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Original Article

Effects of Skp2 expression on the recurrence and prognosis of esophageal squamous cell carcinoma after complete macroscopic resection

Jinjun Ye[1*], Guoren Zhou[2*], Zhi Zhang[3], Lei Sun[4], Xia He[1], Jianwei Zhou[5]

[1]Department of Radiotherapy, Affiliated Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing 210009, China; [2]Department of Chemotherapy, The Affiliated Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing 210009, China; [3]Department of Thoracic Surgery, The Affiliated Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing 210009, China; [4]Department of Radiology, The Affiliated Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing 210009, China; [5]Department of Molecular Cell Biology & Toxicology, Cancer Center, School of Public Health, Nanjing Medical University, Nanjing 211166, China. [*]Equal contributors.

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Abstract: Objective: This study aimed to investigate the effects of Skp2 expression on the recurrence and prognosis of esophageal squamous cell carcinoma (ESCC) after complete macroscopic resection and relationship between Skp2 expression and clinicopathological characteristics of ESCC patients. Methods: ESCC samples were collected from 74 patients and processed for Skp2 expression detection. Postoperative clinical information and follow-up data were collected. The overall survival (OS), disease-free survival (DFS), local recurrence-free survival (LRFS), and distant metastasis-free survival (DMFS) were evaluated. Results: The positive Skp2 expression was found in 47.3% of ESCC tissues. The 3-year and 5-year survival rates of patients with negative Skp2 expression were significantly higher than in patients with positive Skp2 expression. Skp2 expression was associated with lymph node metastasis, infiltration depth, and TNM stage of ESCC ($P<0.01$) but not with the age and sex. In ESCC tissues, Skp2 was associated with tumor size, lymph node metastasis, infiltration depth, and TNM staging (mean $P<0.01$). Univariate Cox regression analysis indicated that infiltration depth, lymph node metastasis, pathological stages, and TNM stage of ESCC were correlated with Skp2 expression whereas age and sex did not correlate with the prognosis ($P>0.05$). Multivariate Cox regression analysis showed that TNM stage, infiltration depth, lymph node metastasis, and Skp2 expression were independent predictors of the survival of ESCC patients. The post-operative OS, DSF, DMSF, and LRFS in ESCC patients with high Skp2 expression were significantly worse than in patients with low Skp2 expression. Conclusion: Skp2 over-expression predicts a poor post-operative prognosis of ESCC patients.

Keywords: Esophageal squamous cell carcinoma, s-phase kinase-associated protein 2, complete macroscopic resection, immunohistochemistry

Introduction

China has a high incidence of esophageal cancer. The esophageal cancer patients in China account for more than half of total esophageal cancer patients worldwide. Moreover, esophageal cancer has been the first leading cause of cancer related death in the world [1]. In China, the thoracic esophageal squamous cell carcinoma (ESCC) accounts for more than 90% of esophageal cancer. The major clinical treatment for esophageal cancer is surgery. In China, intr-operative pathological examination is not usually performed, so, complete macroscopic resection, which means resection of the whole macroscopic tumor, is widely applied for patients with esophageal cancer. Although many patients have received effective surgical treatment, the 5-year survival rate is only 15%-24% after surgery and the post-operative recurrence rate may reach 34-79% [2]. Skp2 is the recognition subunit of the Skp2/SCF (Skp1-Cullin1-F-box) complex. It is associated with the ubiquitination and degradation of cell cycle-associated proteins such as p27 and p21. Over-expression of Skp2 may result in excessive cell proliferation. To date, it has been shown that Skp2 is over-expressed in a variety of cancers, which is associated with the abnormal proliferation of cancer cells and a poor prognosis [3].

This study aimed to investigate the influences of Skp2 on the recurrence and prognosis of esophageal cancer after complete macroscopic resection.

Materials and methods

General information

A total of 74 ESCC specimens were collected from esophageal cancer patients who received surgical intervention between January 2008 and December 2009 in the Department of Pathology, Cancer Hospital of Nanjing Medical University. These patients did not receive chemotherapy, radiotherapy and immunotherapy before surgery. All the tissues were embedded in paraffin and preserved in the Department of Pathology. All the patients had complete medical record and follow-up data and were followed up for a median of five years. There were 46 males and 28 females with the median age of 62.3 years (range: 48-76 years). The inclusion criteria were as follows: 1) Postoperative pathological examination confirmed the primary thoracic ESCC, and 2) all the patients underwent complete macroscopic resection (R0), and 3) they had the Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0-2.

Pathological staging

The tumor locations were determined according to the criteria of the 2009 American Joint Committee on Cancer (AJCC): ESCC was found at the upper thoracic segment in 14 patients, middle thoracic segment in 35 patients, and lower thoracic segment in 25 patients. The pathological stages were determined according to the international TNM classification system for esophageal cancer (7th edition; UICC, 2009 edition): stage 0-I ESCC was found in 22 patients, stage II ESCC in 26, and stage III ESCC in 26. The extent of cancer cell differentiation was also determined: well differentiated ESCC in 19 patients, moderately differentiated ESCC in 26, and poorly differentiated ESCC in 29.

Reagent

Rabbit anti-human Skp2 p45 (H435) polyclonal antibody (sc-7164) was purchased from Santa Cruz Biotechnology Inc. (USA). The secondary antibody and diaminobenzidine (DAB) reagents were from Long Island Shanghai Antibody Diagnostic Reagent Co., Ltd.

Immunohistochemistry

The cancer tissues were fixed in 4% formaldehyde, embedded in paraffin, cut into 0.4-μm sections. The Skp2 expression in the ESCC tissues was detected by immunohistochemistry according to the manufacturer's instructions with the two-step Envision system. The dilution of the working solution of the primary antibody was 1:200. In brief, tissue sections were deparaffinized and hydrated. After antigen retrieval, sections were washed with distilled water, and then treated with 3% hydrogen peroxide for 10 min to block the endogenous peroxidases. After washing with distilled water, the sections were incubated with primary antibody (1:200) for 10 min, followed by washing in TBS for 10 min. Then, the sections were incubated with Envision TM for 10 min, washed with TBS for 10 min, incubated with the chromogenic substrate DAB, mounted and observed under a light microscope. In the negative control group, the primary antibody was replaced with TBS.

Interpretation of results

The Skp2 positive cells had brown particles in the nucleus and the cytoplasm was light-stained. The extent of Skp2 expression was determined according to the method described by Naoki et al. [4]: No positive cells, (-), 1%-20% (±), 21%-80% (+) and > 80% (++). (-) and (±) were defined as negative, and (+) and (++) defined as positive expression.

Statistical analysis

The statistical analysis was performed using SPSS version 18.0. Quantitative data are presented as means ± standard deviation (X±SD). The qualitative data were compared with Pearson's chi-square test. The univariate and multivariate analyses were performed using a Cox proportional risk regression model. Survival rates were compared using the Kaplan-Meier survival analysis and log-rank test. A value of $P<0.05$ was considered statistically significant.

Results

Expression of Skp2 protein in esophageal cancer tissues

The Skp2 protein was mainly localized in the nuclei of cancer cells. The well differentiated

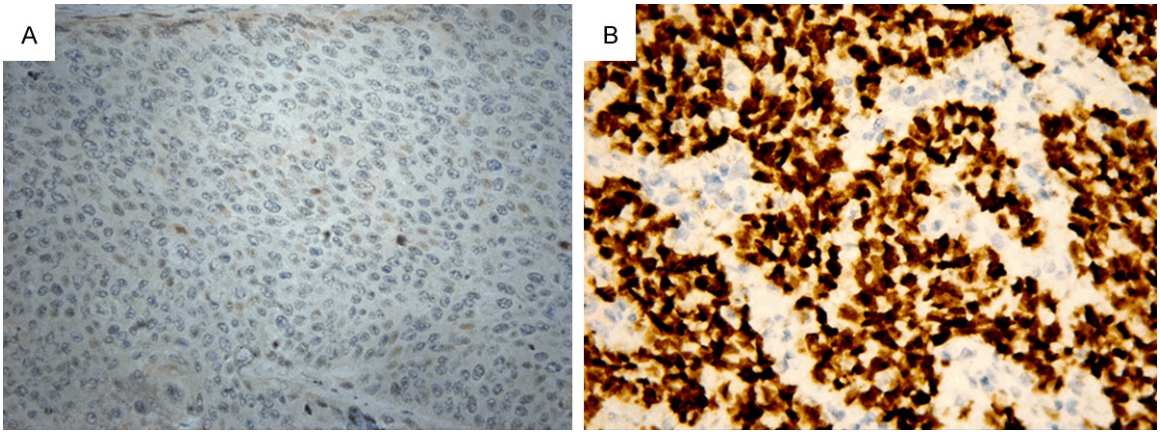

Figure 1. Skp2 expression in ESCC tissues (Immunohistochemistry; Envision method). A. Skp2 expression in well differentiated ESCC (Immunohistochemistry; *400) (+); B. Skp2 expression in poorly differentiated ESCC (Immunohistochemistry; *400) (++).

Table 1. Relationship between Skp2 expression and clinicopathological characteristics of ESCC patients

Variables	N	Skp2 expression		P
		≤10%, n (%)	>10%, n (%)	
Sex				
Male	57			0.595
Female	17			
Age (yr)				
<60	22	10 (54.4)	12 (32.6)	0.417
≥60	52	29 (55.8)	23 (44.2)	
Tumor location				
Upper	14	7 (50.0)	7 (50.0)	0.960
Middle	35	19 (54.3)	16 (45.7)	
Lower	25	13 (52.0)	12 (48.0)	
Cell differentiation				
Well differentiated	19	18 (94.7)	1 (6.3)	<0.001
Moderately differentiated	26	13 (50.0)	13 (50.0)	
Poorly differentiated	29	8 (27.6)	21 (72.4)	
Infiltration depth				
T1	21	14 (66.7)	7 (33.3)	<0.001
T2	19	11 (57.9)	8 (42.1)	
T3	34	14 (41.2)	20 (58.8)	
Lymph node metastasis				
Yes	40	30 (75.0)	10 (25.0)	<0.001
No	34	9 (26.5)	25 (73.5)	
TMN stage				
0-I	22	21 (95.2)	1 (4.8)	<0.001
II	26	14 (53.8)	12 (46.2)	
III	26	4 (15.4)	22 (84.6)	

ESCC tended to have a negative Skp2 expression, whereas the poorly differentiated ESCC tended to have a positive Skp2 expression (**Figure 1**). Immunohistochemistry for Skp2 showed that the median proportion of Skp2 positive cells was 41.24% (range: 26%-78.3%). 47.30% of ESCC tissues were positive for Skp2 expression (35/74).

Relationship between Skp2 expression and clinicopathological characteristics of ESCC patients

The relationship between Skp2 expression and clinical characteristics was further evaluated in 74 ESCC patients. Results showed that the Skp2 expression was associated with infiltration depth, lymph node metastasis, clinical TMN stage, and the degree of cell differentiation. The lower the degree of cell differentiation, the higher the proportion of Skp2 positive cells was ($P<0.001$); the deeper tumor infiltration, the higher the proportion of Skp2 positive cells was ($P<0.05$); the higher the TMN stage, the higher the proportion of Skp2 positive cells was ($P<0.001$). The proportion of Skp2 positive cells in ESCC with lymph node metastasis was higher than in that without lymph node metastasis ($P<0.01$). However, Skp2 expression was not correlated with the sex, age, and tumor location (**Table 1**). The 3-year and 5-year survival rates were 76.5 and 63.3%, respectively in patients with positive Skp2 expression, whereas 23.6

Table 2. Univariate COX regression analysis of factors influencing the prognosis of ESCC patients

Parameters	B	Risk ratio	95% confidence interval	P
Age	-.249	0.779	0.419-1.450	0.431
Sex	.248	1.281	0.661-2.482	0.463
Tumor location	-.004	0.986	0.652-1.522	0.132
Infiltration depth	0.373	1.452	1.012-2.083	<0.001
Cell differentiation	1.333	3.792	2.354-6.108	<0.001
Lymph node metastasis	1.973	7.172	3.695-13.00	<0.001
TMN stage	1.717	5.569	3.268-9.492	<0.001
Skp2 positive expression	1.829	6.225	3.237-11.970	0.001

Table 3. Multivariate Cox regression analysis of factors influencing the prognosis of ESCC patients

Parameters	B	Risk ratio	95% confidence interval		P
			Lower	Upper	
Age	0.382	1.466	0.727	2.954	0.285
Infiltration depth	-0.238	0.788	0.475	1.309	0.358
Tumor location	-0.266	0.766	0.481	1.221	0.263
Cell differentiation	0.636	1.889	1.022	3.494	0.043
Lymph node metastasis	1.263	3.534	1.609	7.765	0.002
Sex	0.044	1.045	0.510	2.144	0.904
TMN stage	1.118	3.057	1.546	6.047	0.001
Skp2 positive expression	0.867	2.380	1.143	4.953	0.020

and 8.85%, respectively, in patients with negative Skp2 expression.

Survival status of ESCC patients after operation

The overall 1-year survival rate was 91.8%, 3-year survival rate was 56.0%, and 5-year survival rate was 37.8% in 74 ESCC patients after complete macroscopic resection. The median survival time was 41.37 months. At the end of follow-up, there were 45 deaths, and 24 patients survived.

Factors affecting the prognosis of ESCC

Factors that influenced the postoperative survival were analyzed with univariate analysis. Results showed that the infiltration depth, degree of cell differentiation, lymph node metastasis, TMN stage and positive Skp2 expression were factors affecting the postoperative survival (**Table 2**). Multivariate analysis was performed using the COX regression and showed only the degree if cell differentiation, lymph node metastasis, TMN stage, and positive Skp2 expression were associated with the survival of ESCC patients (**Table 3**).

Relationship between Skp2 expression and survival of ESCC patients

The survival was analyzed using the Kaplan-Meier method. Results showed that the OS (χ^2=38.061, P<0.001), DFS (χ^2=13.867, P< 0.001), LRFS (χ^2=9.487, P=0.002), and DMFS (χ^2=26.900, P<0.001) of patients with negative Skp2 expression were all significantly higher than in patients with positive Skp2 expression (**Figure 2**).

Discussion

Surgery is still the major treatment for esophageal cancer. However, the 5-year survival rate after surgical treatment remains at a low level. The prognosis of esophageal cancer is associated with clinical characteristics such as TMN stage. Molecular pathological studies have also shown that the abnormal regulation and expression of a variety of genes and proteins such as p27 and hypoxia-inducible factor (HIF) are closely associated with the occurrence, growth, infiltration, and metastasis of esophageal cancer and significantly affect the prognosis of esophageal cancer [5]. The excess proliferation of cancer cells due to the disordered cell cycle regulation also suggests that the invasive cell subpopulation obtained a survival advantage and caused a poor survival prognosis in the tumor patients. It has been confirmed that many cell cycle-associated proteins such as p21, p27, and cyclin D1 are significantly correlated with the growth of esophageal cancer and the development, progression, and prognosis of a variety of cancers [6, 7]. Therefore, to investigate the relationship between these genes and proteins and the postoperative prognosis of esophageal cancer is helpful for understanding the cause of postoperative recurrence in high-risk population and may find therapeutic target for the improve-

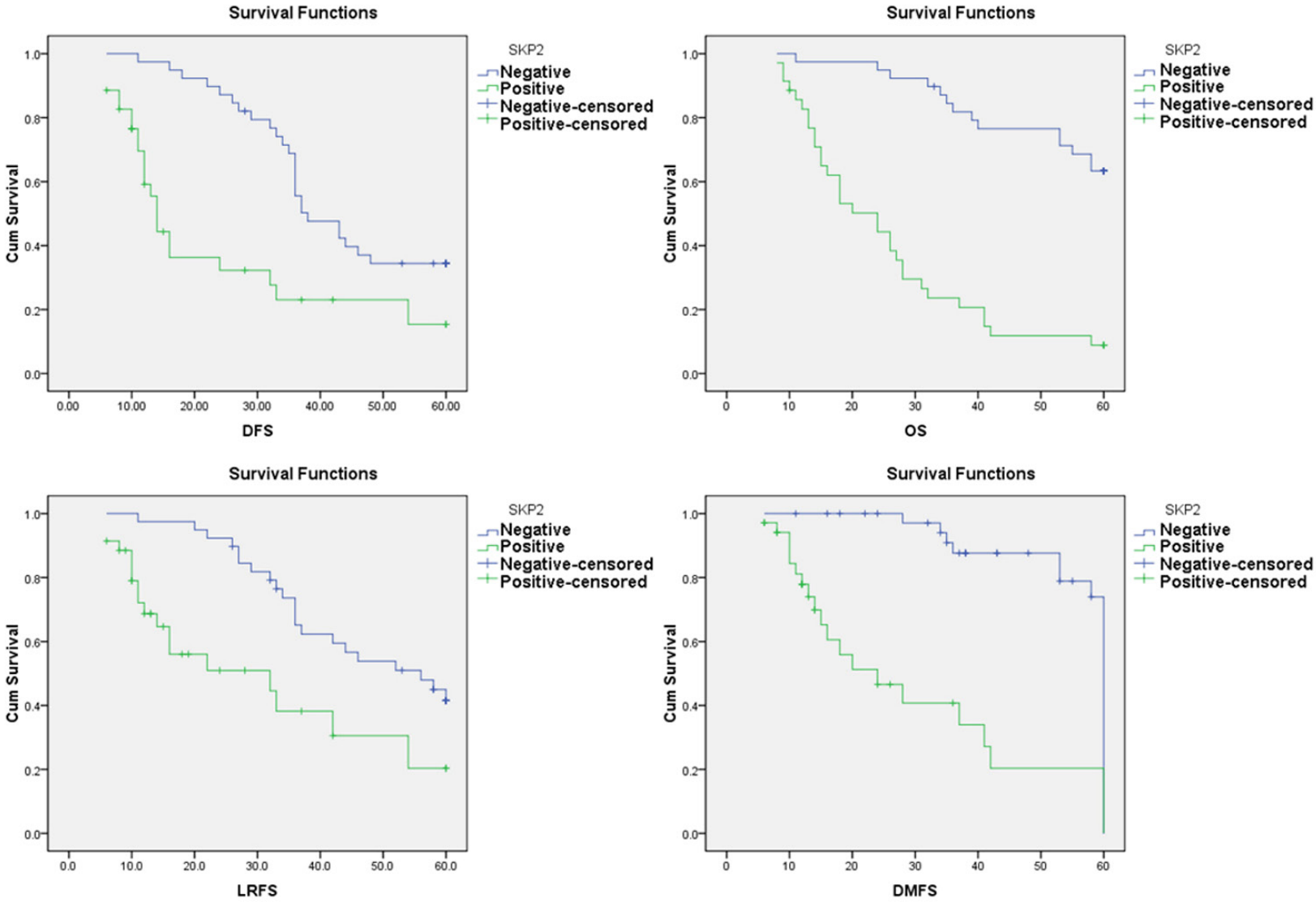

Figure 2. Relationship between Skp2 expression and postoperative OS, DFS, LRFS, and DMFS of ESCC patients.

ment of prognosis of esophageal cancer patients.

Skp2 was first identified as an S-phase Cdk2/Cyclin A-associated protein [8]. It is a member of F-box family and is the recognition subunit of the Skp2/SCF (Skp1-Cullin1-F-box) complex which is responsible for the ubiquitination of proteins to for the degradation by 26 S proteasome. Many negative cell cycle-associated proteins including p21, p57, p27, and p130 are degraded through Skp2-mediated ubiquitination and degradation [8]. Therefore, Skp2 overexpression may promote cell transition fromG1 phase to S phase. A variety of cancers including lung cancer, head and neck cancer, breast cancer, and myxofibrosarcoma [9] display Skp2 over-expression. Studies have confirmed that, in addition to promoting cell proliferation, Skp2 over-expression may be also associated with the invasive phenotypes of a variety of cancers such as cell migration, infiltration, and metastasis [10, 11]. Recent studies indicate that Skp2 is associated with the sensitivity of cancer cells to chemotherapy and radiotherapy [12] and with Akt ubiquitination and degradation, aerobic glycolysis, and cancer development induced by the Erb family. Studies have shown that Skp2 expression is extremely high in a variety of cancers, which is associated with the degree of malignancy and the poor prognosis of cancers [13]. Therefore, Skp2 has been found as an independent factor predicting the poor prognosis of a variety of cancers [14].

In this study, the relationship between Skp2 expression and postoperative prognosis of ESCC was evaluated in 74 patients. Results showed that the overall 1-year, 3-year and 5-year survival rate was 85%, 55%, and 38%, respectively, in these patients. Patients were followed up for a median of 41.37 months, and 45 deaths were found at the end of the follow-up. In addition, Skp2 expression was significantly correlated with the postoperative survival of ESCC patients. The 3-year and 5-year survival rate of patients with negative Skp2 expression was significantly higher than in those with positive Skp2 expression. Skp2 expression was also significantly correlated with tumor size, pathological type, TMN stage, infiltration depth, and lymph node metastasis of ESCC. Recently, it has been shown that Skp2 is associated with the pathological type, infiltration depth, and lymph node metastasis in a variety of cancers. Our results indicated that Skp2 expression was associated with lymph node metastasis in ESCC patients, suggesting that it is associated with the malignant behaviors of esophageal cancer.

Log-rank test was employed to evaluate the relationship of Skp2 expression with OS, DSF, LRSF, and DMFS of ESCC patients. Results showed Skp2 expression was associated with the OS, DSF, LRSF, and DMFS of ESCC patients after complete macroscopic resection. Patients with positive Skp2 expression had significantly lower OS, DSF, LRSF, and DMFS than those with negative expression, suggesting that Skp2 plays an important role in the development and progression of ESCC. This might be ascribed to the Skp2 induced cancer cell proliferation and invasion. Further COX regression analysis revealed that Skp2 was an important factor affecting the overall survival of ESCC patients.

Univariate analysis showed that the degree of cell differentiation, TMN stage, infiltration depth, lymph node metastasis, and Skp2 expression were factors affecting the postoperative survival of ESCC patients. Multivariate analysis indicated that the degree of cell differentiation, lymph node metastasis, TMN stage, and positive Skp2 expression were independent prognotic factors in ESCC patients. Our results were consistent with findings reported by Liang et al. [15]. Therefore, we speculate that Skp2 may be an important factor predicting a poor postoperative prognosis of ESCC after complete macroscopic resection and may serve as an important indicator for the postoperative evaluation of prognosis of ESCC patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Xia He, Department of Radiotherapy, Affiliated Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing 210009, China. E-mail: hexia200302@tom.com; Jianwei Zhou,

Department of Molecular Cell Biology & Toxicology, Cancer Center, School of Public Health, Nanjing Medical University, Nanjing 211166, China. E-mail: jwzhou@njmu.edu.cn

References

[1] Yang L, Parkin DM, Li L and Chen Y. Time trends in cancer mortality in China: 1987-1999. Int J Cancer 2003; 106: 771-783.

[2] Lee SJ, Lee KS, Yim YJ, Kim TS, Shim YM and Kim K. Recurrence of squamous cell carcinoma of the oesophagus after curative surgery: rates and patterns on imaging studies correlated with tumour location and pathological stage. Clin Radiol 2005; 60: 547-554.

[3] Hershko DD. Oncogenic properties and prognostic implications of the ubiquitin ligase Skp2 in cancer. Cancer 2008; 112: 1415-1424.

[4] Luo SW, Peng XB, Zhang Q and Zhang DL. Skp2 structure and its relationship with tumor research progress. J Prac Diagn Ther 2010; 24: 110-112.

[5] Ping W, Sun W, Zu Y, Chen W and Fu X. Clinicopathological and prognostic significance of hypoxia-inducible factor-1alpha in esophageal squamous cell carcinoma: a meta-analysis. Tumour Biol 2014; 35: 4401-4409.

[6] Shiozaki A, Nakashima S, Ichikawa D, Fujiwara H, Konishi H, Komatsu S, Kubota T, Okamoto K, Iitaka D, Shimizu H, Nako Y, Takemoto K, Kishimoto M and Otsuji E. Prognostic significance of p21 expression in patients with esophageal squamous cell carcinoma. Anticancer Res 2013; 33: 4329-4335.

[7] Tong Q, Zhang W, Jin S, Li S and Chen Z. The relationship between p27(kip1) expression and the change of radiosensitivity of esophageal carcinoma cells. Scand J Gastroenterol 2011; 46: 173-176.

[8] Tomiatti V, Istvanffy R, Pietschmann E, Kratzat S, Hoellein A, Quintanilla-Fend L, von Bubnoff N, Peschel C, Oostendorp RA and Keller U. Cks1 is a critical regulator of hematopoietic stem cell quiescence and cycling, operating upstream of Cdk inhibitors. Oncogene 2015; 34: 4347-4357.

[9] Huang HY, Kang HY, Li CF, Eng HL, Chou SC, Lin CN and Hsiung CY. Skp2 overexpression is highly representative of intrinsic biological aggressiveness and independently associated with poor prognosis in primary localized myxofibrosarcomas. Clin Cancer Res 2006; 12: 487-498.

[10] Chan CH, Lee SW, Li CF, Wang J, Yang WL, Wu CY, Wu J, Nakayama KI, Kang HY, Huang HY, Hung MC, Pandolfi PP and Lin HK. Deciphering the transcriptional complex critical for RhoA gene expression and cancer metastasis. Nat Cell Biol 2010; 12: 457-467.

[11] Li CF, Wang JM, Kang HY, Huang CK, Wang JW, Fang FM, Wang YH, Wu WR, Li SH, Yu SC, Lee JC, Lan J, Shiue YL, Wu LC and Huang HY. Characterization of gene amplification-driven SKP2 overexpression in myxofibrosarcoma: potential implications in tumor progression and therapeutics. Clin Cancer Res 2012; 18: 1598-1610.

[12] Wang XC, Tian LL, Tian J and Jiang XY. Overexpression of SKP2 promotes the radiation resistance of esophageal squamous cell carcinoma. Radiat Res 2012; 177: 52-58.

[13] Tian YF, Chen TJ, Lin CY, Chen LT, Lin LC, Hsing CH, Lee SW, Sheu MJ, Lee HH, Shiue YL, Huang HY, Pan HY, Li CF and Chen SH. SKP2 overexpression is associated with a poor prognosis of rectal cancer treated with chemoradiotherapy and represents a therapeutic target with high potential. Tumour Biol 2013; 34: 1107-1117.

[14] Oliveira AM, Okuno SH, Nascimento AG and Lloyd RV. Skp2 protein expression in soft tissue sarcomas. J Clin Oncol 2003; 21: 722-727.

[15] Liang Y, Hou X, Cui Q, Kang TB, Fu JH, Zhang LJ, Luo RZ, He JH, Zeng YX and Yang HX. Skp2 expression unfavorably impacts survival in resectable esophageal squamous cell carcinoma. J Transl Med 2012; 10: 73.