Phosphorylated mammalian target of rapamycin expression is associated with the response to chemoradiotherapy in patients with esophageal squamous cell carcinoma

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Objective: The mammalian target of rapamycin signaling pathway has been implicated in therapeutic resistance in several types of cancer. However, the significance of mammalian target of rapamycin activation in chemoradiotherapy sensitivity and its effect on the prognosis of esophageal squamous cell carcinoma treated with chemoradiotherapy remain unknown. However, this pathway is of particular interest because an effective inhibitor is available.

Methods: By using immunohistochemistry, phosphorylated mammalian target of rapamycin expression was examined in 77 patients with esophageal squamous cell carcinoma treated with preoperative chemoradiotherapy followed by surgery between 1999 and 2009, and correlated with treatment outcome. With the use of CE81T/VGH and TE2 cell lines, cells were treated with chemotherapy, temsirolimus (mammalian target of rapamycin inhibitor), or a combination of chemotherapy and temsirolimus, and investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Results: Pathologic complete response rates were 42% and 16% in patients with negative and positive phosphorylated mammalian target of rapamycin expression, respectively ($P = .01$). The 3-year overall survivals were 57% and 30% in patients with negative and positive phosphorylated mammalian target of rapamycin expression, respectively ($P = .005$). Positive phosphorylated mammalian target of rapamycin expression was independently associated with inferior overall and disease-free survival. In patients who did not achieve pathologic complete response, postchemoradiotherapy esophagectomy specimens showed significantly higher phosphorylated mammalian target of rapamycin expression than pretreatment biopsy specimens. In cell lines, concomitant administration of temsirolimus enhanced the effect of chemotherapy.

Conclusions: Phosphorylated mammalian target of rapamycin expression is independently associated with the response to chemoradiotherapy and prognosis of patients with esophageal squamous cell carcinoma treated with preoperative chemoradiotherapy. Mammalian target of rapamycin inhibition can sensitize esophageal cancer cells to chemotherapy. Our results suggest the potential for mammalian target of rapamycin as a therapeutic target for patients with esophageal squamous cell carcinoma who receive multimodality treatment. (J Thorac Cardiovasc Surg 2012;144:1352-9)

Supplemental material is available online.

The outcome of patients with advanced esophageal squamous cell carcinoma (ESCC) treated with surgery alone is unsatisfactory.^{1,2} In an attempt to improve survival, a multimodality approach using combined chemoradiotherapy with and

without surgery has been advocated for patients.^{3,4} However, the value of the surgery after chemoradiotherapy remains unclear. Recent phase III trials^{3,4} showed that surgery may not be necessary for those patients who respond well to chemoradiotherapy. After preoperative chemoradiotherapy, pathologic complete response is found in only 20% to 40% of patients³⁻⁶ and is associated with improved survival.⁷ However, there is still a large portion of

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Funding: This work was supported in part by grants from the National Science Council, Taiwan (NSC 100-2314-B-182A-044-MY3) and Chang Gung Memorial Hospital (CMRPG890631).

Disclosures: Authors have nothing to disclose with regard to commercial support. Received for publication March 10, 2012; revisions received May 18, 2012; accepted for publication June 18, 2012; available ahead of print July 27, 2012.

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0022-5223/\$36.00

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<http://dx.doi.org/10.1016/j.jtcvs.2012.06.049>

Abbreviations and Acronyms

AJCC	= American Joint Committee on Cancer
CDI	= combination drug index
CI	= confidence interval
CT	= computed tomography
DFS	= disease-free survival
DMEM	= Dulbecco's Modified Eagle Medium
ESCC	= esophageal squamous cell carcinoma
EUS	= endoscopic ultrasound
5-FU	= 5-fluorouracil
mTOR	= mammalian target of rapamycin
OR	= odds ratio
OS	= overall survival
p-mTOR	= phosphorylated mammalian target of rapamycin

patients who do not respond well to chemoradiotherapy. In addition, there are substantial differences in the response to chemoradiotherapy and survival between patients with the same clinical stage. Therefore, there is an urgent need to identify patients who are likely to respond to chemoradiotherapy to spare them the potential perioperative morbidity and mortality. A reliable marker that can precisely predict the tumor response to chemoradiotherapy in patients with ESCC is important, and this marker may serve as a therapeutic target in the future.

The mammalian target of rapamycin (mTOR), also known as "FK506 binding protein 12-rapamycin associated protein 1," is a serine/threonine protein kinase that plays a key role in regulating protein synthesis, ribosomal protein translation, and cap-dependent translation.⁸ Deregulation in mTOR signaling is frequently associated with tumorigenesis, angiogenesis, tumor growth, and metastasis.⁸ In response to extracellular stimuli, mTOR is activated by phosphorylation of Ser2448 through the phosphatidylinositol 3-kinase/AKT signaling pathway.⁸ Activation of mTOR stimulates the phosphorylation of 2 key downstream proteins that regulate protein translation: eukaryotic initiation factor 4E binding protein-1 and protein S6 kinase 1. Through the activation of eukaryotic initiation factor 4E and protein S6 kinase 1, mTORC complex 1 promotes the translation of messenger RNAs that often encode proteins necessary for cell cycle progression, cell growth, and metabolism. A number of studies suggest that activation of the mTOR signaling pathway plays an important role in multiple anticancer drug-resistance mechanisms in several types of cancer.⁹ In particular, a recent development of the mTOR inhibitor temsirolimus has generated considerable excitement in both the clinical and the basic cancer research communities, because it exhibits potent activity against a wide panel of cancers.¹⁰ Therefore, further elucidation

of the mTOR activation in human cancers is critical for anticancer therapy targeting the mTOR signaling pathway. However, the significance of mTOR activation in chemoradiotherapy sensitivity and its effect on the prognosis of patients with ESCC treated with chemoradiotherapy remain unclear.

Thus, we evaluated the mTOR activity with immunohistochemistry and investigated the prognostic role of mTOR activation in 77 patients with ESCC who were treated with preoperative chemoradiotherapy followed by surgery. In addition, we also use ESCC cell lines to determine whether the mTOR inhibitor can enhance the effect of chemotherapy.

MATERIALS AND METHODS

Patient Population

Patients with ESCC who were treated with chemoradiotherapy at Kaohsiung Chang Gung Memorial Hospital between January 1999 and December 2009 were retrospectively reviewed. This study was approved by the institutional review board of Chang Gung Memorial Hospital. All patients selected for this study were required to undergo esophagectomy after chemoradiotherapy to allow pathologic correlation.

During this period, 109 patients with ESCC who were treated with preoperative chemoradiotherapy followed by surgery were identified. Among these 109 patients, 25 (23%) achieved pathologic complete response. Pretreatment specimens of biopsy were available in 77 patients. Patients were evaluated by a multidisciplinary team, including a thoracic surgeon, medical oncologist, radiation oncologist, radiologist, and gastroenterologist. Pretreatment staging evaluation included physical examination, endoscope, contrast-enhanced computed tomography (CT) scans from the neck to upper abdomen, and endoscopic ultrasound (EUS). All 77 patients had CT scans. EUS was performed in 61 patients. In patients who did not receive EUS, T stage was determined by CT scans only. The clinical staging was determined according to the American Joint Committee on Cancer (AJCC) staging system (*AJCC Cancer Staging Manual*, 7th Edition).¹¹

Treatment Plan

Patients were treated with 2 cycles of cisplatin and 5-fluorouracil (5-FU)-based chemotherapy and radiotherapy (36 Gy in 20 fractions) concurrently. The interval between treatment cycles was 3 to 4 weeks. Radiotherapy of 1.8 Gy per day was delivered 5 days per week for 4 weeks. Three-dimensional conformal radiotherapy via a 4-field technique was used in most cases. Within 3 to 4 weeks after the end of irradiation, a CT scan was performed to assess the treatment response. The multidisciplinary team reviewed the clinical information to determine whether the lesions were resectable. If yes, surgery was advised approximately 4 to 6 weeks after the end of preoperative chemoradiotherapy. Patients undergoing surgery had a radical esophagectomy with cervical esophagogastric anastomosis (McKeown procedure) or an Ivor Lewis esophagectomy with intrathoracic anastomosis, reconstruction of the digestive tract with gastric tube, and pylorus drainage procedures. Two-field lymph node dissection was performed in all patients. Pathologic complete response was defined as the complete disappearance of all viable cancer cells in all surgical specimens, including the primary esophageal tumor and lymph nodes.

Patient Follow-up

After the operation, patients were scheduled for follow-ups at 3-month intervals in years 1 and 2, 6-month intervals in years 3 to 5, and annually

thereafter. Overall survival (OS) was calculated from the date of diagnosis to death as a result of all causes. Disease-free survival (DFS) was computed from the time of surgery to the recurrence or death from any cause without evidence of recurrence.

Immunohistochemistry

Immunohistochemistry staining was performed using an immunoperoxidase technique. Staining was performed on slides (4 μ m) of formalin-fixed, paraffin-embedded tissue sections with primary antibodies against phosphorylated mTOR (p-mTOR) (Ser2448, Clone 49F9, 1:50, Cell Signaling Technology, Inc, Danvers, Mass). Briefly, after deparaffinization and rehydration, the retrieval of the antigen was performed by treating the slides in 10 mmol/L citrate buffer (pH 6.0) in a hot water bath (95°C) for 20 minutes. Endogenous peroxidase activity was blocked for 15 minutes in 0.3% hydrogen peroxide. After blocking with 1% goat serum for 1 hour at room temperature, the sections were incubated with primary antibodies for at least 18 hours at 4°C overnight. Immunodetection was performed using the LSAB2 kit (Dako, Carpinteria, Calif) followed by 3-3'-diaminobenzidine for color development and hematoxylin for counterstaining. Incubation without the primary antibody and normal esophageal squamous epithelium¹² were used as a negative control. A normal gastric gland¹² was used as a positive control. The staining assessment was independently carried out by 2 pathologists (S.L.W. and W.T.H) without any information about clinicopathologic features or prognosis. The fraction of tumor cells with cytoplasmic p-mTOR expression (0%-100%) was recorded, and the average value of 2 pathologists was calculated in each patient. Whenever the 2 pathologists disagreed (eg, difference >10% or discordant scores), the slides were reevaluated and discussed, and a consensus was reached by 2 pathologists. Then p-mTOR expression level was scored by using the 3-tier system: negative expression (score 0), less than 10%; low expression (score 1), 10% or more, but less than 50%; and high expression (score 2), 50% or more tumor cells with staining.^{13,14} When scores were classified into 2 groups for statistical analysis, "low" and "high" were combined as "positive."¹²⁻¹⁴

Cell Culture and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Human esophageal cancer cells (CE81T/VGH), a cell line derived from a well-differentiated squamous cell carcinoma of the esophagus, were provided by Dr C-C Yen (Taipei Veterans General Hospital, Taipei, Taiwan) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 ig/mL streptomycin. Human esophageal cancer cells (TE2), a cell line derived from a poorly differentiated squamous cell carcinoma of the esophagus, were provided by Dr W-C Huang (Mackay Memorial Hospital, Taipei, Taiwan) and cultured in DMEM supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 ig/mL streptomycin. The rapamycin analogue CCI-779 (temsirolimus) was provided by Wyeth-Ayerst (King of Prussia, Pa).

To test the effects of cell proliferation of chemotherapy alone and in combination with temsirolimus on cell proliferation, cells were plated into 96-well, flat-bottomed plates at 3×10^3 cells per 100 μ L per well in DMEM containing 10% fetal bovine serum. After overnight incubation, triplicate wells were treated with chemotherapy alone, temsirolimus alone, or chemotherapy in combination with different concentrations of temsirolimus for 24 hours. The relative percentages of metabolically active cells compared with untreated controls were then determined on the basis of mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan. In brief, after incubation, 10 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St Louis, Mo) solution (5 mg/mL) was added to each well for 3 hours, and then the medium was replaced with 150 μ L of dimethyl

sulfoxide per well. Results were assessed in a 96-well format plate reader by measuring the absorbance at a wavelength of 540 nm using a Titertek Multiscan (Thermo, Finland).

Statistical Analysis

For patient data, statistical analysis was performed using the Statistical Package for the Social Sciences 17 software package (SPSS Inc, Chicago, Ill). The chi-square and Fisher exact tests were used to compare data between the 2 groups. Changes in p-mTOR expression during chemoradiotherapy were analyzed by the McNemar-Bowker test. Multivariate analysis of pathologic complete response was performed by logistic regression, and variables with *P* values less than .1 identified at the univariate level were entered into the model. For survival analysis, the Kaplan-Meier method was used for univariate analysis, and the difference between survival curves was tested by a log-rank test. In a stepwise forward fashion, parameters with *P* values less than .1 at the univariate level were entered into the Cox regression model to analyze their relative prognostic importance. For all analyses, 2-sided tests of significance were used.

For ESCC cell line experiments, *t* test was used for the statistical analysis. Data are presented as means $\pm$ standard deviation. Each experiment was carried out independently at least twice, with 3 repeats each. The combination drug index (CDI) was used to evaluate whether the combination of drug A and drug B was synergistic or not, which was calculated according to the following formula¹⁵: CDI = survival % (drug A + drug B)/survival % (drug A) $\times$ survival % (drug B). The value of CDI less than 1 meant synergistic drug interaction.

RESULTS

Patient Characteristics

A total of 77 patients were collected in the study with a median age of 52 years (range, 37-77 years). Among them, 75 were men and 2 were women. The pretreatment analyses of the clinical tumor stage revealed T2 in 4 patients (5%), T3 in 39 patients (51%), and T4 in 34 patients (44%). Additional pretreatment analyses according to the AJCC staging system demonstrated clinical stage II tumors for 16 patients (21%) and clinical stage III for 61 patients (79%). Further analyses of histologic grades showed a grade 1 lesion in 12 patients, grade 2 in 44 patients, and grade 3 in 21 patients. Primary tumor location was found to be upper in 13 patients (17%), middle in 38 patients (49%), and lower in 26 patients (34%).

Among these 77 patients, 21 achieved pathologic complete response after preoperative chemoradiotherapy. The 3-year OS and DFS for these 77 patients were 42% and 38%, respectively. The median periods of follow-up were 73.2 months (range, 28.6-120 months) for the 24 survivors and 22.6 months (range, 3.8-120 months) for all 77 patients.

Correlation Between Clinicopathologic Parameters and Expression of Phosphorylated Mammalian Target of Rapamycin

p-mTOR immunoreactivity was absent in normal squamous cell epithelium (Figure 1, A) but was present in normal gastric glands (Figure 1, B). Among the 77 patients

FIGURE 1. Immunohistochemical staining of p-mTOR. A, p-mTOR immunoreactivity was absent in normal squamous cell epithelium (black arrows). Original magnification $\times 200$. B, p-mTOR immunoreactivity was present in normal gastric glands (black arrows). Original magnification $\times 400$. C, Representative example of negative p-mTOR expression in ESCC. Original magnification $\times 100$. D, Representative example of positive p-mTOR expression in ESCC. Original magnification $\times 100$.

collected, 33 (43%) showed “negative” for p-mTOR expression and 44 (57%) showed “positive” for p-mTOR (Figure 1, C and D). p-mTOR expression was not associated with any clinicopathologic parameters, including age, gender, primary tumor location, histologic grade, keratinizing, AJCC staging, T staging, and N staging (data not shown).

Correlation Between Clinicopathologic Parameters and Pathologic Complete Response

The relationship between the clinicopathologic parameters and the response to chemoradiotherapy is summarized in Table 1. Negative p-mTOR expression ($P = .01$) was significantly associated with pathologic complete response after chemoradiotherapy. The pathologic complete response rate was 42% in patients with negative p-mTOR expression compared with 16% in patients with positive p-mTOR expression. The logistic model showed that negative p-mTOR expression ($P = .012$; odds ratio [OR], 3.891; 95% confidence interval [CI], 1.346-11.236) independently correlated with pathologic complete response after chemoradiotherapy.

Survival Analyses

Correlations of clinicopathologic parameters and p-mTOR expression with OS and DFS are summarized in Table 2. Univariate analyses demonstrated that positive p-mTOR expression (3-year OS rate, 30% vs 57%, $P = .005$; 3-year DFS rate,

25% vs 54%, $P = .015$; Figure 2, A and B), AJCC stage III (3-year OS rate, 34% vs 68%, $P = .015$; 3-year DFS rate, 30% vs 68%, $P = .007$), T4 disease (3-year OS rate, 27% vs 54%, $P = .01$; 3-year DFS rate, 24% vs 49%, $P = .007$), and positive regional lymph nodes (3-year OS rate, 33% vs 61%, $P = .019$; 3-year DFS rate, 28% vs 61%, $P = .005$) were associated with the inferior OS and DFS. In multivariate comparison, positive p-mTOR expression (OS, $P = .001$; OR, 2.814; 95% CI, 1.553-5.097; DFS, $P = .003$; OR, 2.438; 95% CI, 1.368-4.347), T4 disease (OS, $P = .005$; OR, 2.256; 95% CI, 1.276-3.990; DFS, $P = .005$; OR, 2.227; 95% CI, 1.271-3.902), and positive regional lymph nodes (OS, $P = .015$; OR, 2.329, 95% CI, 1.180-4.598; DFS, $P = .003$; OR, 2.749; 95% CI, 1.399-5.402) represented independent adverse prognosticators for OS and DFS. The 3-year OS and DFS were 30% and 25%, respectively, in patients with positive p-mTOR expression, and 57% and 54%, respectively, in patients with negative p-mTOR expression.

Change of the Phosphorylated Mammalian Target of Rapamycin Expression After Chemoradiotherapy

Among the 77 patients collected, 56 did not achieve pathologic complete response after chemoradiotherapy. Immunohistochemical p-mTOR expression scores were compared between the biopsy samples obtained before chemoradiotherapy and the esophagectomy specimens after chemoradiotherapy. Esophagectomy specimens after

TABLE 1. Associations between pathologic complete response and clinicopathologic parameters

Parameters	Pathologic complete response		<i>P</i> value
	Absent	Present	
Age			
<52 y	25	11	.54
≥52 y	31	10	
AJCC stage			
II	9	7	.12
III	47	14	
T stage			
T2+3	28	15	.092
T4	28	6	
N stage			
N0	14	9	.127
N1+2+3	42	12	
Tumor grade			
1+2	38	18	.12
3	18	3	
Primary tumor location			
Upper/middle	38	13	.62
Lower	18	8	
Keratinizing			
Absent	11	4	1.00
Present	45	17	
p-mTOR			
<10%	19	14	.01*
≥10%	37	7	

AJCC, American Joint Committee on Cancer; p-mTOR, phosphorylated mammalian target of rapamycin. *Statistically significant. Chi-square or Fisher exact test was used for statistical analysis.

chemoradiotherapy showed significantly ($P = .004$, Figure 2, C) higher percentages of high p-mTOR scores than the biopsy samples before chemoradiotherapy.

Mammalian Target of Rapamycin Inhibitor Potentiates Growth Inhibition by Chemotherapy in Esophageal Squamous Cell Carcinoma Cell Lines

Our clinical data showed that p-mTOR expression is significantly associated with the response to chemoradiotherapy. Therefore, we tested whether the mTOR inhibitor temsirolimus can potentiate growth inhibition by chemotherapy in ESCC cell lines, CE81T/VGH and TE2. We compared the ability of the mTOR inhibitor temsirolimus to exhibit cooperative effects with cisplatin or 5-FU using doses of chemotherapy that resulted in approximately 15% to 30% growth inhibition when given alone. Temsirolimus alone displayed a growth-inhibitory effect in a dose-dependent manner (Figure E1). In addition, a combination of chemotherapy and temsirolimus showed significantly better growth inhibition than chemotherapy or temsirolimus alone (Figure 3). Synergistic effects were also observed, especially in TE2 ESCC cell lines.

TABLE 2. Results of univariate log-rank analysis of prognostic factors for overall and disease-free survivals in 77 patients with esophageal squamous cell carcinoma receiving preoperative chemoradiotherapy followed by surgery

Factors	No. of patients	OS		DFS	
		3-y OS (%)	<i>P</i> value	3-y DFS (%)	<i>P</i> value
Age					
<52 y	36	44	.51	39	.66
≥52 y	41	39		37	
p-mTOR					
<10%	33	57	.005*	54	.015*
≥10%	44	30		25	
AJCC stage					
II	16	68	.015*	68	.007*
III	61	34		30	
T classification					
T2+3	43	54	.01*	49	.007*
T4	34	27		24	
N status					
N0	23	61	.019*	61	.005*
N1+2+3	54	33		28	
Tumor grade					
1	12	58	.35	58	.34
2+3	65	38		34	
Primary tumor location					
Upper/middle	51	39	.48	35	.49
Lower	26	46		42	
Keratinizing					
Absent	15	33	.44	27	.47
Present	62	44		40	
Pathologic CR					
Absent	56	30	.009*	25	.007*
Present	21	71		71	

OS, Overall survival; DFS, disease-free survival; p-mTOR, phosphorylated mammalian target of rapamycin; AJCC, American Joint Committee on Cancer; CR, complete response. *Statistically significant.

DISCUSSION

Previous studies have revealed that mTOR activation is associated with poor prognosis in numerous human cancers.^{16,17} However, the significance of mTOR activation in chemoradiotherapy sensitivity and its effect on the prognosis of patients with ESCC treated with chemoradiotherapy remain unknown. Thus, we focused on the patients with ESCC who were treated with preoperative chemoradiotherapy and undertook the present study to determine the significance of mTOR activation in chemoradiotherapy sensitivity and patients' survival.

In the present study, a significant inverse correlation between expression of p-mTOR and the response to chemoradiotherapy was found. A total of 14 (42%) of 33 patients with negative p-mTOR expression achieved pathologic complete response after preoperative chemoradiotherapy, but only 7 (16%) of 44 patients with positive p-mTOR expression achieved pathologic complete

FIGURE 3. A and B, Temozolomide inhibits cell growth in combination with cisplatin in CE81T and TE2 ESCC cell lines. CE81T and TE2 ESCC cell lines were treated with cisplatin alone, temsirolimus alone, or a combination of cisplatin and temsirolimus. Cisplatin was given at a single concentration. C and D, Temozolomide inhibits cell growth in combination with 5-FU. CE81T and TE2 ESCC cell lines were treated with 5-FU alone, temsirolimus alone, or a combination of 5-FU and temsirolimus. 5-FU was given at a single concentration. *Significant difference in growth inhibition after combination treatment compared with chemotherapy or temsirolimus alone. Columns, mean; bars, standard deviation. 5-FU, 5-Fluorouracil; S, synergistic effect; Tem, temsirolimus.

hypoxic environment, such as an environment after chemotherapy or radiotherapy. This phenomenon may contribute to the resistance to chemotherapy and radiotherapy. Recent studies demonstrated that mTOR activation is critical for the metabolic switch to aerobic glycolysis of cancer cells.²²

The mTOR inhibitor has been approved for clinical use in the systemic treatment of advanced renal cell carcinoma.^{9,10} Several preclinical studies have also suggested that the mTOR inhibitor can enhance the efficacy of a variety of chemotherapeutic agents in breast,²³ ovarian,²⁴ and colon²⁵ cancers. However, little is known about the combination effect of the mTOR inhibitor and cytotoxic agents in ESCC. Hou and colleagues²⁶ reported that the mTOR inhibitor rapamycin significantly inhibits the growth of ESCC when combined with cisplatin. In the present study, we found that the mTOR inhibitor temsirolimus enhances the effect of cisplatin and 5-FU, which are common chemotherapeutic agents used in ESCC. These results indicate that the mTOR inhibitor can enhance the potentiation of conventional chemotherapy and serve as a new therapeutic tool for patients with advanced ESCC.

Study Limitations

Our study has important limitations. Most of the patients in this study did not have fluorine 18 fluorodeoxyglucose positron emission tomography scans during pretreatment staging or in restaging after neoadjuvant chemoradiotherapy because 18 fluorodeoxyglucose-positron emission tomography scans are not routinely supported by Taiwan's health insurance. Furthermore, our observations were also limited by relatively short follow-up periods and small patient numbers.

CONCLUSIONS

Our study showed that p-mTOR expression is independently associated with the response to chemoradiotherapy and prognosis of patients with ESCC who were treated with preoperative chemoradiotherapy. Furthermore, inhibition of mTOR can sensitize esophageal cancer cells to chemotherapy. Therefore, mTOR may be a target for therapeutic intervention in patients with ESCC who receive multimodality treatment.

The authors thank the Center for Translational Research in Biomedical Sciences, Kaohsiung Chang Gung Memorial Hospital (CLRPG871342) for providing the instruments for this study, and Sheng-Lan Wang (a pathologist from Union Pathology Center, Kaohsiung, Taiwan, ROC) for assessment of immunohistochemical staining.

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FIGURE E1. Temsirolimus alone displayed a growth-inhibitory effect in a dose-dependent manner in 2 ESCC cell lines: CE81T (A) and TE2 (B). *Significant difference in growth inhibition. Columns, mean; bars, standard deviation.