The expression of Wnt/β-cantenin signaling pathway related protein in Kazak esophageal squamous cell carcinoma and its prognostic significance

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Research

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Abstract

Background Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors. Kazak in Xinjiang is a high incidence of esophageal cancer in China. It is known that the occurrence of ESCC is related to the abnormal activation of Wnt/β-cantenin signaling pathway, but the regulatory mechanism of its abnormal activation is not clear. This study aims to study the role of Wnt/β-cantenin signaling pathway related proteins in the development of Kazakh esophageal cancer.

Methods: Methods In this study, 226 paraffin tissues and 29 fresh esophageal squamous carcinoma tissues were included. Immunohistochemistry and RT-PCR were used to detect Wnt/β-cantenin signaling pathway related proteins (Wnt2, β-cantenin, GSK3, The expression of suivivin in ESCC and its corresponding normal esophageal mucosa of Kazak and Han nationality, and the relationship between the expression of each protein and the clinicopathological characteristics and prognosis of Kazak ESCC were analyzed.

Results We found that there was a significant difference between Han nationality and age (p = 0.038) and lymph node metastasis (p = 0.043). The proportion of Kazak patients under 60 years old was higher than that of Han nationality, indicating that the age of Kazak ESCC patients was lower; the proportion of Han nationality with lymph node metastasis was higher, indicating that the recurrence and progression rate was higher; the expression of Wnt2 in ESCC was related to TNM (p= 0.012), I = 9.8%, II = 52.9%, III = 29.4%, IV = 7.8%; the expression of β- Catenin was significantly different in the depth of invasion (p = 0.032), postoperative radiotherapy or chemotherapy (p = 0.049); the expression of GSK3β in ESCC was related to TNM (p = 0.013), in which I = 13.2%, II = 51.5%, III = 29.4%, IV = 5.9%.The mRNA expression of β- cantenin and GSK3β in ESCC was significantly higher than that in normal esophageal mucosa (p < 0.001), but there was no significant difference in Wnt2 and survivin. Lymph node metastasis (p=0.009), nerve invasion (p= 0.001) and radiotherapy and chemotherapy (p < 0.001) can be used as independent factors to affect the Overalll survivalof patients with ESCC; TNM (p= 0.017), nerve invasion (p= 0.028), hematogenous metastasis (p=0.048), radiotherapy and chemotherapy (p = 0.008) and Survivin (p = 0.0029) were all independent influence shadows of squamous cell carcinoma in patients with ESCC. There was no difference between Kazak and Han in Overalll survival(p = 0.929) and progression free survival (p = 0.845).

Discussion In ESCC, the level of β-cantenin, protein in Wnt/β-cantenin signaling pathway is high, while that of GSK-3 is low. The recurrence time of survivin positive patients is shorter than that of negative patients. In addition, the expression of Wnt2,β-cantenin, GSK3βand Survivin were not significantly different between Han and Kazak, and there was no significant difference in the total and progression free survival of Kazak patients with ESCC.

Introduction

Esophageal squamous cell carcinoma(ESCC) is one of the most common upper gastrointestinal cancers in the world. The mortality and incidence rate is eighth and sixth [1] in the world respectively. The distribution of ESCC in China is obviously different from that in the region. The Kazakh people in Xinjiang are the most prevalent ethnic groups in China. The incidence rate is as high as 155.9/10 million, far higher than the national average of 14.95/10 million. The incidence rate of ESCC in Han nationality living in the same area is only 13/10 10000, and its mortality is 2.3 times higher than the national average level [2,3].In addition, Kazak ESCC is a high incidence disease in the region, but there are few studies on Wnt pathway and ESCC. Therefore, it is of practical significance for the clinical treatment of esophageal squamous carcinoma of Kazak nationality in Xinjiang to study the related genes in the process of occurrence and development of esophageal squamous carcinoma of Kazak nationality in Xinjiang and clarify its mechanism of action and explore the potential specific therapeutic target.

Wnt/β-cantenin pathway is the classic pathway of Wnt signaling pathway, and it also plays a major role. Wnt signaling pathway includes multiple oncogenes (β-cantenin, CtBP, c-myc, CyclinD1, survivin, etc.) and tumor suppressor genes (GSK3β, APC, wtx1, Axin, etc.), and the structural connection between the related proteins of this pathway is based on the regulation of oncogenes and tumor suppressor genes. As early as 2012, Katrin hoffmeyer published an article on science entitled Wnt/β-cantenin signaling regulates telomerase of stem cells and cancer cells, laying a foundation for discovering the mechanism of Wnt/β-cantenin signaling pathway in tumor [4].β-cantenin, is the key protein of Wnt pathway. β-cantenin, (β-cantenin,) was first found as a kind of adhesion factor. Later, it was found that β-cantenin, is a multifunctional protein, whose function is mainly to mediate intercellular adhesion and participate in gene expression. It widely exists in various types of cells, such as endothelial cells, fibroblasts, osteoblasts, and participate in the proliferation, differentiation and apoptosis of these cells It has played an important regulatory role[5]. The genes regulating the transfer of β-cantenin, from cell membrane to cytoplasm and nucleus belong to the upstream genes of the signal pathway, such as Wnt2, LRP5 / 6, APC, GSK, CK1, Axin; the genes regulated by β-cantenin, in the nucleus belong to the downstream genes, such as c-myc, CyclinD1, survivin, VEGF. When Wnt is activated abnormally, its signal can inhibit the function of regulatory complex, and then enhance the stability of β-cantenin, and cause its shift. Therefore, we chose Wnt2 and GSK3βas the upstream genes, survivin as the research objects.

Wnt2 is a ligand protein secreted by tumor fibroblasts, secreted in a paracrine manner, which can activate the Wnt/β-cantenin pathway[6]. The main function of GSK3βis to promote the phosphorylation and degradation of β-cantenin,. The expression of GSK3βis significantly down regulated in tumor tissues, and the function of phosphorylated β-cantenin, is weakened [7]. Survivin (encoding gene BIRC5) is a dual functional protein, which can be used as a key inhibitor of apoptosis (IAP) and a key regulator of cell cycle progression. The overexpression of survivin often occurs in various human cancers, and the increase of survivin is related to poor clinical prognosis, tumor recurrence and drug resistance. Because of its selective expression in tumors rather than normal tissues, survivin is considered to be an attractive target for cancer treatment[8].

The purpose of this study is to study the correlation between Wnt/β-cantenin, signal pathway related differential protein and clinicopathological parameters in Kazakh esophageal squamous carcinoma, and to explore whether it can be used as a marker for metastasis and prognosis evaluation of esophageal squamous carcinoma, so as to lay a foundation for the future study of target gene therapy and prognosis monitoring of Kazakh esophageal squamous carcinoma.

Methods

1. Patients and tissue samples

In this study, 226specimens of ESCC and paraffinic normal tissues were collected from the First Affiliated Hospital of Xinjiang Medical University from 2014 to 2018. Among them, 114 were Han and 112 Kazak. At the same time, 29 cases of esophageal cancer and adjacent normal fresh tissue were collected. All the patients were local residents in Xinjiang, and they were not treated with radiotherapy and chemotherapy before operation. They were diagnosed as ESCC by senior diagnostic physician of the first affiliated pathology department of Xinjiang Medical University. At the same time, we collected the basic information of patients, including age (< 60 years old, over 60 years old),gender (male = 160, female = 66), nationality (Han = 114, Kazak = 112), tumor growth site (upper esophagus = 11, middle esophagus = 133, lower esophagus = 82), tumor size (< 3cm = 67, ≥3cm = 159), degree of differentiation (low differentiation = 49, medium differentiation = 120, high differentiation = 57), depth of invasion (mucosal layer = 7, muscular layer 7 = 93; whole layer = 126), clinical stage (stage I = 17; stage II = 141;stage III = 47;stage IV = 21), lymph node metastasis (yes = 75; no = 151), vascular invasion (yes = 42; no = 184), nerve invasion (yes = 48; no = 178), hematogenous metastasis (yes = 30; no = 196), radiotherapy and chemotherapy (yes = 79; no = 147). The patients were followed up to observe the prognosis.

2.Immunohistochemistry

In this study, 229 cases of paraffin embedded ESCC and normal mucosa were made into tissue chips by immunohistochemical technique. Paraffin section was dewaxed and hydrated, 3% hydrogen peroxide blocked endogenous peroxidase, and citric acid (pH = 6) was put into boiling water at 100 ℃, respectively, for antigen repair, calf serum blocked non-specific antigen, and rat anti-human antibody (Wnt2, survivin, GSK3β, β-canteninwas added) was put into the incubator at 37 ℃ for 60 minutes, and then added into the second general antibody of rabbit and mouse respectively. It was put into the oven at 37 ℃ for 30 minutes, and the results were observed under the microscope. The dyeing intensity was divided into 0 (non staining), 1 (weak staining), 2 (medium staining) and 3 (strong staining). The number of positive cells was 0 (< 5%), 1 (5–25%), 2 (26–50%) and 3 (> 50%) respectively. If the multiplier is less than 6, the expression level is defined as "low expression"; otherwise, it is defined as "high expression". Wnt2 is expressed in the stroma of ESCC; β-cantenin, is expressed in the cell membrane of ESCC; suivin is expressed in the nucleus of ESCC, and GSK3β is expressed in the cytoplasm of ESCC.

3. RT-PCR

In the First Affiliated Hospital of Xinjiang Medical University, 29 fresh esophageal cancer tissues and their matched normal mucosa tissues were stored in a refrigerator at − 80 ℃. Design primers are shown in Table 1. Total RNA extraction: take 200 mg tissue, grind it in liquid nitrogen environment and add 1 ml Trizol, and collect it into 1.5 ml EP tube,. Concussion for 30 s. Add 0.2 ml of chloroform, shake vigorously for 30 s, room temperature for 10 min. 12000 × g, centrifuged at 4 ℃, 15 min. Absorb the colorless water phase of the upper layer and transfer it into another EP tube (about 0.5 ml). Add equal volume of isopropanol, room temperature, 10 min. 12000 × g, centrifuged at 4 ℃, 10 min. At the bottom of the tube, a small amount of RNA precipitate can be seen. Discard the supernatant, add 1 ml of 75% ethanol, and shake. 7500 × g, centrifuged at 4 ℃, 10 min. Discard the supernatant, carefully suck the residual liquid with a pipette, and dry at room temperature for 5–10 min. The precipitate was dissolved in 20 μ L DEPC water, 1.5 μ l total RNA concentration was measured in nanodrop and the purity was calculated by od260 / od280. The configured reverse transcription system was put into the PCR instrument, and the reaction conditions were set at 42 ℃ for 60 minutes and 72 ℃ for 5 minutes. After the reverse transcription, the cDNA was stored at − 20 ℃ for standby. SYBR green dye and 7500 real-time PCR system were used to detect the expression of target gene in fresh tissue. β - acting as a reference gene. All reactions were carried out three times under the following conditions: activation of the polymerase for 2 minutes at 50 ℃, initial denaturation for 10 minutes at 94 ℃, followed by 40 denaturation for 15 s at 94 ℃, annealing and elongation for 30 s at 60 ℃. The mRNA expression of the target gene was calculated by ΔΔct.

Table 1
the primer sequence of RT-pCR

primer	sequence
β-actin-F	5′-CATGTACGTTGCTATCCAGGC-3′
β-actin-R	5′-CTCCTTAATGTCACGCACGAT-3′
Wnt2-F	5′-GATGCGTGCCATTAGCCAG-3′
Wnt2-R	5′-AGATTCCCGACTACTTCGGAG-3′
β-cantenin-F	5′-GCGCCATTTTAAGCCTCTCG-3′
β-cantenin-R	5′-CTGAAGCTGCTCCTCAGACC-3′
Survivin-F	5′-AGGACCACCGCATCTCTACAT-3′
Survivin-R	5′-AAGTCTGGCTCGTTCTCAGTG-3′
GSK3β-F	5′-GGCAGCATGAAAGTTAGCAGA-3′
GSK3β-R	5′-GGCGACCAGTTCTCCTGAATC-3′
CyclinD1-F	5′-GCTGCGAAGTGGAAACCATC-3′
CyclinD1-R	5′-CCTCCTTCTGCACACATTTGAA-3′

4.Statistical analysis

SPSS 17 statistical software was used to analyze the clinical characteristics of patients. The relationship between Wnt2, survivin, GSK3β,β-cantenin and the clinicopathological characteristics of ESCC was analyzed by chi square test and Fisher exact test, and the relationship between their combinations was analyzed by Pearson correlation. The Overalll survivaltime and progression free survival time were used to estimate the survival time. Progression free survival time was defined as the time from the diagnosis of ESCC to the time of tumor progression or death. Overalll survival time was defined as the time from the diagnosis of ESCC to the time of death or final follow-up (July 1, 2019). Kaplan Meier method and Cox risk ratio model were used to single out the survival and prognosis of ESCC Variable and multivariate analysis. Differences were indicated statistically significant when p was less than 0.05 and all p values were two-tailed.

Result

1. Differences of clinicopathological parameters between Han and Kazak patients with ESCC

In this study, 226 cases of ESCC were included, including 114 cases of Han nationality and 112 cases of Kazak nationality. Through statistical analysis of the relationship between nationality and clinical pathological parameters(Table 2), we found that there was a significant difference between nationality and age (p = 0.038).The percentage of Kazak patients younger than 60 years old is higher than that of Han, which indicates that the age of Kazak esophageal cancer patients is lower.It was found that there was a significant difference in the incidence of lymph node metastasis(p = 0.043) between ethnic groups. The percentage of Kazak esophageal cancer patients with lymph node metastasis was lower than that of Han patients, suggesting that the probability of Kazak patients with lymph node metastasis was lower than that of Han patients.

Table 2
Characteristics of 226 ESCC patients included in this study

Clinical pathological parameters	Number	Han	Kazakh	p	χ2
	226	114	112		
Age					
≤ 60	84	32(28.1%)	52(46.4%)	0.004	8.153
⩾60	142	82(71.9%)	60(53.6%)		
Gender					
male	160	86(75.4%)	74(66.1%)	0.122	2.398
Female	66	28(24.6%)	38(33.9%)		
Tumor location					
Upper	11	9(7.9%)	2(1.8%)	0.038	6.652
Middle	133	70(61.4%)	63(56.3%)		
Lower	82	35(30.7%)	47(42.0%)		
Tumor size					
< 3 cm	67	35(30.7%)	32(28.6%)	0.726	0.123
≥3 cm	159	79(69.3%)	80(71.4%)		
Differentiation					
Low	49	27(23.7%)	22(19.6%)		
Medium	120	63(55.3%)	57(50.9%)	0.331	2.214
Highly	57	24(21.1%)	33(29.5%)		
Depth of invasion					
Mucosal	7	6(5.3%)	1(0.9%)	0.146	3.855
Muscle	93	44(38.6%)	49(43.8%)		
Full	126	64(56.1%)	62(55.4%)		
TNM					
I	17	9(7.9%)	8(7.1%)	0.555	2.087
II	141	66(57.9%)	75(67.0%)		
III	47	27(23.7%)	20(17.9%)		
IV	21	12(10.5%)	9(8.0%)		
Lymph node metastasis					
No	151	69(60.5%)	82(73.2%)	0.043	4.102
Yes	75	45(39.5%)	30(26.8%)		
vascular invasion					
No	184	92(80.7%)	92(82.1%)	0.781	0.078
Yes	42	22(19.3%)	20(17.9%)		
Nerve invasion					
No	178	92(80.7%)	86(76.8%)	0.472	0.518
Yes	48	22(19.3%)	26(23.2%)		
Hematogenous metastasis					
No	196	98(86.0%)	98(87.5%)	0.734	0.116
Yes	30	16(14.0%)	14(12.5%)		
Radiotherapy and chemotherapy					
No	147	75(65.3%)	40(64.3%)	0.813	0.0.56

Clinical pathological parameters	Number	Han	Kazakh	p	χ2
Yes	79	39(34.2%)	40(35.7%)		

2.Wnt/β-cantenin related protein expression in ESCC

As the key protein of Wnt/β-cantenin signaling pathway, Wnt2 is expressed in the cytoplasm of ESCC (Fig. 1. A), with a positive rate of 45.1% (Table 3), and in the normal mucosa (Fig. 1. B). The results show that the expression of Wnt2 in ESCC is significantly different in TNM (p = 0.012), among which I = 9.8%, II = 52.9%, III = 29.4%, IV = 7.8%.

Table 3

The relationship between the expression of Wnt2, - Catenin, survivin and GSK3 and the clinicopathological para

ββ												
		Wnt2				β-catenin				Survivin		
Clinical pathological parameters	Number	Positive	Negative	P	χ2	Positive	Negative	P	χ2	Positive	Negative	P
	226	102(45.1%)	124(54.9%)			162(71.7%)	64(28.3%)			82(36.3%)	144(63.7%)	
Age												
≤ 60	84	42(41.2%)	42(33.9%)	0.258	1.279	57(35.8%)	27(38.6%)	0.326	0.963	26(31.7%)	58(40.3%)	0.
60	142	60(58.8%)	82(66.1%)			105(64.2%)	37(57.8%)			56(68.3%)	86(59.7%)	
Gender												
male	160	75(73.5%)	85(68.5%)	0.412	0.672	119(73.5%)	41(64.1%)	0.162	1.958	55(67.1%)	105(72.9%)	0.
Female	66	27(26.5%)	39(31.5%)			43(26.5%)	23(35.9%)			27(32.9%)	39(27.1%)	
Nation												
Han	114	54(52.9%)	60(48.4%)	0.496	0.464	37(54.4%)	77(48.7%)	0.434	0.613	40(48.8%)	74(51.4%)	0.
Kazakh	112	48(47.1%)	64(51.6%)			31(45.6%)	81(51.3%)			42(51.2%)	70(48.6%)	
Tumor location												
Upper	11	1(1.2%)	10(6.9%)	0.157	3.699	7(4.3%)	4(6.3%)	0.4	1.834	1(1.2%)	10(6.9%)	0.
Middle	133	50(61.0%)	83(57.6%)			92(56.8%)	41(64.1%)			50(61.0%)	83(57.6%)	
Lower	82	31(37.8%)	51(35.4%)			63(38.9%)	19(29.7%)			31(37.8%)	51(35.4%)	
Tumor size												
<3 cm	67	31(30.4%)	36(29.0%)	0.824	0.05	46(28.4%)	21(32.8%)	0.512	0.429	22(26.8%)	45(31.3%)	0.
≥3 cm	159	71(69.6%)	88(71.0%)			116(71.6%)	43(67.2%)			60(73.2%)	99(68.8%)	
Differentiation												
Low	49	19(18.6%)	30(24.2%)	0.231	2.927	34(21.0%)	15(23.4%)	0.919	0.168	16(19.5%)	33(22.9%)	0.
Medium	120	52(51.0%)	68(54.8%)			87(53.7%)	33(51.6%)			50(61.0%)	70(48.6%)	
Highly	57	31(30.4%)	26(21.0%)			41(25.3%)	16(25.0%)			16(19.5%)	41(28.5%)	
Depth of invasion												
Mucosal	7	3(2.9%)	4(3.2%)	0.153	3.575	3(1.9%)	4(6.3%)	0.032	6.871	0(0%)	7(4.9%)	0.
Muscle	93	35(34.3%)	58(46.8%)			61(37.7%)	32(50.0%)			41(50.0%)	52(36.1%)	
Full	126	64(62.7%)	62(50.0%)			98(60.5%)	28(43.8%)			41(50.0%)	85(59.0%)	
TNM												
I	17	10(9.8%)	7(5.6%)	0.012	11.002	9(5.6%)	8(12.5%)	0.354	3.252	4(4.9%)	13(9.0%)	0.
II	141	54(52.9%)	87(70.2%)			104(64.2%)	37(57.8%)			54(65.9%)	87(60.4%)	
III	47	30(29.4%)	17(13.7%)			34(21.0%)	13(20.3%)			20(24.4%)	27(18.8%)	
IV	21	8(7.8%)	13(10.5%)			15(9.3%)	6(9.4%)			4(4.9%)	17(11.8%)	
Lymph node metastasis												
No	151	60(58.8%)	91(73.4%)	0.021	5.353	105(64.8%)	46(71.9%)	0.31	1.031	56(68.3%)	95(66.0%)	0.
Yes	75	42(41.2%)	33(26.6%)			57(35.2%)	18(28.1%)			26(31.7%)	49(34.0%)	
vascular invasion												
No	184	80(78.4%)	104(83.9%)	0.295	1.094	131(80.9%)	53(82.8%)	0.734	0.115	68(82.9%)	116(80.6%)	0.
Yes	42	22(21.6%)	20(16.1%)			31(19.1%)	11(17.2%)			14(17.1%)	28(19.4%)	

ββ												
Nerve invasion												
No	178	81(79.4%)	97(78.2%)	0.828	0.047	127(78.4%)	51(79.7%)	0.831	0.046	67(81.7%)	111(77.1%)	0.
Yes	48	21(20.6%)	27(21.8%)			35(21.6%)	13(20.3%)			15(18.3%)	33(22.9%)	
Hematogenous metastasis												
No	196	90(88.2%)	106(85.5%)	0.544	0.368	137(84.6%)	59(92.2%)	0.128	2.314	71(86.6%)	125(86.8%)	0.
Yes	30	12(11.8%)	18(14.5%)			25(15.4%)	5(7.8%)			11(13.4%)	19(13.2%)	
Radiotherapy and chemotherapy												
No	147	66(64.7%)	81(65.3%)	0.923	0.009	99(61.1%)	48(75.0%)	0.049	3.892	52(63.4%)	95(66.0%)	0.
Yes	79	36(35.3%)	43(34.7%)			63(38.9%)	16(25.0%)			30(36.6%)	49(34.0%)	

β-cantenin is mainly expressed in the cell membrane of ESCC (Fig. 1C), and its positive expression rate is 69.4%, which is significantly higher than that of 30.6% of ESCC (Table 3). It is negative in normal esophageal mucosa (Fig. 1D). It was found that the expression of β- Catenin in ESCC was significantly different in patients with different depth of invasion (p = 0.032), and there was also a difference in the expression of β- Catenin between patients who received radiotherapy or chemotherapy after operation.

Survivin is mainly expressed in the nucleus of esophageal cancer (Fig. 1. E), and it is negative in normal esophageal mucosa (Fig. 1. F), but the positive expression rate is 36.2% in ESCC. There was a significant difference between the expression of Survivin and the depth of infiltration (p = 0.027). There was no positive expression of Survivin in the mucosa, and 50% in the muscular layer and the whole layer (Table 3).

GSK3βwas mainly expressed in cytoplasm (Fig. 1. G) and was negative in esophageal mucosa (Fig. 1. H). In ESCC, the positive rate of GSK3β was 29.7%, much lower than the negative expression rate of 70.3%, which was significantly different from TNM stage (p = 0.013), in which I = 13.2%, II = 51.5%, III = 29.4%, IV = 5.9% (Table 3). It was considered that the earlier the stage, the higher the expression of GSK3β.

The results showed that the expression of survivin was positively correlated with Wnt2 (r = -0.148, p = 0.026).However, the expression of β- Catenin and GSK3β protein was positively correlated with Wnt2 protein expression(r = 0.156,p = 0.019;r = 0.277，p < 0.001), and there was no significant correlation between other related proteins (Table 4).

Table 3

The relationship between the expression of Wnt2, -cantenin, survivin and GSK3 and the clinicopathological parameters of ESCC

ββ																	
		Wnt2				β-cantenin				Survivin				GSK3β			
Clinical pathological parameters	Number	positive	Negative	p	χ2	positive	Negative	p	χ2	positive	Negative	p	χ2	positive	Negative	p	χ2
	226	102(45.1%)	124(54.9%)			162(71.7%)	64(28.3%)			82(36.3%)	144(63.7%)			68(30.1%)	158(69.9%)		
Age																	
≤ 60	84	42(41.2%)	42(33.9%)	0.258	1.279	57(35.8%)	27(38.6%)	0.326	0.963	26(31.7%)	58(40.3%)	0.200	1.643	23(33.8%)	61(38.6%)	0.495	0.4
⊠60	142	60(58.8%)	82(66.1%)			105(64.2%)	37(57.8%)			56(68.3%)	86(59.7%)			45(66.2%)	97(62.4%)		
Gender																	
male	160	75(73.5%)	85(68.5%)	0.412	0.672	119(73.5%)	41(64.1%)	0.162	1.958	55(67.1%)	105(72.9%)	0.353	0.863	50(73.5%)	110(69.6%)	0.553	0.3
Female	66	27(26.5%)	39(31.5%)			43(26.5%)	23(35.9%)			27(32.9%)	39(27.1%)			18(26.5%)	48(30.4%)		
Nation																	
Han	114	54(52.9%)	60(48.4%)	0.496	0.464	37(54.4%)	77(48.7%)	0.434	0.613	40(48.8%)	74(51.4%)	0.706	0.142	80(49.4%)	34(53.1%)	0.612	0.2
Kazakh	112	48(47.1%)	64(51.6%)			31(45.6%)	81(51.3%)			42(51.2%)	70(48.6%)			82(50.6%)	30(46.9%)		
Tumor location																	
Upper	11	1(1.2%)	10(6.9%)	0.157	3.699	7(4.3%)	4(6.3%)	0.4	1.834	1(1.2%)	10(6.9%)	0.157	3.699	4(5.9%)	7(4.4%)	0.684	0.7
Middle	133	50(61.0%)	83(57.6%)			92(56.8%)	41(64.1%)			50(61.0%)	83(57.6%)			42(61.8%)	91(57.6%)		
Lower	82	31(37.8%)	51(35.4%)			63(38.9%)	19(29.7%)			31(37.8%)	51(35.4%)			22(32.4%)	60(38.0%)		
Tumor size																	
<3 cm	67	31(30.4%)	36(29.0%)	0.824	0.05	46(28.4%)	21(32.8%)	0.512	0.429	22(26.8%)	45(31.3%)	0.484	0.490	19(27.9%)	48(30.4%)	0.713	0.1
≥3 cm	159	71(69.6%)	88(71.0%)			116(71.6%)	43(67.2%)			60(73.2%)	99(68.8%)			49(72.1%)	110(69.6%)		
Differentiation																	
Low	49	19(18.6%)	30(24.2%)	0.231	2.927	34(21.0%)	15(23.4%)	0.919	0.168	16(19.5%)	33(22.9%)	0.919	0.168	12(17.6%)	37(23.4%)	0.495	1.4
Medium	120	52(51.0%)	68(54.8%)			87(53.7%)	33(51.6%)			50(61.0%)	70(48.6%)			36(52.9%)	84(53.2%)		
Highly	57	31(30.4%)	26(21.0%)			41(25.3%)	16(25.0%)			16(19.5%)	41(28.5%)			20(29.4%)	37(23.4%)		
Depth of invasion																	
Mucosal	7	3(2.9%)	4(3.2%)	0.153	3.575	3(1.9%)	4(6.3%)	0.032	6.871	0(0%)	7(4.9%)	0.027	7.199	2(2.9%)	5(3.2%)	0.955	0.0
Muscle	93	35(34.3%)	58(46.8%)			61(37.7%)	32(50.0%)			41(50.0%)	52(36.1%)			29(42.6%)	64(40.5%)		
Full	126	64(62.7%)	62(50.0%)			98(60.5%)	28(43.8%)			41(50.0%)	85(59.0%)			37(54.4%)	89(56.3%)		
TNM																	
I	17	10(9.8%)	7(5.6%)	0.012	11.002	9(5.6%)	8(12.5%)	0.354	3.252	4(4.9%)	13(9.0%)	0.176	1.941	9(13.2%)	8(5.1%)	0.013	10.
II	141	54(52.9%)	87(70.2%)			104(64.2%)	37(57.8%)			54(65.9%)	87(60.4%)			35(51.5%)	106(67.1%)		
III	47	30(29.4%)	17(13.7%)			34(21.0%)	13(20.3%)			20(24.4%)	27(18.8%)			20(29.4%)	27(17.1%)		
IV	21	8(7.8%)	13(10.5%)			15(9.3%)	6(9.4%)			4(4.9%)	17(11.8%)			4(5.9%)	17(10.8%)		
Lymph node metastasis																	
No	151	60(58.8%)	91(73.4%)	0.021	5.353	105(64.8%)	46(71.9%)	0.31	1.031	56(68.3%)	95(66.0%)	0.722	0.127	41(60.3%)	110(69.6%)	0.172	1.8
Yes	75	42(41.2%)	33(26.6%)			57(35.2%)	18(28.1%)			26(31.7%)	49(34.0%)			27(39.7%)	48(30.4%)		
vascular invasion																	
No	184	80(78.4%)	104(83.9%)	0.295	1.094	131(80.9%)	53(82.8%)	0.734	0.115	68(82.9%)	116(80.6%)	0.659	0.194	58(85.3%)	126(79.7%)	0.325	0.9
Yes	42	22(21.6%)	20(16.1%)			31(19.1%)	11(17.2%)			14(17.1%)	28(19.4%)			10(14.7%)	32(20.3%)		
Nerve invasion																	
No	178	81(79.4%)	97(78.2%)	0.828	0.047	127(78.4%)	51(79.7%)	0.831	0.046	67(81.7%)	111(77.1%)	0.414	0.668	53(77.9%)	125(79.1%)	0.843	0.0
Yes	48	21(20.6%)	27(21.8%)			35(21.6%)	13(20.3%)			15(18.3%)	33(22.9%)			15(22.1%)	33(20.9%)		
Hematogenous metastasis																	
No	196	90(88.2%)	106(85.5%)	0.544	0.368	137(84.6%)	59(92.2%)	0.128	2.314	71(86.6%)	125(86.8%)	0.963	0.002	61(89.7%)	135(85.4%)	0.386	0.7
Yes	30	12(11.8%)	18(14.5%)			25(15.4%)	5(7.8%)			11(13.4%)	19(13.2%)			7(10.3%)	23(14.6%)		
Radiotherapy and chemotherapy																	
No	147	66(64.7%)	81(65.3%)	0.923	0.009	99(61.1%)	48(75.0%)	0.049	3.892	52(63.4%)	95(66.0%)	0.698	0.150	47(69.1%)	100(63.3%)	0.400	0.7
Yes	79	36(35.3%)	43(34.7%)			63(38.9%)	16(25.0%)			30(36.6%)	49(34.0%)			21(30.9%)	58(36.7%)		

Table 4

The correlation with Wnt2

	Wnt2		p(⊠0.05)	r
	positive	negative		
Survivin				
positive	29(28.4%)	53(42.7%)	0.026	-0.148
negative	73(71.6%)	71(57.3%)		
β-cantenin				
positive	81(79.4%)	81(65.3%)	0.019	0.156
negative	21(20.6%)	43(34.7%)		
GSK3β				
positive	45(44.1%)	23(18.5%)	0.000	0.277
negative	57(55.9%)	101(81.5%)		

3. mRNA expression of Wnt/β-cantenin pathway related proteins in ESCC

RT-pCR was used to detect the mRNA expression of Wnt / β - cantenin pathway key protein in 29 fresh tissues of ESCC and matched fresh tissues of normal esophageal mucosa. The results showed that the mRNA expression of Wnt 2 (p = 0.752) (Fig. 2. A) in ESCC was not significantly different from that in normal mucosa. The mRNA expression of β-cantenin, (p < 0.001) (Fig. 2. B) and GSK3β (p < 0.001) (Fig. 2. D) was significantly different in esophageal cancer and normal esophageal mucosa (Table 5).Survivin mRNA expression in ESCC was higher than that in normal mucosa, but there was no statistical significance (p = 0.097) (Fig. 2. C).

At the same time, we found that the mRNA expression of β- Catenin and survivinin ESCC was positively correlated with the expression of GSK3β, among which β- Catenin (r = 0.723, p < 0.001), survivin (r = 0.425, p = 0.022), were all positively correlated (Table 6).

Table 5. Wnt/β-cantenin related protein mRNA expression in ESCC and normal mucosa						
	Mean	S.D	S.E	95%CI	t	*p*
Wnt2						
cancerVS.mucosa	-0.024	0.411	0.076	(-0.181,0.132)	-0.319	**0.752**
β-cantenin						
cancerVS.mucosa	1.206	0.960	0.178	(0.841,1.572)	6.770	**0.000**
survivin						
cancerVS.mucosa	0.315	0.986	0.183	(-0.060,0.690)	1.719	0.097
GSK3β						
cancerVS.mucosa	1.499	1.474	0.274	(0.939,2.060)	5.478	**0.000**

Table 6. Correlation of Wnt / β - cantenin pathway related protein mRNA expression in ESCC						
	β-cantenin		survivin		GSK3β	
	r	***p***	**r**	***p***	**r**	***p***
Wnt2	0.221	0.250	0.282	0.139	0.340	0.071
β-cantenin	-		0.363	0.053	**0.723**	**0.000**
survivin	-		-		**0.425**	**0.022**
GSK3β	-		-		-	

4.prognosis analysis

In this study, 226 patients with ESCC were included. The overall survival rate of 114 Han patients was 27.2%, and that of 112 Kazak patients was 30.5%. The results of univariate and multivariate analysis showed that the influence of clinicopathological factors on the survival and prognosis of patients with ESCC (Table 7). There was no significant difference in Overalll survivaltime (P = 0.929) and progression free survival time (P = 0.845) between Kazakh and Han ESCC patients (Fig3). Lymph node metastasis (P = 0.009), nerve invasion (P = 0.001) and radiotherapy and chemotherapy (P < 0.001) can be used as independent factors to affect the Overalll survivalof patients with ESCC. The overall survival of patients with lymph node metastasis, nerve invasion and no radiotherapy and chemotherapy is shorter (Fig 4). TNM stage (P = 0.017), nerve invasion (P = 0.028), hematogenous metastasis (P = 0.048), radiotherapy and chemotherapy (P= 0.008) and Survivin (P = 0.0029) were all independent influence shadows of squamous non progression stage in patients with esophageal squamous carcinoma (Fig 5), which could affect the occurrence and development of patients with esophageal squamous carcinoma. In TNM stage, patients in stage I and stage II did not show disease progression for a longer time than those in stage III and stage IV, patients without nerve infiltration, blood metastasis and postoperative radiotherapy and chemotherapy had a longer progression free survival period, and patients with survivin positive had a shorter recurrence time than those with negative.

Table.7 Effect of clinicopathological factor on prognosis of ESCC									
Clinical pathological parameters	Overall survival				progression-free-survival				
	kaplan-meier	COX			kaplan-meier	COX			
	p	*p*	HR	95%CI	*p*	*p*	HR	95%CI	
Age	0.547	-	-	-	0.499	-	-	-	
Gender	0.128	-	-	-	0.400	-	-	-	
Nation	0.929	-	-	-	0.845	-	-	-	
Tumor location	0.868	-	-	-	0.715	-	-	-	
Tumor size	0.284	-	-	-	0.415	-	-	-	
Differentiation	0.309	-	-	-	0.791	-	-	-	
Depth of invasion	0.646	-	-	-	0.442	-	-	-	
TNM	0.113	-	-	-	**0.065**	**0.017**	1.291	(1.048-1.591)	
Lymph node metastasis	**0.047**	**0.009**	1.562	(1.118-2.184)	**0.046**	-	-	-	
Vascular invasion	0.190	-	-	-	0.846	-	-	-	
Nerve invasion	**0.001**	**0.001**	1.856	(1.299-2.651)	**0.022**	**0.012**	1.566	(1.104-2.192)	
Hematogenous metastasis	0.041	-	-	-	0.153	**0.048**	1.584	(1.004-2.500)	
Radiotherapy and chemotherapy	**0.001**	**<0.001**	0.527	(0.375-0.739)	0.107	**0.008**	0.637	(0.456-0.890)	
Wnt2	0.34	-	-	-	0.490	-	-	-	
β-cantenin	0.677	-	-	-	0.833	-	-	-	
survivin	0.473	-	-	-	**0.040**	**0.029**	0.718	(0.534-0.966)	
GSK3β	0.215	-	-	-	0.259	-	-	-	

Discussion

The main treatment of esophageal cancer is surgical resection, but it is very difficult to treat those who relapse or progress. Therefore, it is of great significance to study the potential biomarkers of ESCC, reveal its potential mechanism, and find a potential effective targeted drug to improve the prognosis of ESCC patients. In recent years, a large number of studies have suggested that Wnt/β-cantenin signaling pathway plays an important role in the occurrence and development of esophageal cancer, so we aimed to find out the role of its key proteins.

Wnt2/β-cantenin signaling pathway plays an important role in regulating the growth and differentiation of cell cycle during the growth and differentiation of tissue and embryo, and also plays an important role in the development of many malignant tumors, such as esophageal cancer [9], breast cancer [10], cervical cancer [11]. Wnt2, β-cantenin, GSK3β and Survivin are key proteins of Wnt pathway, which play an important role in regulating cell proliferation, inhibiting cell differentiation and cell cycle.

In this study, we found that there is a certain relationship between the age of ESCC patients and ethnic groups. The proportion of Kazak patients under 60 years old is higher than that of Han nationality, suggesting that Kazak patients with esophageal cancer are younger, Kazak patients with esophageal cancer lymph node metastasis rate is lower than that of Han nationality, suggesting that Kazak patients with esophageal cancer are less likely to have metastasis than that of Han nationality, considering their own living habits and personal body Physical quality. However, there was no significant difference in total and progression free survival between 112 Kazak and 114 Han ESCC patients.

As a carcinogen, Wnt2 can be detected to increase expression in many cancers and participate in tumor progression. It has also been found that Wnt2 can promote the growth of NSCLC cells by activating Wnt/β-cantenin signaling pathway[12]. In human colorectal cancer (CRC), Wnt2 selectively increases in cancer-related fibroblasts (CAF), leading to increased invasion and metastasis. It is mainly associated with the expression of Wnt2, which leads to the increase of angiogenesis, vascular density and tumor volume[13]. In rectal cancer, Wnt2 is associated with tumor invasion depth, lymph node metastasis, TNM stage, vascular invasion and recurrence[14]. We found that Wnt2 was highly expressed in esophageal cancer, and it was related to TNM stage and lymph node metastasis. High TNM stage or lymph node metastasis was the positive rate of Wnt2, so it was considered that Wnt2 could promote the development of ESCC.

Survivin is a member of the inhibitor apoptosis gene family[15]. The gene may be related to the aggressive behavior in many types of cancer, such as ovarian [16], bladder cancer [17], esophageal [18], [19] and so on. In our study, we found that the high expression of survivin was related to the depth of invasion, and the positive expression of Survivin in patients with esophageal cancer invading into the muscular layer and the whole layer was higher than that in patients only invading into the mucosal layer, suggesting that survivin can be used as a predictor of invasion progress. Moreover, we found that survivin is related to the progression free survival of esophageal cancer patients. The progression free survival of patients with positive survivin expression is shorter than that of patients with negative survivin expression. Therefore, we consider survivin as an independent prognostic factor of esophageal squamous cancer patients. At the same time, it has also been found that the high expression of Survivin in ESCC is related to the degree of tissue differentiation and invasion, and the prognosis of patients with high expression of survivin ESCC is relatively poor[18].It is consistent with our conclusion. The expression of Wnt2 was negatively correlated with the expression of survivin. However, the actual expression rate of survival in esophageal cancer tissue is higher than that in normal mucosa tissue. The reason may be that although the expression rate is higher than that in normal tissue, it may be because the sample size is not enough to lead to the low positive rate, resulting in the phenomenon of negative correlation with Wnt2 expression.

GSK3β has been found to be a tumor suppressor gene in many tumors. FAK / Pyk2 can activate Wnt/β-cantenin signal transduction by phosphorylating GSK3β, and DAX1 can promote the growth and tumorigenicity of cervical cancer cells by phosphorylating GSK3β;mir-135a can activate Wnt/β-cantenin, signaling pathway by down regulating GSK3 β expression, thus accelerating EMT of bladder cancer cells, promoting invasion and migration [22]. In this study, GSK3β was found to be low expression in ESCC, and TNM stage was related, but it was not found that GSK3β had an impact on the prognosis of esophageal cancer. However, in 29 cases of ESCC, the mRNA expression of GSK3β was higher than that of normal mucosa, and the mRNA expression ofβ-cantenin, survivin and GSK3βwere correlated with each other. Considering that they were Wnt signal proteins, there was a relationship of mutual regulation.

β-cantenin, is the key protein of Wnt signaling pathway. It is reported that acly stabilizes β-cantenin, protein through interaction, promotes the transport of CTNNB1 from cytoplasm to nucleus, and then promotes the transcription activity of β-cantenin, and the migration and invasion ability of colon cancer cells [23]. this study found that the expression of β-cantenin, in ESCC was high, which was related to the depth of invasion, radiotherapy and chemotherapy, and was related to the expression of Wnt2 at the protein level. At the transcription level, it was found that the expression of β-cantenin, mRNA was positively correlated with the expression of GSK3β. In Ishiguro Hideyuki's study, it was also found that β-cantenin, was associated with TNM stage and lymph node metastasis in esophageal cancer patients, and was co expressed with CDH1 [24]. It is suggested that there may be a co expression relationship between β-cantenin and Wnt2, GSK3β.

In conclusion, Wnt2,β-cantenin and Survivin are highly expressed in Wnt/β-cantenin signaling pathway in ESCC. Wnt2 is related to TNM stage and lymph node metastasis. β-cantenin, is related to depth of invasion and radiotherapy and chemotherapy. Survivin is related to depth of invasion. All the above proteins promote the development of esophageal carcinoma. GSK-3 is low expression in protein level, TNM stage is related, considering that it can inhibit the development of ESCC, but there may be phosphorylation of GSK3βto promote the development of esophageal cancer. Lymph node metastasis, nerve invasion and hematogenous metastasis can be used as independent factors to affect the overall survival of patients with ESCC. The Overalll survivalof patients with lymph node metastasis, nerve invasion and hematogenous metastasis is shorter. TNM stage, lymph node metastasis, nerve invasion, radiotherapy and chemotherapy, and Survivin are all independent influence shadows of esophageal cancer patients in squamous non progression stage, which can affect the occurrence and development of esophageal cancer patients. In TNM stage, patients in stage I and stage II did not show disease progression for a longer time than those in stage III and stage IV, patients without nerve infiltration, blood metastasis and postoperative radiotherapy and chemotherapy had a longer

progression free survival period, and patients with survivin positive had a shorter recurrence time than those with negative. In addition, the expression of Wnt2, β-cantenin, GSK3β and Survivin were not significantly different between Han and Kazak, and there was no significant difference in the total and progression free survival of Kazak esophageal cancer patients.

Declarations

Funding Information

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Disclosure

The author reports no conflicts of interest in this work.

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26.

Figures

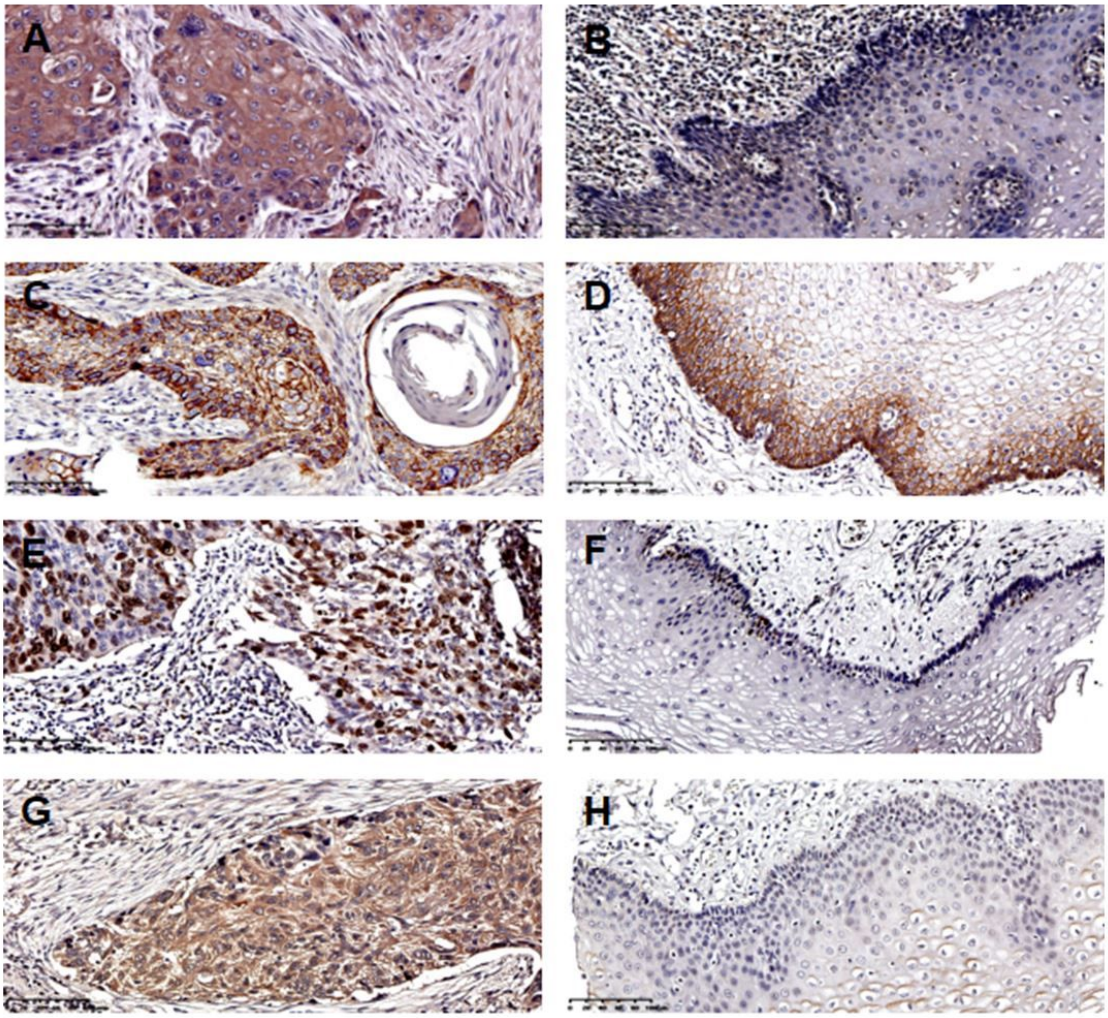

Figure 1

Immunohistochemical expression of Wnt2, β-catenin, GSK3βand Survivin in normal and cancer tissues of esophagus (×200). A: Wnt2 was positive in the stroma of esophageal squamous carcinoma; B: Wnt2 was negative in normal esophageal mucosa; C: β-cantenin, was positive in esophageal squamous carcinoma cell membrane; D: β-cantenin, was negative in normal esophageal mucosa cell membrane; E: survivin was positive in esophageal squamous carcinoma. F: The negative expression of Survivin in normal esophageal mucosa, G: GSK3β in ESCC, H: GSK3β in normal esophageal mucosa.

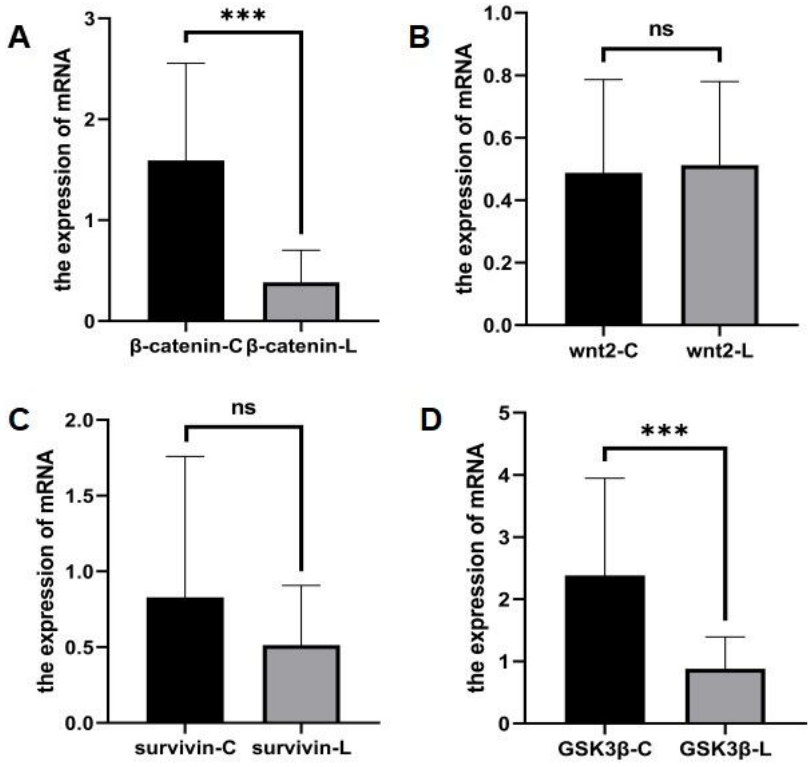

Figure 2

The mRNA expression of Wnt2, β-cantenin, survivin and GSK3β in ESCC and normal esophageal mucosa (***: $p < 0.01$, NS: $p > 0.05$). A:The expression of Wnt2 mRNA in ESCC(Wnt2-C) and normal esophageal mucosa(Wnt2-L) ;B:The expression of β-cantenin mRNA in ESCC(β-cantenin-C) and normal esophageal mucosa(β-cantenin-L);C:The expression of survivin mRNA in ESCC(survivin-C) and normal esophageal mucosa(survivin-L) ; D:The expression of GSK3βmRNA in ESCC(GSK3β-C) and normal esophageal mucosa(GSK3β-L).

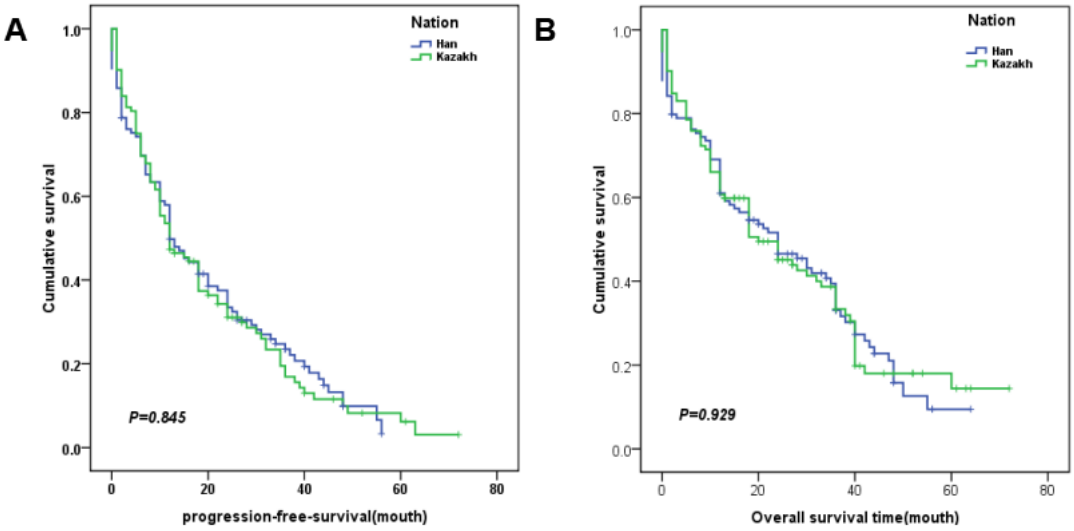

Figure 3

Overalll survival analysis and progression free survival in Kazak and Han ESCC patients.

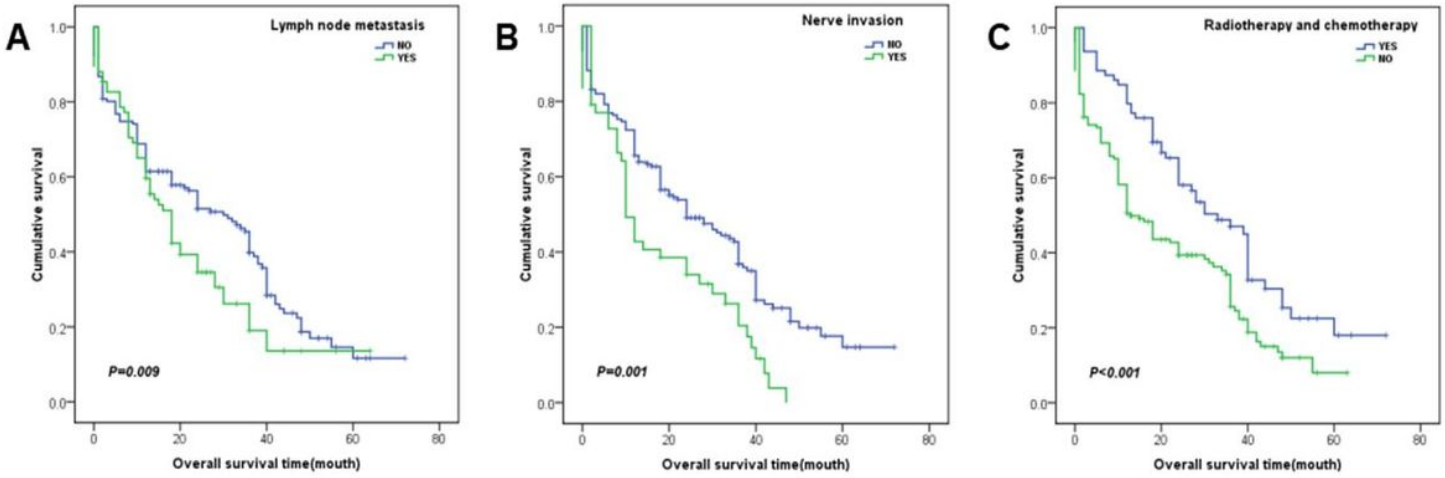

Figure 4

The overall Survival analysis chart of prognsis of ESCC affected by clinicopathological and protein factors.

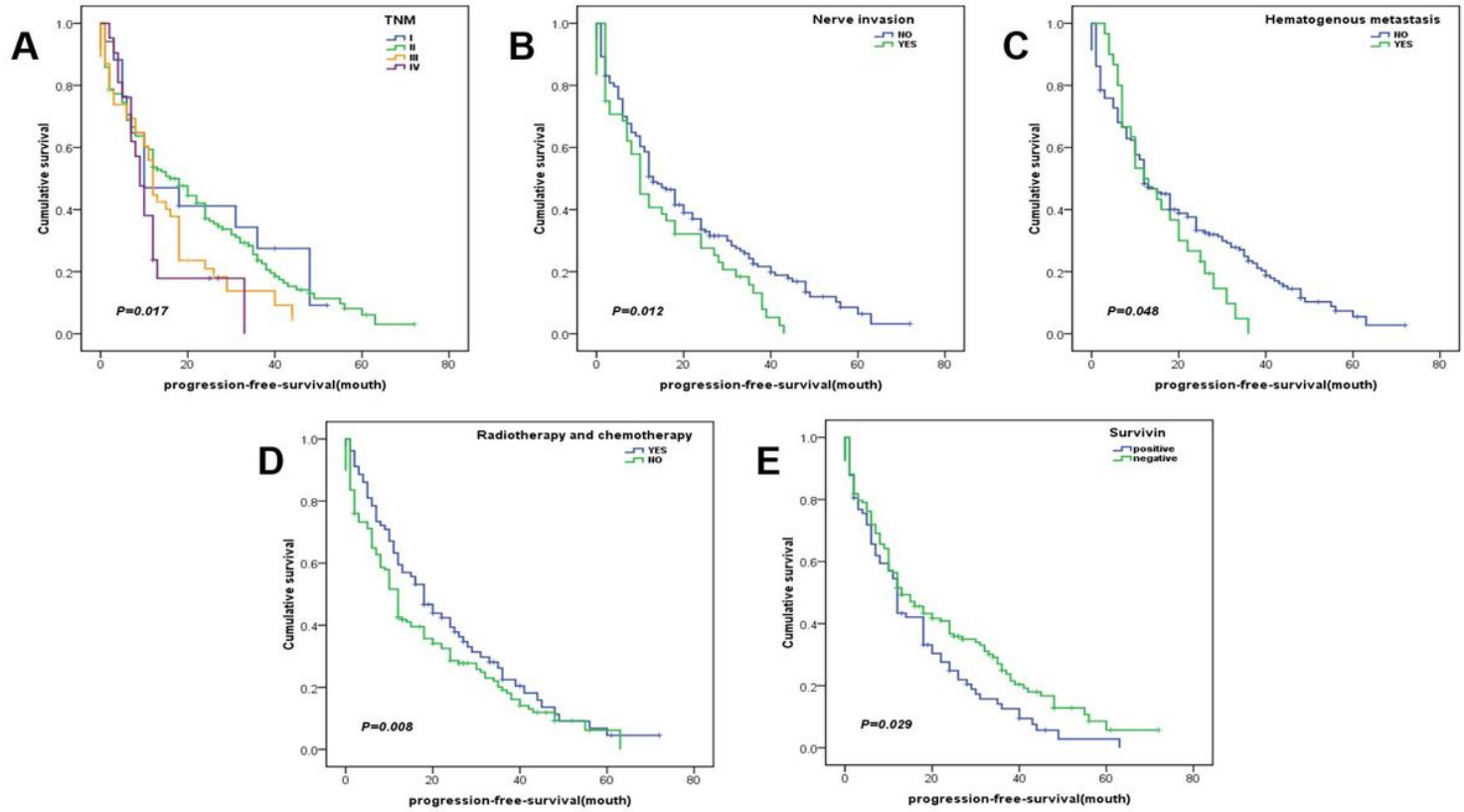

Figure 5

The progression-free-survival analysis chart of prognsis of ESCC affected by clinicopathological and protein factors.