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Original Article

Expression of Wnt11 and Rock2 protein with clinical characteristics of esophageal squamous cell carcinoma in Kazakh and Han patients

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Abstract: Background: Esophageal squamous cell carcinoma (ESCC) is one of the most malignancies with a very poor outcome in China. Wnt11 and Rock2, new identified proteins highly associated with metastasis of many cancers, which were never reported in esophageal squamous cell carcinoma (ESCC). Here we measured the expression levels of Wnt11 and Rock2 in tissues from 265 patients with ESCC. Immunohistochemical staining was employed to detect the correlation of Wnt11 and Rock2 expression with clinicopathological features. Methods: The expression of Wnt11 and Rock2 was detected by immunohistochemistry in esophageal squamous cell carcinomas and normal esophageal tissues. A chi-square test was used to assess the statistical significance of the correlations between Wnt11, Rock2 expression and different clinicopathological parameters, respectively. Results: The high-expression of Wnt11 and Rock2 was observed in ESCCs. Seventy-five cases of ESCC (51.7%) showed a positive expression of Wnt11, which indicated a significant association with the AJCC stage (*P=0.007*). Ninety-eight cases of ESCC (65.5%) showed a positive expression of Rock2, which indicated a significant association with ethnic background. There were no close correlations between Rock2 expression and gender, tumor location, AJCC stage, lymph node metastasis. Specifically, the expression of Rock2 was significantly different between Hans and Kazaks ethnicities (*P=0.000*). In Kaplan-Meier curve analysis, no significant correlation was observed between the expression of Wnt11, Rock-2 and the poor prognosis of ESCCs. Conclusion: Our finding suggests that the over-expression of Rock2 may play an important role in the carcinogenesis and progression, and may become a new underlying molecular marker in the diagnosis and treatment in ESCC.

Keywords: Esophageal squamous cell carcinoma, Wnt11, Rock2, immunohistochemistry, clinicopathologic factors

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most malignancies with a very poor outcome in China [1, 2], particularly in the Chinese Kazakh ethnic population residing in the Xinjiang, northwest china [3-5]. The incidence of Kazak esophageal cancer in Xinjiang is up to 155.9/100000, which is far higher than the national average level (14.95/100000) and Han peoples in the same region (13/100000). Its 5 years of survival rate is only 5% ~ 13%. The five-year survival rate of ESCC is approximately 42% despite recent advancements in clinical treatment from the Worldwide Esophageal Cancer Collaboration [6]. To improve patient outcome, the molecular mechanism of ESCC, should be investigated respectively in Kazakh and Han ethnic population in the Xinjiang. Therefore, it will be of great significance to find the underlying molecular markers as new prognostic and therapy targets in esophageal cancer diagnosis and treatment.

Wnt signaling pathway plays important roles in embryonic development, tissue differentiation, and cancer. In both normal and malignant tissue, members of Wnt family are often expressed combinatorially, although the significance of this is not understood [7]. Wnt molecules trigger gene transcription via at least three signaling pathways: one of them is the canonical or

Table 1. Characteristic of 265 ESCC patients included in this study

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	33 (28.7)	37 (24.5)	70 (26.3)
>3 cm	82 (71.3)	113 (75.5)	195 (73.7)
Tumor location			
upper	3 (2.6)	9 (6.0)	12 (4.5)
middle	31 (27)	70 (46.7)	101 (38.1)
lower	81 (70.4)	71 (47.3)	152 (57.3)
Degree of differentiation			
Well	23 (20)	19 (12.7)	42 (15.8)
moderately	67 (58.3)	95 (63.3)	162 (61.1)
poorly	25 (22.7)	36 (24)	61 (23.1)
AJCC stage			
T0	4 (3.5)	13 (8.6)	17 (6.4)
T1	56 (48.7)	64 (42.4)	120 (45.1)
T2	18 (15.7)	17 (11.3)	35 (13.2)
T3	37 (32.1)	56 (37.7)	93 (35.1)
Lymph node status			
No	71 (61.7)	117 (78)	188 (70.9)
Yes	44 (38.3)	33 (22)	77 (29.1)

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

â-catenin dependent, while the other two are non-canonical pathways. The two non-canonical pathways are JNK/AP1 dependent, planar cell polarity (PCP) pathway and PKC/CAMKII/NFAT dependent Ca^{2}+ pathway [8], Respectively, Wnt11 and Rock2 identified as planar cell polarity (PCP) pathway.

Wnt11 is also a necessary signal molecule in the non-classical WNT signaling pathways. It is a secreted protein that modulates cell growth, differentiation and morphogenesis during development. For example, it is required for convergent extension movements during gastrulation [9] and kidney morphogenesis [10]. Wnt11 promotes increases proliferation [11], migration and transformation of intestinal epithelial cells [12]. A role for Wnt11 in human cancer was first suggested by its high expression in gastric and renal cell carcinoma cell lines, as well as some primary colorectal adenocarcinomas [13]. It is also highly expressed in some colon cancers [14] and many prostatic tumors [15]. However, the molecular mechanism of Wnt11 to affects prechordal plate progenitor movements is still unclear.

The Rho-kinases (ROCKs) are one of the immediate downstream effectors of Rho, one of the small guanosine triphosphatases in Rho family. ROCKs play important roles in many physiological process, including neurite growth retardation [16, 17], prenatal and postnatal development [18-20], and smooth muscle contractions [21]. ROCK activity is regulated by distinct members of Rho guanosine triphosphatases (Rho). The Rho/ROCK pathway also participates in regulating cytoskeletal signaling events, which is crucial in cell motility [22]. Deregulation of Rho/ROCK pathway has been recently implicated in tumor progression, and particularly, tumor metastasis. Over-expression of two members of ROCK family (ROCK1 and ROCK2) was detected in testicular and bladder cancers at the protein level [23, 24]. Although up-regulation of Rho/ROCK is often associated with aggressive tumor behavior [25, 26], the roles of Rho-kinases in ESCC have not been fully elucidated.

Previous studies have identified Wnt11 and Rock2 as markers of poor prognosis in several tumors, but the clinicopathological significance of the expression of Wnt11 and Rock2 in human ESCC remains unclear. The purpose of this study is to clarify the relationship between Wnt11, Rock2 expression and the clinicopathological features of ESCC.

Materials and methods

Patients and tissue samples

Two-hundred and sixty-five patients with esophageal squamous cell carcinoma underwent transthoracic subtotal esophagectomy at the First Affiliated Hospital of Xinjiang Medical

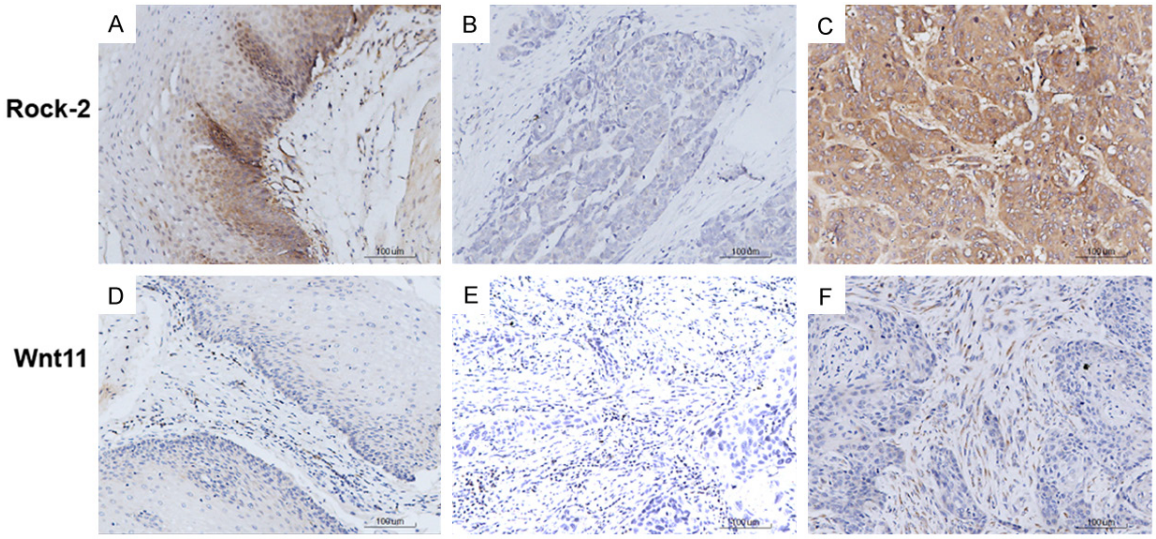

Figure 1. Representative photomicrographs of tissue sections immunostained for Wnt11 and Rock2. A. Rock-2 expression was detected in normal esophageal squamous epithelium (×200). B. Primary esophageal cancer negative for Rock-2 expression in cancer cell nests (×200). C. Primary esophageal cancer with Rock-2 Low-expression detected in the cytoplasm of cancer cell nests (×200). D. Primary esophageal cancer with Rock-2 High- expression detected in the cytoplasm of cancer cell nests (×200). E. Wnt11 expression was detected in normal esophageal squamous epithelium intercellular (×200). F. Primary esophageal cancer negative for Wnt11 expression in cancer cell intercellular (×200). G. Primary esophageal cancer with Wnt11 expression detected in intercellular of cancer cell (×200).

University, between 2007 and 2014. None of these patients received adjuvant therapy prior to the surgery 265 formalin-fixed, paraffin-embedded were applied for histological and immunohistochemical analysis. Normal esophageal mucosal samples were taken from a region >5 cm distant from the cancer, as non-tumor control samples. Tumor staging were carried out according to the American Joint Committee on Cancer (AJCC) [27] classification system based on the tumor size (T), lymph node involvement (N), and distant metastasis (M). Then, the following patient characteristics were collected for the 265 ESCC patients, including: age, gender, tumor location, tumor size, degree of differentiation, ethnic, clinicopathological stage, lymph node status. The characteristics of these 265 patients are listed in **Table 1**. Clinical follow-up information was obtained by telephone. Clinical follow-up information was obtained by telephone or from the outpatients' records.

Immunohistochemistry

Before antigen repairing, the tissue sections were deparaffinized for 30 min with formaldehyde, and then fixed with 100% ethanol for 5 minutes. To block endogenous peroxidase, the slides were incubated in 3% hydrogen peroxide. The sections were incubated at 4°C overnight with the rabbit polyclonal antibody for Wnt11 (1:400, rabbit monocolonal antibody, Abcam, UK), and the rabbit polyclonal antibody for ROCK2 (1:200, rabbit monocolonal antibody, Abcam, UK), respectively. After adding secondary antibodies (ZSGB, China), the slides were incubated in 37°C incubator for 35 minutes. Immunostaining was visualized with a labeled streptavidin-biotin (LSAB) method using DAB as a chromogen.

Scoring of immunostaining for Wnt11 and Rock2

Stained sections were observed under a BX40 microscope (Olympus, Japan). the expression of Wnt11 and Rock2 were evaluated by a blinded observer, unaware of the corresponding clinical information. The final score for Rock2 was the average of the scores obtained by the two observers. The intensity and extent of the staining were used as criteria of evaluation. The staining intensity was scored as 0 (no staining), 1 (light yellow), 2 (yellow brown), or 3 (brown). Extent of staining was scored as 0 (0%), 1

Table 2. Associations between the expression of Wnt11 and the clinicopathological features in the number of ESCC

Characteristic	Kazakh		*P*	Han		*P*	Total		*P*
	Low (%)	High (%)		Low (%)	High (%)		Low (%)	High (%)	
Tumor size			0.360			0.054			0.352
≤3 cm	18 (15.7)	8 (7.0)		69 (46)	6 (4.0)		87 (32.8)	14 (5.3)	
>3 cm	43 (37.3)	46 (40)		62 (41.3)	13 (8.7)		105 (39.6)	59 (22.3)	
Tumor location			0.354			0.800			0.666
Upper	0 (0)	0 (0)		8 (5.3)	8 (5.3)		8 (3.0)	8 (3.0)	
Middle	6 (5.2)	9 (7.8)		41 (27.3)	37 (24.7)		47 (17.7)	46 (17.3)	
Lower	60 (52.2)	40 (34.8)		18 (12)	38 (25.2)		78 (29.5)	78 (29.5)	
Degree of differentiation			0.784			0.788			0.646
Well	17 (14.8)	8 (7.0)		32 (21.3)	20 (13.3)		49 (18.5)	28 (10.6)	
moderately	36 (31.3)	31 (27.0)		39 (26)	35 (23.3)		75 (28.3)	66 (44)	
poorly	15 (13.0)	8 (6.9)		16 (10.7)	8 (53.4)		31 (11.7)	16 (5.9)	
AJCC stage			0.710			0.007			0.524
T0	3 (2.6)	3 (2.6)		20 (13.3)	4 (2.6)		23 (8.7)	7 (2.7)	
T1	26 (22.6)	26 (22.6)		36 (24)	28 (18.7)		62 (23.3)	54 (20.4)	
T2	6 (5.2)	6 (5.2)		16 (10.7)	0 (0.0)		22 (8.3)	6 (2.2)	
T3	32 (27.8)	13 (11.4)		14 (9.3)	32 (21.4)		46 (17.4)	45 (16.8)	
Lymph node metastasis			0.452			0.317			0.654
No	47 (40.8)	30 (26.1)		75 (50)	47 (31.3)		122 (46.0)	77 (29.1)	
Yes	20 (17.4)	18 (15.7)		12 (8)	16 (10.7)		32 (12.1)	34 (12.8)	

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

(<5%), 2 (5-25%), 3 (26-50%), 4 (51-75%), or 5 (76-100%). Staining index was calculated as the multiplication of staining intensity score and the proportion of Rock2-positive tumor cells. We evaluated Rock2 expression in benign esophageal tissue and malignant tissue on the basis of the staining index values, with scores of 0, 1, 2, 3, 4, 5, 6, 8, 9, 10, 12, and 15. An standard value was identified: a staining index score of ≥5 was considered as high Rock2 expression, whereas a staining index score of ≤4 was considered as low Rock2 expression; Wnt11 immunoreactivity was assessed as follows: "high expression" was defined as >5% positively stained cells under 200× microscopic field; ≤5% was defined as "low expression".

Statistical analysis

The Fisher's exact test or χ^2 test was used to assess the statistical significance of the correlations between Wnt11, Rock2 expression and the different clinicopathological parameters respectively. The patients were followed-up Overall survival (OS) was defined as time from surgery to the time of death or the last follow-up time. Overall survival was calculated using the Kaplan-Meier method and curves were compared using log-rank test. Overall survival was defined as the time from the date of surgical resection to the date of death. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc, Chicago, IL). A significant difference was considered statistically when *P* value was <0.05.

Results

Immunohistochemical expressions of Wnt11 and Rock2 in ESCC

In normal esophageal epithelium, the staining of Wnt11 was localized in the intercellular substance, while Rock2 was localized in the squamous epithelium. Represepontative results of immunohistochemistry for Wnt11 and ROCK2 in normal ESCC tissue samples are shown in **Figure 1**. In normal esophageal tissues, 29.8% were identified as Wnt-positive in 265, 44.4% were identified as Wnt11-positive in 150 Han , 26.3% were identified as Wnt-positive in 115 Kazakhs; 12.3% were identified as Rock2-positive in 265, 14.1% were identified as

Table 3. Associations between the expressions of Rcok2 and the clinicopathological features in the number of ESCC

Characteristic	Kazakh		*P*	Han		*P*	Total		*P*
	Low (%)	High (%)		Low (%)	High (%)		Low (%)	High (%)	
Tumor size			0.419			0.592			0.352
≤3 cm	18 (15.7)	19 (16.5)		20 (13.3)	26 (17.3)		38 (14.3)	45 (17.0)	
>3 cm	38 (33.0)	40 (34.8)		34 (29.7)	70 (46.7)		72 (27.2)	110 (41.5)	
Tumor ocation			0.611			0.266			0.124
upper	2 (1.7)	2 (1.7)		3 (2)	6 (4)		5 (1.8)	8 (3.0)	
middle	17 (14.8)	17 (14.8)		24 (16)	44 (39.3)		41 (15.4)	61 (23.0)	
lower	38 (33.0)	39 (34)		27 (18)	46 (30.7)		63 (23.8)	85 (32.0)	
Degree of differentiation			0.694			0.280			0.235
Well	12 (10.4)	12 (10.4)		7 (4.6)	12 (8.0)		18 (6.7)	24 (9.1)	
moderately	33 (28.7)	34 (29.7)		34 (22.7)	63 (42)		67 (25.3)	95 (35.9)	
poorly	12 (10.4)	12 (10.4)		11 (7.4)	23 (15.3)		23 (8.7)	35 (13.3)	
AJCC stage			0.414			0.083			0.364
T0	2 (1.7)	2 (1.7)		5 (3.3)	8 (5.3)		7 (2.7)	10 (3.8)	
T1	28 (24.3)	28 (24.3)		23 (15.3)	41 (27.3)		51 (19.2)	69 (26.0)	
T2	9 (7.8)	9 (7.8)		5 (3.3)	12 (8.0)		14 (5.3)	21 (7.9)	
T3	18 (15.6)	19 (16.6)		16 (10.7)	40 (26.8)		34 (12.8)	59 (22.3)	
Lymph node metastasis			0.809			0.185			0.991
No	36 (31.3)	37 (32.2)		43 (28.7)	76 (50.7)		79 (29.8)	113 (42.6)	
Yes	20 (17.4)	22 (19.1)		11 (7.3)	20 (13.3)		31 (11.7)	42 (15.9)	
Ethnic									0.000
	56 (48.7)	59 (51.3)		54 (36.0)	96 (64.0)		110 (41.5)	155 (58.5)	

Rock2-positive in 150 Han, 10.8% were identified as Rock2-positive in 115 Kazakhs; In the tumor tissues, Wnt11 immunostaining was positive in the tumor interstitium and Rock was positive in the endochylema of tumor cells. Rock2-positive staining was localized in the cytoplasm of tumor cells. The expression of Wnt11 and Rock2 were investigated in 265 ESCCs by immunohistochemistry in **Figure 1**. In 150 Hans, 38 (25%) ESCCs were Wnt11-positive, whereas 112 (75%) ESCCs were Wnt11-negative; in 115 Kazakhs, 32 (27.5%) ESCCs were regarded as Wnt11-positive compared to 83 (72.5%) Wnt11- negative ESCCs. According to the scoring criteria of ROCK2, the rate of Rock2-positive was 50.5% (58/115), whereas the rate of Rock2- negative was 49.5% (57/115) in Kazak patients. However, 98 (65.5%) ESCCs were Rock2-positive, but 52 (35.5%) ESCCs were Rock2-negative in Han patients.

Correlations between Wnt11, Rock2 protein expression and clinical-pathologic factors

The correlations between Wnt11 expression in ESCC and the 8 clinicopathological characteristics of patients (age, gender, tumor size, location of tumor, differentiation, tumor depth, lymphatic invasion, venous invasion, ethnic) are shown in **Table 2**. The results showed that Wnt11 expression was significantly associated with pathologic T category (*P=0.007*) in Han patients. No significant correlation was observed between Wnt11 expression and patient's tumor size, tumor location, Degree of differentiation, lymph node metastasis. As summarized in **Table 3**, to our astonishment, we accidentally found that Rock2 expression was significantly associated with ethnic (*P=0.000*). No significant correlation was observed between Rock2 expression and patient's gender, tumor location, AJCC stage, lymph node metastasis.

Prognostic significance of Wnt11 and Rock2 expression in ESCC patients

100 follow-up patients during follow up periods of 1-72 months (median, 15months) were analyzed by Survival dates. The overall survival rate of ESCC patients with Wnt11-positive expression was lower than the patients with Wnt11-

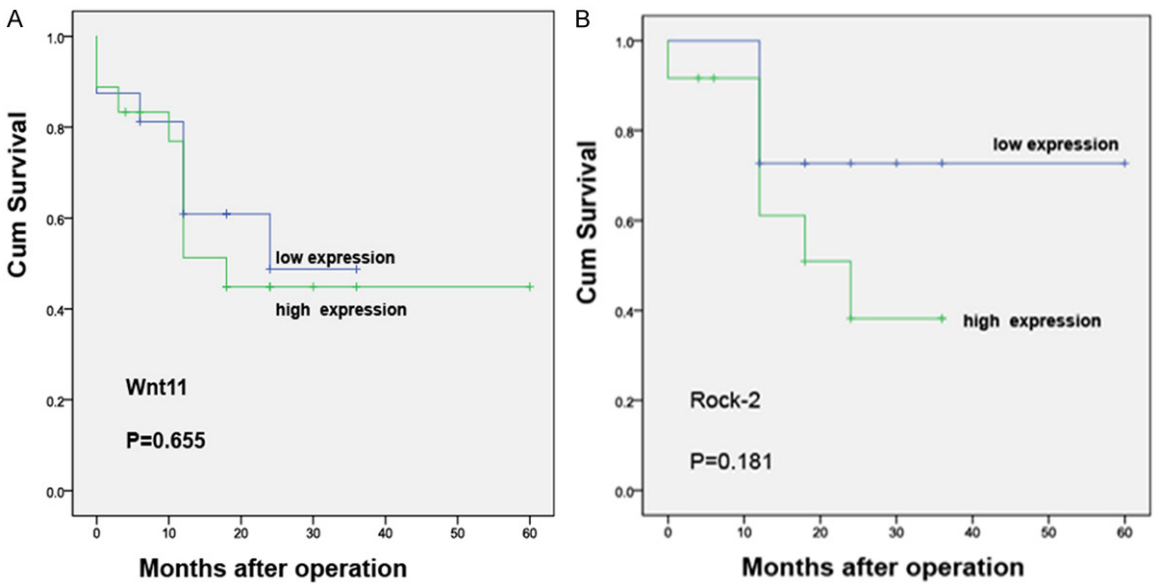

Figure 2. Postoperative survival according to Wnt11 and Rock2 expression. Overall survival rates of ESCC patients with Wnt11 and Rock2-positive expression.

negative expression, but not statistically significant (*P>0.05*) (**Figure 2A**); The 5-year survival rates for the patients was 3.7%. the Kaplan-Meier survival curves showed that the patients with positive expression of Rock2 had a poorer prognosis than those with negative expression of Rock2, but not statistically significant (*P>0.05*) (**Figure 2B**).

Discussion

Esophageal squamous cell carcinoma (ESCC) is one of the most virulent malignancies worldwide with the 5-year survival rate less than 30% [24]. ESCC is a highly malignant tumor of the digestive system, thus, the key role involved in the development and progression of the disease must be investigated. One of the key findings in this study is that the expression of Rock2 was significantly different between Hans and Kazaks; it is highly unlikely that this staining heterogeneity is due to genetic alterations or the alteration in the microenvironment. We elucidated that the protein expression of ROCK2 was higher in Kazakh ESCC tissues than Han. Therefore, further study with a larger sample size is needed to obtain more confidential evidences.

ROCK2 is the main downstream effector of Rashomologous (Rho) family of GTPases [25, 26]. It serves as a molecular switch in many cellular functions, such as cell proliferation, apoptosis, invasion, and metastasis [28], cancer cell migration is essential to the process of metastasis. A specific ROCK inhibitor was found to suppress the tumor growth and metastasis [29, 30]. These observations suggested that the ROCK2 might be a molecular target for preventing cancer invasion and metastasis. Metastasis is the main cause of death in most cancer patients. In particular, lymph node metastasis also results in a poor prognosis in patients with ESCC [31].

In our study, Rock2 was founded express higher in ESCC tissues than in normal tissues. Overexpression of Rock2 promotes invasion and metastasis in many solid tumors, such as hepatocellular, breast, and colon cancers [32-35]. However there were still some limitations in the study. One critical finding in this study is that there was a statistically significant difference between Rock2 expression and ethnic in patients with ESCC. The results may be caused by the silencing of target genes, gene mutation, but at the same time, owing to the differences among dietary habit, regions and individuals the differences still exist. A larger sample size is required to be investigated for the result. We only investigated the Kazakhs and Hans in Xinjiang, so we still did not know whether the same rule could be used for other Chinese.

In this study, there was no statistically significant difference between Rock2 expression and the two groups of the 150 Han patients (high-expression group and low-expression group). Nevertheless, according to the rate of high-expression group of Rock2 protein (63.2%, 64.2%, 67.7% respectively), It is not hard to find the positive rate of Rock2 in ESCCs showed an increasing trend with the increase of differentiated degree. The trend is consistent with the findings of E. Lock [36], who indicated the function of Rock2 in epithelial differentiation. This may be attributed to the variation in population heterogeneity and the influence of tissue specific or gene mutation in ESCC carcinogenesis. Further studies need to be conducted with larger ESCC samples sizes.

The role of Wnt11 in cell motility was reported to relate with the directional movements of the cells in the assembling ventricular wall by regulating cell adhesion in connection with changes in the cytoskeleton [37]. A common theme is that Wnt11 acts locally to control the turnover of proteins involved in cell-cell and cell-substrate adhesion and thereby facilitate coordinated cell migration [38, 39]. Wnt11 increased the migration of LNCaP and PC3 prostate cancer cells [39, 40]. The abnormal Wnt11 expression in the protein level of other genes was also involved in the progress of ESCC. However, the mechanism of Wnt11involved in translation was unclear. The small sample size, tissue specific, gene mutation and no standard or universally accepted scoring criteria might generate different results and so on.

Our study has shown that the positive expression of Wnt11 association with the AJCC stage. Moreover, the abnormal expression of Wnt11 did not show significant correlation with the postoperative recurrence rate or survival rate, such results could be limited by the small sample sizes. To investigate the potential role of Wnt11 in ECSS warrants, further studies with enlarged sample sizes or functional analysis in cell or animal models should be done.

It was easily found the low rate of follow-up in our study, which limited the prognosis results. The communication difficulties with most of Kazakhs, who usually spoke his ethnic languages and live, scattered from the First Affiliated Hospital, Xinjiang Medical University, leaded to lose contact or received some incorrect message. Moreover, Due to the poor prognosis of esophageal cancer, many patients and their families' phone numbers has changed 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 Wnt11, Rock2 may be investigated by further study with a large sample size.

It indicates that the role of Wnt11 and Rock2 in esophageal squamous carcinoma is different from Wnt11 and Rock2 in other neoplasm. Moreover, the tissue specificity provides evidence for the critical role and mechanism of Wnt11, Rock2 in esophageal squamous carcinoma implicated the ethnic difference. It also provides a molecular basis for the clinical diagnosis and therapy targeted in this signaling pathway. Although Wnt11, Rock2 may not be an independent prognostic factor, it should be further verified by prospective analysis and more comprehensive follow-up. Mutational changes in Wnt11 and Rock2 genes were not detected by the scope in this study. This should be investigated in the future.

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Disclosure of conflict of interest

None.

Abbreviations

ESCC, Esophageal squamous cell carcinoma; AJCC, American Joint Committee on Cancer staging system; TF, tumor fibroblasts.

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