37.1) | <0.001* |
| *Chemotherapy* | | | | |
| Absent | 1.00 | | 1.00 | |
| Positive | 0.878 (0.5–1.4) | 0.594 | 0.753 (0.5–1.2) | 0.265 |
| *Radiation therapy* | | | | |
| Absent | 1.00 | | 1.00 | |
| Positive | 1.942 (1.6–3.0) | 0.003* | 1.799 (1.138–2.9) | 0.014* |
| *p-S6/S6* | | | | |
| <0.75 | 1.00 | | 1.00 | |
| ≥0.75 | 2.404 (1.5–3.9) | <0.001* | 2.135 (1.3–3.5) | 0.003* |

Abbreviations: CI, confidence interval; HR, hazard ratio.
*$P<0.05$.

was 2.135 ($P=0.003$; Table 3). These results indicate that p-S6/S6 is superior to p-S6 as a potential prognostic indicator in esophagus squamous cell carcinoma.

### Depletion of S6 or S6K1 Attenuates Viability of Esophageal Cancer Cells

Based on the fact that S6 phosphorylation is associated with cell growth capacity, which affects cell size and viability,[8,10,17] we determined whether S6 and S6 phosphorylation regulate viability of esophageal cancer cells. S6 knockdown led to a reduction in viability of TE8 or TE10 esophageal cancer cells. In addition, knockdown of S6K1, the upstream kinase for S6 phosphorylation, had a similar inhibitory effect on viability of both TE8 and TE10 cells (Figure 3a and Supplementary Figure S2a).

Considering that cell cycle proteins are critical to regulating cell viability, and cyclin D1 overexpression is significantly associated with poor outcome in esophagus squamous cell carcinoma,[18] we next determined whether S6 or S6K1 affects the expression of cell cycle regulators in G1/S phase transition. Depletion of S6 or S6K1 resulted in a sharp decrease in cyclin D and cdk 2 levels, whereas levels of p21 and p27, which are cyclin-dependent kinase 4/6 inhibitors, were increased, indicating that both S6 and S6K1 regulate cell cycle progression of TE8 and TE10 esophageal cancer cells (Figure 3b and Supplementary Figure S2b).

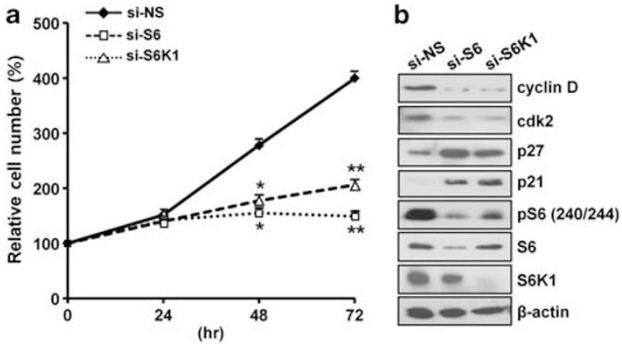


**Figure 3** Depletion of S6 or S6K1 attenuates viability of TE8 esophageal cancer cells. TE8 cells were transfected with non-silencing siRNA (NS), si-S6, or si-S6K1 during the indicated times. (**a**) Cell viability was determined by trypan blue assay. (**b**) Cell lysates were analyzed by immunoblot using the indicated antibodies. Assays and blots are representative of three independent experiments. Values are mean ± s.e.m. (ANOVA, *$P<0.05$, **$P<0.01$).

### Depletion of S6 or S6K1 Attenuates Migration, Invasion, and Focal Adhesion Formation of Esophageal Cancer Cells

Given that migration and invasion are critical steps in metastasis during the progression of esophageal cancer,[1] and in view of the finding that high levels of p-S6 expression were seen in invading cells (Figure 1d), we next assessed whether S6 knockdown affected migration and invasion of TE8 or TE10 esophageal cancer cells. Knockdown of S6 or S6K1 led to inhibition of migration and invasion of both TE8

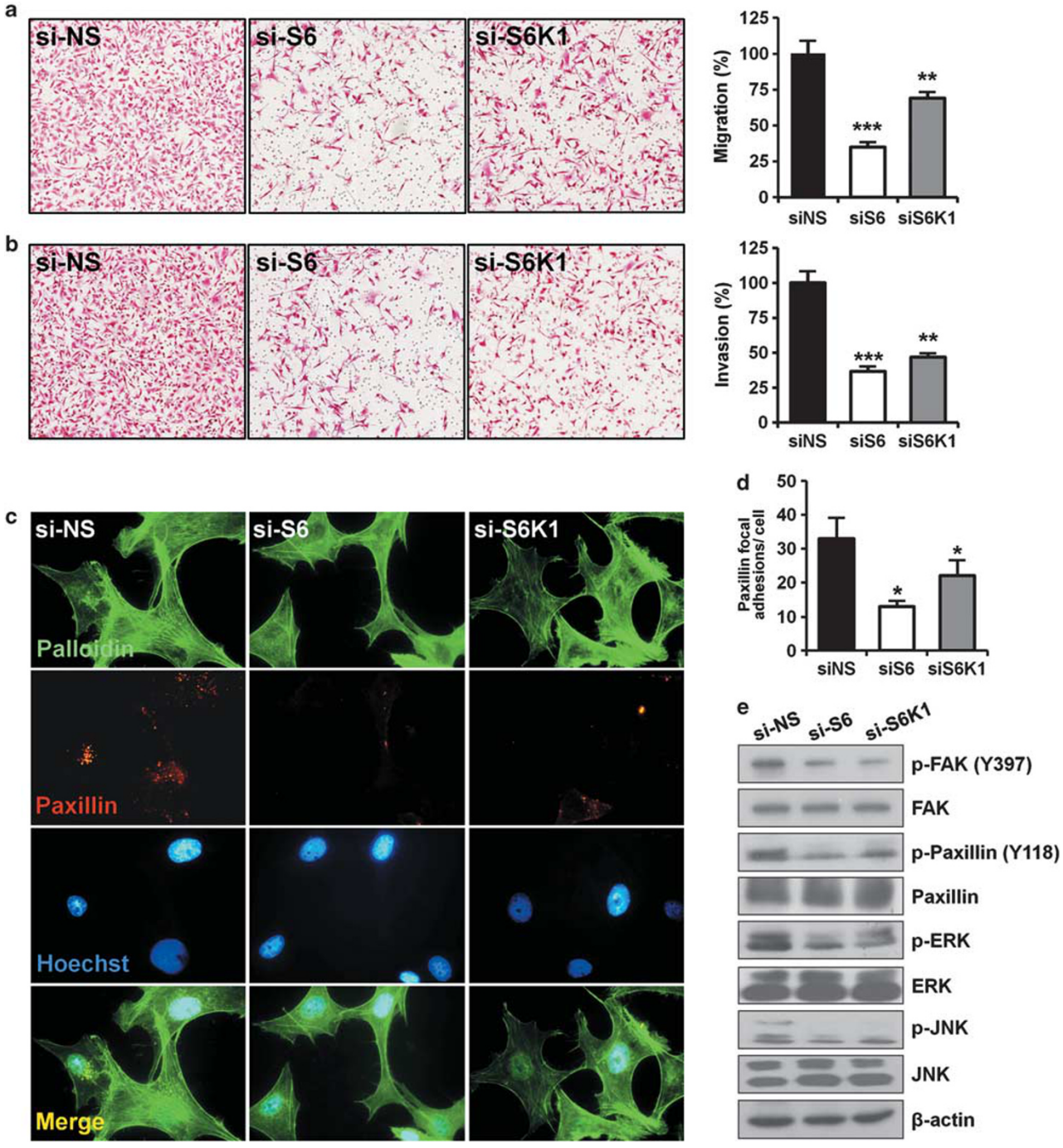


**Figure 4** Depletion of S6 or S6K1 attenuates migration, invasion, and formation of focal adhesion of TE8 esophageal cancer cells. TE8 cells were transfected with non-silencing siRNA (NS), si-S6, or si-S6K1, and then evaluated by (**a**) transwell migration assay or (**b**) invasion assay. (**a**, **b**) Cells were fixed and stained with hematoxylin and eosin and the slides of filters of migrated cells or invaded cells were scanned using an Aperio scanner (original magnification $\times 100$). (**c**) The cells were immunostained using antibody to paxillin and rhodamine-labeled secondary antibody, stained with Hoechst and Alexa 488 phalloidin, and then viewed by fluorescence microscopy. (**d**) The numbers of paxillin focal adhesions per cell were determined. (**e**) Cell lysates were analyzed by immunoblot using the indicated antibodies. Assays and blots are representative of three independent experiments. Values are mean ± s.e.m. (ANOVA, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

and TE10 cells (Figures 4a and b, Supplementary Figures S3a and b), indicating the relevance of S6K1 and S6 to aggressive behavior of esophageal cancer cells.

Based on the fact that tumor invasiveness and lymph node metastasis in esophagus squamous cell carcinoma were associated with FAK overexpression,[19] we attempted to determine whether focal adhesion was influenced by S6 or S6K1 depletion. We found that S6 or S6K1 depletion led to pronounced inhibition of focal adhesion formation as revealed by less densely stained paxillin, which mediates the interaction between the actin

cytoskeleton and integrins (Figures 4c and d).[20,21] Thus, these data suggest that S6K1-S6 signaling is critical to focal adhesion formation, which is essential for esophageal cancer cell motility. To explore the molecular mechanism underlying S6-mediated focal adhesion and invasion, we examined the status of signaling pathways that mediate cell motility. The phosphorylation of FAK and paxillin was markedly suppressed by S6 or S6K1 knockdown in TE8 and TE10 cells (Figure 4e and Supplementary Figures S3c–e). Moreover, knockdown of S6 and S6K1 resulted in inhibition of ERK and JNK phosphorylation, which are downstream regulators of FAK in cellular motility.

Together, these results suggest that S6K1 and S6 mediate migration and invasion of esophagus squamous cell carcinoma by regulating the FAK/ERK/JNK pathway.

## Discussion

Despite advances in therapeutic approaches, the 5-year survival rate of patients with esophagus squamous cell carcinoma remains poor,[1] underscoring need for identifying signaling pathways for early detection of high-risk patients with esophagus squamous cell carcinoma. In this study, we demonstrate that S6 phosphorylation, the downstream target of S6Ks, is significantly associated with shortened patient survival in esophagus squamous cell carcinoma. In addition, we found that the p-S6/S6 ratio was an independent prognostic factor, indicating that elevated levels of p-S6 in esophagus squamous cell carcinoma were not merely due to S6 overexpression. The combination of measurements of phosphorylated and total forms of S6 may provide a more accurate prediction of clinical outcome than a single biomarker in esophagus squamous cell carcinoma. Consistent with our results, recent studies revealed that phosphorylated mTOR was associated with poor prognosis of patients with esophagus squamous cell carcinoma,[22] although the effect of downstream effectors of mTOR on survival of patients with esophagus squamous cell carcinoma and their functional relevance to esophageal cancer progression were not investigated.

We further found that knockdown of S6 or S6K1 led to suppression of cell cycle progression by inhibiting the expression of cyclin D and cdk 2. Relevant to this, recent studies have shown that genetic deletion of S6K1 resulted in a delay in entry into S phase after hepatectomy. As well, the S6K1 pathway is related to Gli1-mediated proliferation of esophageal adenocarcinoma cells, supporting the role of S6K1 in cancer cell proliferation.[23,24] In addition, cell cycle progression was severely blocked in liver-specific S6 knockout mice after hepatectomy,[25] indicating the critical role of S6 in cell cycle progression. At present, it remains unclear how S6 and its phosphorylation control cell cycle regulators in esophageal cancer. Intriguingly, recent studies have shown that depletion of S6, which impairs 40S ribosome biogenesis, leads to p53 induction resulting in cell cycle arrest in hepatocytes,[26] suggesting that S6 regulates cell cycle checkpoints such as p53, dysregulation of which may contribute to esophageal cancer development.

Considering that cell motility and invasion are critical factors for advanced esophageal cancer,[1] p-S6 positive cells may have more metastatic characteristics. Indeed, S6 knockdown resulted in reduction of migration and invasion of esophagus squamous cell carcinoma cells. In line with this, elevated levels of p-S6 are associated with metastasis in lung adenocarcinoma[27] and renal angiomyolipoma[11] similar to our results in esophagus squamous cell carcinoma, indicating the relevance of p-S6 in invasion of cancer cells. We further demonstrated that lowering levels of S6K1 and S6 led to impairment of focal adhesion formation, which was paralleled by a reduction in phosphorylation of FAK and paxillin, which control dynamic changes in cell adhesion. Consistent with this, a recent study demonstrated that rapamycin, an mTOR inhibitor, leads to impairment of F-actin reorganization by suppressing phosphorylation of focal adhesion proteins, including FAK and paxillin in glioblastoma and prostate carcinoma cells, although the clinical significance were not studied.[28] Further studies will be needed to address how S6 signaling mediates cell adhesion and invasion, and whether S6 signaling has an impact on *in vivo* metastasis.

Cho *et al*[29] revealed that p-S6 expression in patients with advanced renal carcinoma was highly associated with the response to temsirolimus, an inhibitor of mTOR-S6K signaling. In addition, p-S6 expression was predictive of the early sarcoma response to AP23573, an inhibitor of mTOR,[30] suggesting that the status of p-S6 in solid tumors influences the response to anticancer therapy. In this regard, our findings extend the clinical role of p-S6 by demonstrating that elevated p-S6 and an elevated p-S6/S6 ratio are associated with poor clinical outcome of patients with early stage esophagus squamous cell carcinoma. Our results also reveal the role of S6 and S6K1 in migration and invasion of esophageal cancer cells. Thus, our findings suggest that the expression status of p-S6 and the p-S6/S6 ratio may be relevant to tumor progression and may be helpful in the development of potential therapeutic strategies for the treatment of esophagus squamous cell carcinoma.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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