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ORIGINAL ARTICLE

Prognostic relevance of β-*catenin* expression in T2-3N0M0 esophageal squamous cell carcinoma

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Abstract

AIM: To study the expression of β-*catenin* in esophageal squamous cell carcinoma (ESCC) at stage T2-3N0M0 and its relation with the prognosis of ESCC patients.

METHODS: Expression of β-*catenin* in 227 ESCC specimens was detected by immunohistochemistry (IHC). A reproducible semi-quantitative method which takes both staining percentage and intensity into account was applied in IHC scoring, and receiver operating characteristic curve analysis was used to select the cut-off score for high or low IHC reactivity. Then, correlation of β-*catenin* expression with clinicopathological features and prognosis of ESCC patients was determined.

RESULTS: No significant correlation was observed between β-*catenin* expression and clinicopathological parameters in terms of gender, age, tumor size, tumor grade, tumor location, depth of invasion and pathologi-cal stage. The Kaplan-Meier survival curve showed that the up-regulated expression of β-*catenin* indicated a poorer post-operative survival rate of ESCC patients at stage T2-3N0M0 (*P* = 0.004), especially of those with T3 lesions (*P* = 0.014) or with stage ⅡB diseases (*P* = 0.007). Multivariate analysis also confirmed that β-*catenin* was an independent prognostic factor for the overall survival rate of ESCC patients at stage T2-3N0M0 (relative risk = 1.642, 95% CI: 1.159-2.327, *P* = 0.005).

CONCLUSION: Elevated β-*catenin* expression level may be an adverse indicator for the prognosis of ESCC patients at stage T2-3N0M0, especially for those with T3 lesions or stage ⅡB diseases.

INTRODUCTION

Esophageal carcinoma, an aggressive tumor with a poor prognosis, is one of the most common malignant tumors in Asia, especially in certain areas of China, and South

America[1]. Esophageal squamous cell carcinoma (ESCC) accounts for over 90% of all esophageal cancers worldwide[2]. Despite advances in imaging technologies enabling earlier diagnosis of ESCC, surgery and response rates of radiotherapy and chemotherapy, the clinical outcome of ESCC patients remains unsatisfactory. Even in the developed world, more than 85% of ESCC patients die within 2 years after its diagnosis[3]. In China, the esophageal cancer mortality rate ranks fourth of cancer-related deaths[4]. Thus, improvement in the efficacy of ESCC treatment is a major public health goal. New modalities based on a better understanding of the ESCC biology are indispensable. Since the classical staging criteria fail to differentiate the prognostic characteristics of ESCC patients adequately, molecular tumor analysis may provide a necessary means for defining the prognosis of ESCC patients. In order to further improve the survival rate of ESCC patients, it is essential to identify the relevant biomarkers with adverse prognostic significance, and to modify the therapeutic strategies for individual patients according to their molecular tumor status.

β-catenin is an 88kDa versatile protein that has at least two different cellular functions[5-7]. First, *β-catenin* is an important structural component of both normal epithelium and malignant cells. Together with a structurally homologous *γ-catenin*, *β-catenin* participates in cell-cell and cell-matrix adhesion by binding to the intracellular domain of *E-cadherin*, a homotypic cell-to-cell interaction molecule ubiquitously expressed in epithelial cells[6,8,9]. In addition, these catenins play an important role in cell polarity by binding to the actin filament network of cytoskeleton through *a-catenin* as a linker[6,8]. Unlike other known catenins, *β-catenin* is also a key mediator in the Wingless/Wnt signal transduction pathway[7,8,10]. In cytoplasm of normal cells, the amount and location of *β-catenin* are controlled exquisitely through its association with the adenomatous polyposis coli (*APC*) tumor-suppressor gene product, a scaffolding protein *Axin*, and a glycogen synthetase kinase (*GSK-3β*) enabling phosphorylation and degradation of free *β-catenin*[6-10]. Once located in nuclei, *β-catenin* can act as a transcription factor by serving as a coactivator of the lymphoid enhancer factor/TCF family of DNA-binding proteins[10]. Activation of Wnt signaling involves the inhibition of catenin degradation by proteasomes, resulting in its cytoplasmic and nuclear accumulation and transcriptional activation of the target gene[5-7,10]. It is believed that *β-catenin* integrity impairment -related intracellular network may be closely associated with the dedifferentiation, hyperproliferation, invasion and metastatic potential of malignancy[10,11]. This biomarker has thereby been extensively studied in a variety of neoplasms, such as hepatocellular carcinoma[12], colorectal carcinoma[13], gastric cancer[14,15], pancreatic cancer[16], ovarian cancer[17], lung cancer[18-20], breast cancer[21], nasopharyngeal carcinoma[22], prostate cancer[23] and even lymphoma[24], with regard to its potential role as a prognostic factor in cell polarity. The present findings in ESCC are controversial in the literature[25-28].

The role of *β-catenin* in development of ESCC and its prognostic significance remain to be defined. In the present study, the expression level of *β-catenin* was measured in specimens from a relatively homogeneous cohort of ESCC patients with no lymph node involved, which was correlated with the clinical outcome of ESCC patients.

MATERIALS AND METHODS

Patients and tissue samples
The study was approved by the Ethics Committee of Sun Yat-Sen University Cancer Center. A total of 227 consecutive patients with node-negative ESCC at stage I B-II B who underwent curative surgery from January 1993 to August 2004 were enrolled in this study. *β-catenin* expression level was measured in resected specimens with immunohistochemistry (IHC). Patients were followed up prospectively and their survival data were recorded through October 2009. The inclusion criteria were patients with histopathologically-proven ESCC, those with esophageal cancer at T2-3N0M0 based on the seventh edition of the American Joint Committee on Cancer staging system[29], those with at least 15 lymph nodes to be removed for pathological evaluation, those at the age of at least 18 years, those with no evidence of metastatic disease as determined by history, physical examination, and blood chemistry analysis or routine computed tomography, those with no history of adjuvant therapy. Patients with a history of previously treated cancer other than basal or squamous cell carcinoma of the skin, preoperative chemotherapy and/or radiotherapy, or with unknown causes of death in follow-up were excluded from the study.

Immunohistochemistry
β-catenin (CAT-5H10, Fuzhou Maxim Inc., Fuzhou, Fujian province, China) was diluted at 1:100. ESCC tissue was cut into 4 μm-thick paraffin sections, which were stained with immunoperoxidase. The sections were deparaffinized in xylene, hydrated prior to antigen retrieval by microwaving in sodium citrate buffer (pH 6.0), and incubated with a peroxidase block followed by primary antibody. After washed with PBS, the sections were incubated first with secondary antibody followed by 3,3'-diaminobenzidine, and then counterstained with hematoxylin (Hematoxylin 7211; Richard-Allen Scientific, Kalamazoo, Michigan, USA). The peroxidase block, secondary antibody and 3,3'-diaminobenzidine were all obtained from the Dako-Cytomation EnVision System (Glostrup, Denmark).

Immunohistochemical scoring
β-catenin was scored with IHC using a semi-quantitative system as previously described[30,31]. Each section was assigned a score and the score of tumor cell staining was multiplied by the score of staining intensity. Tumor cell staining was scored using a semi-quantitative six-category grading system: 0 = no tumor cell staining, 1 = 1%-10% of tumor cells staining, 2 = 11%-25% of tumor cells staining, 3 =

26%-50% of tumor cells staining, 4 = 51%-75% of tumor cells staining, 5 = over 75% of tumor cells staining. Stain intensity was scored using a semi-quantitative four-category grading system: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. Two experienced pathologists blinded to the clinical follow-up data independently scored the 400 ESCC samples including the cases used in this study. The complete score agreement of the two pathologists was 87% of all cases, indicating that the scoring method is reasonably reproducible. A third blinded pathologist intervened and evaluated the patients with different IHC scores. If the third pathologist agreed with one of the previous scores, it was used for analysis. The three pathologists were asked to reach an agreement on the cases from which three different scores were obtained.

Selection of cut-off scores

Cut-off scores for *β-catenin* expression were selected based on receiver operating characteristic (ROC) curve analysis. ROC curve was plotted for the outcome of ESCC patients under study by calculating the sensitivity and specificity on its points. The score closest to the points (0.0, 1.0) on the curve with a maximum sensitivity and specificity was selected as the cut-off score leading to the greatest number of tumors classified with or without clinical outcome. The area under the ROC curve was calculated to estimate the discriminatory power of *β-catenin* over the entire range of scores for overall survival (OS) rate of ESCC patients. The ROC curve was generated and analyzed using the MedCalc statistical software package 11.0.1 (MedCalc Software bvba, Belgium).

Statistical analysis

Association between categorical variables was analyzed by χ^2 test. Survival curves were calculated with the Kaplan-Meier method and compared by the log-rank test. Time of death was calculated from the date of surgery to the date of death. The time variable was censored on the date of last follow-up of event-free subjects. Multivariate analysis of prognostic factors was performed using the Cox's regression model. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS 13.0 for Windows software system (SPSS Inc., Chicago, IL).

RESULTS

Characteristics of patients and expression of β-catenin

The demographic and clinicopathological parameters of ESCC patients included in this study are listed in Table 1. Various intensities of positive *β-catenin* reaction were detected in cytoplasm and membrane of cancer cells (Figure 1). According to the ROC curves for OS rate, a threshold value of 1.3333 was the optimal point for maximum sensitivity and specificity, and selected as the cut-off score (Figure 2). The 227 ESCC specimens were then catego-

Table 1 Demographic and clinicopathological parameters of esophageal squamous cell carcinoma patients included in this study n (%)

Demographic and clinicopathological parameters	Patients with ESCC
Sex	
Male	165 (72.7)
Female	62 (27.3)
Age (yr)	
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 (7th ed)	
I B	12 (5.3)
II A	83 (36.6)
II B	132 (58.1)

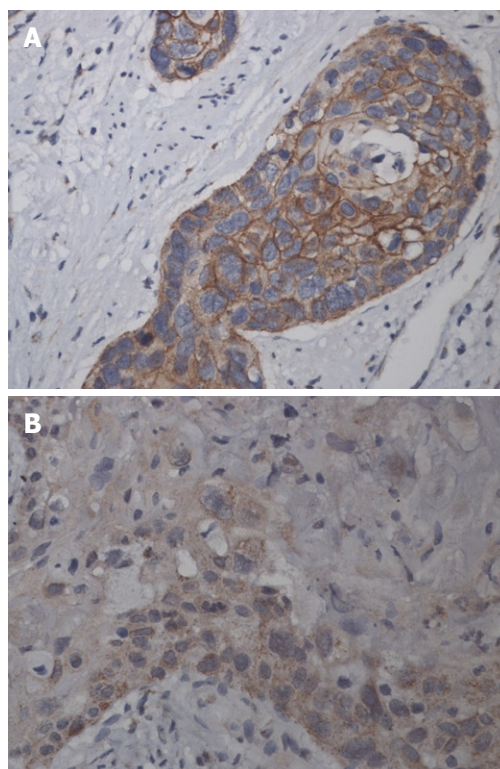ESCC: Esophageal squamous cell carcinoma; AJCC: American Joint Committee on Cancer.

Figure 1 Expression of *β-catenin* in esophageal squamous cell carcinoma tissue samples (magnification × 400). A: Immunohistochemical staining of *β-catenin* in cytoplasm and membrane of cancer cells (immunohistochemistry score: 15); B: Immunohistochemical staining of *β-catenin* in cytoplasm of cancer cells (immunohistochemistry score: 3).

Table 2 *β-catenin* expression in esophageal squamous cell carcinoma patients *n* (%)

Parameters of ESCC patients	*n*	Expression of *β-catenin*		*P*-value
		Low	High	
Sex				
Male	165	57 (34.5)	108 (65.5)	0.354
Female	62	26 (41.9)	36 (58.1)	
Age (yr)				
≤ 60	138	49 (35.5)	89 (64.5)	0.778
> 60	89	34 (38.2)	55 (61.8)	
Tumor size (cm)				
≤ 5.0	155	54 (34.8)	101 (65.2)	0.461
> 5.0	72	29 (40.3)	43 (59.7)	
Tumor grade				
Grade 1	57	17 (29.8)	40 (70.2)	
Grade 2	114	44 (38.6)	70 (61.4)	0.473
Grade 3	56	22 (39.3)	34 (60.7)	
Tumor location				
Upper	25	11 (44.0)	14 (56.0)	
Middle	152	52 (34.2)	100 (65.8)	0.545
Lower	50	20 (40.0)	30 (60.0)	
Depth of invasion				
T2	88	34 (38.6)	54 (61.4)	0.672
T3	139	49 (35.3)	90 (64.7)	
AJCC staging system (7th ed)				
Ⅰ B	12	3 (25.0)	9 (75.0)	
Ⅱ A	83	31 (37.3)	52 (62.7)	0.694
Ⅱ B	132	49 (37.1)	83 (62.9)	

ESCC: Esophageal squamous cell carcinoma; AJCC: American Joint Committee on Cancer.

Figure 2 Receiver operating characteristic curve analysis of *β-catenin* and selection of cut-off score.

Sensitivity: 71.0
Specificity: 52.8
Criterion > 1.333
AUC: 0.597
P = 0.0138

Figure 3 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma at stage T2-3N0M0 according to *β-catenin* expression.

rized into high and low *β-catenin* expression groups. The expression level of *β-catenin* was up-regulated in 144 cases (63.4%) and down-regulated in 83 cases (36.6%).

Correlation between β-catenin expression and clinicopathological features

The correlation between *β-catenin* expression in and clinicopathological features of ESCC patients are shown in Table 2. No significant correlation was identified between *β-catenin* expression and any clinicopathological parameters, including gender, age, tumor size, tumor grade, tumor location, depth of invasion and pathological stage based on the seventh edition of AJCC staging system[29].

β-catenin expression and survival rate

At the time of data analysis (October 2009), 72 patients (31.7%), with a median follow-up time of 32 mo (range 5-138 mo), remained alive and 155 patients (68.3%) died. The overall 1-, 3- and 5-year survival rates for the patients were 58%, 39%, and 33%, respectively.

The Kaplan-Meier survival curves (Figure 3) showed that the post-operative survival rate of patients with a low *β-catenin* expression level was significantly higher than that of those with a high *β-catenin* expression level (*P* = 0.004). Further stratified analysis split by depth of invasion (Figure 4) showed that the expression of *β-catenin* had a statistically significant influence on the survival rate of patients with T3 diseases (*P* = 0.014) rather than on the survival rate of
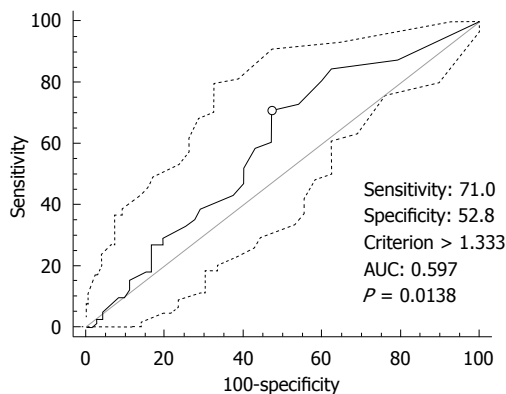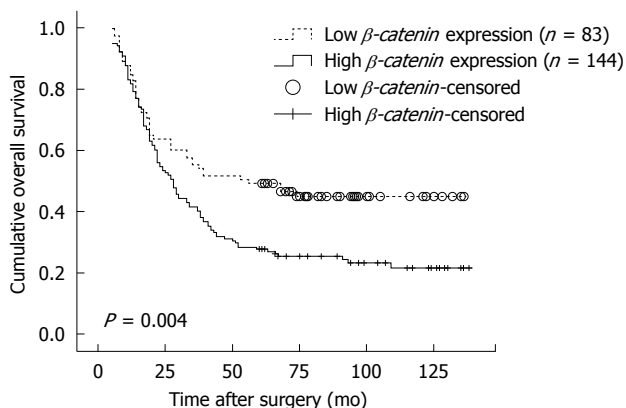
those with T2 lesions (*P* = 0.145). Furthermore, the stratified analysis split by pathological stage based on the new staging system (Figure 5) revealed that *β-catenin* expression had a significant influence on the prognosis of patients with ESCC at stage Ⅱ B (*P* = 0.007) but not on the prognosis of those with Ⅱ A diseases (*P* = 0.253). Patients with ESCC at stage Ⅰ B were not included in this analysis due to a small sample size.

Factors involved in OS rate of ESCC patients were identified using the Cox proportional hazards model (Table 3). Univariate analysis showed that tumor grade, depth of invasion and *β-catenin* expression were found to be the significant prognostic indicators for the OS rate of ESCC patients, and thereby selected as the parameters to be included in the same Cox regression model. Further multivariate analysis also confirmed that *β-catenin* expression (relative risk = 1.642, 95% CI: 1.159-2.327, *P* = 0.005), tumor grade (relative risk = 1.549, 95% CI: 1.095-2.190, *P* = 0.013) and depth of invasion (relative risk = 1.493, 95% CI: 1.066-2.089, *P* = 0.020) were the independent prognostic factors for the OS rate of ESCC patients.

DISCUSSION

To date, several IHC studies have been performed in order

Table 3 Univariate and multivariate analysis of overall survival rate of esophageal squamous cell carcinoma patients with Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Age (yr)						
≤ 60 *vs* > 60	1.023	0.740-1.414	0.889			
Gender						
Male *vs* female	0.692	0.474-1.009	0.056			
Tumor size (cm)						
≤ 5 *vs* > 5	0.987	0.704-1.386	0.942			
Grade						
G1 *vs* G2 and 3	1.498	1.060-2.116	0.022[a]	1.549	1.095-2.190	0.013[a]
Tumor location						
Upper and middle *vs* lower	0.954	0.657-1.385	0.804			
Depth of invasion						
T2 *vs* T3	1.461	1.045-2.043	0.026[a]	1.493	1.066-2.089	0.020[a]
AJCC staging system (7th ed)						
ⅠB and ⅡA *vs* ⅡB	1.192	0.865-1.643	0.284			
β-catenin						
Low *vs* high	1.644	1.161-2.329	0.005[b]	1.642	1.159-2.327	0.005[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* univariate analysis. AJCC: American Joint Committee on Cancer; CI: Confidence interval.
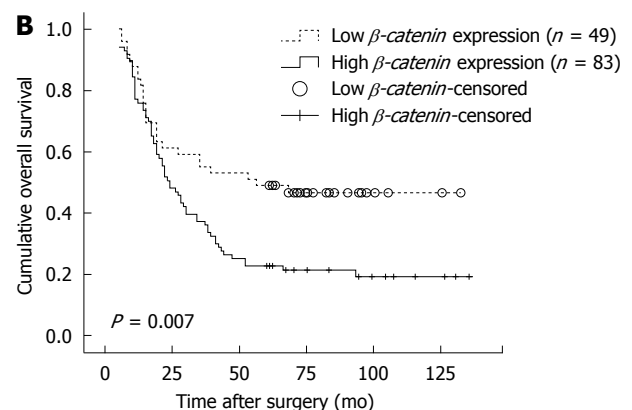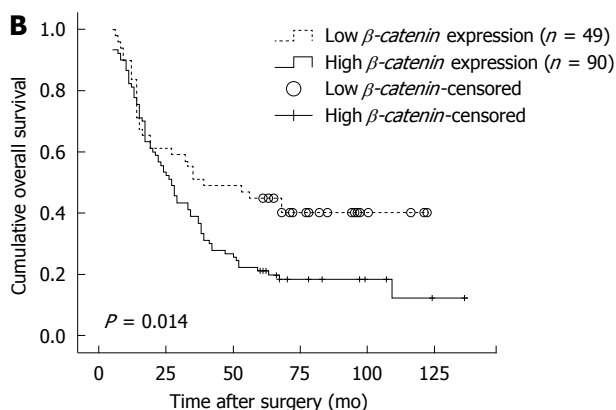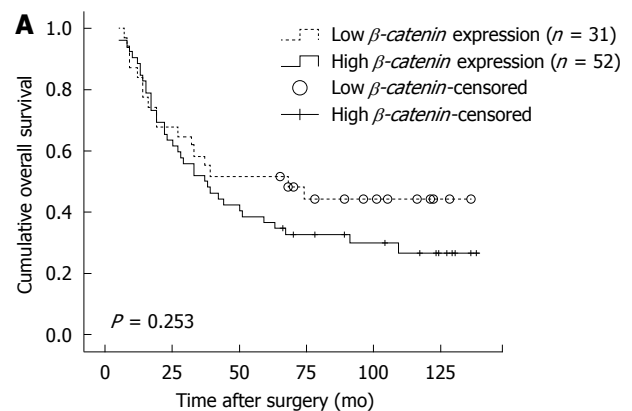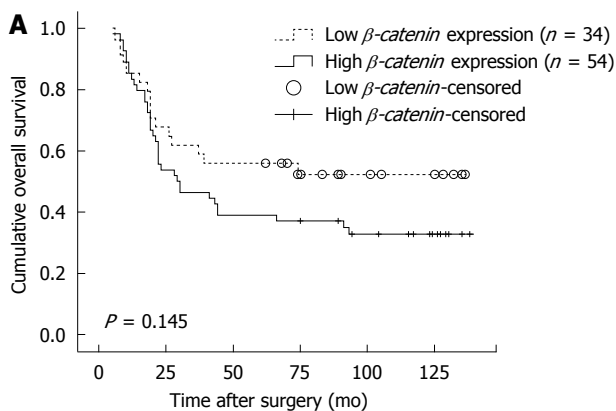

Figure 4 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma according to *β-catenin* expression. A: Correlation between *β-catenin* expression and post-operative survival rate of patients with T2 lesions; B: Correlation between *β-catenin* expression and post-operative survival rate of patients with T3 lesions.

Figure 5 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma stratified for pathological stage according to *β-catenin* expression. A: Correlation between *β-catenin* expression and post-operative survival rate of patients with ⅡA diseases; B: Correlation between *β-catenin* expression and post-operative survival rate of patients with ⅡB diseases.

to elucidate the role of *β-catenin* in ESCC, but the current findings in terms of its expression pattern and potential involvement in formation and progression of ESCC are contradictory in the literature[25-28]. One problem faced by

researchers is the determination of tumor immunohistochemical positivity for *β-catenin* which is clinically and biologically relevant. Previous studies have applied different scoring systems in predetermination of cut-off scores

which might be set arbitrarily[30-33]. A lack of consistent, widely applicable methodology may have been primarily responsible for the contradictory results of these studies evaluating *β-catenin* and its prognostic value in ESCC patients. Therefore, our study used a reproducible scoring method which takes both staining percentage and intensity into account, and the cut-off score was selected based on ROC curve analysis, so that the trade-off between sensitivity and specificity was the smallest, leading to the greatest overall number of correctly classified tumors with and without clinical outcome.

In the present study, univariate and multivariate analyses showed that high expression level of *β-catenin* in completely resected samples from patients with ESCC at stage T2-3N0M0 was significantly correlated with the worse post-operative survival rate of ESCC patients, which is consistent with the reported findings[27]. Krishnadath *et al*[34] reported that low expression level of *β-catenin* is significantly correlated with the poor prognosis of esophageal adenocarcinoma patients, especially at early-stage. It has been shown that the higher the expression level of *β-catenin* is, the better the outcome of esophageal adenocarcinoma patients is, and the *β-catenin* expression level is higher in more invasive tumors than in superficial tumors[35]. However, Zhao *et al*[26] and Lin *et al*[28] demonstrated that *β-catenin* expression does not imply more aggressive malignant behaviors of ESCC or predict the poor prognosis of ESCC patients. Krishnadath *et al*[34] and Osterheld *et al*[35] showed a different histological type of tumor (adenocarcinoma) when they analyzed these contradictory results. In addition, esophageal carcinoma at stages I -IV was involved in the studies[26,28,34,35], suggesting that different therapeutic strategies including adjuvant or neoadjuvant chemotherapy and radiotherapy for more advanced disease may introduce confounding factors affecting the application of molecular analysis in assessing the prognosis of ESCC patients.

β-catenin protein not only serves as a pivotal component of *E-cadherin/catenin* complex which participates in cell-cell and cell-matrix adhesion[6,8,9], but also as a key mediator in the Wingless/Wnt signal transduction pathway[5,7], indicating that disruption of *E-cadherin/catenin* complex or physical and functional loss of *β-catenin* protein can lead to loosening of cell-cell contact and promote tumor invasion and metastasis. On the other hand, *β-catenin* can be oncogenically activated either by direct gene mutation[36] and inactivation of the *APC* tumor suppressor[37], or by activation of the Wingless/Wnt signal transduction pathway[38], thus resulting in post-translational stabilization of *β-catenin* protein. Excess cytoplasmic accumulation of *β-catenin* protein can then increase the influx of this molecule into nuclei, leading to over-expression of tumor-promoting genes, such as *cyclin-D1* and *c-myc*[5,6], and promote cell mitosis and growth[39,40]. Therefore, either up-regulated or down-regulated expression of *β-catenin* contributes to invasive and metastatic potentials of esophageal cancer. Obviously, the question is which physiopathological process takes the advantage in different circumstances, such

as different histological types or different pathological stages. Further analysis of the role of *β-catenin* gene and its products in formation and progression of esophageal carcinomas may provide a better understanding of this pathogenic process.

In this study, further stratified analysis split by pathological stage and depth of invasion showed that *β-catenin* exhibited its effect on the prognosis of patients with ESCC at stage ⅡB or with T3 lesions, indicating that this biomarker is more valuable in predicting the outcome of ESCC patients at advanced stages, which is consistent with the findings in other studies[2,22]. Further study is needed to verify this trend. In this study, no significant correlation was found between *β-catenin* expression and prognostic parameters, including tumor grade, tumor location, depth of invasion and pathological stage. Multivariate survival analysis of all potential prognostic variables also confirmed that *β-catenin* was an absolutely independent prognostic factor, which is in accordance with the reported findings[25,28]. However, other studies showed that *β-catenin* is significantly correlated with the accepted prognostic parameters of ESCC[26,27,41]. Further study with a large sample size is needed to obtain a clearer picture.

In conclusion, elevated *β-catenin* expression level is an adverse prognostic factor for ESCC patients at stage T2-3N0M0, especially for those with T3 lesions or with stage ⅡB diseases. However, further study with a larger cohort of patients is required to verify this observation, especially in view of the contradictory results.

COMMENTS

Background
Esophageal squamous cell carcinoma (ESCC), an aggressive tumor with a poor prognosis, is one of the most common malignant tumors in Asia, especially in certain areas of China. Despite advances in early diagnosis and therapies, the clinical outcome of ESCC patients remains unsatisfactory. Since the classical staging criteria fail to differentiate prognostic characteristics of ESCC patients adequately, many efforts have been made to identify relevant biomarkers with adverse prognostic significance, and to modify therapeutic strategies for individual patients. *β-catenin*, as a prognostic factor for ESCC, has been extensively studied in a variety of neoplasms. However, the exact role of *β-catenin* and its prognostic significance in ESCC remain to be defined.

Research frontiers
β-catenin protein not only serves as a pivotal component of *E-cadherin/catenin* complex which participates in cell-cell and cell-matrix adhesion, but also as a key mediator in the Wingless/Wnt signal transduction pathway. The hotspot in molecular tumor analysis of *β-catenin* is whether *β-catenin* involves and how it involves in the formation and progression of esophageal carcinoma.

Innovations and breakthroughs
Previous immunohistochemistry studies showed contradictory results in *β-catenin* expression pattern and its prognostic value for ESCC, due to lack of consistent, widely applicable methods. Therefore, the present study used a reproducible scoring method which takes both staining percentage and intensity into account, and the cut-off score was selected based on receiver operating characteristic (ROC) curve analysis so that the trade-off between sensitivity and specificity was the smallest, leading to the greatest overall number of correctly classified tumors with and without clinical outcome. This is the first study to evaluate *β-catenin* expression in ESCC patients with this novel method, showing that elevated *β-catenin* expression is an adverse prognostic factor for ESCC patients at stage T2-3N0M0, especially for those with T3 lesions or stage ⅡB diseases.

Applications
The novel methods used in this study can be applied in treatment of ESCC patients at stage T2-3N0M0.

Terminology
ROC curve analysis: In signal detection theory, a ROC curve is a graphical plot of the sensitivity, or true positivity *vs* (1-specificity), or false positivity for a binary classifier system as its discrimination threshold is varied. The ROC curve can also be represented equivalently by plotting the fraction of true positivity (TPR = true positive rate) *vs* the fraction of false positivity (FPR = false positive rate). ROC curve analysis provides tools to select possibly optimal models and to discard suboptimal ones independently from (and prior to specifying) the cost context or the class distribution. ROC curve analysis is related in a direct and natural way to cost/benefit analysis of diagnostic decision making. *β-catenin*: *β-catenin* protein was originally identified as a component of adherence junction, a multi-protein complex supporting tight cell-cell contacts in the presence of extracellular calcium. However, *β-catenin* also plays a key role in the Wnt signaling transduction pathway.

Peer review
The authors studied the expression of *β-catenin* in ESCC at stage T2-3N0M0 and its prognostic significance by analyzing the expression of *β-catenin* in 227 ESCC specimens with IHC and ROC curve analysis to select the cut-off score for high or low IHC reactivity. Then, they correlated the *β-catenin* expression with clinicopathological features of ESCC patients and its relation with the prognosis of ESCC patients. No significant correlation was observed between *β-catenin* expression in and clinicopathological parameters of ESCC patients, but multivariate analysis confirmed that *β-catenin* was an independent prognostic factor for the overall survival rate of ESCC patients at stage T2-3N0M0. The manuscript reads nicely and is easy to follow. Tables are legible and easy to understand. I believe that the method used in this study is plausible and the conclusion is well supported by the data. This manuscript adds to the current knowledge on this topic and it is a pleasure to read it.

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