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Original Article

The clinicopathological significance and prognostic value of β-catenin Ser45-phosphorylation expression in esophageal squamous cell carcinoma

Xinning Pan[1*], Lili Ma[2*], Jinsheng Wang[1]

[1]Department of Pathology, Changzhi Medical College, Changzhi, China; [2]Department of Pathology, Heping Hospital Affiliated to Changzhi Medical College, Changzhi, China. [*]Equal contributors.

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Abstract: β-Catenin is a multifunctional protein which plays a central role in physiological homeostasis, and it acts both as an adaptor protein for intracellular adhesion and a transcriptional co-regulator. As a pivotal component of the Wnt signaling pathway, the accumulation of β-catenin in the cytoplasm/nucleus leads to many diseases including cancer. The phosphorylation at Ser45 of β-catenin causes the degradation of β-catenin, which makes β-catenin at a very low level. It has been shown that phosphorylation at Ser45 of β-catenin is closely related to the occurrence and development of tumors. However, little is known about the exact role of β-catenin Ser45-phosphorylation in esophageal squamous cell carcinoma (ESCC). Purpose: The present study was aimed at exploring the role and the prognostic value of the expression of β-catenin Ser45-phosphorylation in ESCC. Methods: The expression of phosphorylation at Ser45of β-catenin was detected by immunohistochemistry in 90 cases of ESCC and their corresponding adjacent nonneoplastic esophageal tissues (n = 90). We then evaluated the relationships among the expressions of phosphorylation at Ser45 of β-catenin, the clinicopathological parameters, and the prognoses of the ESCC patients. Results: The expression level of phosphorylation at Ser45 of β-catenin in ESCC was 65.6% (59/90), significantly lower than the expression level in nonneoplastic esophageal tissues, where it was 88.9% (80/90), (X^2 = 10.340, P = 0.003). The expression of β-catenin Ser45-phosphorylation was significantly related to the degree of tumor cell differentiation, but not to age, gender, tumor size, AJCC clinical stage, or lymphatic metastasis. In univariate and multivariate Cox regression analyses, we found that lymphatic invasion and depth of invasion were independent risk factors for the poor prognosis of ESCC patients. Furthermore, a survival analysis revealed that the positive expression of β-catenin Ser45-phosphorylation had a significantly better survival date than the negative group after curative surgery. Conclusion: β-catenin Ser45-phosphorylation may play an important role in the pathogenesis and development of ESCC and may provide clinically useful prognostic information.

Keywords: β-catenin, phosphorylation at Ser45 of β-catenin, esophageal squamous cell carcinoma (ESCC), immunohistochemistry (IHC), prognosis

Introduction

Esophageal cancer (EC) is the eighth most common cancer worldwide, with an estimated 456,000 new cases diagnosed per year, and it is the malignancy with the sixth highest mortality rate [1, 2]. Esophageal squamous cell carcinoma (ESCC) is the major histological subtype of EC, which is recognized as a prevalent cancer with a high morbidity rate in China, especially in Taihang Mountain [3]. The current treatment for ESCC is still ineffective because of invasion and migration. Therefore, deepening the understanding of the pathogenesis of EC and identifying novel, targeted therapeutic strategies in ESCC are urgently needed.

In differentiated normal cells, β-catenin is mainly located in the cell membrane, and the free β-catenin in the cytoplasm is maintained at a very low level by the regulation of the Wnt signal. β-catenin is a critical structural role of cell junctions and is important in maintaining the polarity and integrity of the epithelium [4]. In addition, it is involved in the compound formed with glycogen synthase kinase3β (GSK3β), Casein kinase1 (CK1), APC, and Axin [5, 6]. The phosphorylation of β-catenin occurs in this

"polyprotein compound". The occurrence and development of cancer is closely related to Wnt/β-catenin signaling. As a pivotal molecule of the Wnt signaling pathway, the accumulation of β-catenin in the cytoplasm/nucleus is the key to the activation of the signaling pathway. The cause of cytoplasmic/nuclear accumulation of-catenin varies from tumor to tumor. It has been shown that the phosphorylation at serine 45 (Ser45) of β-catenin initiates the degradation of β-catenin [7]. Although the β-catenin phosphorylations play positive roles in the pathogenesis of many tumors, the expression and function of β-catenin Ser45-phosphorylation in tumors remain unclear. In the present study, we explored the expression of phosphorylation at Ser45 of β-catenin in ESCC and analyzed the relationships among β-catenin Ser45-phosphorylation expression, the clinicopathological parameters, the survival rate, and prognostic information.

Materials and methods

Patients and tissues

The tumor tissue samples evaluated in our experiment were obtained from 90 patients (41 female, 49 male; median age 60 years; range 36-76 years) who had undergone radical esophagectomy in the First Affiliated Hospital of Changzhi Medical College (Changzhi, Shanxi, China) from January 2012 to October 2013. The patients were selected at their first diagnosis and none of them received radiotherapy, chemotherapy and/or immunotherapy before the radical surgical treatment. All samples were matched with the corresponding adjacent normal mucosa (> 3-10 cm). The sample tissues collected immediately were made into liquid nitrogen snap-frozen specimens and paraffin blocks until use. All samples, with a histopathologic diagnosis of ESCC and the corresponding adjacent normal tissues, were confirmed by two independent pathologists who were blinded to the original diagnosis. No metaplasia, dysplasia, or atypical hyperplasia were in the nonneoplastic esophageal tissues, which are the strict evaluation criteria used for diagnosis. Clinicopathologic data were collected, including age, gender, degree of differentiation, AJCC clinical stage and lymphatic metastasis. Tumors were staged using the American Joint Committee on Cancer (AJCC) staging system.

The follow-up was carried out continuously after the surgeries until April 2019. The regular assessment of the survival status occurred over a 5.5-7.3 year period. The patients' medical follow-up records were updated every month during the first year after surgery, then trimonthly during the second year, and then every six months thereafter. All the patients who died of a cause not related to ESCC or from accidents were excluded from this study.

Tissue microarray (TMA)

The tissue microarray was made in collaboration with the Shanghai Biochip Company, (Shanghai, China). We marked all cases HE sliced lesions scope and matched them to the corresponding paraffin wax block. A small core of tissue (2 mm) was drilled out of the original block. This was then transferred to the recipient block. The block was gently warmed to anneal and cooled before the sections are cut. Then, 180 TMA block tissues with formalin-fixed, paraffin-embedded (90 cases of ESCC and 90 cases of corresponding adjacent normal mucosa tissues) were prepared.

Immunohistochemistry

Paraffin sections (4 um thick) obtained from the TMA blocks were cut for the immunohistochemical reactions. The slides were dewaxed, hydrated, then immersed in a 3% hydrogen peroxide solution for 10 min. In order to retrieve the antigens, the sections were hyperbarically heated in an ethylene diaminetetra acetic acid (EDTA) buffer (pH 9.0) at 100°C for 8 min. After being incubated with 10% normal goat serum at room temperature for 30 min, the slides were incubated with β-catenin Ser45-phosphorylation rabbit polyclonal antibody (1:300 dilution, Abcam, Cambridge, UK) overnight at 4°C. After we washed them three times with a phosphate buffer solution (PBS), the sections were treated with a corresponding second antibody (Zhongshan Golden Bridge Corporation, Beijing, China), then treated with a peroxidase-conjugated streptavidin. Diaminobenzidine (DAB) was dropped on the sections for a color reaction. The gastric carcinoma slides were utilized as positive controls. PBS, as the substitute for the primary antibody, was used as a negative control.

Section evaluation of immunohistochemical staining

A semiquantitative method based on the distribution and intensity of the staining was used

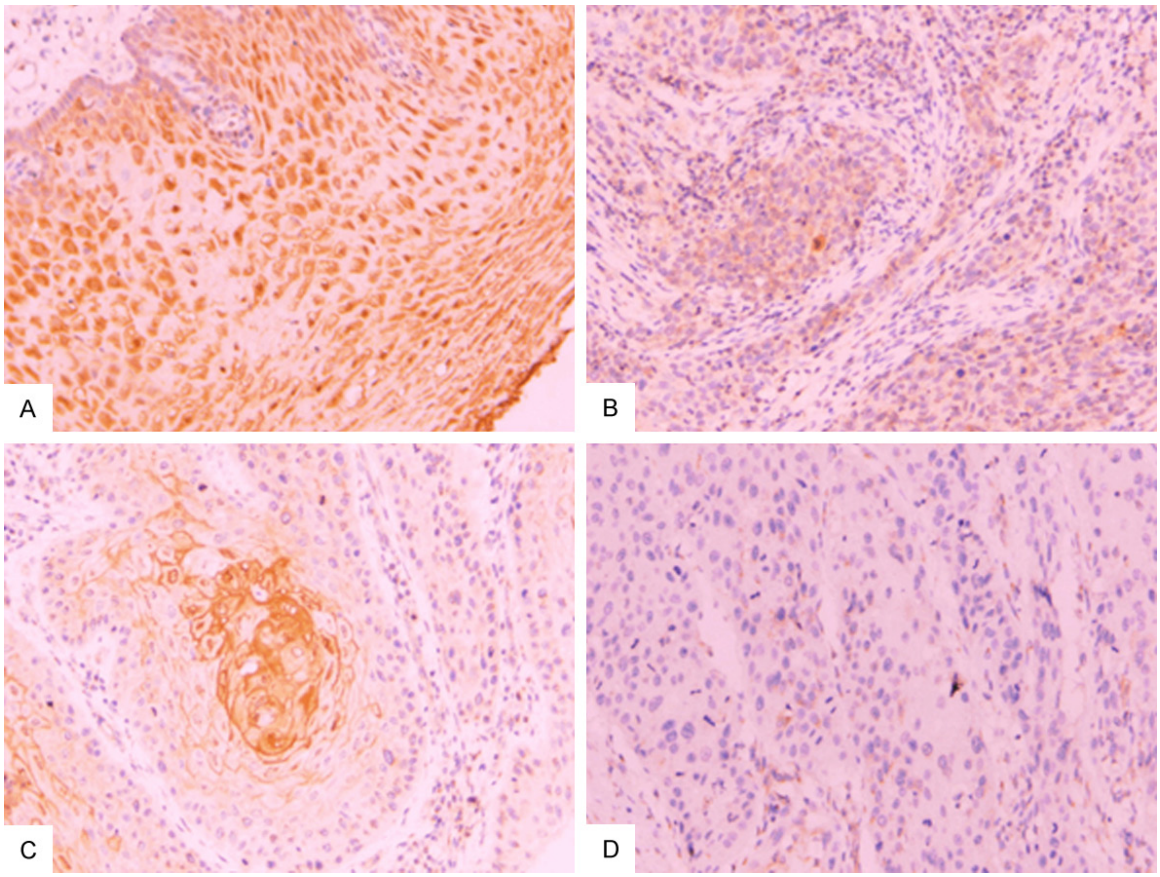

Figure 1. A. Expression of β-catenin Ser45-phosphorylation in nonneoplastic esophageal tissue (SP × 200); B. Expression of β-catenin Ser45-phosphorylation in ESCC (SP × 200); C. Expression of β-catenin Ser45-phosphorylation in high grade ESCC (SP × 200); D. Expression of β-catenin Ser45-phosphorylation in low grade ESCC (SP × 200).

to grade the β-catenin Ser45-phosphorylation immunostaining. Two pathologists without knowledge of the clinical outcomes assessed the degree of immunostaining. When yellow or brown particles were located in the cell nuclei and (or) cytoplasms, we considered them a β-catenin Ser45-phosphorylation positive specimen. The intensity of staining was graded as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The percentage of positive cells was graded as follows: 1 (0-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The expression of phosphorylation at Ser45 of β-catenin was defined according to the final one obtained from the intensity grade multiplied by the score of the percentage of positive cells: negative (0-3), weakly positive (4-5), or strongly positive (6-7) [8].

Statistical analysis

The statistical analysis was performed using SPSS 18.0 for Windows (SPSS Inc., Chicago, USA). A chi-square test was used to analyze the correlation between β-catenin Ser45-phosphorylation expression and the clinicopathological parameters. Survival curves were drawn using Kaplan-Meier. A log-rank test was used to determine the comparative analysis of the subgroups. A univariate proportional hazards regression was used to estimate the dependence of survival on each variable. The multivariate survival analysis was based on the Cox proportional hazard model to test the variables selected by the univariate analysis as having prognostic value. A value of $P < 0.05$ was considered statistically significant.

Results

The relationship between β-catenin Ser45-phosphorylation expression and the clinicopathological parameters of ESCC

It was found that the β-catenin Ser45-phosphorylation expression was in the cell cytoplasms, as well as in a few of the nuclei (**Figure 1A**, **1B**). The β-catenin Ser45-phosphorylation

Table 1. Correlation of β-catenin Ser45-phosphorylation expression with the clinicopathological parameters

Variable		n	P-Ser45-β-catenin Expression (%)		X^2	*P*-value
			+	-		
Overall frequency	Nonneoplastic	90	80 (88.9)	10 (11.1)	10.340	0.003*
	ESCC	90	59 (65.6)	31 (34.4)		
Gender	Male	49	16 (32.7)	33 (67.3)	0.361	0.548
	Female	41	11 (26.8)	30 (73.2)		
Age (yr) at surgery	≥ 60	58	20 (34.5)	38 (65.5)	1.561	0.212
	< 60	32	7 (21.9)	25 (78.1)		
Tumor size (cm)	< 4	54	18 (33.3)	36 (66.7)	2.224	0.338
	4-7	32	7 (21.9)	25 (78.1)		
	≥ 8	4	2 (50.0)	2 (50.0)		
Cell differentiation	High-grade	15	10 (66.7)	5 (33.3)	7.811	0.021*
	Middle-grade	64	16 (25.0)	48 (75.0)		
	Low-grade	11	2 (18.2)	9 (81.8)		
Depth of invasion	T1	8	1 (12.5)	7 (87.5)	2.981	0.358
	T2	48	14 (29.2)	34 (70.8)		
	T3	31	10 (32.3)	21 (67.7)		
	T4	3	2 (66.7)	1 (33.3)		
Lymphatic invasion	(-)	67	21 (31.3)	46 (68.7)	0.225	0.635
	(+)	23	6 (26.1)	17 (73.9)		
AJCC clinical stage	I + II	70	22 (31.4)	48 (68.6)	0.306	0.580
	III + IV	20	5 (25.0)	15 (75.0)		

*P < 0.05.

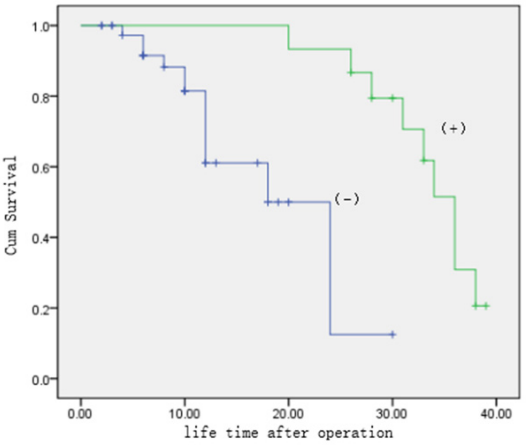

Figure 2. Overall survival curves of patients with ESCC according to the status of β-catenin Ser45-phosphorylation expression. (X^2 = 10.165, P = 0.001).

expression in ESCC was significantly lower than it was in the nonneoplastic esophageal tissues. Among the 90 primary ESCC cases, the incidence of a positive expression of Ser45-phosphorylationwas 65.6% (59/90), and the positive rate of nonneoplastic esophageal tissues was 88.9% (80/90). A significant down-regulation of β-catenin Ser45-phosphorylation immunoreactivity was found between ESCC and the nonneoplastic esophageal tissue (X^2 = 10.340, P = 0.003). The statistical analyses also indicated the relationship between β-catenin Ser45-phosphorylation expression and the clinicopathological parameters. A decreasing trend of β-catenin Ser45-phosphorylation expression from high grade to low grade was found. We also demonstrated a significant difference among them (P = 0.021) (**Figure 1C**, **1D**). However, no statistically significant correlations between β-catenin Ser45-phosphorylation expression and gender (P = 0.548), age (P = 0.212), depth of invasion (P = 0.358), tumor size (P = 0.338), lymphatic invasion (P = 0.635), or AJCC clinical stage (P = 0.580) were found (**Table 1**).

Survival analysis

Kaplan-Meier survival curves were drawn for all 90 patients so as to determine whether β-catenin Ser45-phosphorylation expression is a prognostic factor. The median survival time for the patients with positive β-catenin Ser45-

Table 2. Univariate analysis of predictive factors for survival

Prognostic factors	Relative risk (95% CI)	*P*-value
Univariate		
CK1α (+) (-)	0.209 (0.697-6.794)	0.086
Gender (Male) (Female)	0.598 (0.246-1.451)	0.618
Age (≥ 60 years) (< 60 years)	0.375 (0.119-1.178)	0.340
Tumor size (< 4 cm) (4-7 cm) (≥ 8 cm)	0.153 (0.670-1.083)	0.061
Cell differentiation (High-grade) (Middle-grade) (Low-grade)	1.390 (0.659-2.789)	0.921
Depth of invasion (T1) (T2) (T3) (T4)	1.101 (1.309-2.686)	0.024
Lymphatic invasion (-) (+)	1.305 (1.028-1.164)	0.043
AJCC clinical stage (I + II) (III + IV)	1.912 (0.508-2.680)	0.089

Table 3. Multivariate analysis of predictive factors for survival

Prognostic factors	Relative risk (95% CI)	*P*-value
Lymphatic invasion	1.273 (1.120-2.625)	0.038
Depth of invasion (T1) (T2) (T3) (T4)	1.365 (2.326-3.095)	0.023

phosphorylation expression was 36 (95% CI: 33.3-38.7) months and 19 (95% CI: 12.9-23.1) months for the group with negative β-catenin Ser45-phosphorylation expression. A significantly better survival rate was observed in the β-catenin Ser45-phosphorylation positive expression group than in the negative group (X^2 = 10.165, P = 0.001) (**Figure 2**). Univariate and multivariate Cox-regression analyses were used to determine independent predictors for survival. Age, gender, degree of differentiation, AJCC clinical stage and lymphatic metastasis entered into the analysis. And both the univariate and multivariate survival analysis indicated lymphatic metastasis and depth of invasion were poor prognostic factors (P < 0.05, **Tables 2**, **3**).

Discussion

The N-terminal phosphorylation of β-catenin leads to its degradation, which plays positive roles in the pathogenesis of many tumors [9]. But little is known about the expression and function of β-catenin Ser45-phosphorylation in tumors. Vaid et al. indicated that silymarin, an inhibitor of the Wnt/β-catenin pathway, enhanced the levels of casein kinase 1α, glycogen synthase kinase-3β, and phosphorylated-β-catenin on the critical residues Ser45, Ser33/37, and Thr41, decreased the accumulation of nuclear β-catenin and the inhibition of MMP-2 and MMP-9 levels, which significantly inhibited cell migration of the Mel 1241 cells [10]. Kaneda et al. demonstrated that (23R, 24E)-23-acetoxymangiferonic acid (23R-AMA) promoted ser45/thr41 phosphorylation of β-catenin and suppressed its intranuclear accumulation, which was thought to be related to the inhibition of MITF expression [11]. These results lead to inhibiting the growth and migration of B16-F10 melanoma. Gao et al. determined that protein disulfide isomerase family 6 (PDIA6) overexpression resulted in decreased phosphorylation of β-catenin at Ser45, while it increased β-catenin nuclear accumulation, and the upregulation of the Wnt/β-catenin signaling target genes cyclinD1 and c-myc, and it promoted the proliferation of HeLa cells in the end [12]. Park et al. suggested that calotropin, a cardenolide from *Calotropis gigantea*, inhibited Wnt signaling by decreasing both nuclear and cytosolic β-catenin in a dose-dependent manner and promoted the degradation of β-catenin by increasing the phosphorylation of β-catenin at Ser45 through casein kinase 1α (CK1α) in colon cancer cells [13]. Yan et al. suggested that all-transretinoic acid (ATRA) induced the differentiation of Colorectal CSCs and inhibited their self-renewing ability by increasing the degradation of β-catenin Thr41/Set45 phosphorylation and suppressing the Wnt/β-catenin pathway [14].

However, unlike the previous findings, our study found that the APC/phospho-β-catenin complex in cell protrusions appears to be distinct from the APC/axin/β-catenin destruction complex, and phosphorylation at Ser45 of β-catenin does not necessarily lead to its degradation, but instead regulates cell migration and/or adhesion processes in tumor cells [15]. In addition, Huang et al. showed that N-terminally phosphorylated β-catenin, in a distinct function, modulates microtubule regrowth at centrosomes [16]. Medrek's work implicated that

Ser-45 phosphorylation of β-catenin is related to Wnt5a-induced β-catenin/E-cadherin association, and the inhibitory effect of Wnt-5a on breast epithelial cell invasion is reduced upon the mutation of β-catenin-Ser-45, then Wnt-5a-CKIα-induced Ser-45 phosphorylation does not lead to the degradation of β-catenin [17]. Iaconelli et al. found that HDAC6 inhibitors increased Ser45 phosphorylation and Lys49 acetylation but did not affect Ser33, Ser37, and Thr41 phosphorylation, which could not affect the total levels of β-catenin, and it resulted in the increased membrane localization of β-catenin [18]. Maher's study reported that the phosphorylation of β-catenin at Ser45 appears to be involved in a distinct nuclear function and is spatially separate from the phosphorylation at Ser33/37/41 [19]. These studies highlight the possibility that Ser-45 phosphorylation of β-catenin may not necessarily lead directly to its degradation.

In our present study, we examined the expression of phosphorylation at Ser45 of β-catenin in ESCC and in nonneoplastic esophageal tissues, and we analyzed the clinicopathological data in order to determine the prognosis of the patients. Our study showed that the deregulation of phosphorylation of β-catenin at Ser45 was lower in ESCC than in the nonneoplastic esophageal tissues, so the expression of phosphorylation at Ser45 of β-catenin was significantly correlated with the differentiation degree of ESCC. So, the expression of phosphorylation at Ser45 of β-catenin may play an important role in the carcinogenesis and tumor progression of ESCC.

The analysis of survival data revealed that positive expression of phosphorylation at Ser45 of β-catenin was significantly associated with a better prognosis of ESCC. Furthermore, univariate and multivariate Cox-regression analyses indicated that lymphatic invasion and depth of invasion were poor prognostic factors for the ESCC patients. These research findings suggest that the phosphorylation at Ser45 of β-catenin could be a useful prognostic indicator for ESCC.

In summary, our preliminary research demonstrates that the phosphorylation of β-catenin at Ser45 provides clinically useful prognostic information in cases of ESCC. The expression of phosphorylation at Ser45 of β-catenin might be a helpful indicator and a potential therapeutic target in ESCC. However, current evidence shows that the role of phosphorylation at Ser45 of β-catenin in tumors is still controversial. Therefore, further investigation is still required to explore the molecular mechanisms of phosphorylation at Ser45 of β-catenin in ESCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jinsheng Wang, Department of Pathology, Changzhi Medical College, Changzhi, China. E-mail: tigergv@163.com

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