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Original Article

Expression and clinical significance of B cell translocation gene 2 in esophageal squamous cell carcinoma

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Abstract: Esophageal squamous cell carcinoma (ESCC) is widely known as a highly fatal cancer, and thus it is important to identify tumor-specific and radiosensitivity-specific markers in ESCC. B cell translocation gene 2 (BTG2) has been considered a novel tumor suppressor gene or radiotherapy sensitivity-associated gene. However, the relationship between BTG2 and ESCC development and radiotherapy sensitivity is uncertain. The present study aims to explore the expression and clinical significance of B cell translocation gene 2 (BTG2) in ESCC by analyzing the RNAseq data from the TCGA and immunohistochemical staining of ESCC samples. We found that the level of BTG2 mRNA was significantly decreased in ESCC patients, and further decreased significantly in radiotherapy resistant patients compared to sensitive patients. The positive expression rate of BTG2 protein was 56.0% (103/184) in 184 ESCC tissue samples and 84.0% (42/50) in normal esophageal mucosal samples, respectively. The positive ratios of BTG2 expression in radiotherapy-sensitive group and radiotherapy resistant group were 57.9% (22/38) and 23.5% (4/17), respectively. Furthermore, the analysis indicates that the expression level of BTG2 significantly correlated with lymph node metastasis and clinical staging in ESCC patients. A multivariate analysis with Cox regression model showed that BTG2 level was an independent risk factor affecting the prognosis of ESCC patients. Above all, the downregulation of BTG2 may be used as a molecular marker to identify and predict ESCC progression and radiosensitivity.

Keywords: Esophageal squamous cell carcinoma (ESCC), B cell translocation gene 2 (BTG2), radiosensitivity, prognosis

Introduction

Esophageal cancer is one of the most common malignant tumors of the digestive system [1, 2]. There are almost 572,000 cases of esophageal cancer and 508,000 deaths worldwide every year. Furthermore, in China, esophageal cancer is the fourth deadliest cancer with 307,000 cases and 283,000 deaths per year, of which esophageal squamous cell carcinoma (ESCC) accounts for about 90% [3]. Although diagnosis and treatment of ESCC are improving, many patients are found at an advanced stage due to its high invasion and early metastasis [4, 5]. For patients with advanced ESCC who are unsuitable for surgical treatment, palliative treatment is a irreplaceable therapeutic schedule but its effect is not ideal. The 5-year survival rate is only 10%-30%, due to factors such as the radiotherapy resistance of ESCC [6]. Besides, ESCC radiotherapy resistance is also considered to be an important cause of tumor recurrence and radiotherapy failure, which seriously affects the prognosis of ESCC patients [7]. Therefore, it is important to identify tumor-specific and radiosensitivity-specific markers in ESCC.

B cell translocation gene 2 (BTG2) is a member of the BTG family and in light of the anti-proliferation function of this family, BTG2 is also known as BTG anti-proliferation factor 2 [8]. Briefly, BTG2 is an important downstream target of tumor suppressor and radiosensitive gene p53 and is expressed in a variety of tissues and

organs as a transient early reactive protein [8, 9], and widely involved in a variety of biologic activities such as cell differentiation, proliferation, and apoptosis. Therefore, BTG2 is considered to be a novel tumor suppressor gene for many types of cancer, such as laryngeal cancer [10] gastric cancer [11], liver cancer and breast cancer [12]. Moreover, over-expression of BTG2 in breast cancer cells can improve their radiotherapy sensitivity [13]. However, the relationship between BTG2 and ESCC development and radiotherapy sensitivity has not been reported so far. Therefore, this study intends to analyze the transcriptome data of ESCC patient in the Cancer Genome Atlas (TCGA) and combining with the immunohistochemical staining analysis to explore the expression and clinical significance of BTG2 in ESCC. This study will provide a new theoretical basis and method for the diagnosis and prognosis of ESCC.

Materials and methods

BTG2 mRNA analysis

Esophageal expression data sets and clinical data (ID: TCGA.ESCA.SampleMap/HiSeqV2.) from TCGA database were downloaded from the UCSC genome browser (http://xena.ucsc.edu/welcome-to-ucsc-xena/). The data set was based on IlluminaHiSeq_RNASeqV2 high-throughput RNA sequencing platform, and the reads were the relative values of expression values normalized by computer programming language. The BTG2 transcriptase sequencing data of 81 ESCC patients with clinical data and 11 control tissues were extracted for subsequent analysis. Among the selected expression spectrum of ESCC patients, the variable "measure of response" was used as the evaluation index of radiotherapy response, of which "complete response" was identified as the sensitive group and "radiographic progression disease" was considered as the resistance group.

Human ESCC tissue samples

This study was conducted with the knowledge of all patients and approved by the Ethics Committee of Lianshui County People's Hospital affiliated to Kangda College of Nanjing Medical University. Retrospective collection and analysis was done of the pathologic data and paraffin-embedded pathologic tissue of confirmed ESCC patients in Lianshui County People's Hospital from January 2013 to December 2015. Case selection criteria: 1. ESCC confirmed by pathology department; 2. Neither surgical patients nor patients with radical chemoradiotherapy received chemoradiotherapy before taking samples; 3. No history of infection or blood-borne diseases before treatment. Case exclusion criteria: 1. Recent history of blood transfusion or incomplete clinical data; 2. No follow-up records; 3. Severe infection or autoimmune disease. A total of 184 patients aged 36-86 years were included in this study, including 127 males and 27 females, as detailed in **Table 1**. Among them, 54 patients with ESCC received radical radiotherapy in the radiotherapy department, and 50 cases of normal esophageal mucosa adjacent to cancer were selected as the control. For the detection of patient survival, the start date of survival time was set as the date of biopsy and the end date was set as the date of death or the time of the last follow-up. The deadline for follow-up was March 2019.

Evaluation of short-term effects

To evaluate tumor responses, esophageal barium meal examination and chest CT were carried out for one month, following the completion of a 4 weeks' RT after radiotherapy. According to the Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 standard, patients were divided into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). In the present study, CR and PR were selected as the defined sensitive group, and SD and PD were defined as the radiotherapy resistant group.

Immunohistochemical staining and scoring standard

BTG2 rabbit antibody was purchased from Abcam (USA). Briefly, tissues were fixed in 4% neutral formalin solution, then embedded in paraffin. The paraffin-embedded tissues were cut into 4 μm-thick sections for immunohistochemical staining. The slices were heated at 65°C for 30 min, then dewaxed and washed with PBS in turn. After adding primary antibody, the slices were placed in a wet box at 4°C overnight, and then placed at room temperature for 1 h. The slices were washed with PBS solution for 3 times, 3 min each time. Biotin-labeled secondary antibodies were added and incubated at

Table 1. Association between BTG2 protein expression and clinicopathologic characteristics of ESCC patients

Item	N	BTG2 protein		x^2	*P*
		Positive	Negative		
Gender					
Male	157	91	66	1.708	0.191
Female	27	12	15		
Age					
≤ 60 years	91	57	34	3.240	0.071
> 60 years	93	46	47		
Location					
neck/upper thoracic	56	36	20	2.254	0.133
mid/lower thoracic	128	67	61		
Grade					
G1/G2	125	71	54	0.107	0.744
G3	59	32	27		
T					
T1/T2	79	43	36	0.135	0.714
T3/T4	105	60	45		
N					
N0	79	51	28	4.134	0.042
N1	105	52	53		
M					
M0	175	100	75	1.210	0.290
M1	9	3	6		
Stage					
I+II	106	67	39	5.303	0.021
III+IV	78	36	42		
Radiosensitivity					
Sensitive	38	22	16	5.565	0.018
Resistant	17	4	13		

37°C for 30 min, then washed with PBS solution for 3 times, 3 min each. Horseradish peroxidase was added and incubated at 37°C for 30 min, followed by washing with PBS, DAB chromogenic, hematoxylin re-staining of the nuclei, and sealing the slices.

Evaluation of immunohistochemical staining

The staining intensity of tumor cells and the proportion of positive cells were scored. Staining intensity score: 0 for non-staining, 1 for light yellow, 2 for brownish, and 3 for tan. Percentage of positive cells score: < 10% was 0, 10%~29% was 1, 30%~60% was 2, > 60% was 3. Points for staining intensity and the percentage of positive cells were added, and the tissue specimens were classified into two groups according to their overall score: Negative expression for 0-4 points and positive expression for 4-9 points [14].

Statistical analysis

Statistical analyses were carried out using SPSS 22.0, and Graphpad Prism 5 was used to draw statistical graphs. Measurement data were presented as mean ± SD. Levene test was used to detect the homogeneity of variance, and Student *t* test was used to analyze the statistical significance of the comparison between the two groups according to the homogeneity of variance. Enumeration data were represented by frequency or percentage (%) and were tested by x^2 test. For the four-lattice table, if the occurrence frequency was less than 5, Fisher's exact probability method was used to calculate the corresponding x^2 value and *P* value. All *P* values were double-tailed tests, and a *P* value of less than 0.05 was considered significant.

Results

The diagnostic value of BTG2 mRNA expression in ESCC and radiotherapy resistance

Analysis of ESCC mRNA expression profile in the TCGA database showed that the relative expression of BTG2 in ESCC tissues was 5.08±1.06, and that in 11 control tissues was 5.91±1.29 (**Figure 1A**). The difference was statistically significant (t = 2.387, *P* = 0.019). The receiver operating characteristic (ROC) analysis results revealed that the area under the curve (AUC) was 0.679 (**Figure 1C**), which had little value for ESCC diagnosis. The relative mRNA expression level of BTG2 in 23 radiotherapy sensitive patients and 8 radiotherapy resistant patients was 5.44±0.73 and 3.82±0.97, respectively (**Figure 1B**). The difference between the two groups of patients was significant (t = 4.935, *P* < 0.001). ROC results suggested that BTG2 mRNA expression had diagnostic value in differentiating radiosensitive patients and radiosensitive patients (AUC = 0.902, **Figure 1D**), with a truncation value of 4.352, a specificity of 95.65%, and a sensitivity of 75%.

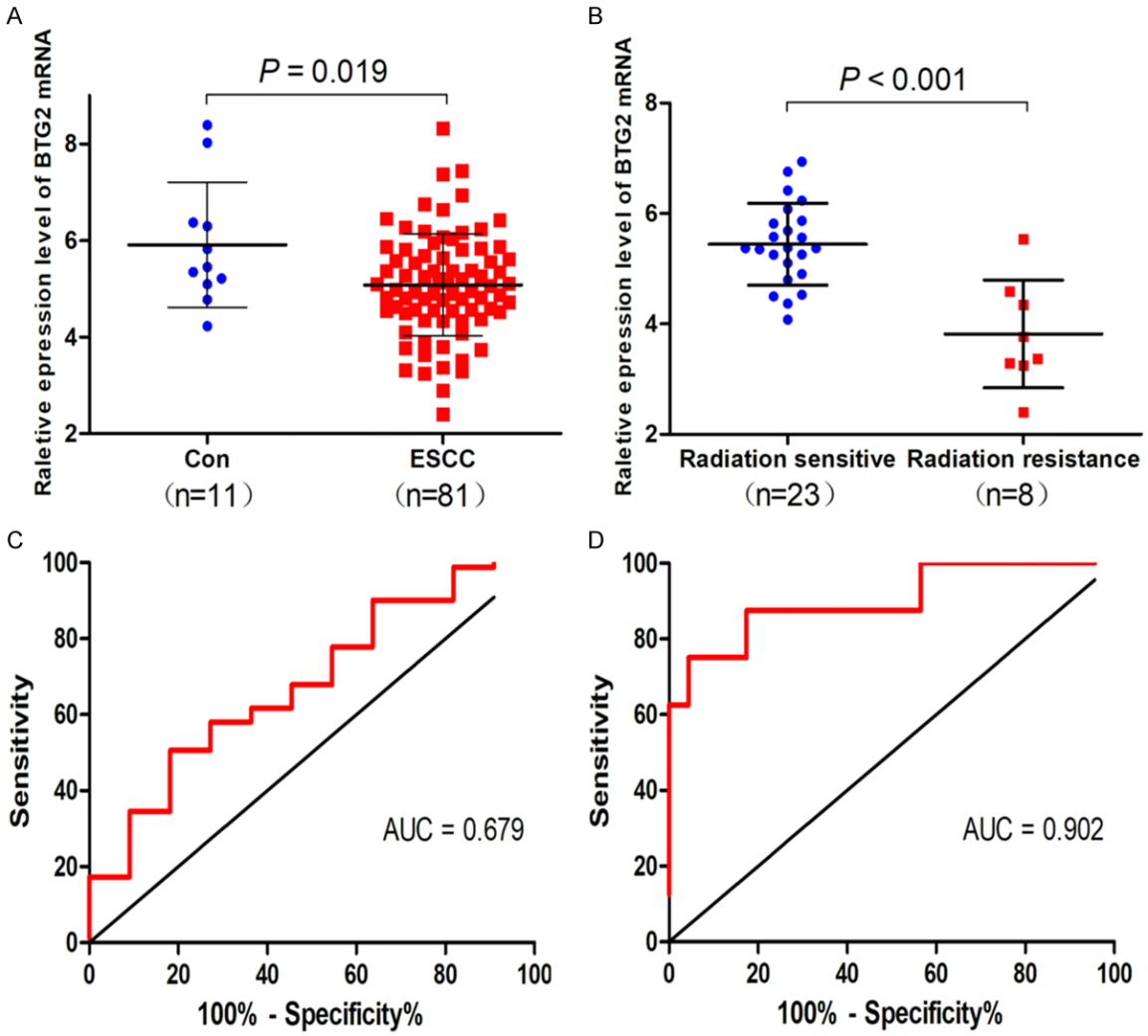

Figure 1. Tissue expression levels of BTG2 mRNA. A: Expression levels of BTG2 mRNA in ESCC patients and controls. B: Expression levels of BTG2 mRNA in radiotherapy sensitive group and radiotherapy resistant group. C: Receiver operating characteristic (ROC) curves for BTG2 mRNA in discriminating ESCC patients with controls. D: ROC curves for BTG2 mRNA in discriminating radiotherapy sensitive group from radiotherapy resistant group.

Protein expression of BTG2 in ESCC

Immunohistochemical staining results showed that there were 42 cases (84.0%) with positive BTG2 protein expression (**Figure 2A**) and 8 cases (16.0%) with negative BTG2 protein expression (**Figure 2B**) in 50 normal esophageal mucosal tissues. Further, among 184 ESCC tissues, 103 (56.0%) cases were positive for BTG2 expression (**Figure 2C**) and 81 (44.0%) cases were negative for BTG2 expression (**Figure 2D**). The positive expression rate of BTG2 in ESCC tissues was lower than that of normal esophageal mucosal tissues, and the difference was significant (x^2 = 13.10, P < 0.001).

Relation between BTG2 and the clinicopathologic features and radiotherapy sensitivity of ESCC patients

The relation between BTG2 protein expression and clinical disease characteristics of ESCC patients is shown in **Table 1**. There were no significant differences in BTG2 protein expression levels with age (x^2 = 3.240, P = 0.071), gender (x^2 = 1.708, P = 0.191), tumor location (x^2 = 2.254, P = 0.133), degree of differentiation (x^2 = 0.107, P = 0.744), T staging (x^2 = 0.135, P = 0.714), or M staging (x^2 = 1.210, P = 0.290) of ESCC patients, but it was correlated with N staging (x^2 = 4.134, P = 0.042) and clinical

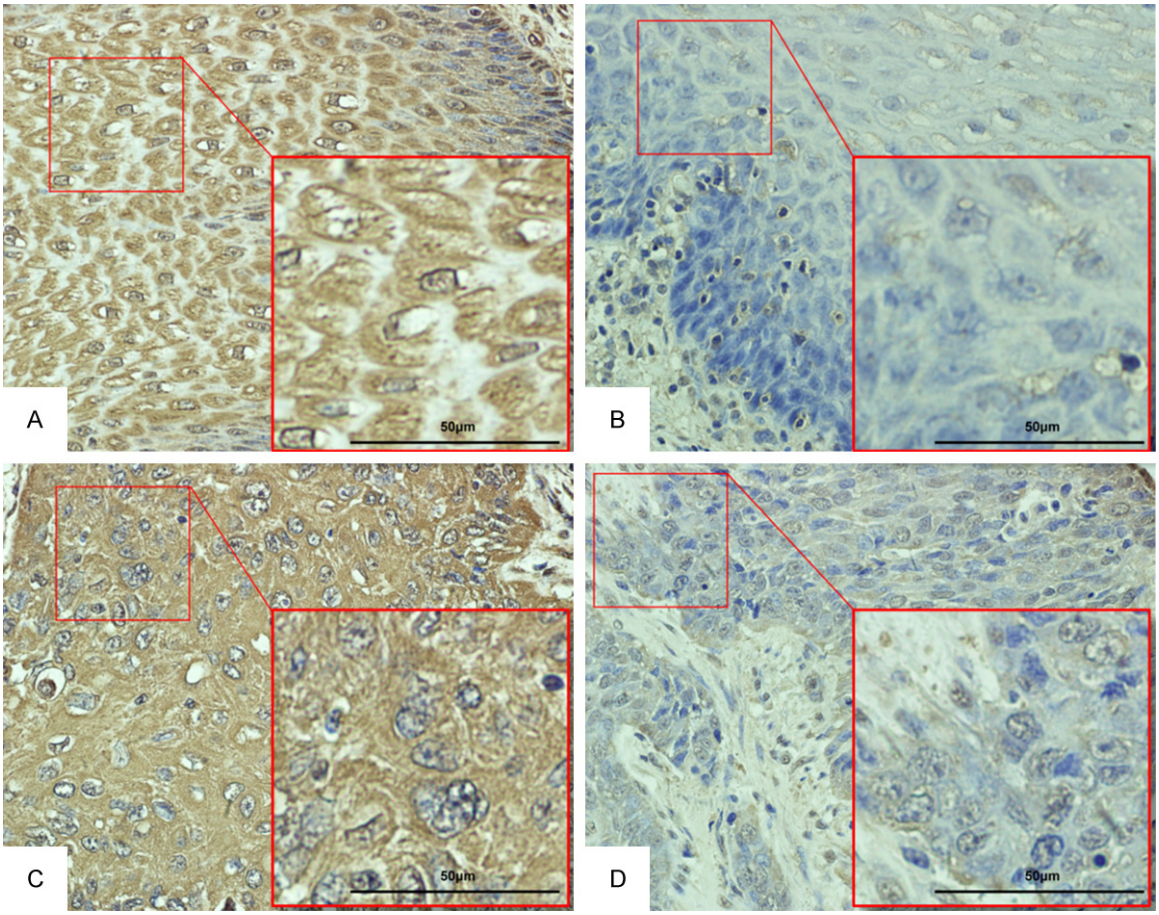

Figure 2. Protein expression of BTG2. A: The positive expression of BTG2 protein in normal tissue samples. B: The negative expression of BTG2 protein in normal tissue samples. C: The positive expression of BTG2 protein in ESCC samples. D: The negative expression of BTG2 protein in ESCC samples.

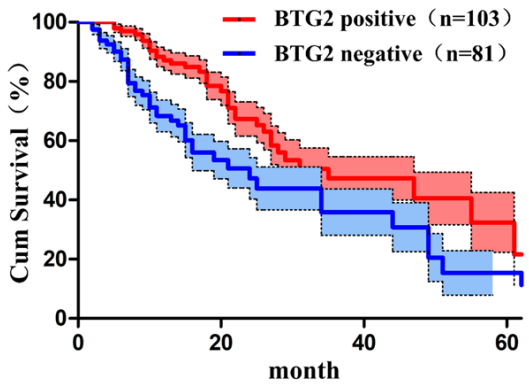

Figure 3. Overall survival (OS) curves of ESCC patients classified by positive and negative expression of BTG2 protein.

staging (x^2 = 5.565, P = 0.018). Among the 55 patients who had successfully completed radical radiotherapy, 22 patients were BTG2 positive and 16 patients were BTG2 negative in the radiotherapy sensitive group. However, in the radiation resistant group, BTG2 was positive in 4 cases and negative in 13 cases, with significant differences compared to the radiotherapy sensitive group (x^2 = 5.565, P = 0.018).

Association between survival outcome and BTG2 expression

The study was followed up for 2 to 73 months, and the median survival time for ESCC patients was 24.16 months. The overall survival rates at the first, third, and fifth year were 78.80%, 42.11% and 23.62%, respectively, with a median survival of 35 months. The survival rates of BTG2-positive patients at the first, third, and fifth year were 87.17%, 47.27% and 21.61%, respectively, while the survival rates of BTG2-negative patients at the first, third, and fifth year were 68.35%, 35.92%

Table 2. Univariate and multivariate analysis of overall survival (OS)

Item		univariate				multivariate		
		HR	95% CI	*P*	1	HR	95% CI	*P*
Gender	1	2.224	0.965~5.125	0.061	1	-		
Age	1	1.008	0.641~1.583	0.974	1	-		
Location	1	0.861	0.486~1.525	0.608	1	-		
Grade	1	0.500	0.317~0.790	0.003	1	0.613	0.381~0.987	0.044
T	1	0.643	0.404~1.023	0.063	1	-		
N	1	0.275	0.157~0.479	0.000	1	0.507	0.259~0.991	0.047
M	1	0.317	0.151~0.665	0.002	1	-		
Stage	1	0.269	0.167~0.434	0.000	1	0.504	0.278~0.916	0.025
BTG2	1	1.956	1.242~3.079	0.003	1	1.608	1.011~2.558	0.045

and 15.39%, respectively, with a median survival of 24 months. The survival curves of the BTG2-positive group and the BTG2-negative group were constructed by Kaplan-Meier method respectively (**Figure 3**). According to the logrank test, the cumulative survival rate of the BTG2-positive group was significantly higher than that of the BTG2-negative group (HR = 0.489, 95% CI: 0.305~0.782, *P* = 0.003).

Univariate analysis by Cox regression model revealed that the degree of tumor differentiation (HR = 0.5, 95% CI: 0.317-0.790, *P* = 0.003), N stage (HR = 0.275, 95% CI: 0.157~0.479, *P* < 0.001), M staging (HR = 0.489, 95% CI: 0.151~0.665, *P* = 0.002), clinical staging (HR = 0.269, 95% CI: 0.167~0.434, *P* < 0.001) and BTG2 expression (HR = 1.956, 95% CI: 1.242~3.079, *P* = 0.003) were closely correlated with patient prognosis (**Table 2**), while there were no significant differences in gender, age, or lesion site with patient survival rate (*P* > 0.05). Furthermore, significant variables by univariate analysis were incorporated into the multivariate analysis model. As shown in **Table 2**, Cox multivariate analysis confirmed that N stage (HR = 0.507, 95% CI: 0.259-0.991, *P* = 0.047), degree of tumor differentiation (HR = 0.613, 95% CI: 0.381-0.987, *P* = 0.044), clinical stage (HR = 0.504, 95% CI: 0.278-0.916, *P* = 0.025), and BTG2 expression (HR = 1.608, 95% CI: 1.011~2.558, *P* = 0.045) were independent prognostic risk factors for the overall survival rate of ESCC patients.

Discussion

Esophageal cancer is occurs in the esophageal mucosal epithelium under the action of multiple carcinogenic factors such as environment and genetics [15]. Genetic studies have shown that ESCC is a progressive pathologic process, caused by activation of a series of oncogenes and/or inactivation of tumor suppressor genes [16]. Although findings from previous molecular biology studies have improved the understanding of the pathogenesis of ESCC, its appropriate biomarkers or high-risk population screening, clinical diagnosis and prognosis have not yet been identified [7]. In the present study, we first used the TCGA database to analyze the differentially expressed mRNAs. The results showed that the expression of BTG2 mRNA in ESCC tissues was decreased markedly compared to normal tissues (5.08±1.06 vs 5.91±1.29, t = 2.387, *P* = 0.019), which preliminarily indicated that BTG2 might be involved in the occurrence of ESCC as a tumor suppressor gene. More importantly, since radiotherapy is a major approach for ESCC non-surgical treatment, tumor radiotherapy sensitivity is an important factor affecting the curative effect of radiotherapy. The tumor radiotherapy sensitivity is a complex and highly individualized problem. At present, there are no quantitative indicators to accurately determine the sensitivity of patients with esophageal cancer to radiotherapy or chemoradiotherapy. However, our results suggest that BTG2 mRNA expression level may predict radiotherapy sensitivity in ESCC patients, with a truncated value of 4.352 of BTG2 mRNA expression (specificity of 95.65%, sensitivity of 75%, AUC = 0.902).

Composed of 158 amino acid residues, BTG2 is originally defined as a transient early reactive protein and plays an important role in the development and progression of many cancers [17]. Previous research has shown that a low expres-

sion level of BTG2 in breast cancer [18], lung cancer [19], bladder cancer [20] or other cancer, is related to tumor grade, size, metastasis, recurrence and survival rate. Our study confirmed that, in addition to the transcription level, the expression level of BTG2 protein in normal esophageal mucosal tissues and ESCC tissues was significantly different, of which the positive rate of BTG2 protein expression in ESCC tissues (56.0%) was remarkably lower than that in normal esophageal mucosal tissues (84.0%). The relation between clinical data and BTG2 expression was further analyzed, and the results showed that the BTG2 protein expression level had no statistical significance with age, gender, tumor location, degree of differentiation, T stage, and M stage of ESCC patients, but was significantly correlated with N stage ($x^2 = 4.134$, $P = 0.042$) and clinical stage ($x^2 = 5.565$, $P = 0.018$). These results preliminarily indicate that BTG2 may be involved in the initiation and development of ESCC and the evolution of malignant biologic behaviors of ESCC cells.

In clinical practice, many ESCC patients have no indication for surgery or cannot tolerate surgery at the time of diagnosis. Therefore, radiotherapy with a high dose of ionizing radiation has become an important treatment method for esophageal cancer. However, some ESCC patients have poor therapeutic effect and cannot get benefits from it. Therefore, to predict and evaluate sensitivity to radiotherapy before radiotherapy may be of great significance in guiding clinicians to develop individualized treatment regimens. In addition to the function of tumor suppressor genes, BTG2 is also valued as a new ionizing radiation-induced gene. It has been shown that the expression of BTG2 can be increased when cells are treated with various factors which can cause DNA damage, such as ionizing rays, ultraviolet rays, and Adriamycin [21, 22]. As an important downstream gene of the p53 gene, the study has found that when the expression of p53 protein was increased, the expression of BTG2 was also up-regulated. Further study identified that p53 can protein activates the up-regulation of BTG2 during DNA damage, then BTG2 gene inhibits cyclin D1 transcription, thus reducing the expression level of cyclin D1 protein, and cells are blocked at G1 phase through pRb pathway [23]. In this study, we also found that there were 22 BTG2 positive cases and 16 negative cases in the radiotherapy sensitive group, while there were 4 BTG2 positive cases and 13 negative cases in the radiation-resistant group the difference was significant ($x^2 = 5.565$, $P < 0.05$), and the result was consistent with the biologic function of BTG2 in other cancers previously reported [24].

Considering the characteristics of BTG2 tumor suppressor gene and radiosensitive gene, univariate analysis and multivariate analysis of Cox regression model showed that the degree of tumor differentiation, N stage, M stage, clinical staging, and BTG2 level were closely related to the prognosis of patients. In addition, this study also showed that the expression level of BTG2 was an independent risk factor affecting the prognosis of ESCC and BTG2, and might be used as a new target for ESCC treatment. Regulation of BTG2 expression level and its mediated cell signaling pathway in ESCC is conducive to the discovery of novel drugs for the treatment of ESCC, and can provide theoretical reference for the diagnosis and prognosis assessment of ESCC. However, ESCC is a systemic multigene disease, and the exact role of BTG2 in ESCC is still unclear, and needs to be further clarified through ESCC cell lines and molecular biologic methods.

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Disclosure of conflict of interest

None.

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