

Expression and association of *CD44v6* with prognosis in T2-3N0M0 esophageal squamous cell carcinoma

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Aim: To investigate the expression of *CD44v6* in stage T2-3N0M0 esophageal squamous cell carcinoma (ESCC) and its prognostic significance.

Methods: The expression of *CD44v6* in a series of 227 ESCC specimens was evaluated by immunohistochemistry (IHC). A reproducible semiquantitative method which took both staining percentage and intensity into account was applied for IHC scoring, and receiver operating characteristic (ROC) curve analysis was utilized to select the cut-off score for high or low IHC reactivity. Then, the correlations of *CD44v6* expression with clinicopathological features of patients and its prognostic relevance were determined.

Results: In the present study, the proportion of low *CD44v6* expression was found significantly lower in Grade 3 of ESCC, than that of Grade 1 and Grade 2 of ESCC. There are no significant correlations between *CD44v6* expression and other clinicopathological parameters including gender, age, tumor size, tumor location, depth of invasion and pathological stage. The Kaplan-Meier survival curves showed that up-regulated expression of *CD44v6* indicated a poorer post-operative survival for ESCC patients of stage T2-3N0M0 (P=0.009), especially for those with T2 lesions (P=0.044) or with stage IIB diseases (P=0.005). Multivariate analysis also confirmed that *CD44v6* expression [relative risk, 1.639; 95% confidence interval (CI): 1.142-2.354, P=0.007] and depth of invasion (relative risk, 1.487; 95% CI: 1.063-2.080, P=0.020) were independent prognostic factors.

Conclusions: Elevated *CD44v6* expression may be an adverse prognostic indicator for patients with stage T2-3N0M0 ESCC, especially for those with T2 lesions or stage IIB diseases.

Keywords: Esophageal squamous cell carcinoma (ESCC); *CD44v6*; prognosis; receiver operating characteristic curve; immunohistochemistry (IHC)



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Introduction

Esophageal cancer is one of the most common lethal malignancies in the world (1-3), causing more than 400,000 deaths per year (4). In Asian countries, esophageal squamous cell carcinomas (ESCC) accounts for over 90% of esophageal cancers (5). Despite tremendous advances in diagnosis and multimodality therapy, the prognosis of ESCC remains poor and the overall 5-year survival

is still less than 15% (1,6). More and more evidence has revealed that classical staging criteria provide insufficient prognostic characterization of ESCC patients, and molecular tumor analysis may provide an effective and feasible means of better defining prognosis (7,8). Therefore, to further improve the survival rate in patients with ESCC, novel prognostic and molecular targets for therapeutic intervention are critically needed for patients with ESCC.

CD44 forms a family of cell surface proteins involved in cell migration, cell adhesion, tumor progression and metastatic spread (9-11). The CD44 gene is 50-60 kDa in size, located on chromosome 11p13, and is known to be composed of at least 20 exons. *CD44v6* is an important isoform of CD44 family (12-14). Related studies have shown that the expression of *CD44v6* correlates with tumor progression and metastasis development in several human malignancies, such as lung cancer, gastrointestinal cancer, breast cancer, and so on (10,13,15-17). However, reports on the relationship between *CD44v6* expression and prognosis are contradictory. Some authors have reported that the *CD44v6* is an independent prognostic factor (15,18), but others have failed to draw a similar conclusion (19,20). Therefore, we examined the expression of *CD44v6* in T2-3N0M0 ESCC to elucidate its significance in clinical prognosis.

Materials and methods

Patients and tissues

The study was approved by the Research Ethics Committee of the Cancer Center of Sun Yat-Sen University. From January 1993 to August 2004, we enrolled 227 consecutive patients with stage IB-IIIB node-negative ESCC who received surgical treatment with curative intent, and the resected specimens were assessed for *CD44v6* expression level via immunohistochemistry (IHC). Patients were followed prospectively and their survival data was recorded through October 2009. Patients were included in this study based on the following eligibility criteria: (I) histopathologically proven ESCC; (II) disease stage of T2-3N0M0 based on the seventh edition of the American Joint Committee on Cancer (AJCC) staging system for esophageal cancer (21); (III) at least 15 lymph nodes to be removed for pathological evaluation; (IV) age at least 18 years; (V) no evidence of metastatic disease as determined by history, physical examination, and blood chemistry analysis or routine computed tomography; and (VI) no history of adjuvant therapy. Patients were excluded based on the following criteria: (I) history of previously treated cancer other than basal or squamous cell carcinoma of the skin; (II) history of preoperative chemotherapy and/or radiotherapy; (III) unknown cause of death in follow-up.

Immunohistochemistry (IHC)

Immunoperoxidase stain for *CD44v6* (1:50 dilution;

Novocastra Laboratories, Ltd., Newcastle upon Tyne, United Kingdom) was done on 4- μ m-thick paraffin sections. The slides were deparaffinized in xylene then hydrated prior to antigen retrieval by microwaving in sodium citrate buffer (pH 6.0). The slides were then incubated with a peroxidase block and then the primary antibody. After a PBS wash, the slides were incubated first with the secondary antibody and then with 3,3'-diaminobenzidine, then counterstained with hematoxylin (Hematoxylin 7211; Richard-Allen Scientific, Kalamazoo, Michigan, United States of America). The peroxidase block, secondary antibody and 3,3'-diaminobenzidine were all obtained from the DakoCytomation EnVision System (Glostrup, Denmark). After a hematoxylin counterstain (Hematoxylin 7211; Richard-Allen Scientific, Kalamazoo, MI), the slides were coverslipped.

Immunohistochemical scoring

The immunohistochemical scoring of *CD44v6* was performed using a semiquantitative system as demonstrated previously (7,15,22). Each slide was assigned a score: the score of tumor cell staining multiplied by the score of staining intensity. Tumor cell staining was assigned a score using a semiquantitative six-category grading system: 0, no tumor cell staining; 1, 1% to 10% of tumor cells staining; 2, 11% to 25% of tumor cells staining; 3, 26% to 50% of tumor cells staining; 4, 51% to 75% of tumor cells staining; 5, more than 75% of tumor cells staining. Stain intensity was assigned a score using a semiquantitative four-category grading system: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. Two experienced pathologists independently scored 400 ESCC samples including the cases used in this study blinded to clinical follow-up data. The complete score agreement between these two pathologists is 87% of cases, indicating that the scoring method was reasonably reproducible. A third blinded pathologist intervened and evaluated cases with different IHC scores. If the third pathologist agreed with one of the previous scorings, that score was used for analysis. For the cases in which three different scores were obtained, the three pathologists were asked to reach agreement on the case.

Selection of cut-off score

The cut-off scores for *CD44v6* expression were selected based on receiver operating characteristic (ROC) curve

Table 1 Characteristics of esophageal squamous cell carcinoma (ESCC) patients and tumors

Characteristic	Patients with ESCC (N=227)
Sex (%)	
Male	165 (72.7)
Female	62 (27.3)
Age (years)	
Median	58
Range	33-77
Tumor size (cm)	4.92±2.046
Tumor grade (%)	
Grade 1	57 (25.1)
Grade 2	114 (50.2)
Grade 3	56 (24.7)
Tumor location (%)	
Upper	25 (11.0)
Middle	152 (67.0)
Lower	50 (22.0)
Depth of invasion (%)	
T2	88 (38.8)
T3	139 (61.2)
AJCC staging system (7 th edition) (%)	
IB	12 (5.3)
IIA	83 (36.6)
IIB	132 (58.1)

AJCC, American Joint Committee on Cancer.

analysis. The sensitivity and specificity for the outcome under study were plotted, thus generating a ROC curve. The score closest to the point with both maximum sensitivity and specificity [i.e., the point (0.0, 1.0) on the curve] was selected as the cut-off score leading to the greatest number of tumors classified correctly as having or not having the clinical outcome. The area under the ROC curve (AUC) was calculated to estimate the discriminatory power of *CD44v6* over the entire range of scores for overall survival. Both generation and analysis of the ROC curve were performed by a MedCalc statistical software package 11.0.1 (MedCalc Software bvba, Belgium).

Statistical analysis

Associations between categorical variables were analyzed using the Chi-square test. Survival curves were calculated

by the Kaplan-Meier method and were compared by the log-rank test. Time to event (death) was calculated from date of surgery to date of event. In event-free subjects, the time variable was censored at date of last follow-up. Multivariate analysis of prognostic factors was performed using Cox's regression model. A significant difference was defined as a two-tailed P-value of less than 0.05. All of the statistical analyses were performed using the SPSS 13.0 for Windows software system (SPSS Inc, Chicago, IL, USA).

Results

Characteristics of all patients and expression of *CD44v6*

Table 1 provided all the patients' clinicopathological features. Among the 227 patients included in this study, 162 (72.7%) were male patients and 62 (27.3%) were female patients. The median age was 58 years (range from 33 to 77 years). In cancer cells, it was generally the cytoplasm and membrane that revealed various intensity of positive reaction for *CD44v6* (*Figure 1*). According to the ROC curve in our study (*Figure 2*), threshold value of 14 was the closest to the point with both maximum sensitivity and specificity, and thereby selected as the cut-off score. The AUC of our ROC curve analysis was 0.550 [95% confidence interval (CI): 0.483-0.616]. Patients were thus divided into two groups, namely high expression group (n=50) and low expression group (n=177).

Correlation between the expression of *CD44v6* and clinicopathological features

The association between *CD44v6* expression and clinicopathological variables of ESCC patients is shown in *Table 2*. Low *CD44v6* expression was found in 66.1% of Grade 3 of ESCC, significantly lower than that of Grade 1 and Grade 2 of ESCC. There are no significant correlations between *CD44v6* expression and other clinicopathological parameters including gender, age, tumor size, tumor location, depth of invasion and pathological stage based on the seventh edition of AJCC staging system (21).

CD44v6 expression and survival

At the time of data analysis (October 2009), with a median follow-up of 32 months (range, 5-138 months), 72 patients (31.7%) remained alive and 155 patients (68.3%) died from cancer-related causes. The 1-, 3- and 5-year overall survival

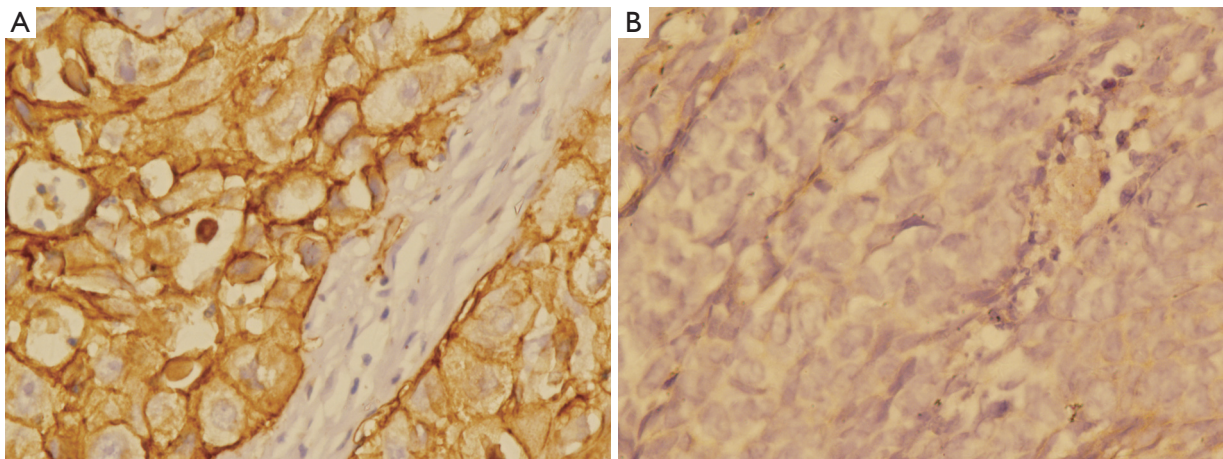


Figure 1 Expression of *CD44v6* in esophageal squamous cell carcinoma (ESCC) tissue (Envision, $\times 400$). (A) Immunohistochemical staining of *CD44v6* in cytoplasm and membrane (IHC score: 15); (B) Immunohistochemical staining of *CD44v6* in cytoplasm (IHC score: 3). IHC, immunohistochemistry.

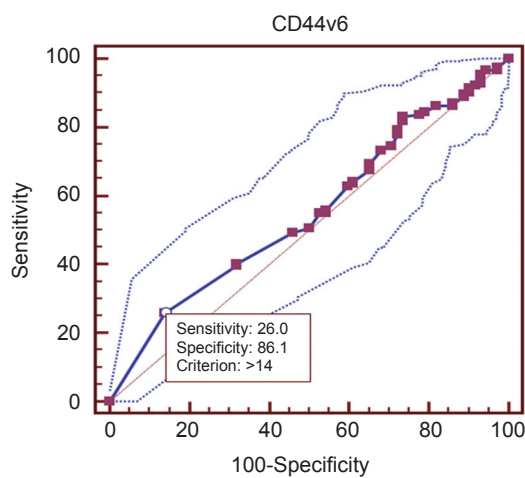


Figure 2 Receiver operating characteristic (ROC) curve analysis for *CD44v6* and the selection of cut-off score.

probabilities for the entire group were 58%, 39% and 33%, respectively.

The Kaplan-Meier survival curves (Figure 3) showed that patients with low *CD44v6* expression experienced significantly better post-operative survival than those with high *CD44v6* expression ($P=0.009$). In further stratified analysis split by depth of invasion (Figure 4), *CD44v6* expression had a statistically significant influence on the survival of patients with T2 diseases ($P=0.044$) rather than on the survival of those with T3 lesions ($P=0.056$). Furthermore, the stratified analysis split by pathological stage based on the new staging system (Figure 5) revealed

that the prognostic significance of *CD44v6* expression remained on the survival of patients with stage IIB ESCC ($P=0.005$) but not on the survival of those with IIA diseases ($P=0.421$). Patients with stage IB ESCC were not included in this analysis in view of the small sample size.

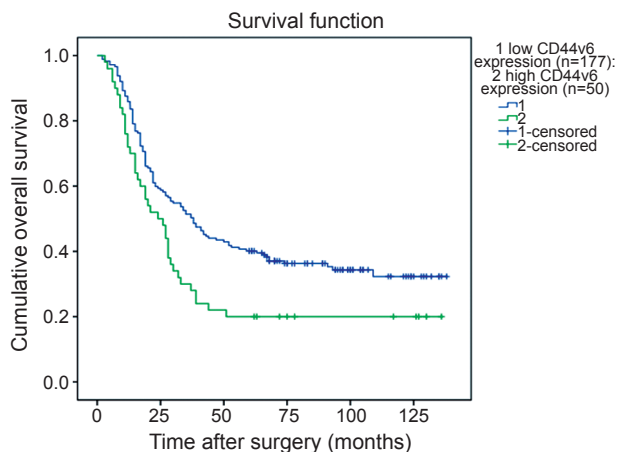
We performed analysis using the Cox proportional hazards model to identify factors involved in OS of T2-3N0M0 ESCC patients (Table 3). The univariate analysis showed that depth of invasion and *CD44v6* expression were significant prognostic indicators for OS, and thereby selected as the parameters to be included in the same Cox regression model. Further multivariate analysis also confirmed that *CD44v6* expression (relative risk, 1.639; 95% CI: 1.142-2.354, $P=0.007$) and depth of invasion (relative risk, 1.487; 95% CI: 1.063-2.080, $P=0.020$) were independent prognostic factors.

Discussion

Numerous studies have been performed in order to elucidate the role of *CD44v6* in ESCC; however, data for this prognostic marker in ESCC are quite controversial (15,18-20). One problem faced by researchers is the determination of the extent of tumor IHC positivity for *CD44v6* which is clinically and biologically relevant. Several previous studies had applied different scoring systems and predetermined cut-off scores which might be set arbitrarily. This may primarily be responsible for the contradictory results of these studies evaluating *CD44v6* and its prognostic

Table 2 Correlation of *CD44v6* with clinicopathological variables

	n	<i>CD44v6</i>		P-value
		Low n (%)	High n (%)	
Sex				
Male	165	127 (77.0)	38 (23.0)	0.552
Female	62	50 (80.6)	12 (19.4)	
Age				
≤60 years	138	104 (75.4)	34 (24.6)	0.237
>60 years	89	73 (82.0)	16 (18.0)	
Tumor size (cm)				
≤5.0	155	123 (79.4)	32 (20.6)	0.461
>5.0	72	54 (75.0)	18 (25.0)	
Tumor grade				
Grade 1	57	48 (84.2)	9 (15.8)	0.041 ^a
Grade 2	114	92 (80.7)	22 (19.3)	
Grade 3	56	37 (66.1)	19 (33.9)	
Tumor location				
Upper	25	15 (60.0)	10 (40.0)	0.067
Middle	152	121 (79.6)	31 (20.4)	
Lower	50	41 (82.0)	9 (18.0)	
Depth of invasion				
T2	88	67 (76.1)	21 (23.9)	0.595
T3	139	110 (79.1)	29 (20.9)	
AJCC staging system (7th edition)				
IB	12	12 (100.0)	0 (0)	0.152
IIA	83	65 (78.3)	18 (21.7)	
IIB	132	100 (75.8)	32 (24.2)	

AJCC, American Joint Committee on Cancer; ^aP<0.05.**Figure 3** Kaplan-Meier survival function for patients with T2-3N0M0 esophageal squamous cell carcinoma (ESCC) according to *CD44v6* expression.

value in ESCC. Therefore, our study utilized a reproducible scoring method which took both staining percentage and intensity into account, and selected the cut-off score based on ROC curve analysis for *CD44v6* expression evaluation, where the score closest to the point with both maximum sensitivity and specificity was chosen. This method allowed the greatest number of tumors to be correctly classified as carrying or not carrying the clinical outcome (23). In addition, all patients contained in our series were all free of lymph node metastasis and received no other therapy before or after the resection.

In the present study of T2-3N0M0 ESCC patients, high expression of *CD44v6* was significantly correlated with the worse post-operative survival rate, both by univariate and multivariate analysis, which is consistent with the reported findings. Nozoe *et al.* (24) reported that the over-expression

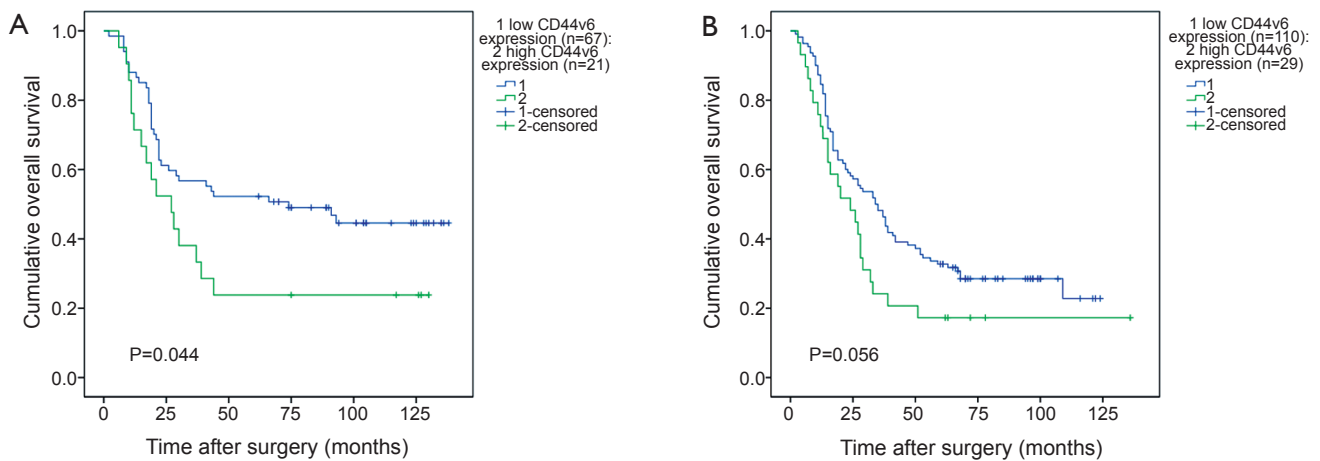


Figure 4 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma (ESCC) stratified for depth of invasion according to *CD44v6* expression. (A) Correlation between *CD44v6* expression and post-operative survival of patients with T2 lesions; (B) Correlation between *CD44v6* expression and post-operative survival of patients with T3 lesions.

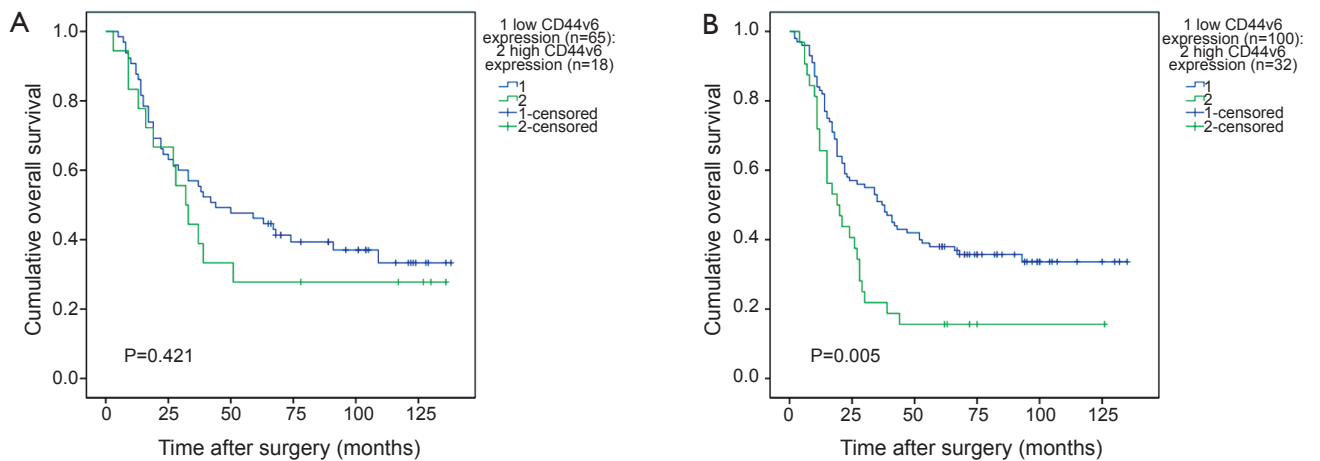


Figure 5 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma (ESCC) stratified for pathological stage according to *CD44v6* expression. (A) Correlation between *CD44v6* expression and post-operative survival of patients with IIA diseases; (B) Correlation between *CD44v6* expression and post-operative survival of patients with IIB diseases.

level of *CD44v6* in ESCC patients is significantly correlated with the higher proportions of the incidence of lymph node metastasis, lymphatic permeation, blood vessel invasion, more advanced stage and therefore poorer prognosis. Similarly, Shen *et al.* (25) received a comparable conclusion that *CD44v6* expression in ESCC was significantly higher than that in adjacent normal tissue and was correlated with distant metastasis and TNM stage.

It has been known that well differentiation of ESCC which is deeply related to apoptosis to the tumor cells is likely to influence the less invasive potential of ESCC (26,27). Several investigations suggest the correlation of *CD44v6* with

the histologic grade in SCC of the head and neck (28,29). In our study, we draw a similar conclusion that the proportion of low *CD44v6* expression was found significantly lower in Grade 3 of ESCC, than that of Grade 1 and Grade 2 of ESCC, which was consistent with Shen *et al.* (25).

Moreover, *CD44v6* expression has been reported to be an indicator to predict the sensitivity to neoadjuvant chemotherapy in cervical carcinoma (30); hence, an assessment of an IHC *CD44v6* expression might possibly be an auxiliary information regarding the response of ESCC to the adjuvant therapies. Also, Koyama *et al.* (31) reported that the expression of *CD44v6* was significantly reduced

Table 3 Univariate and multivariate overall survival analysis of ESCC patients by Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age						
≤60 vs. >60 yrs	1.023	0.741-1.414	0.889			
Gender						
Male vs. female	0.691	0.474-1.008	0.055			
Tumor size						
≤5 vs. >5 cm	0.987	0.704-1.386	0.941			
Grade						
G1 vs. G2&3	1.145	0.788-1.664	0.477			
Tumor location						
Upper & middle vs. lower	0.953	0.657-1.384	0.802			
Depth of invasion						
T2 vs. T3	1.462	1.046-2.044	0.026 ^a	1.487	1.063-2.080	0.020 ^a
AJCC staging system (7 th edition)						
IB & IIA vs. IIB	1.193	0.865-1.644	0.282			
<i>CD44v6</i>						
Low vs. high	1.608	1.120-2.308	0.010 ^a	1.639	1.142-2.354	0.007 ^a

AJCC, American Joint Committee on Cancer; CI, confidence interval; ^aP<0.05.

in the irradiated primary ESCC, which might imply that *CD44v6* could be a factor on the response of ESCC to radiotherapies.

In conclusion, the study elucidated that *CD44v6* might be correlated with the histologic grade and its high expression may be a potential worse prognosis indicator for the patients with T2-3N0M0 ESCC, especially for the patients with T2 lesions or stage IIB diseases. However, there are still many unanswered questions regarding *CD44v6* expression in ESCC. Additional studies with randomization and longer follow-up are needed for the establishment of more effective management plan, which will aid in improving prognosis.

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