

Association between RNF2+P-AKT expression in pretreatment biopsy specimens, and poor survival following radiotherapy in patients with esophageal squamous cell carcinoma

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Abstract. The protein expression levels of Ring finger protein 2 (RNF2) and phosphor-protein kinase B (P-AKT) were determined in esophageal squamous cell carcinoma (ESCC) tissues, and the association between patient clinicopathological characteristics and survival time following definitive intensity-modulated radiotherapy was assessed. Cancerous biopsy tissues were collected from patients with ESCC at The Fourth Affiliated Hospital of Hebei Medical University between January 2010 and December 2013. Of these 99 cases, 83 were used to analyze the protein expression level of RNF2 (89.2% positive), 85 for P-AKT (65.9% positive) and 80 for RNF2+P-AKT protein expression levels (62.5% both positive). The expression levels of RNF2 protein in ESCC were associated with tumor volume ($P=0.024$), whilst those of P-AKT and RNF2+PAKT were associated with sex ($P=0.041$ and $P=0.003$, respectively). There were no significant differences in overall survival (OS) or progression-free survival (PFS) rate between the RNF2⁻ and the RNF2⁺⁺⁺ groups ($P=0.134$ and $P=0.366$, respectively), or between the P-AKT⁻ group and P-AKT⁺⁺⁺ group ($P=0.468$; $P=0.580$, respectively). The 1-, 3- and 5-year OS rates were 68.0, 28.0, and 20.0%, and 86.7, 53.3, and 31.1%, in the RNF2/P-AKT⁺ group and Other group, respectively ($\chi^2=4.205$; $P=0.040$). Multivariate analysis revealed that age, T stage and RNF2+P-AKT expression were independent prognostic factors for ESCC ($P=0.010$, $P=0.008$ and $P=0.010$, respectively). The expression of RNF2+P-AKT combined was an independent prognostic factor affecting survival rate, and

therefore presents a potential prognostic indicator for patients with ESCC, treated with definitive radiotherapy.

Introduction

Esophageal cancer is a common malignancy of the digestive tract and the sixth for cancer death worldwide (1). The predominant histopathological types are squamous cell carcinoma and adenocarcinoma, and in China, squamous cell carcinoma is most frequently exhibited (2). Although there are numerous treatments available for esophageal cancer, the 5-year survival rate remains at ~20% (3). The discovery of improved predictive markers, early detection and improved treatment options are key to increasing the overall survival (OS) time of patients with esophageal cancer.

There are ≥ 2 types of nuclear protein complex in the polycomb group family, namely polycomb-repressive complex 1 (PRC1) and PRC2 (4). Human PRC1 includes polycomb (PC), polyhomeotic (PH), B-cell-specific Moloney murine leukemia virus integration site-1 (BMI1), RING1a and RING1b (5). PRC2 includes embryonic ectoderm development (EED), enhancer of zeste homolog 2 (EZH2) and suppressor of zeste 12 homolog (SUZ12) (6). In the PRC1 complex, BMI1 and RNF2 heterodimers form E3 ubiquitin ligase in the N-terminal area of the RING (7-9). BMI1 expression is upregulated in hepatocellular carcinoma (10) and pancreatic cancer (11), and RNF2 is overexpressed in many different types of tumors, such as gastrointestinal tumors, lymphomas (12), breast cancer (13), ovarian tumor tissues (14). A previous biological study revealed that silencing Ring finger protein 2 (RNF2) in esophageal cancer cells may lead to defects in DNA damage pathways, and therefore increase sensitivity to radiotherapy (15). Another previous study demonstrated that a high expression level of BMI1 promoted the expression of phosphor-protein kinase B (P-AKT) following radiotherapy, and that the phosphoinositide 3-kinase (PI3K)/AKT pathway was involved in resistance to radiotherapy (16). However, the potential association between P-AKT and the RNF2 expression level, and the survival of patients with esophageal squamous cell carcinoma (ESCC) treated with radiotherapy, requires further investigation.

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Therefore, in the present study, it was hypothesized that an increased expression of RNF2 and P-AKT may result in increased resistance to radiotherapy in patients with ESCC. Immunohistochemistry was used to detect RNF2 and P-AKT protein expression in patients prior to radiotherapy, and the association between RNF2+P-AKT protein expression and clinicopathological features and survival prognosis was retrospectively analyzed, in order to investigate the role of RNF2+P-AKT protein expression in patients with ESCC following radiotherapy.

Patients and methods

Patient sample selection. Between January 2010 and December 2013, 99 I-IVa (American Joint Committee on Cancer, 2010) (17) stage ESCC patients, with a mean age of 66 years (range, 48-87) and a male:female ratio of 1.61 (61/38), were selected from The Fourth Affiliated Hospital of Hebei Medical University, and their tumor samples (formalin-fixed and paraffin-embedded) were taken for the analysis of RNF2 and P-AKT. Patients who had received any anti-cancer treatment prior to diagnosis were excluded. The inclusion criteria were as follows: i) Histological evidence of invasive squamous-cell carcinoma of the esophagus; and ii) informed written consent to receive radiotherapy (RT). Detailed clinical and follow-up data was obtained from all patients, and written consent was obtained for the collection of tissue specimens. The study protocols were approved by the Ethics Committee for Clinical Research of the Fourth Affiliated Medical University.

Immunohistochemistry (IHC). IHC was performed using conventional methods. Briefly, paraffin-embedded ESCC tissues were sliced into 4- μ m-thick sections, deparaffinized in xylene at 55°C, and rehydrated in a descending alcohol series (100% alcohol first time, 100% alcohol second time, 95% alcohol, 80% alcohol) for 10 min respectively. Endogenous peroxidases were blocked using 3% hydrogen peroxide for 20 min at 37°C, and antigen retrieval was conducted using citrate buffer (Thermo Fisher Scientific, Inc.) in a microwave oven for 15 min at 98°C. The sections were incubated with primary antibodies against RNF2 (1:100; cat. no. ab101273; Abcam) and P-AKT (1:40; cat. no. ab81283; Abcam) at 4°C overnight, followed by subsequent incubation with horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1:100; cat. no. SP-9000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 30 min at 37°C. The sections were then processed with 3,3'-diaminobenzidine (DAB) for 5 min at 37°C and counterstained with hematoxylin for 16 min at 37°C and assessed under a light microscope (Olympus Corporation) by two pathologists who were blinded to the clinical parameters of the patients.

Reactivity scoring and interpretation of IHC. The expression of RNF2 and P-AKT was microscopically observed using a light microscope, and this was identified to be predominantly located in the nucleus. The IHC results were independently evaluated by 2 pathologists with no prior knowledge of the patients' clinicopathological data. If different scores were assigned for the same sample, the sample was reevaluated and, if required, further discussed

to determine a final score. For positive staining in the nuclei, the most intensively stained region was initially selected with a low-power magnification (x100). The percentage of positively stained cells was then calculated from the observation of 5 random sections at a higher magnification (x200). A total of 100 tumor cells were counted in each section, and the number and intensity classification of the positively-stained cells was determined. The immunoreactive score (IRS) system was used. The staining intensity classification (18,19) was as follows: i) 0, unstained; ii) 1, light yellow; iii) 2, brownish yellow; and iv) 3, tan. The percentage score for positive cells was classified as follows: i) 0, positive cells $\leq 5\%$; ii) 1, positive cells 6-25%; iii) 2, positive cells 26-50%; iv) 3, positive cells 51-75%; and v) 4, positive cells $>75\%$. The total sum of the staining intensity and percentage-positive scores was indicated as follows: i) 0-1, negative (-); ii) 2-3, weakly positive (+); iii) 4-5, moderately positive (**); and iv) 6-7, strongly positive (+++). Where the degree of positive staining is not being addressed, the word 'positive' has been used to indicate positive staining in general, in place of the percentage-positive scoring symbols, which is also suitable for the negative staining.

Statistical analysis. SPSS 19.0 statistical software (IBM Corp.) was used for all statistical analyses. The relationship between RNF2, P-AKT and patient clinicopathological parameters was analyzed using the χ^2 test, extended Fisher's exact test, linear-by-linear association and the Goodman-Kruskal γ test. The Kaplan-Meier method was used to analyze survival prognosis, in addition to the log-rank test. Univariate and multivariate analysis of survival prognosis was performed using cox regression analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data and characteristics. IHC analysis was performed on 99 patients with ESCC, pathologically diagnosed at The Fourth Affiliated Hospital of Hebei Medical University. Due to incomplete clinicopathological or IHC data, or discontinued radiotherapy, a number of patients were excluded from the study. As a result, a total of 83 patients were assigned to the RNF2 group, 85 to the P-AKT group, and 80 to the RNF2+P-AKT group. Detailed clinical data are outlined in Table I.

Influence of RNF2 protein expression level on ESCC. IHC analysis of patients' specimens revealed that the expression of RNF2 protein was primarily localized to the nuclei of tumor cells (Fig. 1). Among the 83 cases of ESCC, 9 (10.8%) were RNF2-negative, while 74 (89.2%) exhibited positive expression levels of RNF2; of these RNF2-positive patients, 20 (24.1%), 18 (21.7%) and 36 (43.4%) demonstrated weak, moderate and strong-positive expression levels, respectively. Furthermore, there was a significant association between RNF2 expression and tumor volume ($P < 0.05$), whereas no significant association was revealed for any other clinicopathological feature, including age, sex, tumor site, tumor length and Tumor-Node-Metastasis (TNM) stage (17) (Table II).

Table I. Demographic, baseline variables and treatment characteristics of the study population.

Variables	RNF2 (n=83) (%)	P-AKT (n=85) (%)	RNF2/P-AKT (n=80) (%)
Sex			
Male	51 (61.4)	53 (62.4)	49 (61.2)
Female	32 (38.6)	32 (37.6)	31 (38.8)
Age (years)			
≤66	44 (53.0)	45 (52.9)	43 (53.8)
>66	39 (47.0)	40 (47.1)	37 (46.3)
Tumor location			
Cervical	6 (7.2)	6 (7.1)	6 (7.5)
Upper thoracic	24 (28.9)	25 (29.4)	24 (30.0)
Middle thoracic	37 (44.6)	38 (44.7)	34 (42.5)
Lower thoracic	16 (19.3)	16 (18.8)	16 (20.0)
Tumor length (cm)			
≤3	4 (4.8)	4 (4.7)	4 (5.0)
3-5	33 (39.8)	33 (38.8)	32 (40.0)
5-7	24 (28.9)	25 (29.4)	23 (28.8)
>7	22 (26.5)	23 (27.1)	21 (26.3)
Tumor volume (cm ³)			
≤25	25 (30.1)	26 (30.6)	24 (30.0)
>25	58 (69.9)	59 (69.4)	56 (70.0)
T stage			
1+2	29 (34.9)	29 (34.1)	27 (33.8)
3+4	54 (65.1)	56 (65.9)	53 (66.3)
N stage			
0	34 (41.0)	35 (41.2)	33 (41.3)
+	49 (59.0)	50 (58.8)	47 (58.8)
Tumor- Node-Metastasis stage (17)			
I+II	36 (43.4)	37 (43.5)	34 (42.5)
III+IV	47 (56.6)	48 (56.5)	46 (57.5)
Radiation field			
IFI	45 (54.2)	46 (54.1)	43 (53.8)
ENI	38 (45.8)	39 (45.9)	37 (46.3)
Radiation dose (Gy)			
≤60	42 (50.6)	43 (50.6)	40 (50.0)
>60	41 (49.4)	42 (49.4)	40 (50.0)
Chemotherapy			
No	41 (49.4)	43 (50.6)	38 (47.5)
Yes	42 (50.6)	42 (49.4)	42 (52.5)

RNF2, Ring finger protein 2; P-AKT, phosphor-protein kinase B; IFI, involved field radiation; ENI, elective nodal irradiation.

Influence of P-AKT protein expression on ESCC. The assessment of the P-AKT expression level was determined for 85 tumor samples, and this was identified to be predominantly present in the nuclei of tumor cells (Fig. 2). A total of 29 (34.1%) patient samples were classified as expression-negative, whereas 56 (65.9%) exhibited positive P-AKT expression. In the P-AKT-positive group, 39 (45.9%), 16 (18.8%) and 1 (1.2%) samples exhibited weak, moderate and strong-positive expression levels, respectively.

There was also a significant association between P-AKT expression and the sex of the patient ($P<0.05$), whereas no such association was observed for the other clinicopathological features, including age, tumor site, tumor length and volume, T, N and TNM stage (Table III).

Influence of RNF2+P-AKT protein expression on ESCC. Among the 80 samples used to assess the level of RNF2+P-AKT expression, 5 patients (6.25%) were RNF2-negative/P-AKT-negative,

Table II. Association between RNF2 expression and the clinicopathological variables of patients with esophageal squamous cell carcinoma.

Variables	RNF2 expression								P-value
	(-) (n=9)	%	(+) (n=20)	%	(++) (n=18)	%	(+++) (n=36)	%	
Sex									
Male	4	44.4	12	60.0	14	77.8	21	58.3	0.635
Female	5	55.6	8	40.0	4	22.2	15	41.7	
Age, years									
≤66	6	66.7	13	65.0	7	38.9	18	50.0	0.254
>66	3	33.3	7	35.0	11	61.1	18	50.0	
Tumor location									
Cervical	1	11.1	2	10.0	2	11.1	1	2.8	0.579
Upper thoracic	2	22.2	6	30.0	5	27.8	11	30.6	
Middle thoracic	4	44.4	8	40.0	9	50.0	16	44.4	
Lower thoracic	2	22.2	4	20.0	2	11.1	8	22.2	
Tumor length, cm									
≤5	6	66.7	7	35.0	11	61.1	13	36.1	0.269
>5	3	33.3	13	65.0	7	38.9	23	63.9	
Tumor volume, cm ³									
≤25	5	55.6	6	30.0	8	44.4	6	16.7	0.024
>25	4	44.4	14	70.0	10	55.6	30	83.3	
T stage									
1+2	4	44.4	7	35.0	7	38.9	11	30.6	0.467
3+4	5	55.6	13	65.0	11	61.1	25	69.4	
N stage									
0	4	44.4	8	40.0	10	55.6	12	33.3	0.503
+	5	55.6	12	60.0	8	44.4	24	66.7	
Tumor-Node-Metastasis stage (17)									
I+II	4	44.4	9	45.0	7	38.9	16	44.4	0.992
III+IV	5	55.6	11	55.0	11	61.1	20	55.6	

RNF2, Ring finger protein 2.

21 (26.25%) were RNF2-positive/P-AKT-negative, 4 (5.00%) were RNF2-negative/P-AKT-positive, and 50 patients (62.50%) were RNF2-positive/P-AKT-positive. Furthermore, there was a significant association between RNF2-positive/P-AKT-positive expression and sex ($\chi^2=9.132$; $P=0.003$), and an association with T stage ($\chi^2=7.240$; $P=0.065$), yet no significant association with age, tumor length, N stage or TNM stage (Table IV).

Association between RNF2 and P-AKT protein expression level and OS. The patient follow-up date was December 2016, with a median follow-up time of 80.78 months (95%CI, 61.06-80.61 months) and a loss rate of 4.0% (4/99) due to loss of contact. Kaplan-Meier survival curves were generated to estimate the survival rates of these patients. The results revealed no significant differences in OS and progression-free survival (PFS) rates between the

RNF2⁻ and RNF2⁺⁺⁺⁺ groups ($\chi^2=2.244$, $P=0.134$; $\chi^2=0.818$, $P=0.366$), or between the P-AKT⁻ and P-AKT⁺⁺⁺⁺ groups ($\chi^2=0.526$, $P=0.468$; $\chi^2=0.306$, $P=0.580$; Fig. 3A and B; Table V).

Furthermore, the results illustrated a median survival time of 15.7 and 36.0 months; the 1, 3 and 5-year survival rates were 68.0, 28.0 and 20.0%, and 86.7, 53.3 and 31.1% ($\chi^2=4.205$; $P=0.040$) in the RNF2+P-AKT-positive expression group and the Other group, respectively (Fig. 4). PFS was not significantly different between the RNF2+P-AKT-positive expression and Other group ($\chi^2=3.407$; $P=0.065$; Table V).

Univariate analysis showed that age, T stage, tumor volume, RNF2 protein expression, P-AKT protein expression and RNF2+P-AKT expression were prognostic factors affecting OS ($P=0.036$, 0.023, 0.039, 0.007, 0.003 and 0.040, respectively). Furthermore, multivariate analysis demonstrated that age, T stage and RNF2+P-AKT expression were

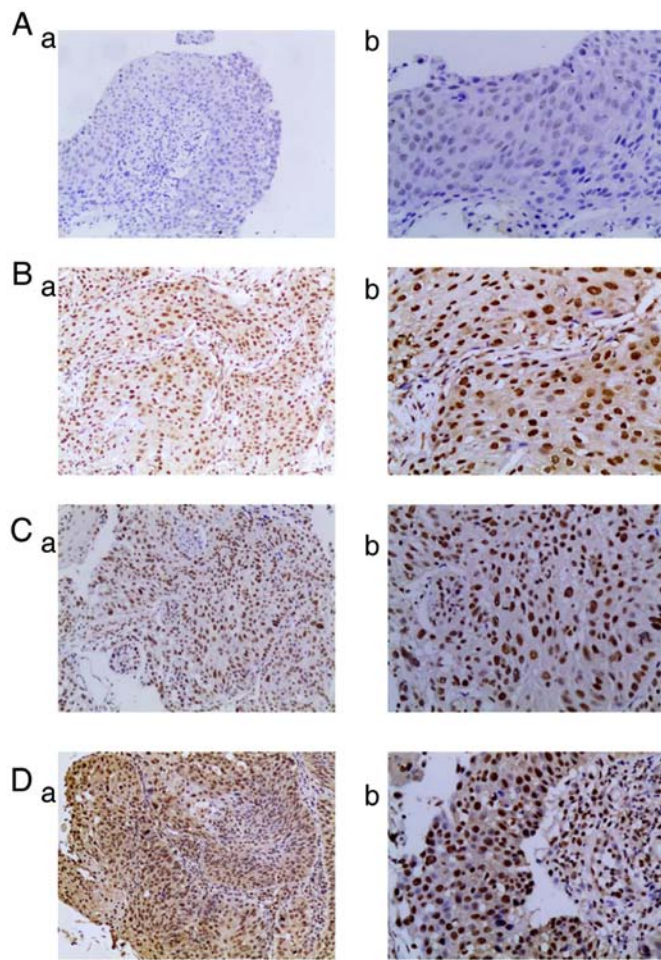


Figure 1. Expression levels of RNF2 protein in ESCC tissues. (A) No RNF2 expression in ESCC tissues; (a) magnification, x100 and (b) magnification, x200. (B) Weak expression of RNF2 in ESCC tissues; (a) magnification, x100 and (b) magnification, x200. (C) Moderate expression of RNF2 in ESCC tissues; (a) magnification, x100 and (b) magnification, x200. (D) Strong expression of RNF2 in ESCC tissues; (a) magnification, x100 and (b) magnification, x200. RNF2, Ring finger protein 2; ESCC, esophageal squamous cell carcinoma.

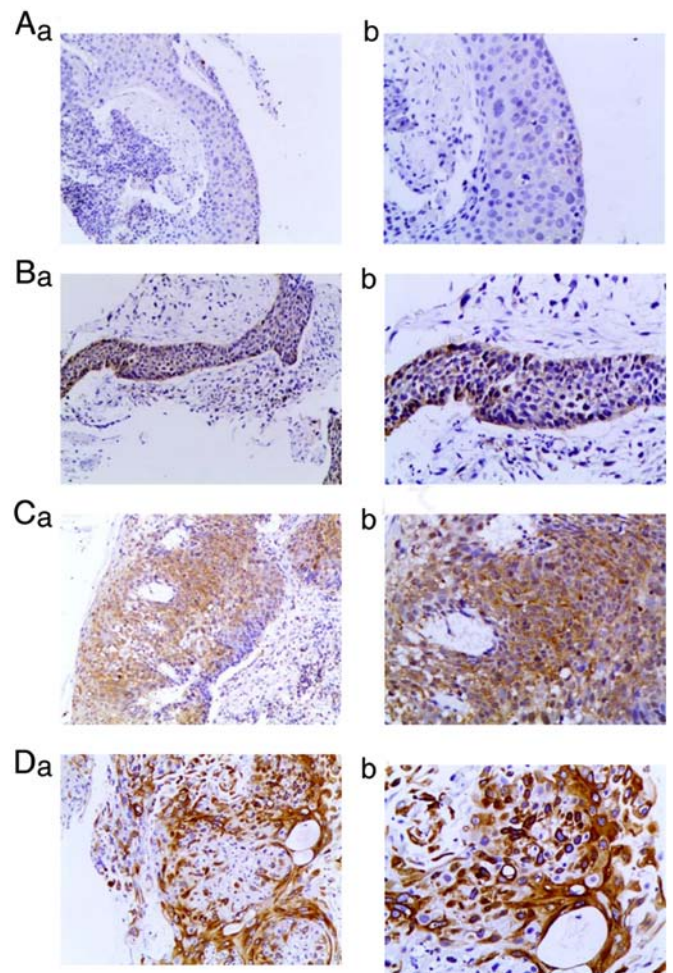


Figure 2. Expression levels of P-AKT protein in ESCC tissues. (A) No P-AKT expression in ESCC tissue; (a) magnification, x100 and (b) magnification, x200. (B) Weak expression of P-AKT in ESCC tissues; (a) magnification, x100 and (b) magnification, x200. (C) Moderate expression of P-AKT in ESCC tissues; (a) magnification, x100 and (b) magnification, x200. (D) Strong expression of P-AKT in ESCC tissues; (a) magnification, x100 and (b) magnification, x200. P-AKT, phosphor-protein kinase B; ESCC, esophageal squamous cell carcinoma.

independent prognostic factors for ESCC ($P=0.010$, 0.008 and 0.010 , respectively; Table VI).

Discussion

In the present study, IHC analysis was performed on the pathological tissues of patients with ESCC, which revealed that RNF2 and P-AKT protein expression levels were high in ESCC tissues. Furthermore, patient survival rates in the RNF2+P-AKT-positive expression group were lower compared with that in the other group, suggesting that the expression of RNF2+P-AKT in patients with ESCC who had previously received radiotherapy was associated with poor prognosis.

The overexpression of RNF2 has been reported in numerous types of solid tumor, such as gastrointestinal tumors, lymphomas (12), breast cancer (13) and ovarian tumor tissues (14), and is associated with poor prognosis in esophageal cancer (15). The present study identified that the positive expression rate of RNF2 in ESCC was 89.2%, and that localization of this protein to the nucleus was consistent with

previously published work (20). The upregulation of RNF2 expression in other tumors has also been associated with tumor size, pathological grade and poor patient prognosis (21). In the present study, a high RNF2 expression level was associated with tumor volume, prompting the hypothesis that, in ESCC, RNF2 serves an important role in early metastasis and tumor formation; this has also been confirmed by other studies (22-24).

The PI3K signaling pathway is involved in numerous cellular activities and sensitivity to radiotherapy, in addition to tumor metastasis and apoptosis (25-27). The phosphorylation of AKT to P-AKT is central to the PI3K pathway, and its activation stimulates the proliferation of tumor cells (28). Yoshioka *et al* (29) reported that the prognosis of a low P-AKT-expression group of patients with ESCC treated with chemotherapy had improved prognoses compared with those exhibiting high P-AKT expression levels. In addition, Schmitz *et al* (30) revealed a negative correlation between the P-AKT expression level and patient survival. However, the results of the present study revealed no significant differences

Table III. Association between phosphor-protein kinase B expression and the clinicopathological variables of patients with esophageal squamous cell carcinoma.

Variables	P-AKT expression								P-value
	(-) (n=29)	%	(+) (n=39)	%	(++) (n=16)	%	(+++) (n=1)	%	
Sex									
Male	13	44.8	28	71.8	11	68.8	1	100.0	0.041
Female	16	55.2	11	28.2	5	31.3	0	0.0	
Age, years									
≤66	14	48.3	21	53.8	9	56.3	1	100.0	0.456
>66	15	51.7	18	46.2	7	43.8	0	0.0	
Tumor location									
Cervical	1	3.4	3	7.7	2	12.5	0	0.0	0.248
Upper thoracic	6	20.7	13	33.3	6	37.5	0	0.0	
Middle thoracic	15	51.7	18	46.2	5	31.3	0	0.0	
Lower thoracic	7	24.1	5	12.8	3	18.8	1	100.0	
Tumor length, cm									
≤5	10	34.5	19	48.7	7	43.8	1	100.0	0.281
>5	19	65.5	20	51.3	9	56.3	0	0.0	
Tumor volume, cm ³									
≤25	8	27.6	12	30.8	6	37.5	0	0.0	0.611
>25	21	72.4	27	69.2	10	62.5	1	100.0	
T stage									
1+2	14	48.3	22	56.4	3	18.7	0	0.0	0.117
3+4	15	51.7	17	43.6	13	81.3	1	100.0	
N stage									
0	12	41.4	19	48.7	4	25.0	0	0.0	0.309
1	17	58.6	20	51.3	12	75.0	1	100.0	
Tumor-Node-Metastasis stage (17)									
I+II	11	37.9	25	64.1	6	37.5	0	0	0.688
III+IV	18	62.1	14	35.9	10	62.5	1	100.0	

P-AKT, phosphor-protein kinase B.

Table IV. Association between RNF2+P-AKT expression and the clinicopathological variables of patients with esophageal squamous cell carcinoma.

Variables	RNF2/P-AKT expression				P-value
	RNF2/P-AKT both positive		Other		
	(n=50)	%	(n=30)	%	
Sex					
Male	37	74.00	12	40.00	0.003
Female	13	26.00	18	60.00	
T stage					
1	0	0.00	2	6.67	0.065
2	17	34.00	8	26.67	
3	6	12.00	8	26.67	
4	27	54.00	12	40.00	

RNF2, Ring finger protein 2; P-AKT, phosphor-protein kinase B; Other, including patients with RNF2/P-AKT both negative, RNF2-P-AKT⁺⁺⁺ and RNF2⁺⁺⁺-P-AKT.

between the survival rates of the P-AKT-positive and -negative expression groups, which may result from differences in clinical data and treatment methods between the 2 groups. The small sample size, particularly the P-AKT⁺⁺⁺ number, was another factor that may have affected the results; however, to a certain extent, the bias caused by small sample size could be decreased by the analysis of P-AKT⁽⁺⁺⁻⁺⁺⁺⁾ expression. A larger sample size may clarify this.

BMI1 is an important predictor of tumor progression (13) that is able to promote cellular activity, induce resistance to apoptosis, and increase the likelihood of metastasis (14). It may also be associated with treatment failure in a number of malignancies, including breast and prostate cancer, and hepatocellular carcinoma (31-33). Previous studies have demonstrated that the overexpression of BMI1 and RNF2 was associated with tumor cell transformation in multiple types of tumor tissue (12,34). BMI1 is also essential for the ubiquitination of histone H2AX (35); when BMI1 is present, the capacity of RNF2 to ubiquitinate H2AX is enhanced (36), while H2AX itself is associated with the radiotherapeutic sensitivity of esophageal cancer cells (37). RNF2 serves as an E3 ubiquitin ligase (35,38,39); BMI1 is

Table V. Survival rates of patients with esophageal squamous cell carcinoma with different RNF2+P-AKT expression levels.

Expression levels	Overall survival (%)			χ^2	P-value	Progression-free survival (%)			χ^2	P-value
	1-year	3-year	5-year			1-year	3-year	5-year		
RNF2 expression										
Negative	77.8	55.6	55.6	2.244	0.134	66.7	33.3	33.3	0.818	0.366
Positive	75.7	37.8	21.6			62.2	25.7	15.5		
P-AKT expression										
Negative	82.8	48.3	24.7	0.526	0.468	69.0	27.6	11.0	0.306	0.580
Positive	69.6	32.1	17.1			55.4	25.0	20.6		
RNF2/P-AKT expression										
Positive	68.0	28.0	20.0	4.205	0.040	52.0	20.0	14.4	3.407	0.065
Other	86.7	53.3	31.1			76.7	33.3	18.3		

RNF2, Ring finger protein 2; P-AKT, phosphor-protein kinase B; Other, including patients with RNF2-negative/P-AKT-negative, RNF2-positive/P-AKT-negative and RNF2-negative/P-AKT-positive.

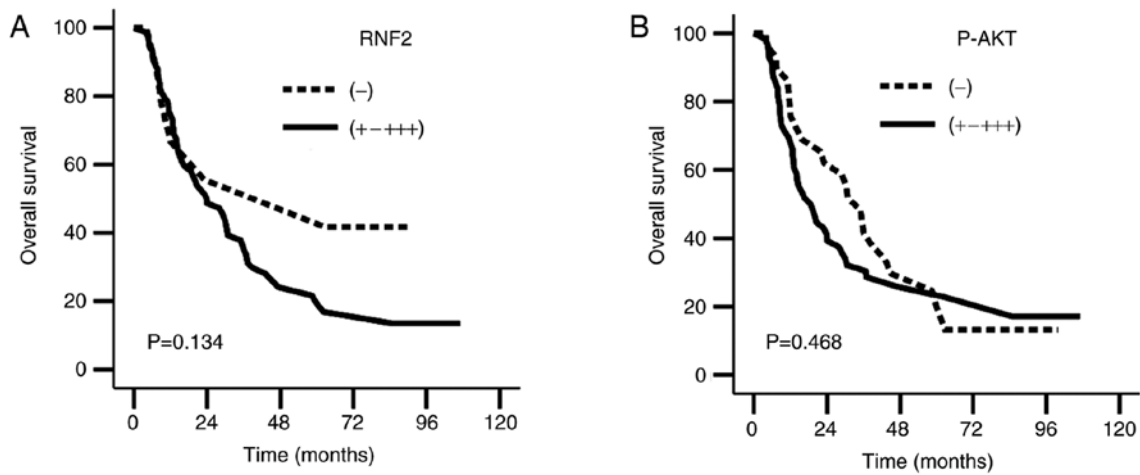


Figure 3. Overall survival curve of patients with esophageal squamous cell carcinoma with RNF2+P-AKT expression. (A) Overall survival curves of the RNF2⁻ vs. the RNF2^{+/-++} groups. (B) Overall survival curves of the P-AKT⁻ vs. the P-AKT^{+/-++} groups. RNF2, Ring finger protein 2; P-AKT, phosphor-protein kinase B.

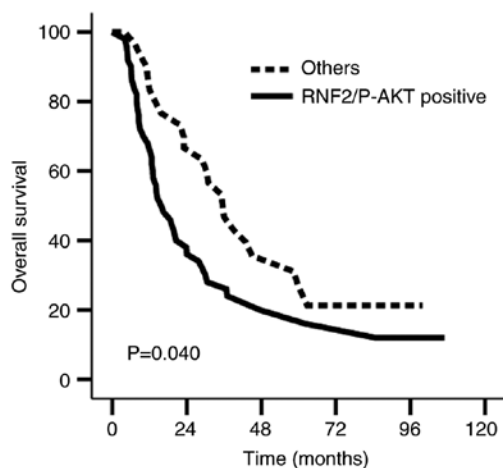


Figure 4. Overall survival curve of patients with esophageal squamous cell carcinoma in the RNF2+P-AKTpositive expression group compared with other group. RNF2, Ring finger protein 2; P-AKT, phosphor-protein kinase B; Other, including patients with RNF2-negative/P-AKT-negative, RNF2-positive/P-AKT-negative, RNF2-negative/P-AKT-positive.

essential for the activity of this E3 ubiquitin ligase in the PRC1 complex (40), and the ubiquitination activity of RNF2 requires that BMI1 be present. In a previous study, it was revealed that the knockdown of BMI1 resulted in a DNA-damage response defect, and that the PI3K/AKT pathway could be perturbed to increase sensitivity to radiotherapy (16). Therefore, it was hypothesized that RNF2 overexpression may increase radiotherapeutic resistance by activating the PI3K/AKT pathway. IHC analysis was conducted using tissue samples from patients with ESCC, which revealed that the survival rate of those in the RNF2+P-AKT-positive expression group was significantly lower compared with that of the other group. In addition, multivariate analysis revealed that RNF2+P-AKT-positive expression was an independent prognostic factor affecting survival. To a degree, these results reflected the synergistic effects of RNF2 and P-AKT in ESCC. Therefore, it was hypothesized that the expression of RNF2 and P-AKT in ESCC was an important prognostic indicator, though the association between these proteins requires further experimental confirmation.

Table VI. Univariate and multivariate analysis for the 80 patients with esophageal squamous cell carcinoma with RNF2+P-AKT positive expression.

Variables	Univariate analysis				Multivariate analysis			
	Overall survival		Progression-free survival		Overall survival		Progression-free survival	
	HR	95.0% CI	P-value	HR	95.0% CI	P-value	HR	95.0% CI
Age (≤ 66 / > 66 years)	1.704	1.030-2.819	0.036	1.477	0.908-2.402	0.116	1.957	1.175-3.259
Tumor volume (≤ 25 / > 25 cm ³)	1.586	1.298-2.802	0.039	1.863	1.058-3.279	0.031	0.856	0.423-1.735
T stage (1+2/3+4)	1.871	1.081-3.239	0.023	1.932	1.133-3.296	0.016	2.125	1.222-3.698
RNF2 ⁻ -RNF2 ⁺⁺⁺	2.752	1.319-5.740	0.007	0.888	0.706-1.119	0.315	0.833	0.622-1.115
P-AKT ⁻ -P-AKT ⁺⁺⁺	0.045	0.005-0.420	0.003	1.025	0.755-1.392	0.872	0.739	0.513-1.065
RNF2 and P-AKT Expression (other/both positive)	1.713	1.017-2.886	0.040	1.596	0.966-2.636	0.068	2.010	1.183-3.415
RNF2, Ring finger protein 2; P-AKT, phosphor-protein kinase B; Other, including patients with RNF2-negative/P-AKT-negative, RNF2-positive/P-AKT-negative, RNF2-negative/P-AKT-positive; HR, hazard ratio; CI, confidence interval.								

There were several limitations associated with the present study. Firstly, it was a single-center retrospective study with a small number of cases, and the results require additional confirmation from a larger prospective sample study. Secondly, the tissue samples were all from biopsy specimens, and therefore the tissues were small and not sufficient to fully evaluate the grading of ESCC. Therefore, the association between the degree of RNF2 and P-AKT expression, and the classification of tumor tissues, was not analyzed. Finally, the association between RNF2 and P-AKT was not assessed in molecular *in vitro* studies, which would strengthen the conclusions drawn from the present study.

In conclusion, the present study revealed that the RNF2 and P-AKT proteins may be important oncogenes in the prognosis of patients with ESCC, and that their expression was associated with tumor volume and OS rate, indicating their use as important molecular markers of ESCC. Therefore, RNF2 and P-AKT are predicted to be novel prognostic indicators for radical radiotherapy in patients with ESCC.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QL, SZ and XY analyzed and interpreted the patient data regarding the research. QL, SZ and CS made substantial contributions to conception and design. QL and CS drafted the manuscript. QL, SL and CS were major contributors in writing the manuscript. SL and XZ performed the histological examination of the esophagus. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent to participate prior to the collection of tissue specimens and radiotherapy and/or chemotherapy. The present study was approved by the Medical Ethics Committee of the Clinical Research of the Fourth Affiliated Hebei Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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