SPC18 expression is an independent prognostic indicator of patients with esophageal squamous cell carcinoma

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Short title: SPC18 expression in esophageal cancer

Key words: SPC18, SEC11A, EGFR, Esophageal cancer, TGF-α, Akt

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Abstract

Objectives: Esophageal cancer is the sixth most common malignancy worldwide. Signal peptidase complex 18 (SPC18) protein, which is encoded by the *SEC11A* gene, is one of the subunits of the signal peptidase complex and plays an important role in secretion of proteins including transforming growth factor-α (TGF-α). In the present study, we investigated the significance of SPC18 expression in human esophageal squamous cell carcinoma (ESCC). ***Methods:*** SPC18 expression was examined by immunohistochemistry. RNA interference was used to inhibit SPC18 expression in ESCC cell lines. To examine cell viability, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Effects of SPC18 inhibition on the epidermal growth factor receptor (EGFR) signaling were analyzed by western blotting. ***Results:*** In total, 46 (50%) of 92 ESCC cases were positive for SPC18. SPC18 staining was observed more frequently in stage II/III/IV cases than in stage I cases ($P = 0.028$). We found that SPC18 expression was significantly associated with increased cancer-specific mortality ($P = 0.006$, log-rank test). SPC18 expression was frequently found in EGFR-positive cases compared with EGFR-negative cases. Cell proliferation and EGFR signaling were inhibited by SPC18 knockdown. ***Conclusion:*** Specific inhibitors of SPC18 may be promising anticancer drugs for patients with ESCC.

194 words

Introduction

Esophageal cancer is the sixth most common malignancy worldwide [1]. The two predominant forms of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. Globally, ESCC accounts for more than 90% of esophageal cancer. Most ESCC is diagnosed at an advanced stage, and even superficial ESCC that appears to extend no further than the submucosa metastasizes to the lymph nodes in 50% of cases [2]. For localized ESCC, surgery is the primary therapeutic option. However, the prognosis is unsatisfactory, even in curatively resected patients, and the 5-year survival rate is <50% after surgery [3]. Several prognostic markers, such as nodal status and tumor stage, are currently accepted for clinical use, and we also previously identified several ESCC -associated genes [4–6]. However, these genes cannot completely identify which patients are at low or high risk for disease recurrence. Therefore, there is an urgent need for new prognostic markers and therapeutic targets for ESCC.

Transforming growth factor (TGF)-α is a mitogenic polypeptide that has a wide range of biological activities [7]. TGF-α activates epidermal growth factor receptor (EGFR) and stimulates multiple signaling pathways involved in cell proliferation, anti-apoptosis, and other processes [8]. Previously, we have reported that signal peptidase complex 18 (SPC18) induces TGF-α secretion in human gastric cancer cells [9]. SPC18 protein, which is encoded by the *SEC11A* gene, is one of the subunits of the signal peptidase complex. Most secretory proteins contain amino terminal or internal signal peptides that direct their sorting to the endoplasmic reticulum (ER) [10]. From the ER, proteins are transported to either the extracellular space or plasma membrane. The ER signal peptides are then cleaved by the signal peptidase complex. It has been reported that the signal peptidase complex purified from canine microsomes has five distinct subunits [11]. Two of these subunits, SPC18 and SPC21, are presumed to have catalytic activity [12], indicating that SPC18 overexpression induces

TGF-α secretion. SPC18 overexpression is also associated with tumor progression of colorectal and urinary bladder cancers [13, 14]. These data support the notion that SPC18 protein may be a potential novel marker for a wide variety of malignancies. However, expression of SPC18 has not been examined in ESCC.

In the present study, we analyzed the expression and distribution of SPC18 protein in human ESCC by immunohistochemistry and examined the relationship between SPC18 protein expression and clinicopathological characteristics. We also examined the association between EGFR and SPC18 expression, and the effect of inhibiting SPC18 expression by RNA interference (RNAi) on cell growth, invasiveness, and EGFR signaling in ESCC cells.

Materials and methods

Tissue samples

In a retrospective study design, 92 primary tumors were collected from patients diagnosed with ESCC, who underwent surgery between April 2008 and March 2013 at Hiroshima University Hospital (Hiroshima, Japan). All patient samples were obtained with informed consent. The present study was approved by the Ethics Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan). All patients underwent curative resection. Only patients without preoperative radiotherapy or chemotherapy and without clinical evidence of distant metastasis were enrolled in the study. Operative mortality was defined as death within 30 days of the patient leaving the hospital. These patients were excluded from the analysis. Postoperative follow-up was scheduled every 1, 2, or 3 months during the first 2 years after surgery and every 6 months thereafter unless more frequent follow-ups were deemed necessary. Chest X-ray, chest computed tomography scanning, and serum chemistry analyses were performed at each follow-up visit. Patients were followed by the patients' physician until the patient's death or the date of the last documented contact.

Archival formalin-fixed, paraffin-embedded tissues from 92 patients who had undergone surgical excision for ESCC were examined by immunohistochemical analysis. One or two representative tumor blocks, including the tumor center, invading front, and tumor-associated non-neoplastic mucosa, was examined from each patient by immunohistochemistry. In cases of large, late-stage tumors, two different sections were examined to include representative areas of the tumor center as well as the lateral and deep tumor invasive front. Tumor staging was determined according to the TNM classification system [15].

Immunohistochemistry

Immunohistochemical analysis was performed with a Dako Envision+ Rabbit or Mouse Peroxidase Detection Systems (Dako Cytomation, Carpinteria, CA, USA). Antigen retrieval was conducted by microwave heating in citrate buffer (pH 6.0) for 30 min. Peroxidase activity in sections was blocked by incubation with 3% H_2O_2 in methanol for 10 min, followed by incubation with normal goat serum (Dako Cytomation) for 20 min to block nonspecific antibody-binding sites. Sections were incubated with an anti-SPC18 antibody [9] or anti-EGFR antibody (1:20; Novocastra, Newcastle, UK) for 1 h at room temperature, followed by incubation with Envision+ anti-rabbit or anti-mouse peroxidases for 1 h. For color development, sections were incubated with DAB Substrate-Chromogen Solution (Dako Cytomation) for 10 min. Sections were counterstained with 0.1% hematoxylin. Negative controls were prepared by omission of the primary antibody.

Expression of SPC18 and EGFR was scored in all tumors as positive or negative. When more than 10% of tumor cells were stained, the immunostaining was considered as positive. Using this definition, two surgical pathologists (NO and KS) without knowledge of the clinical and pathological parameters or the patient outcomes independently reviewed the

immunoreactivity of each specimen. Interobserver differences were resolved by consensus review under a double-headed microscope after independent review.

Cell lines

Four cell lines derived from human esophageal cancer (TE-1, TE-5, TE-10, and TE-11) were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cell line identity was verified by short tandem repeat profiling (Promega, Madison, MD, USA). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) in a humidified atmosphere with 5% CO_2 at 37°C.

Western blot analysis

Cells were lysed as described previously [16]. The lysates (40 μg protein) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose membrane. Western blot procedures were performed as described previously [16]. An anti-SPC18 antibody was prepared as described previously [9]. Anti-Akt, anti-phosphorylated Akt (Ser473), anti-Erk, and anti-phosphorylated Erk1/2 (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). β-Actin (Sigma Chemical, St. Louis, MO, USA) was also stained as a loading control.

RNAi and cell proliferation assay

Short interfering RNA (siRNA) oligonucleotides targeting SPC18 and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). We used three independent SPC18 siRNA oligonucleotide sequences. Transfection was performed using Lipofectamine

RNAiMAX (Invitrogen) as described previously [17]. Briefly, 60 pmol siRNA and 10 μL Lipofectamine RNAiMAX were mixed in 1 mL RPMI 1640 medium (10 nmol/L final concentration). After 20 min of incubation, the mixture was added to the cells, and then the cells were plated in culture dishes. At 48 h after transfection, the cells were analyzed.

Cell proliferation was monitored after 1, 2, and 4 days by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [9]. Three independent experiments were carried out. The mean ± standard error (SE) was calculated for each experiment.

Measurement of TGF-α

The levels of TGF-α in culture media were measured by enzyme-linked immuno sorbent assay (ELISA). The TGF-α ELISA kit was purchased from R&D Systems. Forty-eight hours after siRNA transfection, cells were serum-starved for 72 hours in serum-free RPMI1640, during which time the media were changed every 24 hours. Cell culture media were collected, and cells were centrifuged at 1300 rpm for 10 min. Pellets were discarded, and the supernatant was tested for levels of TGF-α as per the manufacturer's instructions.

Statistical methods

Associations between clinicopathological parameters and SPC18 expression were analyzed by Fisher’s exact test. Kaplan-Meier survival curves were constructed for SPC18-positive and -negative patients. Differences between survival curves were tested for statistical significance by the log-rank test. Univariate and multivariate Cox regressions were used to evaluate the associations between clinical covariates and cancer-specific mortality. The hazard ratio (HR) and 95% confidence interval (CI) were estimated from Cox proportional hazard models. Differences between the two groups (SPC18 siRNA- and negative control

siRNA-transfected cells) were tested by the Student's t-test. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Expression and distribution of SPC18 protein in ESCC tissues

Immunohistochemical analysis was performed using whole paraffin-embedded blocks to analyze in detail the expression and distribution of SPC18 protein in ESCC tissues. In non-neoplastic esophageal mucosa, staining of SPC18 was either weak or absent in epithelial and stromal cells, whereas corresponding ESCC tissue showed relatively stronger, more extensive staining (**Fig. 1a**). SPC18 was detected in the cytoplasm of tumor cells (**Fig. 1b**). The percentage of SPC18-stained tumor cells ranged from 0% to 90%. When more than 10% of tumor cells were stained, the immunostaining was considered as positive for SPC18. In total, 46 (50%) of 92 ESCC cases were positive for SPC18. SPC18 staining was observed more frequently in stage II/III/IV cases than in stage I cases ($P = 0.028$, Fisher's exact test, **Table 1**). We found that SPC18 expression was significantly associated with increased cancer-specific mortality ($P = 0.006$, log-rank test, **Fig. 1d**).

Next, we analyzed expression of EGFR by immunohistochemistry. The percentage of EGFR-stained tumor cells ranged from 0% to 90%. When more than 10% of tumor cells were stained, the immunostaining was considered positive for EGFR. In total, 19 (21%) of 92 ESCC cases were positive for EGFR. SPC18 expression was frequently found in EGFR-positive cases than in EGFR-negative cases (**Table 1**). In both SPC18 and EGFR-positive cases, SPC18-stained ESCC cells were also stained for EGFR (**Fig. 1b and c**). However, a statistically significant association was not found between SPC18 and EGFR expression ($P = 0.068$, Fisher's exact test, **Table 1**).

The univariate analysis indicated that expression of SPC18 (HR, 3.85; 95% CI, 1.43-

12.12; $P = 0.006$), T classification (HR, 7.44; 95% CI, 2.41-32.57; $P = 0.001$), N classification (HR, 19.88; 95% CI, 4.05-359; $P = 0.001$), lymphatic invasion (HR, 5.04; 95% CI, 1.43-32.02; $P = 0.009$), vascular invasion (HR, 4.37; 95% CI, 1.69-12.24; $P = 0.002$), and EGFR expression (HR, 3.01; 95% CI, 1.11-7.67; $P = 0.032$) were associated with survival, while age, sex, and histological classification were not (**Table 2**). Moreover, in the multivariate model, SPC18 expression (HR, 3.17; 95% CI, 1.14-10.46; $P = 0.027$) and N classification (HR, 9.35; 95% CI, 1.45-190; $P = 0.015$) were independent prognostic indicators (**Table 2**).

3.2. Effect of SPC18 inhibition on the proliferation of ESCC cells

We examined the effect of SPC18 inhibition on cell proliferation by MTT assay. SPC18 protein expression was clearly detected in in TE-1, 5, 10, and 11 cell lines (**Fig. 2a**). Western blotting showed that the expression of SPC18 in both TE-1 and TE-5 cells was substantially suppressed by treatment with siRNA1 and siRNA3 (**Fig. 2b**, **c**). Thus, siRNA1 and siRNA3 were used for the following experiments. Cell proliferation was analyzed by MTT assays. SPC18 siRNA1- and siRNA3-transfected TE-1 cells showed significantly reduced cell proliferation relative to negative control siRNA-transfected TE-1 cells (**Fig. 2d**). We also performed an MTT assay at 4 days after siRNA transfection of TE-5 cells, and similar results were obtained (**Fig. 2e**).

Effect of SPC18 inhibition on EGFR signaling was examined because SPC18 induces TGF-α secretion [9]. It is well known that TGF-α phosphorylates EGFR that subsequently stimulates multiple signaling pathways involved in cellular proliferation, anti-apoptosis, and other cellular processes. We analyzed the phosphorylation of Akt and Erk in TE-5 cells with SPC18 inhibition. The levels of phosphorylated Akt and Erk in cells transfected with SPC18 siRNA1 and siRNA3 were lower than those in cells transfected with

negative control siRNA (**Fig. 3**).

Previously, we have reported that the levels of TGF-α in culture media from gastric cancer cells are reduced by knockdown of SPC18. Thus, effect of SPC18 inhibition on TGF-α secretion was examined by ELISA in TE-5 cells. However, TGF-α was not detected in culture media from the TE-5 cells (data not shown).

Discussion

In the present study, we analyzed the expression and distribution of SPC18 protein in human ESCC by immunostaining. In non-neoplastic esophagus, weak or no staining of SPC18 was observed in epithelial and stromal cells, whereas ESCC cells showed stronger, more extensive staining. The percentage of SPC18-stained tumor cells ranged from 0% to 90%, and 50% of ESCC cases were positive for SPC18. SPC18 staining was observed more frequently in stage II/III/IV cases than in stage I cases. These results indicate that SPC18 plays a major role in the progression of ESCC. Furthermore, multivariate analysis demonstrated that SPC18 expression is an independent prognostic indicator. In addition to clinical stage information, immunohistochemical analysis of SPC18 can facilitate prediction of patient survival.

We have reported overexpression of SPC18 in gastric, colorectal, and urinary bladder cancers [9, 13, 14]. SPC18 expression is correlated with patient survival in these cancers. SPC18 is also upregulated in ovarian cancer, and SPC18 expression is associated with tumor progression and patient survival. These results suggest that SPC18 participates in the malignant behaviors of a wide variety of human cancers. Functional analysis of SPC18 is important to understand tumor progression. In the present study, we showed that inhibition of SPC18 reduced cell proliferation. SPC18 protein is one of the subunits of the signal peptidase complex [10]. Increased activity of the signal peptidase complex caused by SPC18 protein

overexpression induces secretion of several kinds of growth factors including TGF-α [9]. However, TGF-α was not detected in culture media from the TE-5 cells. We confirmed that the levels of phosphorylated Akt and Erk, both of which are activated by EGFR, were reduced by SPC18 knockdown, suggesting that other growth factors may activate EGFR. These results suggest that SPC18 is involved in activation of EGFR signaling in ESCC cells.

Because SPC18 knockdown reduced cell proliferation in the present study, we did not analyze effect of SPC18 knockdown on invasive activity. We found that the levels of phosphorylated Akt and Erk were reduced by SPC18 knockdown. It has been reported that phosphorylated Akt and Erk increase the invasive activity of ESCC cell. TRIM44 promotes ESCC progression via the Akt pathway [18]. MicroRNA148a regulates the Erk signaling pathway and suppresses the development of ESCC by targeting MAP3K9 [19]. Taken together, these findings suggest that SPC18 activates ESCC cell invasiveness by phosphorylation of Akt and Erk.

During the last decade, immunotherapy has induced clinical responses in cancer patients [20]. The discovery of tumor antigens can improve anti-tumor T-cell responses and T-cell-based immunotherapy. In fact, it has been reported that neoantigen-based immunotherapy is effective for lung SCC [21]. Importantly, one of the signal peptidase functions is epitope processing [22]. Because expression of SPC18 is upregulated in ESCC cells, several alterations of epitope processing may occur in SPC18-positive cases. Associations between immune responses, such as lymphocytic cell infiltration, and SPC18 expression should be analyzed in the future.

In summary, we demonstrate that SPC18 expression is associated with the tumor stage and patient survival. We also found that SPC18 knockdown inhibits tumor cell proliferation and phosphorylation Akt and Erk. Specific inhibitors of SPC18 may be promising anticancer drugs for patients with basal-like BC.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (B-15H04713) and for Challenging Exploratory Research (26670175 and 16K15247) from the Japan Society for the Promotion of Science and by the Takeda Science Foundation. We thank Edanz Group (www.edanzediting.com) for editing a draft of this manuscript.

Statement of Ethics

This study was approved by the Ethics Committee for Human Genome Research of the Hiroshima University (Hiroshima, Japan). Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis.

Disclosure Statement

The authors declare no conflict of interest.

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Table 1. Relationship between SPC18 expression and clinicopathological characteristics of ESCC patients

		SPC18 expression		
		Positive	Negative	*P* value[a]
Age				
	< 65 years	19(21%)	19	1.000
	≥ 65 years	27(29%)	27	
Sex				
	Male	42(46%)	37	0.135
	Female	4(4%)	9	
T classification				
	T1a	3(3%)	11	0.020
	T1b/2/3/4	43(47%)	35	
N classification				
	N0	16(17%)	25	0.059
	N1/2/3	30(33%)	21	
Stage				
	I	11(12%)	21	0.028
	II/III/VI	35(38%)	25	
Lymphatic invasion				
	Negative	15(16%)	17	0.662
	Positive	31(34%)	29	
Vascular invasion				
	Negative	29(32%)	34	0.262
	Positive	17(18%)	12	
Histological classification				
	Well, moderately	36(39%)	32	0.342
	poorly	10(11%)	14	
EGFR expression				
	Negative	33(36%)	40	0.068
	Positiive	13(14%)	6	

[a]Chi-squared test

Table 2. Univariate and multivariate Cox regression analyses of SPC18 expression and survival of ESCC patients

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age				
≤ 65 years	1 (Ref.)	0.365		
> 65 years	0.65 (0.25-1.67)			
Sex				
Female	1 (Ref.)	0.138		
Male	3.51 (0.72-63.3)			
T classification				
T1	1 (Ref.)	0.002	1 (Ref.)	0.331
T2/3/4	7.44 (2.41-32.54)		2.07 (0.50-11.42)	
N classification				
N0	1 (Ref.)	0.001	1 (Ref.)	0.015
N1/2/3	19.88 (4.05-99.21)		9.35 (1.45-190.84)	
Lymphatic invasion				
Negative	1 (Ref.)	0.009	1 (Ref.)	0.706
Positive	5.04 (1.43-32.02)		1.41 (0.25-11.23)	
Vascular invasion				
Negative	1 (Ref.)		1 (Ref.)	0.592
Positive	4.37 (1.69-12.27)	0.002	1.39 (0.42-5.34)	
Histological classification				
Well, moderately	1 (Ref.)			
poorly	0.83 (0.28-2.23)	0.733		

EGFR expression				
Negative	1 (Ref.)		1 (Ref.)	
Positive	3.01 (1.11-7.67)	0.031	2.65 (0.91-7.36)	0.072
SPC18 expression				
Negative	1 (Ref.)		1 (Ref.)	
Positive	3.85 (1.43-12.13)	0.006	3.17 (1.14-10.46)	0.027

HR: hazard ratio; CI: confidence interval

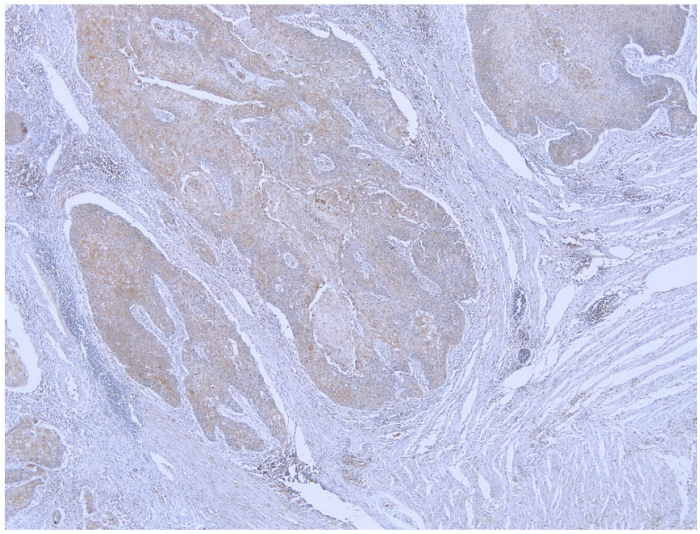
Figure Legends

Fig. 1. Expression of SPC18 in ESCC. **a** Immunostaining of SPC18 in ESCC. Original magnification, ×10. **b** Immunostaining of SPC18 in ESCC. Original magnification, ×400. **c** Immunostaining of EGFR in the same ESCC case shown in panel b. Original magnification, ×400. **d** Kaplan-Meier plot of ESCC patient survival according to SPC18 expression.

Fig. 2. Effect of SPC18 inhibition on ESCC cell lines. **a** Western blot analysis of SPC18 in esophageal cancer cell lines. **b** Western blot analysis of SPC18 in TE-1 cells transfected with negative control siRNA or SPC18 siRNAs. **c** Western blot analysis of SPC18 in TE-5 cells transfected with negative control siRNA or SPC18 siRNAs. **d** Effect of SPC18 knockdown on TE-1 cell proliferation. Cell proliferation was assessed by MTT assays at days 1, 2, and 4 after seeding in 96-well plates. **e** Effect of SPC18 knockdown on TE-5 cell proliferation. Cell proliferation was assessed by MTT assays at days 1, 2, and 4 after seeding in 96-well plates.

Fig. 3. Western blot analysis of SPC18, Akt, phosphorylated Akt (pAkt), Erk1/2, and phosphorylated Erk1/2 (pErk1/2) in lysates of TE-5 cells transfected with the negative control siRNA or SPC18 siRNAs.

Fig. 1



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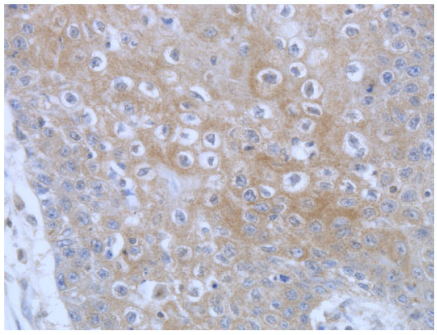

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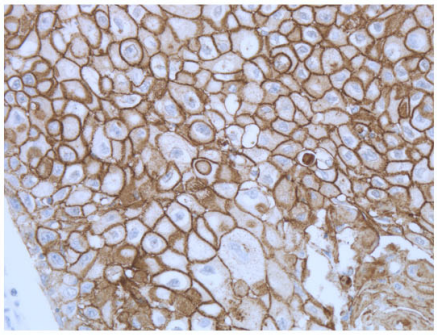

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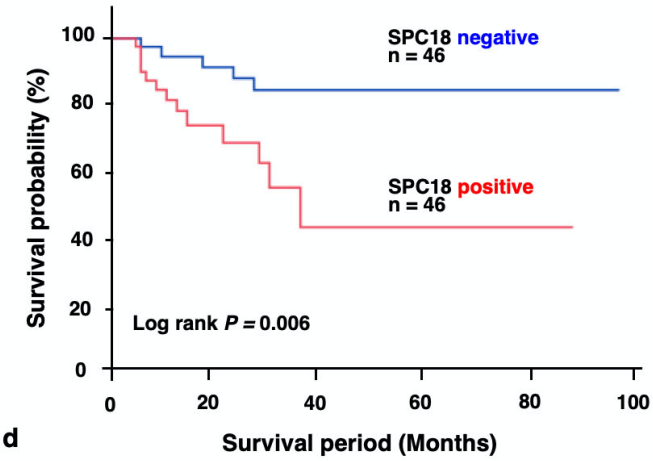
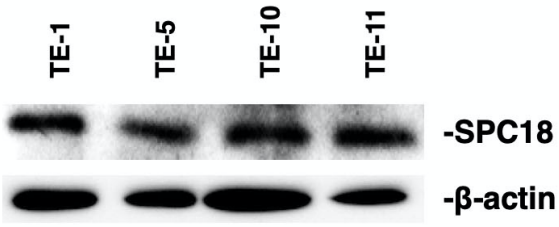
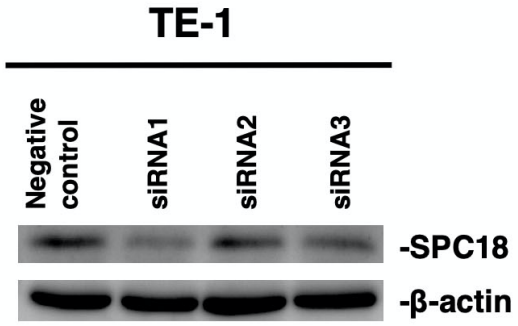

100
80
60
40
20
0
Survival probability (%)
SPC18 negative
n = 46
SPC18 positive
n = 46
Log rank P = 0.006
0
20
40
60
80
100
Survival period (Months)

d

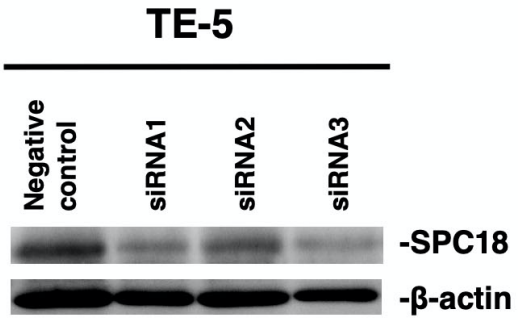
Fig. 2


TE-1
TE-5
TE-10
TE-11
-SPC18
-β-actin

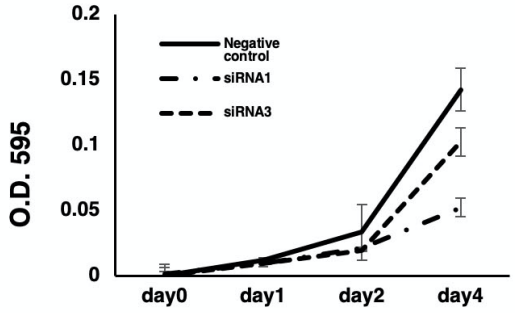
a


TE-1
Negative control
siRNA1
siRNA2
siRNA3
-SPC18
-β-actin

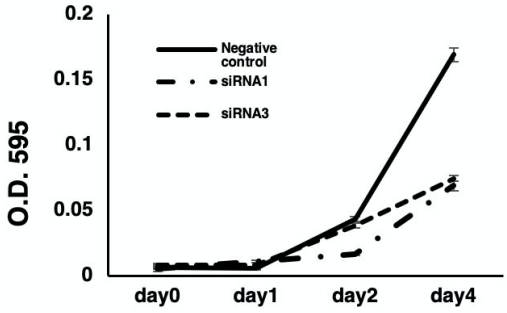
b


TE-5
Negative control
siRNA1
siRNA2
siRNA3
-SPC18
-β-actin

c

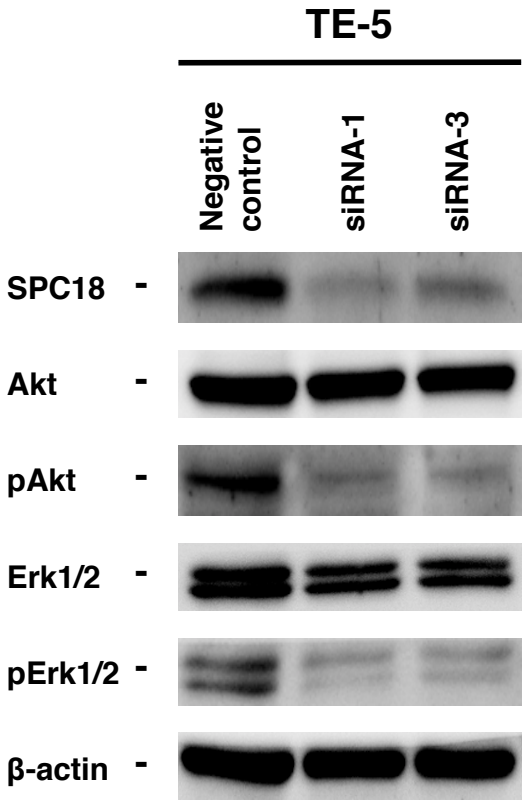

O.D. 595
0.2
0.15
0.1
0.05
0
Negative control
siRNA1
siRNA3
day0
day1
day2
day4

d


O.D. 595
0.2
0.15
0.1
0.05
0
Negative control
siRNA1
siRNA3
day0
day1
day2
day4

e

Fig. 3


TE-5
Negative control
siRNA-1
siRNA-3
SPC18
Akt
pAkt
Erk1/2
pErk1/2
β-actin