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Original Article

Clinicopathological significance of wnt/β-catenin signaling pathway in esophageal squamous cell carcinoma

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Abstract: Background/Aim: Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors. It has been reported that Wnt signaling pathway plays an important role in Esophageal Cancer progression, metastasis and invasion. However the clinicopathological significance of Wnt2, GSK3β, and β-catenin in ESCC has been little reported. In the present study, the aim of this study was to investigate the clinicopathologic and prognosis roles of Wnt2, GSK3β, and β-catenin in ESCC tissue. Methods: 265 ESCC samples were analyzed by immunohistochemistry using Wnt2, GSK3β, and β-catenin antibodies. Then, correlation of Wnt2, GSK3β, and β-catenin expression with clinicopathological features and prognosis of ESCC patients was statistically analyzed. Results: Cytoplasmic Wnt2 overexpression was detected in 55.5% (147 of 265) ESCCs, which was significantly correlated with the degree of differentiation ($P = 0.031$). Cytoplasmic GSK3β overexpression was detected in 7.2% (19 of 265) ESCCs, and aberrant β-catenin expression was identified in 54.3% (144 of 265) of ESCCs. The positive rate of Wnt2 significantly increased with the malignant degree of Kazak ESCC patients. The aberrant β-catenin expression in GSK3β-negative ESCC was significantly associated with the ethnic, tumor size, tumor location, degree of differentiation, AJCC stage, lymph node status. Furthermore, the expression of β-catenin implicated the ethnic difference ($P = 0.019$). In Kaplan-Meier curve analysis, no significant correlation was observed between the expression of Wnt2, GSK3β, β-catenin and the poor prognosis of ESCCs. Conclusion: The aberrant β-catenin expression could be an adverse underlying factor in carcinogenesis and progression of ESCC. There was a different statistical signification for β-catenin in Kazakhs to compare with Hans.

Keywords: Esophageal squamous cell carcinoma, immunohistochemistry, Wnt2, GSK3β, β-catenin, clinicopathology

Introduction

Esophageal cancer (EC) is one of the most common malignant tumors, and is the sixth most common cause of cancer death worldwide [1]. Despite progress in the multimodality treatment of esophageal cancer in the past several decades, the prognosis for esophageal cancer remains poor [2]. More than 90% of esophageal cancer is squamous cell carcinoma (ESCC) [3]. Xinjiang is one of the higher incidence areas of esophagus cancer in China. The highest incidence of ESCC is Kazakhs in Xinjiang [4]. The previous study shows that Wnt signaling pathway plays an important role in esophageal cancer progression, metastasis and invasion [5]. In addition, in our previous study, LC-ESI-MS was used in fresh tumor samples to detect the gene expression profiles in ESCC tissues matched adjacent non-cancerous samples. It was found that the protein expression of Wnt2 and β-catenin was high expression in ESCC compared with normal esophageal tissue, while the protein expression of GSK3β was low expression in ESCC compared with normal esophageal tissue. However, the clinicopathological significance of Wnt2, GSK3β, and β-catenin in ESCC has been little reported. Thus, further study was designed to investigate the impacts on the clinicopathological features and prognosis of patients with ESCC.

Wnt signaling pathway not only plays an important role in embryonic development but also in

cancer biology [6, 7]. It is consisted of the canonical Wnt pathway, the planar cell polarity pathway and the Wnt/Ca^{2+} pathway [8]. the canonical Wnt pathway was considered as a central mechanism in cancer biology [8]. Canonical Wnt signaling activation is multistep complex process involving in Wnt2, GSK3β, Axin, APC, β-catenin, TCF, c-myc and cyclin D1 [6].

Wnt2, a ligand protein, is secreted by tumor fibroblasts (TF) in a paracrine fashion [9]. Wnt2 in the different cells can activate different signaling pathways, including WNT2/β-catenin pathway, also called the canonical Wnt pathway [10]. WNT2/β-catenin pathway promotes β-catenin expression and induces β-catenin translocation from the cell membrane to the cytoplasm even to the nucleus [7, 11]. β-catenin is a multifunctional protein which mediates cell-extracellular matrix adhesion and promotes tumor proliferation and metastasis [12-14]. β-catenin not only has been extensively identified and studied in embryonal development [6], but also plays a vital role in tumor progression by affecting E-cadherin linking cell adhesion and Wnt signaling pathway [13, 14]. Wnt2 signaling pathway can enhance the stability of β-catenin and cause the accumulation of free β-catenin in the nucleus [15]. β-catenin, which combines with T-cell factor/lymphoid enhancer factor (TCF/LEF) family, stimulates the transcription of specific genes which include in oncogenic transformation [16, 17]. GSK3β is one of the few signaling mediators that play central roles in a diverse range of signaling pathways, including Wnt signaling pathway [18]. GSK3β mediates phosphorylation triggers β-catenin destabilization. However, Wnt signal inhibits GSK3β activity and increases free β-catenin level [19].

The purpose of this study was to investigate the clinicopathological significance of Wnt2, GSK3β, and β-catenin and to analyze their correlations with the prognosis.

Materials and methods

Patients and tissue samples

Informed consent was obtained from every patient prior surgery. The study was approved by Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University. Between 2007 and 2014, 265 specimens of human ESCC and paired adjacent normal esophageal squamous epithelium were obtained from patients of different grades who underwent esophagectomy at First Affiliated Hospital of Xinjiang Medical University. At the same time, all patients never received any radiotherapy or chemotherapy prior to surgery. The patients consisted of 115 Kazakhs who was defined as experimental group and 150 Hans who was defined as control group. Diagnosis was performed at the Department of Pathology, First Affiliated Hospital of Xinjiang Medical University. All of the samples were histopathologically diagnosed as ESCC, and the following information was recorded for each patient: age, gender, ethnic, tumor location, tumor size, degree of differentiation, clinicopathological stage based on the seventh edition of the American Joint Committee on Cancer staging system (AJCC) [20], and lymph node status.

Immunohistochemistry

Formalin-fixed, paraffin-embedded primary tissues were cut 3 um thick. Tissue slides were blocked in xylene for 30 minutes and in graded ethanol for 5 minutes. To enhance antigen retrieval, the slides were autoclaved for 20 minutes in 1% sodium citrate buffer (PH 6.0) and then left at room temperature. Then the slides was incubated with 3% H_2O_2 in methanol for 15 min to quench the endogenous peroxidase activity .Slides were incubated with the primary antibodies at 4°C overnight against Wnt2 (1:200, rabbit monocolonal antibody, Abcam, UK), β-catenin (1:300, rabbit monocolonal antibody, Abcam, UK), and GSK3β (1:150, rabbit monocolonal antibody, Abcam, UK). The slides were incubated with the corresponding secondary antibodies (ZSGB, China) for 30 minutes at 37°C. The slides were immersed in the prepared DAB solution. Finally, the slides were counterstained with hematoxylin, dehydrated through increasing graded alcohol, coverslipped with mounting.

Immunohistochemical scoring

The expression of Wnt2, GSK3β, β-catenin was assessed by two independent pathologists who had no previous knowledge of clinical data. Positive expression of Wnt2, primarily a cytoplasmic pattern, was defined when five or more Wnt2-positive tumor fibroblasts (TF) in the stro-

Table 1. Characteristics of 265 ESCC patients included in this study n (%)

Characteristic	Kazakh	Han	Total
Age			
Median	59.2	64.41	62.15
Range	60.00 ± 9.019	65 ± 8.712	63.00 ± 9.201
Sex			
Male	80 (69.6)	109 (72.2)	189 (71.1)
Female	35 (30.4)	41 (27.2)	76 (28.6)
Tumor size			
≤ 3 cm	36 (31.7)	37 (24.5)	78 (29.7)
> 3 cm	79 (68.3)	97 (64.2)	187 (70.3)
Tumor location			
upper	3 (2.6)	10 (6.6)	13 (5.0）
middle	33 (27)	70 (46.7)	103 (38.9)
lower	79 (68.6)	70 (46.7)	149 (56.1)
Degree of differentiation			
Well-differentiated	23 (20)	19 (12.7)	42 (15.8)
moderately differentiated	67 (58.3)	96 (64.0)	163 (61.6)
poorly differentiated	25 (21.7)	35 (23.3)	60 (22.6)
AJCC stage			
T0	4 (3.5)	13 (8.6)	17 (6.4)
T1	58 (50.4)	64 (42.4)	122 (46.0)
T2	18 (15.7)	17 (11.3)	35 (13.2)
T3	35 (30.4)	56 (37.1)	91 (34.2)
Lymph node status			
No	75 (65.2)	120 (80.0)	195 (70.7)
Yes	40 (34.8)	30 (20.0)	70 (25.6)

ESCC: esophageal squamous cell carcinoma; AJCC: American joint committee on cancer.

mal compartment were detected per microscopy field (20 × in magnification) [9]. Regarding the β-catenin expression, we used the classification of staining patterns as follows: (1) preserved expression pattern, if > 70% cancer epithelial cells were stained in the cell membranes; (2) reduced expression pattern, if the cancer cells stained were ≤ 70%; (3) translocation expression pattern, if immunoreactivity was present in the cytoplasm and/or nuclear in more than 10% of the cancer cells [21]. Reduced expression pattern and translocation expression pattern were defined as the aberrant expression of β-catenin. The intensity score of cytoplasmic GSK3β was evaluated as follows: 0, negative; 1, weak staining; 2, moderate staining; 3, strong staining. The score of 0 or 1 was considered low-expression of GSK3β, and the score of 2 or 3 was considered high-expression of GSK3β.

Statistical analysis

All statistical analyses were performed using the software package from SPSS version 16.0 for Windows (SPSS Inc, IL, USA). The continuous variables were expressed as means ± SEM. The associations between canonical Wnt signaling pathway proteins expression and different clinical characteristics were estimated using Fisher's exact test or χ^2 test. The patients were routinely followed-up clinically Overall survival (OS) was defined as time between date of surgery and date of death or the date of last follow-up. Overall survival was calculated using the Kaplan-Meier method. Overall survival was defined as the time from the date of surgical resection to the date of death. Differences were indicated statistically significant when *P* was less than 0.05 and all *P* values were two-tailed.

Results

Clinical characteristics of ESCC patients

The patients consisted of 189 (71.1%) males and 76 (28.6%) females with the median age at diagnosis were 62.15 years. The median age of Hans at diagnosis was 64.41. However, the median age of Kazakhs at diagnosis was 59.2, which was smaller than the median age of Hans. Most of the cases were moderately differentiated, included 67 Kazakhs and 96 Hans. The 265 patients were classified according to AJCC as follow: pathological stage T0, 17 (6.4%); pathological stage T1, 122 (46%); pathological stage T2, 35 (13.2%); pathological stage T3, 91 (34.2%). In addition, the rate of lymph node metastasis in the Kazakhs (34.8%) was higher than the rate of lymph node metastasis in the Hans (20.0%). Patients detailed characteristic included in this study were summarized in **Table 1**. The patients were routinely followed-up clinically after surgery for a median period of 15 months.

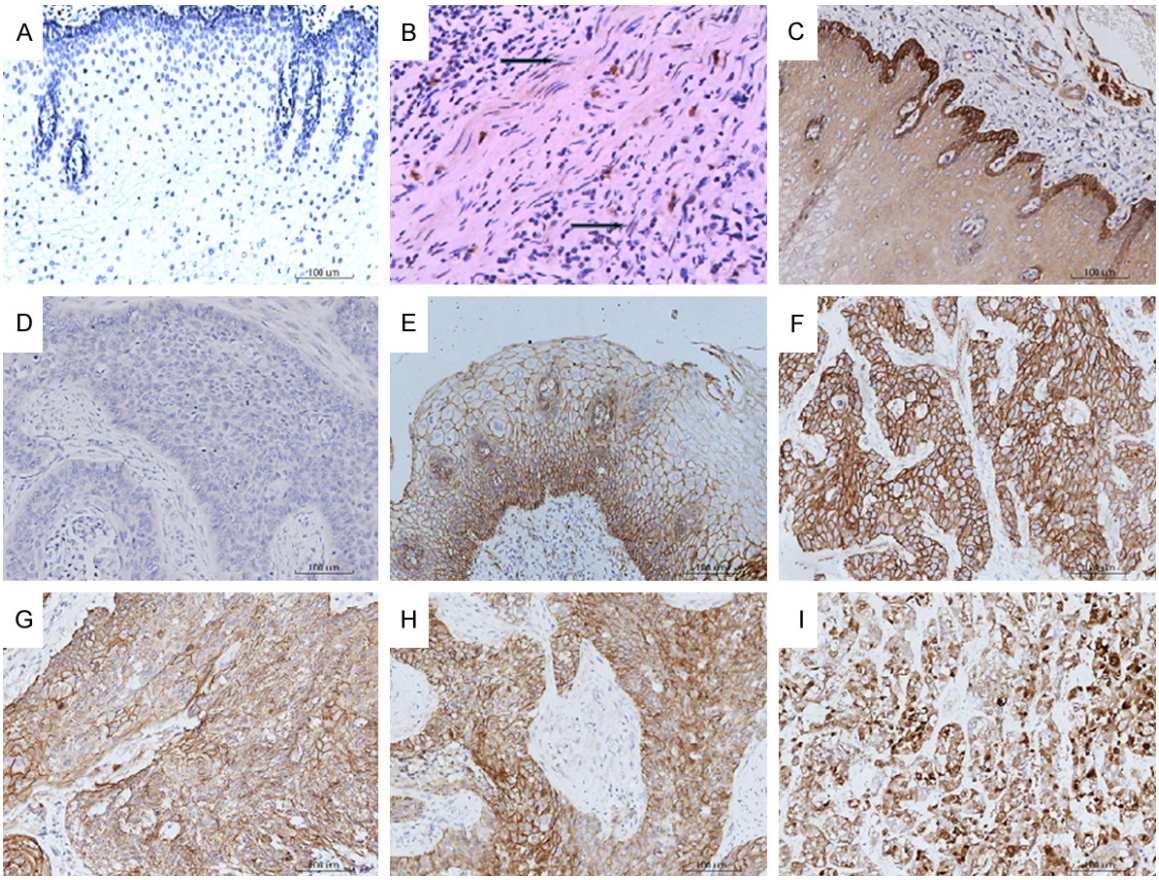

Figure 1. Immunohistochemical expressions of Wnt2, GSK3β, β-catenin in normal and cancer tissues of the esophagus (Original magnification ×200). A. Wnt2-negative staining in normal esophageal tissue; B. Cytoplasmic Wnt2-positive staining in ESCC tissue; C. Cytoplasmic GSK3β-positive staining in normal esophageal tissue; D. GSK3β-negative staining in ESCC tissue; E. Membranous β-catenin-positive staining in normal esophageal tissue; F. Membranous-cytoplasmic β-catenin-positive staining in ESCC tissue; G. Cytoplasmic β-catenin-positive staining in ESCC tissue; H. Cytoplasmic-nuclear β-catenin-positive staining in ESCC tissue; I. Nuclear β-catenin-positive staining in ESCC tissue.

Wnt pathway proteins expression in ESCCs

Wnt2 expression appeared in the form of a cytoplasmic staining pattern (**Figure 1A**). In most (147/265) ESCC cases, the Wnt2 expression was positive in tumor fibroblasts. However, the Wnt2 expression was negative in normal esophageal epithelial tissues (**Figure 1B**). The expression of Wnt2 in the tumors in Kazakhs was 60.0% (69/115), which was higher than that in the tumors in Hans (52%, 78/150), but there was no statistical significant difference ($P > 0.05$). The positive rate of Wnt2 in Kazakh ESCC patients with various differentiation degree were: Well-differentiated, 52.2% (12/23); moderately differentiated, 55.2% (37/67); poorly differentiated, 80% (20/25). The result indicated the positive rate of Wnt2 significantly increased with the malignant degree of Kazak ESCC patients ($P = 0.031$). Furthermore, no significant differences were observed according to the tumor size, tumor location, degree of differentiation, AJCC stage, lymph node status (**Table 2**).

The expression of GSK3β presented in the form of a cytoplasmic staining pattern (**Figure 1C**, **1D**). Regarding the GSK3β expression, in all patients, 19 (7.2%) carcinomas were high-expression compared to 144 (54.2%) normal esophageal tissues. The GSK3β high-expression of Kazaks and Hans in tumors was 8.9% (13/115) and 6.2% (7/150) respectively. The statistic intimated that the expression status of GSK3β was closely associated with the tumor location ($P = 0.02$), but not associated with the ethnic, tumor size, degree of differentiation, AJCC stage, lymph node status (**Table 2**).

The β-catenin expression exhibited four staining patterns (**Figure 1D-I**). The β-catenin

Table 2. Associations between Wnt2, GSK3β, β-catenin and the clinicopathological characteristic in ESCC

	Kazakh			Han			Total		
	Wnt2	GSK3β	β-catenin	Wnt2	GSK3β	β-catenin	Wnt2	GSK3β	β-catenin
Positive rate	60.0%	8.9%	54.8%	52.0%	6.2%	60%	56.3%	7.2%	55.5%
P (Tumor location)	0.270	0.109	0.568	0.681	0.087	0.701	0.605	0.002	0.314
P (Tumor size)	0.176	0.348	0.116	0.954	0.632	0.738	0.773	0.294	0.075
P (Degree of differentiation)	0.031	0.394	0.636	0.854	0.666	0.72	0.060	0.707	0.584
P (AJCC stage)	0.542	0.815	0.057	0.165	0.789	0.260	0.974	0.543	0.091
P (Lymph node metastasis)	0.214	0.666	0.134	0.590	0.571	0.212	0.234	0.858	0.117
P (nation)							0.659	0.625	0.401

Table 3. Relationship between the β-catenin in GSK3β-negative and clinicopathological characteristics

	Kazakh			Han			Total		
	β-catenin		*P* value	β-catenin		*P* value	β-catenin		*P* value
	preserved	aberrant		preserved	aberrant		preserved	aberrant	
Tumor location									
Upper	2	2		1	0		3	2	
Middle	13	14	0.964	29	28	0.621	42	42	0.000
Lower	36	48		48	44		84	92	
Tumor size									
≤ 3 cm	15	13	0.686	28	22	0.614	43	35	0.002
> 3 cm	36	51		50	50		86	101	
Degree of differentiation									
Well	13	13		13	0		26	13	
Moderately	24	37	0.699	48	53	0.230	72	90	0.000
Poorly	14	14		18	18		32	32	
AJCC stage									
T0	0	4		2	8		2	12	
T1	22	31	0.05	40	30	0.152	62	61	0.000
T2	16	5		0	8		16	13	
T3	13	24		40	22		53	46	
Lymph node metastasis									
No	39	33	0.181	70	53		109	86	
Yes	13	30		9	18	0.387	22	48	0.000

expression is localized to the membrane in normal epithelium, but aberrant expression was shown in 144 (54.3%) carcinoma tissues. The altered β-catenin expression rates in Kazak patients and Han patients were 54.8% (63/115) and 60.0% (90/150).

When the GSK3β expressions were down-regulated, the rate of the altered β-catenin expression in Kazak patients and Han patients were 55.6% and 47.1%. Obviously, the expression of β-catenin implicated the nation difference (P = 0.019). Among the GSK3β low-expressions, in Kazak ESCC patients, altered β-catenin expression was closely correlated with the AJCC stage (P = 0.05), but not with tumor size, tumor location, degree of differentiation, lymph node status. At the same time, the data showed the altered β-catenin expression in all GSK3β-negative samples was significantly correlated with the tumor location, tumor size, degree of differentiation, AJCC stage, lymph node status (**Table 3**). However, the aberrant expression of β-catenin in Han GSK3β-negative ESCC was not associated with ethnic group, tumor size, tumor location, degree of differentiation, AJCC stage and lymph node status (**Table 3**).

Significant prognostic value of GSK3β and β-catenin expression patterns for ESCC

Survival dates were analyzed for 100 follow-up patients during follow-up periods of 1-72 months (median, 15 months). The ESCC-5-year

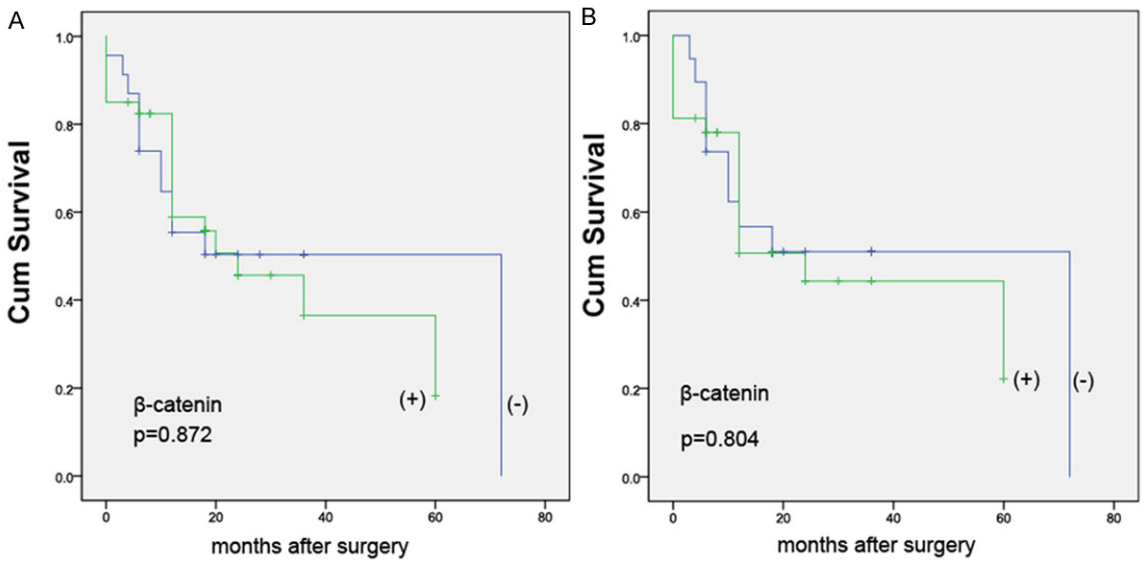

Figure 2. The relationship between the expression of β-catenin and survival curve. A. The aberrant β-catenin expression was not correlated with poor prognosis (P = 0.872); B. In the GSK3β-negative ESCCs, the prognosis of the β-catenin-altered-patients was no obvious difference with the β-catenin-preserved-patients (P = 0.804).

survival rate was 3.7%. Overall Survival curves based on GSK3β and β-catenin expression were constructed using the Kaplan-Meier method. The β-catenin-altered-patients had significantly poorer survival in comparison to the β-catenin-preserved-patients (**Figure 2A**), but was not found statistically significant (P > 0.05). In addition, when the GSK3β was low-expression, the prognosis of the β-catenin-altered-patients was no obvious difference with the β-catenin-preserved-patients (**Figure 2B**).

Discussion

Most patients with ESCC are diagnosed at an advanced stage [22]. Despite recent improvements in its treatment, the clinical outcome of ESCC patients remains unsatisfactory [23]. It is urgently needed to investigate the underlying molecular markers to improve the outcome of patients with ESCC. In recent years, numerous studies have demonstrated that Wnt canonical signaling pathway played an important role in carcinogenesis and progression in ESCC [23-25]. In the current study, we found that Wnt2 and β-catenin was up-regulated while GSK3β was down-regulated in ESCC tissue. Our result was concordant with the findings by Fu et al, He et al, and Wang et al [9, 24, 26].

Wnt2, a ligand protein, has been reported as an oncogene with the potential to activate the Wnt canonical signaling pathway [9]. The Wnt pathway is a central mechanism in cancer biology [27]. High-expression of Wnt2 has been observed in colorectal cancer, gastric cancer and esophageal adenocarcinoma [28-30], while there are a few studies concerning Wnt2 expression in ESCC. In our study, Wnt2 was not expressed in normal esophageal epithelial tissues. However, 56.3% (147/265) ESCCs were Wnt2 positive. It suggested that Wnt2 regulated ESCC progression by activating the Wnt canonical signaling pathway. Consistent with the idea that higher expression of Wnt2 in moderate- and high-grade malignant in comparison to low-grade malignant [31], we found that the positive rate of Wnt2 increased with the malignant degree.

GSK3β is an essential component of the Wnt signaling pathway and plays a critical role in sequestrating β-catenin, GSK3β has joined APC, Axin as a part of β-catenin destruction complex [32, 33]. The main function of the destruction complex is to promote phosphorylacstion of β-catenin. The positive rate of GSK3β in ESCC tissues was 7.2%, but the normal esophageal tissue was 54.2%. It suggested that GSK3β poorly expressed in ESCC, which reconfirmed the findings by He et al [26]. Here, we novel found that the expression status of GSK3β was closely associated with the tumor location. Further studies on the relationship

between the expression of GSK3β and tumor location would be very rewarding. GSK-3 is an essential component of the Wnt signaling pathway and plays important roles in regulating cell proliferation, differentiation, and apoptosis [34]. However, the GSK3β expression showed no significant association with tumor size, degree of differentiation, AJCC stage, lymph node status in ESCC.

β-catenin is not only an important structural component of both normal epithelium and malignant cells, but also a pivotal component of the canonical Wnt signaling pathway [35]. In normal esophageal, membrane β-catenin blinding to membrane E-cadherin forms a complex that promotes cell adhesion. The β-catenin expression in the cytoplasm and/or nuclear could be defined as the aberrant expression. The aberrant β-catenin accumulates the nucleus to interact with TCF/LEF transcription factors which are involved in oncogenic transformation [26, 36]. Then, it was reported that β-catenin was significantly correlated with invasion depth and lymph node status [37]. This study also found that in GSK3β-negative ESCCs the aberrant expression of β-catenin significantly correlated with the clinicopathological features. The results demonstrated that the aberrant expression of β-catenin can be regarded as an indication for activated, oncogenic, Wnt signaling and β-catenin/TCF transcription. As Ninomiya et al. reported, GSK3β mediated phosphorylation function as a switch in regulating the stabilization of β-catenin [38]. However, the results never referred to the correlation between GSK3β and β-catenin. To further verify the relationship between GSK3β and β-catenin, further study should be reformed by other methods. There was a subgroup of patients who had alteration of β-catenin but negative GSK3β .One reason might be that β-catenin activated through other pathways besides Wnt2. Moreover, One critical findings in this study that the aberrant expression of β-catenin in GSK3β-negative ESCCs was significantly higher in Kazak ESCC patients. It was thought that there was compared difference of β-catenin expression in Kazak and Han. So the further research with a large sample size would be worth doing.

In this study, we did not investigate that patients survival was correlated with the expressions of GSK3β, β-catenin. The limitation of our study is high loss to follow-up, which may influence the results. The communication difficulties with most of Kazakhs, who usually spoke ethnic languages and lived in remote country from the First Affiliated Hospital, Xinjiang Medical University, leaded to lose contact or received some error message. Moreover, the wrong or missing phone numbers also caused some ESCCs could not be contacted. For these reasons, only 100 (37.7%) patients of ESCC were followed up. Therefore, the relationship between the prognoses with the Wnt canonical signaling pathway may be investigated by further study with a large sample size.

In summary, we found the positive rate of Wnt2 significantly increased with the malignant degree of Kazak ESCC patients. Our present study also demonstrated that in GSK3β-negative ESCCs, β-catenin was significantly correlated with the tumor location, tumor size, degree of differentiation, AJCC stage, lymph node metastasis. This report provided a novel indication that the expression of β-catenin implicated the ethnic difference. These finding suggests that the aberrant β-catenin expression could be an adverse underlying factor in carcinogenesis and progression of ESCC. Although Wnt2, GSK3β, β-catenin may not be an independent prognostic factor, it should be further verified by prospective analysis and more comprehensive follow-up.

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Disclosure of conflict of interest

None.

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