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High Expression of SGTA in Esophageal Squamous Cell Carcinoma Correlates With Proliferation and Poor Prognosis

Xiaojing Yang,[1,2] Lei Cheng,[3] Mei Li,[2] Hui Shi,[4] Hanru Ren,[4] Zongmei Ding,[1] Fang Liu,[1] Yuchan Wang,[1*] and Chun Cheng[1*]

[1]*Department of Immunology, Medical College, Nantong University, Nantong, Jiangsu 226001, People's Republic of China*

[2]*Department of Oncology, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, People's Republic of China*

[3]*Department of Laparoscopic Surgery, The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011, People's Republic of China*

[4]*Department of Pathology, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, People's Republic of China*

ABSTRACT

Receptor tyrosine kinases (RTKs) expression and the growth factor such as platelet-derived growth factor (PDGF) and their receptors have been considered relevant in the process of angiogenesis and dissemination in esophageal squamous cell carcinoma (ESCC). Small glutamine-rich tetratricopeptide repeat-containing protein alpha (SGTA) downstream of RTK signaling was a critical regulator of PDGF receptors (PDGFR) stability. The aim of the present study was to examine the expression of SGTA and to elucidate its clinicopathologic significance in ESCC. Immunohistochemistry and western blot analysis were performed for SGTA in ESCC samples. SGTA was up-regulated in ESCC as compared with the adjacent normal tissue. High expression of SGTA was associated with tumor grade ($P < 0.01$), and SGTA was positively correlated with proliferation marker Ki-67 ($P < 0.05$). Univariate analysis showed that SGTA expression did has a remarkable prediction for poor prognosis ($P = 0.016$). Knockdown or overexpression of SGTA affected ESCC cells proliferation and cell cycle. Additionally, after ESCC cells silenced for SGTA were treated with cisplatin (an anti-ESCC agent), the cell growth was down-regulated. These findings suggested that SGTA was involved in the pathogenesis of ESCC and might indicate a poor prognosis for ESCC patients. J. Cell. Biochem. 115: 141–150, 2014.

KEY WORDS: ESOPHAGEAL SQUAMOUS CELL CARCINOMA (ESCC); SGTA; PROLIFERATION; PROGNOSIS

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies throughout the world [Kuwano et al., 2005]. ESCC distributes a general poor prognosis due to lack of a singular effective clinical method for early diagnosis. The overall 5-year survival rate for ESCC is approximately 15% [Polednak, 2003]. The etiology of ESCC is a complex process that involves cumulative mutations in multiple genes, but its exact pathogenesis is still unclear. Thus, the identification of effective therapeutic, diagnostic, and/or prognostic marker genes for ESCC is a critically imminent issue.

Genetic alterations of receptor tyrosine kinases (RTKs) in ESCC were first reported in 2002 [Miyazaki et al., 2003]. Gockel et al. [2008] found that a high rate of RTKs expression in ESCC in 2008. At the same time, the growth factor such as platelet-derived growth factor (PDGF) and their receptors have been considered relevant in the

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*Correspondence to: Yuchan Wang and Chun Cheng, Department of Immunology, Medical College, Nantong University, 19 Qi-Xiu Road, Nantong, Jiangsu 226001, People's Republic of China. E-mail: ychan.wang@163.com (Y.W.), cheng_chun@yahoo.com.cn (C.C.)

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process of angiogenesis and dissemination in ESCC. As part of the tyrosine kinase family, PDGF receptors (PDGFR) are involved in multiple tumor-associated processes, such as enhancing tumor angiogenesis by the recruitment and regulation of tumor fibroblasts and pericytes [Jain and Booth, 2003]. It has been recently reported that protein chaperone small glutamine-rich tetratricopeptide repeat (TPR)-containing protein alpha (SGTA) downstream of RTK signaling was a critical regulator of PDGFR stability and SGTA might participate in a regulatory loop acting to enhance cancer cell sensitivity to chaperone inhibitors [Moritz et al., 2010]. Human small glutamine-rich TPR-containing protein (SGT) regulates the cell cycle, protein folding, transcription, protein transport, ubiquitin-proteasomes, hormone receptor signaling, and several other pathways [D′Andrea and Regan, 2003]. It was demonstrated that the presence of human SGT protein in a panel of human cell lines and throughout the cell cycle [Winnefeld et al., 2004]. These findings arose the question that whether deregulation of SGTA contributes to ESCC development and progression?

Therefore, in this study, we aimed to investigate SGTA expression in ESCC and to explore the relationship of SGTA expression with ESCC prognosis. We compared the SGTA expression in ESCC tissues and the adjacent normal tissues using immunohistochemical and western blot methods. We also investigated its associations with clinical and pathologic factors, as well as the prognostic implications. Our study first reported that SGTA affected the proliferation of human ESCC cells and it might be of great value for experimental therapies in ESCC.

MATERIALS AND METHODS

PATIENTS AND TISSUE SAMPLES

The tissue specimens examined in our study were removed from 10 cases of non-tumor esophageal tissues and 110 patients with thoracic ESCC who had undergone surgery at the Affiliated Hospital of Nantong University between 2007 and 2011. Written informed consent to participate in the study was obtained from each patient before surgery, according to the ethical guidelines of Nantong University. All patients underwent potentially curative surgery without preoperative therapy. The tumor stage was classified according to the 5th edition of the TNM classification of the International Union against Cancer (UICC). The mean post-operative follow-up period for the 110 patients was 31.6 months (range: 7.5–48.7 months).

IMMUNOHISTOCHEMICAL (IHC) STAINING AND EVALUATION

The procedures were carried out similarly to previously described methods [Li et al., 2013]. The sections were incubated with anti-SGTA (diluted 1:100; Santa Cruz Biotechnology), and anti-Ki-67 (diluted 1:100; Santa Cruz Biotechnology). Stained sections were observed under a microscope.

Two observers (X.J.Y. and Y.C.W.) independently evaluated the immunostaining results, similar results were obtained in these samples. For assessment of SGTA and Ki-67, five high power fields in each specimen were selected randomly, and cytoplasma (nuclear) staining was examined under high power magnification. More than 500 cells were counted to determine the labeling index, which represented the percentage of immunostained cells relative to the total number of cells. Scores representing the percentage of tumor cells stained positive were as follows: 0, <5% positive tumor cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Staining intensity was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown) and 3 (strong staining = brown). A final score was calculated by multiplying the scores for percentage and intensity, resulting in scores of 0, 1, 2, 3, 4, 6, 9, or 12. For statistical analysis, 0–4 were counted as low expression, while 6, 9, and 12 were counted as overexpression [Yu et al., 2009].

CELL CULTURE AND CELL LYSIS

Human ESCC cell lines ECA109 and TE1 were purchased from China Academy of Science cell library. Cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin, within a humidified atmosphere containing 5% CO_2 at 37°C.

Cells were lysed in ice-cold RIPA lysis buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate). Directly before addition of the lysis buffer, complete protease inhibitors (Roche, Switzerland) and 1 mM PMSF were added. Following the clearing of lysates by centrifugation for 10 min at 4°C, the lysates were adjusted to the same volume and concentration of total protein as determined by a Non-Interfering Total Protein assay (G Biosciences). Lysates were incubated for 15 min at 90°C in SDS–PAGE loading buffer supplemented with β-mercaptoethanol. Samples were stored at −20°C.

WESTERN BLOT ANALYSIS

Western blot was performed according to methods as described previously [Zhang et al., 2013], using primary antibodies including SGTA (1:1,000), Myc (1:1,000), CDK2 (1:1,000), Cyclin A (1:1,000), PCNA (1:1,000) and Ki-67 (1:1,000, all the above antibodies from Santa Cruz Biotechnology), GAPDH (1:1,000, Sigma Chemicals). ImageJ (NIH) was used to compare the density of bands on western blot. Mean densitometry data from independent experiments were normalized by GAPDH. The data were presented as the mean ± SD.

FLOW CYTOMETRIC ANALYSIS

For cell cycle analysis, cells were fixed in 70% ethanol for 1 h at 4°C and then incubated with 1 mg/ml RNase A for 30 min at 37°C. Subsequently, cells were stained with propidium iodide (50 μg/ml PI) (Becton–Dickinson) in phosphate-buffered saline (PBS), 0.5% Tween-20, and analyzed using a Becton–Dickinson flow cytometer BD FACScan (Becton–Dickinson).

CELL PROLIFERATION ASSAY

After treatment according to the protocols, cells were seeded at 3×10^4/well in 100 μl medium in 96-well plates and incubated overnight to allow cell adherence. Cells were then exposed to various concentrations of cisplatin for 48 h, Counting Kit-8 reagents (Dojindo, Japan) was added to each well, and the culture plate was incubated at 37°C for 1 h. Absorbance was measured at 490 nm.

COLONY FORMATION ASSAYS

Cells were plated on 60 mm plates (0.5×10^3 cells per plate) and cultured for 10 days. The colonies were stained with 1% crystal violet for 30 s after fixation with 10% formaldehyde for 5 min.

PLASMID CONSTRUCTION AND TRANSFECTION

Full-length cDNA of SGTA was generated through PCR with 0.1 μg of human fetal liver cDNA library [Zhang et al., 2002] as the template and the primers used were primer 1 (sense) (5′-GATGAATTCATGGACAACAAGAAGCGCC-3′) and primer 2 (anti-sense) (5′-GATCTCGAGTCACTCCTGCT GGTCGTC-3′), the PCR product was inserted in-frame into pcDNA3.0 vector via *Eco*RI and *Xho*I restriction sites. The cDNA of SGTA without the stop code was cloned to pcDNA3.1-myc-His-A–vector (Invitrogen) via *Eco*RI and *Bam*HI restriction sites. The shRNA sequences targeting the SGTA (5′-GCAGAACCCAGAGTTGATA-3′) was ligated into the pSilencer4.1-CMV plasmid (Invitrogen). Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, IL). The association between Ki-67 and SGTA expression and clinicopathological features was analyzed using χ^2 test. Ki-67 and SGTA expression in human ESCC was studied using the Spearman rank correlation test because the data were not normally distributed. Survival curves were calculated using the Kaplan–Meier method, and the log-rank test was used for analysis. Multivariate analysis was performed using Cox's proportional hazards model. Error bar was performed using Spearman rank correlation test. All other data were analyzed with Student's *t*-test. The results are expressed as the mean ± SE. $P < 0.05$ was considered statistically significant.

RESULTS

SGTA AND Ki-67 PROTEIN EXPRESSION AND CORRELATION WITH CLINICOPATHOLOGICAL PARAMETERS IN ESCC

The results of SGTA expression in ESCC were presented in Figure 1 and summarized in Tables I and II. SGTA was expressed mainly in the cytoplasm of ESCC. The positive ratio of SGTA and Ki-67 expression were both increased from TNM I to III (Fig. 1), showing a statistical significance ($P < 0.001$). In addition, we evaluated the association of SGTA expression with clinicopathologic variables. SGTA over-expression was significantly associated with tumor grade ($P < 0.01$), but it is not associated with patients' age ($P = 0.438$), gender ($P = 0.381$) and lymph node metastasis ($P = 0.461$; Table II). As continuous variables, SGTA expression was positively associated with Ki-67 expression (Spearman's r = 0.836, $P < 0.05$) in all cases of ESCC analyzed (Fig. 2C).

To confirm the specificity of the IHC results, we characterized SGTA, PCNA, as well as GAPDH as a loading control, in eight ESCC tissues and adjacent normal tissues for which freshly frozen materials were available. As shown in Figure 2A and 2B, an immunoreactive band of SGTA was seen in all eight cases of ESCC and SGTA expression was significantly higher in tumors than in adjacent

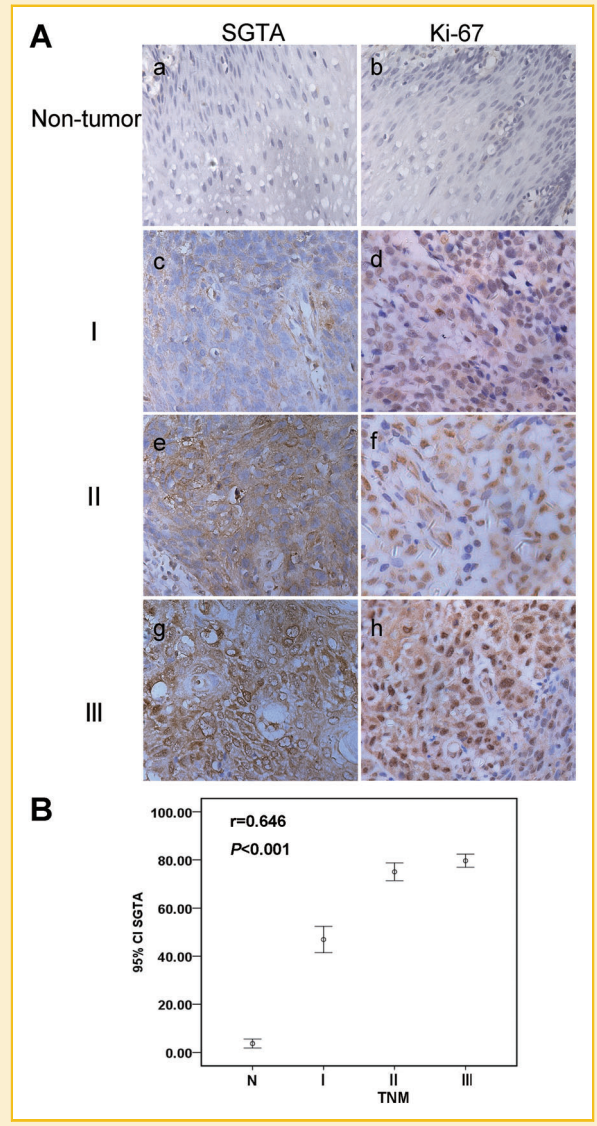

Fig. 1. Immunohistochemical analysis of SGTA and Ki-67 expression in different stages of ESCC and non-tumor esophageal tissues. A: (a and b) showed there were no SGTA and Ki-67 staining in non-tumor tissues of esophagus. (c and d) showed SGTA and Ki-67 staining in ESCC with TNM stage I, respectively. (e and f) showed SGTA and Ki-67 staining in ESCC with TNM stage II, respectively. (g and h) showed SGTA and Ki-67 staining in ESCC with TNM stage III, respectively (400× magnification). B: The error bar for the immunohistochemical analysis, as measured using the Spearman rank correlation test (r = 0.646, $P < 0.001$).

TABLE I. SGTA Expression in Non-Tumor and Tumor Tissues of ESCC Specimens

		SGTA		
Type	Total	Score ≤4, n (%)	Score ≥6, n (%)	*P*-value
Non-tumor	10	10 (100)	0 (0)	0.000*
Tumor	110	21 (19.1)	89 (80.9)	

Statistical analyses were performed by Pearson χ^2 test.
*$P < 0.05$ was considered significant.

TABLE II. SGTA Expression and Clinicopathological Parameters in 110 ESCC Specimens

Parameters	Total	SGTA Score ≤4, n (%)	SGTA Score ≥6, n (%)	*P*-value
Age (years)				
<60	43	9 (20.9)	34 (79.1)	0.438
≥60	67	12 (17.9)	55 (82.1)	
Gender				
Famale	32	5 (15.6)	27 (84.4)	0.381
Male	78	16 (20.5)	62 (79.5)	
Tumor grade				
I	20	18 (90.0)	2 (10.0)	0.000*
II	51	3 (5.9)	48 (94.1)	
III	39	0 (0.0)	39 (100.0)	
Metastasis				
Presence	80	16 (20.0)	64 (80.0)	0.461
Absence	30	5 (16.7)	25 (83.3)	
Tumor size (cm)				
<5	84	15 (17.9)	69 (82.1)	0.369
≥5	26	6 (23.1)	20 (76.9)	
Tumor invasion (T)				
T1	11	2 (18.2)	9 (81.8)	0.654
T2	21	5 (23.8)	16 (76.2)	
T3	27	3 (11.1)	24 (88.9)	
T4	51	11 (21.6)	40 (78.4)	

Statistical analyses were performed by Pearson χ^2 test.
*$P < 0.05$ was considered significant.

normal tissues. The expression was coincided with PCNA (Fig. 2A). As expected, there was a strong expression of SGTA in the ESCC tissues.

PROGNOSTIC SIGNIFICANCE OF SGTA EXPRESSION

Survival analysis was restricted to 110 patients with follow-up data by IHC. Only 43 of 89 (48.3%) patients in the group of high expression of SGTA were alive versus 17 of 21 (81.0%) in the group of low expression of SGTA (Table III). When all variables were compared separately with survival status, SGTA ($P = 0.007$), Ki-67 ($P = 0.019$), and tumor grade ($P = 0.001$) significantly influenced survival (Table III). In univariate analysis, the Kaplan–Meier survival curves of high SGTA expression showed a poor survival with statistical significance (Fig. 3). Multivariate analysis using the Cox′s proportional hazards regression model proved that SGTA expression and tumor grade were independent prognostic indicators of overall survival in ESCC patients (Table IV).

THE EXPRESSION OF SGTA IN PROLIFERATING ESCC CELLS

The results of our findings in human ESCC suggested that high SGTA expression might correlate with oncogenesis. We detected the expression of SGTA in ESCC cell lines ECA109 and TE1 by western blot (Fig. 4A). To examine SGTA during cell cycle progression, we analyzed the cell cycle after serum starvation and refeeding with serum. Both ECA109 and TE1 cells were arrested in the G1 phase by serum deprivation for 48 and 72 h, and the G1 phase increased from 55.67% to 78.94% and 53.51% to 74.07%, respectively (Fig. 4B, C). On serum addition, the cells were released from the G1 phase and reentered the S phase. As expected, the expression of SGTA was increased as early as 4 h after serum stimulation and reached the peak at 8 h in ECA109 cells (Fig. 4D). The expression of CDK2 and Cyclin A were low in the G0 and early G1 phases and high in the S phase (Fig. 4D). Similar results were obtained in TE1 cells (Fig. 4E). These results indicated that SGTA played a central role in the regulation of cell proliferation.

THE BIOLOGIC CONSEQUENCES OF THE EXPRESSION OF SGTA IN ESCC CELL LINES

To further study the potential effects of SGTA on ESCC cells proliferation, ECA109 and TE1 cells were transfected with SGTA-shRNA or control shRNA for 48 h, and the efficiency of transfection

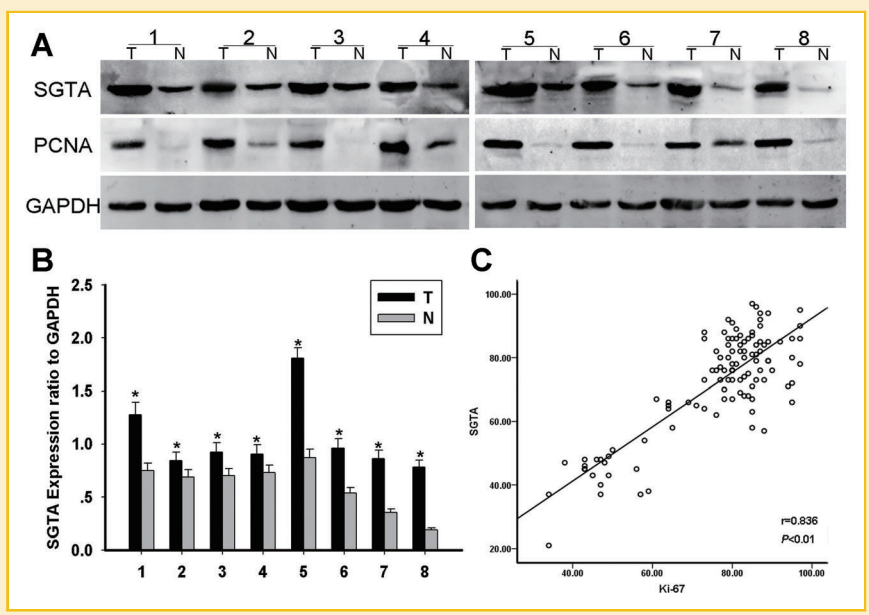

Fig. 2. Expression of SGTA and PCNA in human ESCC. A: Expression of SGTA and PCNA in eight representative paired samples of ESCC (T) and adjacent normal tissues (N). The same experiment was repeated at least three times. B: The bar chart demonstrates the ratio of SGTA protein to GAPDH for the above by densitometry. Three independent experiments were performed for each assayed variable. The data are mean ± SEM (*$P < 0.01$, compared with adjacent normal tissues). C: Relation between SGTA and Ki-67 expression in ESCC. Scatterplot of SGTA versus Ki-67 with regression line showing correlation using the Spearman coefficient ($r = 0.836$, $P < 0.01$).

TABLE III. Survival Status and Clinicopathological Parameters in 110 ESCC Specimens

		Survival status		
	Total	Dead, n (%)	Alive, n (%)	*P*-value
Age (years)				
<60	43	18 (41.9)	25 (58.1)	0.544
≥60	67	32 (47.8)	35 (52.2)	
Genger				
Male	78	37 (47.4)	41 (52.6)	0.515
Female	32	13 (40.6)	19 (59.4)	
Tumor grade				
I	20	4 (20.0)	16 (80.0)	0.001*
II	51	20 (39.2)	31 (60.8)	
III	39	26 (66.7)	13 (33.3)	
Metastasis				
Presence	80	36 (45.0)	44 (55.0)	0.876
Absence	30	14 (46.7)	16 (53.3)	
Tumor size (cm)				
<5	84	36 (42.9)	48 (57.1)	0.325
≥5	26	14 (53.8)	12 (46.2)	
Tumor invasion (T)				
T1	11	4 (36.4)	7 (63.6)	0.823
T2	21	10 (47.6)	11 (42.3)	
T3	27	11 (40.7)	16 (59.3)	
T4	51	25 (49.0)	26 (51.0)	
SGTA				
Score ≤4	21	4 (19.0)	17 (81.0)	0.007*
Score ≥6	89	46 (51.7)	43 (48.3)	
Ki-67				
Low expression	19	4 (21.1)	15 (78.9)	0.019*
High expression	91	46 (50.5)	45 (49.5)	

Statistical analyses were performed by Pearson χ^2 test.
*$P < 0.05$ was considered significant.

was detected (Fig. 5A, B). The growth rates of cells transfected with SGTA-shRNA were significantly decelerated (Fig. 5C, D), which consistently with the down-regulation of the cell proliferation marker PCNA (Fig. 5A, B). Besides, knockdown of SGTA also elicited a down-regulation of CDK2 and Cyclin A in ECA109 and TE1 cells (Fig. 5A, B).

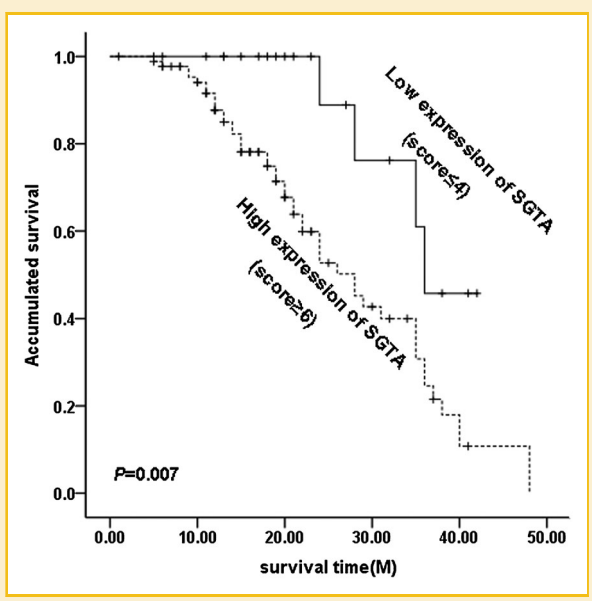

Fig. 3. Cumulative survival curves according to SGTA expression. Patients were divided into high expression of SGTA expressers (score ≥6) and low expression of SGTA expressers (score ≤4). Patients in the group of high expression of SGTA had significantly shorter overall survival.

Previous research showed that SGTA had different expression level in different phase of cell cycle. This led us to evaluate the effects of SGTA on cell cycle progression. This phenomenon was further confirmed by a colony formation assay (Fig. 5E, F). In consistent with above-mentioned results, the biological function of SGTA on proliferation was further confirmed in TE1 and ECA109 cells. FACS analysis of cell cycle distribution revealed an accumulation of cells in the G0/G1 phase, with a concomitant reduction in the number of cells in S phase compared with control shRNA (Fig. 5G, H), suggesting that SGTA may promote G0/G1-S transition of cell cycle and thus the cell growth. To further address the hypothesis, a SGTA expression plasmid was transient transfected into ESCC cells which caused an increase in the expression of PCNA (Fig. 6A, B). In addition, overexpression of SGTA promoted the ESCC cells growth rate by cell proliferation assay (Fig. 6C, D). In summary, these results confirmed that the down-regulation of SGTA decreased the growth rate of ESCC cells and overexpression of SGTA increased the growth rate.

TABLE IV. Contribution of Various Potential Prognostic Factors to Survival by Cox Regression Analysis in 110 Specimens

	Relative ratio	95% Confidence interval	*P*-value
Age (years)	1.468	0.816–2.641	0.200
Gender	0.911	0.480–1.762	0.774
Tumor size	1.011	0.540–1.890	0.973
Metastasis	1.166	0.625–2.174	0.630
Tumor invasion	1.080	0.812–1.435	0.597
Tumor grade	2.169	1.428–3.396	0.000*
SGTA	3.513	2.161–9.791	0.016*

Statistical analyses were performed by Cox test.
*$P < 0.05$ was considered significant.

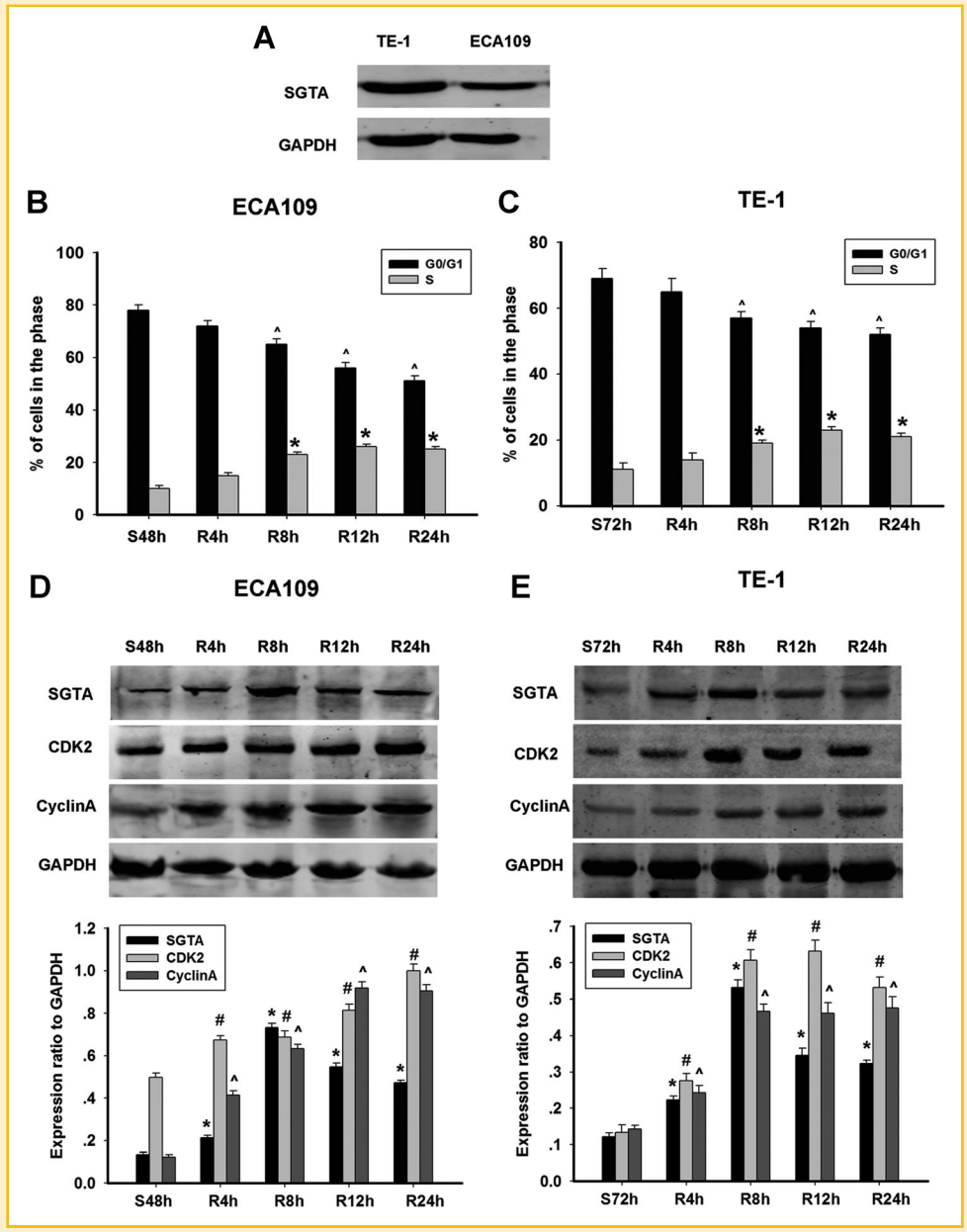

Fig. 4. The expression of SGTA in proliferating ESCC cells. A: Western blot analysis of endogenous SGTA abundance in two human ESCC cell lines: ECA109 and TE1. GAPDH was used as a loading control. The same experiment was repeated at least three times. B and C: Cells synchronized at G1 then induced into the cell cycle by serum after 0, 4, 8, 12, 24 h. Following the cell cycle progression, most of the cells were in S phase. Mean ± SD of three independent experiments. ^, * $P < 0.01$ compared with control starved of serum for 48 h (S48 h) or 72 h (S72 h), respectively. D and E: ECA109 and TE1 cells were S48 h or S72 h and on serum refeeding, cell lysates were prepared and analyzed by western blot using antibodies directed against SGTA, CDK2, Cyclin A and GAPDH (a control for protein load and integrity). The bar charts below demonstrate the ratio of SGTA, CDK2, Cyclin A protein to GAPDH for each time point by densitometry. The data are means ± SEM (n = 3, *, #, ^, $P < 0.05$, compared with control: S48 h or S72 h).

AFFECTION OF DOWN REGULATE OF SGTA ON CHEMOTHERAPY SENSITIVITY

Cisplatin is an anti-tumor agent that can inhibit DNA replication [Salles et al., 1983; Ciccarelli et al., 1985], RNA transcription [Mello et al., 1995], and induce cell cycle arrest at the G2-phase and apoptosis [Sorenson and Eastman, 1988; Sorenson et al., 1990]. It is used for the treatment of various human cancers [Masuda et al., 2001; Siddik, 2003; Feng et al., 2007]. We tested whether down expression of SGTA affected the response to cisplatin treatment in ESCC cells. Cell proliferation assay was used to study the cytotoxicity of cisplatin at various concentrations. The sequel of strengthening the cytotoxicity of cisplatin in ESCC cells by low-expressed of SGTA was examined. The cell proliferation assay results were summarized in Figure 6E, F. At concentrations higher than 10 μmol/L, the survival rates were much lower in the knockdown of SGTA cells than in mock and control cells. Proliferation index PCNA and Ki-67 were detected in these cells which were treated with cisplatin or not (Fig. 6G, H). Meanwhile, the CDK2 and Cyclin A proteins in cell cycle reduced after

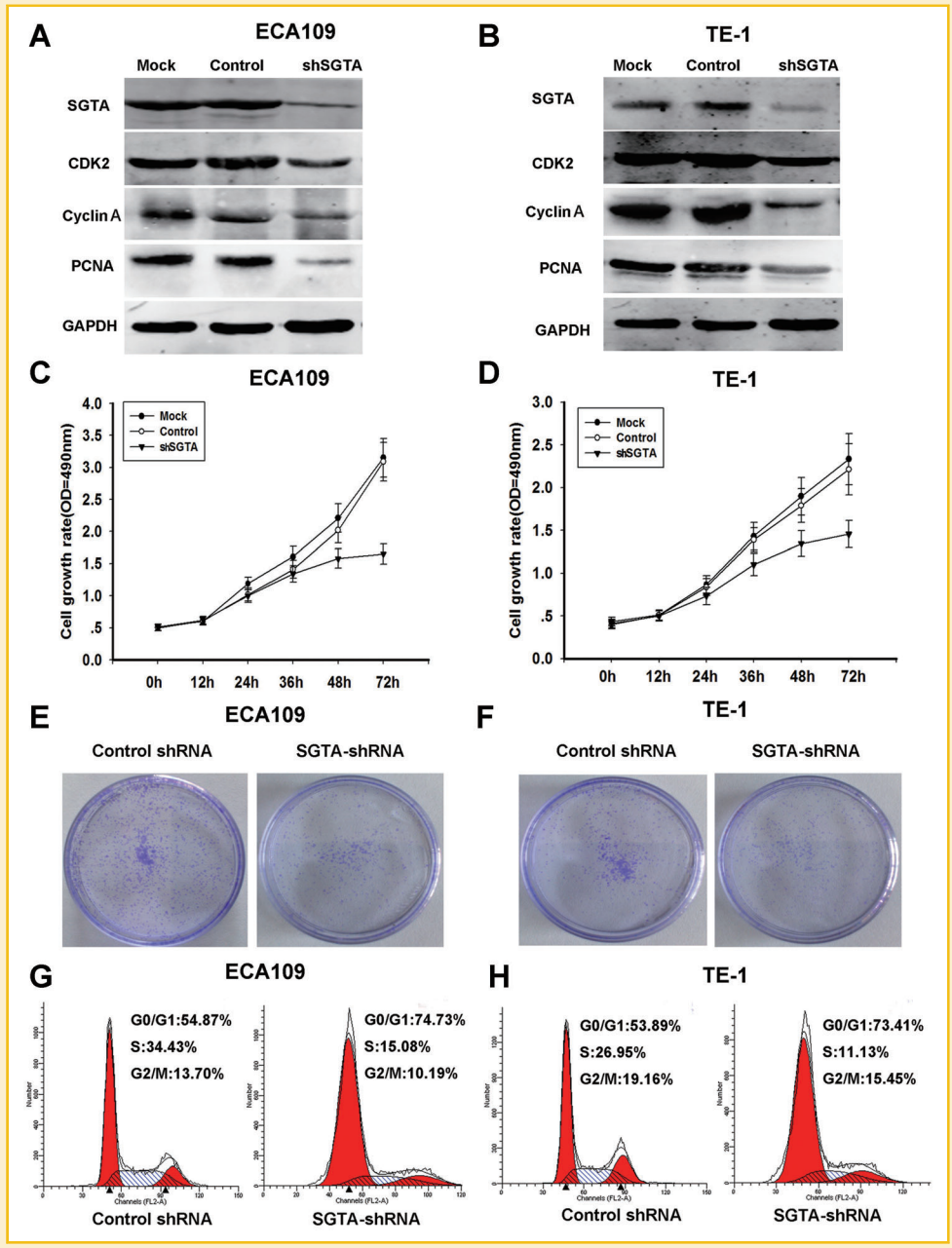

Fig. 5. Expression of SGTA affected ESCC cells proliferation. A and B: ECA109 and TE1 cells were transiently transfected with shRNA targeting either SGTA or a scrambled sequence (control shRNA) as described above for 48 h. Western blot analysis of SGTA, PCNA, CDK2, Cyclin A and GAPDH were performed. C and D: In vitro cell growth was examined by cell proliferation assay at the indicated time. The data are means ± SEM ($n = 3$, $^{*}P < 0.05$, compared with mock and control cells). E and F: Silencing endogenous SGTA inhibits cell growth as determined by colony formation assays. G and H: 48-h post-transfection, cells transfected, as described above, were stained with PI for DNA content analysis by FACS. Details of the experiments are given in "Materials and Methods Section."

interfered of SGTA or treated with cisplatin (Fig. 6G, H). In summary, SGTA was related to the proliferation of ESCC. It can be used as a favorable poor prognostic parameter for ESCC.

DISCUSSION

Diagnosis and therapy of ESCC has been studied for years, but its incidence and mortality rates have decreased very little [Zhu et al., 2011]. ESCC is generally associated with poor prognosis, so we attempt to improve the survival rates of patients with ESCC. In this study, we examined the expression of SGTA in normal and ESCC tissues and found that SGTA was frequently upregulated in human ESCC tissues. SGTA overexpression was correlated with increased tumor grade. Furthermore, SGTA expression was closely associated with poor survival in both univariate and multivariate analysis ($P < 0.01$) which revealed that SGTA expression level was an independent and significant risk factor for survival of ESCC. These

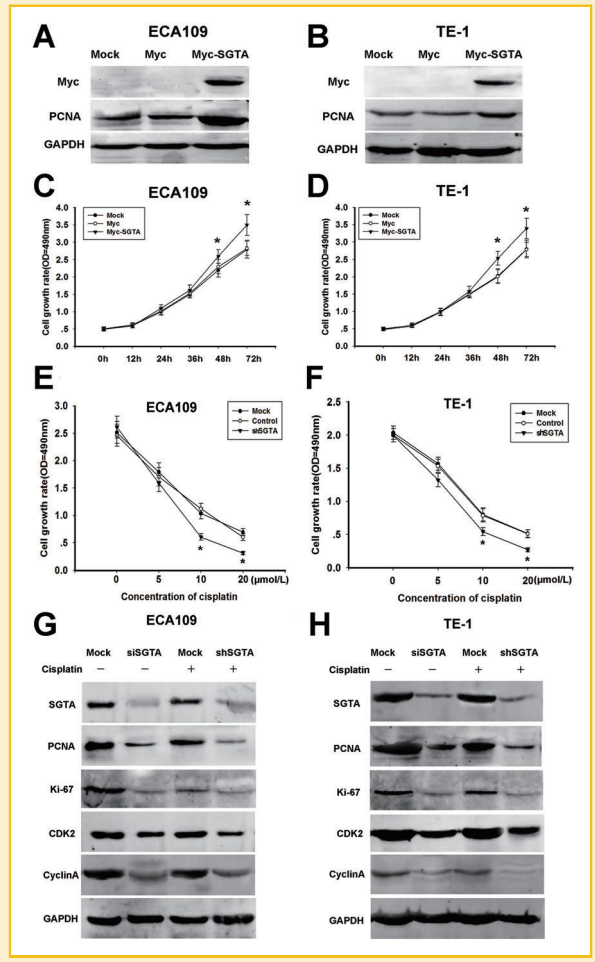

Fig. 6. The effects of SGTA on ESCC cell proliferation and the sensitiveness to chemotherapy drugs. A and B: ECA109 and TE1 cells were transiently transfected with pcDNA3.1-myc or Myc-SGTA as described above for 48 h. Western blot analysis of Myc, PCNA, and GAPDH were performed. C and D: In vitro cell growth was examined by cell proliferation assay at the indicated time. The data are means ± SEM ($n = 3$, $^{*}P < 0.05$, compared with mock and myc-transfected cells). E and F: Treat ECA109 and TE1 cells with cisplatin for 48 h at 5, 10, 20 μmol/L, respectively, after transfected of control shRNA and SGTA-shRNA for 48 h. Data represent mean ± SEM from three independent experiments. $^{*}P < 0.05$, compared with mock and control cells. G and H: Western blot analysis proliferation of ECA109 and TE1 cells when SGTA is down regulated and with or without treatment of cisplatin. Results are the mean ± SEM of three independent experiments.

clinical data suggested that SGTA contributed to the malignant progression of ESCC and might be an useful prognostic biomarker.

Current experimental and clinical evidence indicating SGTA's involvement in human tumors is limited. It has been revealed that increased expression of SGTA was found in the patients with prostate cancer, which was associated with tumor severity [Buchanan et al., 2007]. Our results revealed that SGTA was linked to prognosis of ESCC patients. The IHC results showed that SGTA was not related with invasive of ESCC, while the reasons such as a small amount of specimens should be considered. Plenty of specimens will be needed in the further research. Moritz et al. [2010] discovered that SGTA siRNA had little effect on epidermal growth factor receptor (EGFR) abundance in H1703 cell. As we all known, EGFR promotes invasion in numerous cancers [Dan et al., 2012; Jin et al., 2012; Talasila et al., 2013]. In other words, SGTA might have little relationship with invasion, and our result was consistent with it. However, further investigation is required to elucidate the precise mechanism of SGTA in cancer invasion. High SGTA expression strongly associated with tumor stage, which indicated that SGTA was associated with the prolifertion and progression of ESCC. Moreover, the relationship between SGTA expression and patient prognosis revealed that the SGTA expression in ESCC corresponded remarkably with patients' survival time. Our results suggested that the high expression of SGTA might play an important role in the development and progression of ESCC tumorigenesis, although its exact mechanisms remain need to be studied.

Further studies were planned on exploring the function of SGTA and the mechanism for its up-regulation in ESCC tumor, and to clarify whether SGTA modulated cisplatin sensitivity in ESCC. We detected the expression of SGTA during cell cycle progression in ESCC cells. Upon serum addition and releasing from G1, the SGTA content significantly increased, as well as Cyclin A and CDK2. Moreover, we attempted to investigate how exogenously expressed SGTA affected ESCC cells biologic behavior. Knockdown of SGTA decreased the protein levels of Cyclin A and CDK2, and decelerated cell cycle progression of ECA109 and TE1 cells. To see the affection of SGTA on

chemotherapy sensitivity, we investigated the effects of cisplatin on cell proliferation when the cells transfected with SGTA-shRNA or control-shRNA.

The consequences of RTKs such as PDGFRα high expression in ESCC are tumor cell proliferation, dissemination or angiogenesis [Gockel et al., 2008]. According to Moritz et al. [Moritz et al., 2010] the protein chaperone SGTA at Ser^{305} is essential for PDGFRα stabilization and cell survival in PDGFRα-dependent cancer cells. They highlighted the importance of pathways regulating RTK abundance on survival of cancer cells. At the same time, transfection of a non-small cell lung cancer cell line H1703 with SGTA shRNA resulted in decreased PDGFRα abundance and enhanced cell death. Obviously, SGTA is related to PDGFRα, and PDGFRα signaling pathway can increase cell proliferation [Liu and Zhang, 2012]. So we speculated that SGTA might play the role in cell proliferation and survival by regulating PDGFRα in ESCC. Overexpression of SGTA in tumor tissue led to its amplified function which resulted in over-proliferation of the cells. In the SGTA silenced experiment, cells could prolifer at a low speed when SGTA was interfered. The results indicated that low expression of SGTA in normal tissue could also promote cell growth, and SGTA might play a similar role in normal tissue as in tumor tissue. The discovery of SGTA as a critical regulator of PDGFRα stability could have important therapeutic implications.

In conclusion, our studies confirmed the role of SGTA in the growth of ESCC cells. We demonstrated that changes in SGTA expression might contribute to the deregulation of cell cycle and were involved in the pathogenesis of ESCC. The changes above indicated that SGTA might be a new tool for therapeutic or preventive intervention for ESCC. This may be helpful in further dissecting the debate regarding the role of SGTA as a prognostic marker in ESCC.

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REFERENCES

Buchanan G, Ricciardelli C, Harris JM, Prescott J, Yu ZC, Jia L, Butler LM, Marshall VR, Scher HI, Gerald WL, Coetzee GA, Tilley WD. 2007. Control of androgen receptor signaling in prostate cancer by the cochaperone small glutamine rich tetratricopeptide repeat containing protein alpha. Cancer Res 67:10087–10096.

Ciccarelli RB, Solomon MJ, Varshavsky A, Lippard SJ. 1985. In vivo effects of cis- and trans-diamminedichloroplatinum(II) on SV40 chromosomes: Differential repair, DNA-protein cross-linking, and inhibition of replication. Biochemistry 24:7533–7540.

D′Andrea LD, Regan L. 2003. TPR proteins: The versatile helix. Trends Biochem Sci 28:655–662.

Dan L, Jian D, Na L, Xiaozhong W. 2012. Crosstalk between EGFR and integrin affects invasion and proliferation of gastric cancer cell line, SGC7901. Onco Targets Ther 5:271–277.

Feng FY, Varambally S, Tomlins SA, Chun PY, Lopez CA, Li X, Davis MA, Chinnaiyan AM, Lawrence TS, Nyati MK. 2007. Role of epidermal growth factor receptor degradation in gemcitabine-mediated cytotoxicity. Oncogene 26:3431–3439.

Gockel I, Moehler M, Frerichs K, Drescher D, Trinh TT, Duenschede F, Borschitz T, Schimanski K, Biesterfeld S, Herzer K, Galle PR, Lang H, Junginger T, Schimanski CC. 2008. Co-expression of receptor tyrosine kinases in esophageal adenocarcinoma and squamous cell cancer. Oncol Rep 20: 845–850.

Jain RK, Booth MF. 2003. What brings pericytes to tumor vessels? J Clin Invest 112:1134–1136.

Jin W, Chen BB, Li JY, Zhu H, Huang M, Gu SM, Wang QQ, Chen JY, Yu S, Wu J, Shao ZM. 2012. TIEG1 inhibits breast cancer invasion and metastasis by inhibition of epidermal growth factor receptor (EGFR) transcription and the EGFR signaling pathway. Mol Cell Biol 32:50–63.

Kuwano H, Kato H, Miyazaki T, Fukuchi M, Masuda N, Nakajima M, Fukai Y, Sohda M, Kimura H, Faried A. 2005. Genetic alterations in esophageal cancer. Surg Today 35:7–18.

Li M, Yang X, Zhang J, Shi H, Hang Q, Huang X, Liu G, Zhu J, He S, Wang H. 2013. Effects of EHD2 interference on migration of esophageal squamous cell carcinoma. Med Oncol 30:396.

Liu T, Zhang J. 2012. [Role of PDGF-A/PDGFR-alpha in proliferation and transdifferentiation of fibroblasts from skin lesions of patients with systemic sclerosis]. Nan Fang Yi Ke Da Xue Xue Bao 32:496–501.

Masuda M, Suzui M, Weinstein IB. 2001. Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines. Clin Cancer Res 7:4220–4229.

Mello JA, Lippard SJ, Essigmann JM. 1995. DNA adducts of cis-diamminedichloroplatinum(II) and its trans isomer inhibit RNA polymerase II differentially in vivo. Biochemistry 34:14783–14791.

Miyazaki T, Kato H, Fukuchi M, Nakajima M, Kuwano H. 2003. EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. Int J Cancer 103:657–663.

Moritz A, Li Y, Guo A, Villen J, Wang Y, MacNeill J, Kornhauser J, Sprott K, Zhou J, Possemato A, Ren JM, Hornbeck P, Cantley LC, Gygi SP, Rush J, Comb MJ. 2010. Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. Sci Signal 3:ra64.

Polednak AP. 2003. Trends in survival for both histologic types of esophageal cancer in US surveillance, epidemiology and end results areas. Int J Cancer 105:98–100.

Salles B, Butour JL, Lesca C, Macquet JP. 1983. cis-Pt(NH3)2Cl2 and trans-Pt (NH3)2Cl2 inhibit DNA synthesis in cultured L1210 leukemia cells. Biochem Biophys Res Commun 112:555–563.

Siddik ZH. 2003. Cisplatin: Mode of cytotoxic action and molecular basis of resistance. Oncogene 22:7265–7279.

Sorenson CM, Barry MA, Eastman A. 1990. Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. J Natl Cancer Inst 82:749–755.

Sorenson CM, Eastman A. 1988. Influence of cis-diamminedichloroplatinum (II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. Cancer Res 48:6703–6707.

Talasila KM, Soentgerath A, Euskirchen P, Rosland GV, Wang J, Huszthy PC, Prestegarden L, Skaftnesmo KO, Sakariassen PO, Eskilsson E, Stieber D, Keunen O, Brekka N, Moen I, Nigro JM, Vintermyr OK, Lund-Johansen M, Niclou S, Mork SJ, Enger PO, Bjerkvig R, Miletic H. 2013. EGFR wild-type amplification and activation promote invasion and development of glioblastoma independent of angiogenesis. Acta Neuropathol 125:683–698.

Winnefeld M, Rommelaere J, Cziepluch C. 2004. The human small glutamine-rich TPR-containing protein is required for progress through cell division. Exp Cell Res 293:43–57.

Yu C, Chen K, Zheng H, Guo X, Jia W, Li M, Zeng M, Li J, Song L. 2009. Overexpression of astrocyte elevated gene-1 (AEG-1) is associated with

esophageal squamous cell carcinoma (ESCC) progression and pathogenesis. Carcinogenesis 30:894–901.

Zhang J, Yang X, Wang Y, Shi H, Guan C, Yao L, Huang X, Ding Z, Huang Y, Wang H, Cheng C. 2013. Low expression of cyclin H and cyclin-dependent kinase 7 can decrease the proliferation of human esophageal squamous cell carcinoma. Dig Dis Sci. 58:2028–2037.

Zhang S, Cai M, Xu S, Chen S, Chen X, Chen C, Gu J. 2002. Interaction of p58 (PITSLRE), a G2/M-specific protein kinase, with cyclin D3. J Biol Chem 277:35314–35322.

Zhu H, Wang Q, Hu C, Zhang W, Quan L, Liu M, Xu N, Xiao Z. 2011. High expression of survivin predicts poor prognosis in esophageal squamous cell carcinoma following radiotherapy. Tumour Biol 32:1147–1153.