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Original Article

Increased WNT6 expression in tumor cells predicts unfavorable survival in esophageal squamous cell carcinoma patients

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Abstract: WNT proteins are a family of secreted, cysteine-rich proteins containing 19 members. Signaling through WNT proteins is reported to be involved in carcinogenesis and cancer progression of gastrointestinal tumors, such as gastric cancer and colon cancer. The expression status of WNT6 in ESCCs and their clinico-prognostic significances remain to be elucidated. In this study, One-hundred and thirty-six patients with ESCC were explored. Paraffin-embedded tumor sections were stained with WNT6 antibody. The correlations between WNT6 expression and survival parameters were analyzed. The overall frequency of WNT6 over-expression was 50.7% (69/136) of advanced EC patients. For DMS and OS, over-expression of WNT6 remained the independent factor for worse prognosis (hazard ratio (HR), 2.425; 95% CI, 1.631-3.605; $P < 0.001$ for OS and HR, 2.238; 95% CI, 1.507-3.323; $P < 0.001$ for DMS, respectively). To conclude, our results support the concept that WNT6 may play a role in tumor progression. WNT6 over-expression inversely correlates with the poor long-term survival in ESCC patients. WNT6 can be considered as a predictor for recurrence.

Keywords: ESCC, WNT6, prognosis

Introduction

Esophageal squamous cell carcinoma (ESCC), one of the major histopathological subtypes of esophageal cancer, is the fourth most prevalent malignancy in China and a leading cause of cancer-related death. The prognosis of ESCC is generally unfavorable, with a five-year survival rate of less than 30% [1, 2]. Despite general advances in diagnosis and treatment, the prognosis of ESCC patients remains poor because of high rates of recurrence/metastasis and resistance to adjuvant therapy [3, 4]. Therefore, there is an urgent clinical need to explore novel prognostic markers for ESCC patients.

In addition to the traditional prognostic factors determined at diagnosis, such as TNM stage and cell differentiation, the molecular markers related to tumor cell apoptosis, epithelial-mesenchymal transition (EMT), the function of infiltrated immune cells and angiogenesis in tumor microenvironments have been evaluated for their contribution to the prognoses of ESCC patients in recent studies [5-8]. However, reliable markers are still lacking in ESCC.

WNT proteins are a family of secreted, cysteine-rich proteins containing 19 members [9]. Signaling through WNT proteins is reported to be involved in carcinogenesis and cancer progression of gastrointestinal tumors, such as gastric cancer and colon cancer [10-12]. It is believed that the epithelium formation, adhesion, and cell–cell communication functions associated with WNT6 are mediated through the canonical (WNT/β-catenin signaling) pathway [5, 13].

To date, however, the expression status of WNT6 in ESCCs and their clinico-prognostic significances remain to be elucidated [14-16].

Material and methods

Study population

Paraffin-embedded tumor tissue samples were obtained from 136 ESCC patients who under-

Table 1. Clinicopathologic characteristics of 136 patients with ESSC

Parameter	No. (%)
Total cases	136
Gender	
Male	111 (81.6%)
Female	25 (18.4%)
Age [years]	
≤ 50	71 (52.2%)
> 50	65 (47.8%)
WHO degree	
G1	40 (29.4%)
G2	59 (43.4%)
G3	37 (27.2%)
WNT6 expression	
Low	67 (49.3%)
High	69 (50.7%)
Tumor (T) status	
T1	8 (5.9%)
T2	36 (26.5%)
T3	88 (64.7%)
T4	4 (2.9%)
Lymphoid nodal (N) status	
N0	69 (50.7%)
N1	67 (49.3%)
Distant metastasis (M) status	
M0	130 (95.6%)
M1	6 (4.4%)
Recurrence	
No	31 (22.8%)
Yes	105 (77.2%)
Clinical stage	
1	6 (4.4%)
2	60 (44.1%)
3	8 (5.9%)
4	56 (41.2%)
5	6 (4.4%)

Abbreviations: ESCC = Esophageal squamous cell carcinoma.

went surgery at Sun Yat-Sen University Cancer Center in Guangzhou City of China from November of 2000 to December of 2002. None of the patients had received anticancer treatment prior to surgery, and all of the patients had histologically confirmed primary ESCC in this retrospective study. Each patient had provided written informed consent. The follow-up data from the 136 patients with ESCC in this study were available and complete. All of the records such as clinical and pathological features were collected before blood sampling with no patients having received any treatment, for instance radiotherapy or chemotherapy. The clinicopathological features of the patients included gender, age, smoking history, drinking history, UICC stage, tumor size (T category), lymph node status (N category), pathological type (WHO type), and WNT6 expression level. All patients were restaged by seventh edition of UICC Staging System for esophageal cancer. This study was approved by the Research Ethics Committee of the Sun Yat-Sen University Cancer Center.

Immunohistochemical analyses

The paraffin-embedded tissues were sectioned continuously into 4-μm-thick sections. The tissue sections were de-waxed in xylene, rehydrated and rinsed in graded ethanol solutions. The antigens were retrieved by heating the tissue sections at 100°C for 30 min in citrate (10 mmol/L, pH 6.0) or EDTA (1 mmol/L, pH 8.0) solution when necessary. The sections were then immersed in a 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS) for 5 min, and incubated with the primary antibody mouse anti-human WNT6 (ab50030; Abcam, Cambridge, MA, USA) at 4°C overnight. A negative control was performed by replacing the primary antibody with a normal murine IgG antibody. The sections were then incubated with a horseradish peroxidase-labeled against a mouse/rabbit secondary antibody (Envision; Dako, Glostrup, Denmark) at room temperature for 30 min. Finally, the signal was developed for visualization with 3, 3'-diamino-benzidine tetrahydrochloride (DAB), and all of the slides were counterstained with hematoxylin.

Evaluation of immunohistochemical staining

Two independent observers blinded to the clinicopathological information scored the HIF-1α expression level in tumor cells by assessing (a) the proportion of positively stained cells (0, < 5%; 1, 6 to 25%; 2, 26 to 50%; 3, 51 to 75%; 4, > 75%) and (b) the intensity of staining (0, negative staining; 1, mild staining; 2, moderate staining; 3, strong staining). The score was the product of a × b. The levels of WNT6 expression in tumor tissue were obtained by counting the positively and negatively stained cells in five to

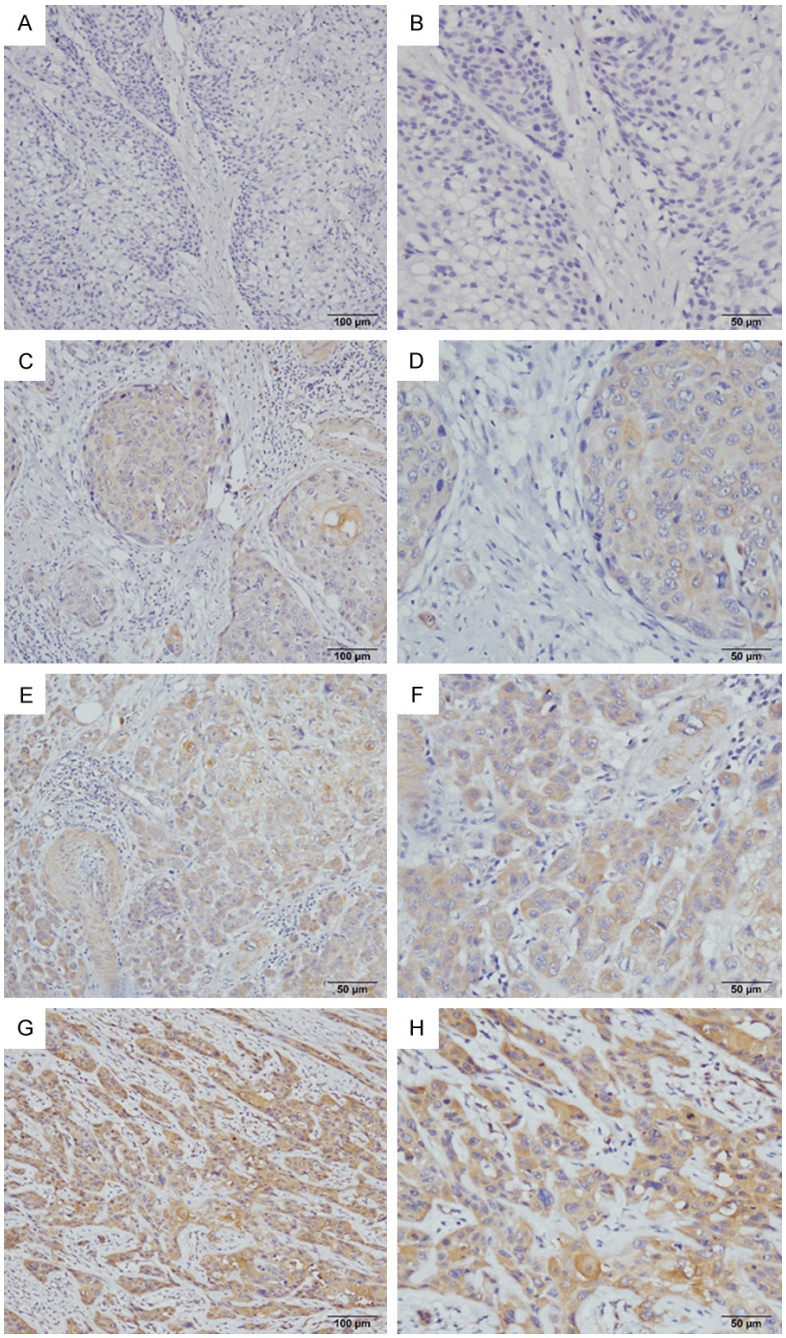

Figure 1. The expression of WNT6 in ESSC tissues by IHC. A. Negative expression of WNT6 protein (100 ×). C. Weak expression of WNT6 protein (100 ×). E. Moderate expression of WNT6 protein (100 ×). G. Intense expression of WNT6 protein (100 ×). B, D, F and H demonstrated the higher magnification (200 ×) from the area of the box in A, C, E and G, respectively.

ten separate 400 × high-power microscopic fields and calculating the mean percentage of positively stained cells among the total cells per field. A 5% proportion of positive tumor cells which were defined as the score were ≥ 5 have been used as cutoff for WNT6 high expression.

Statistical analyses

SPSS 20.0 software was used for the statistical analysis. Continuous variables were divided into different categories as mentioned above. All the cut-off values were obtained by X-tile software (Version 3.6.1, Yale University, New Haven, CT), taking clinical expertise into consideration. The OS analyses were estimated with Kaplan-Meier method and log-rank test to assess the possible individual risk factors related with survival. Further investigations of multivariable analyses were performed by Cox regression for factors that were significantly associated in univariate survival analyses. Results were reported with hazard ratio (HR), corresponding 95% confidence intervals (CI). A *P*-value < 0.05 was considered statistically significant.

Results

Clinicopathological characteristics of the patients

A total of 136 eligible patients with esophageal cancer were included in the present study (**Table 1**). The mean age at diagnosis was 62.1 years (range, 35-90 years). Twenty-five (18.4%) of the patients were female and one hundred and eleven (81.6%) were male. Three (5.9%) and fifty-six (41.2) patients were diagnosed at stage III and stage IV, respectively. In the current research, 69 patients harbored high-level expression of WNT6 and the remaining 71 patients were low-expression group. With regard to metastasis status, 6 patients proved to be distantly metastatic and 130 patients were not. 12 were uncommon mutation.

Table 2. Clinicopathological associations of Wnt6 expression levels in 136 patients with ESCC

Parameter	Category	Total case	Low level of WNT6	*P* value	High level of WNT6	*P*-value
Gender	Male	111	51	< 0.001*	60	< 0.001*
	Female	25	16		9	
Age	≤ 50	71	31	< 0.001*	40	< 0.001*
	50	65	36		29	
WHO degree	G1	40	19	< 0.001*	21	< 0.001*
	G2	59	27		32	
	G3	37	21		16	
T status	T1	8	6	< 0.001*	2	< 0.001*
	T2	36	17		19	
	T3	88	42		46	
	T4	4	2		2	
N status	N0	69	40	0.083	29	0.083
	N1	67	27		40	
M status	M0	130	64		66	
	M1	6	3		3	
Recurrence	No	31	23	< 0.001*	8	< 0.001*
	Yes	105	44		61	
Clinical stage	1	6	5	< 0.001*	1	< 0.001*
	2	60	33		27	
	3	8	2		6	
	4	56	24		32	
	5	6	3		3	

*$P < 0.05$, as determined by Pearson's X^2 test.

Correlations between WNT6 expression and baseline characteristics

Immunohistochemical staining for WNT6 was found in the cytoplasm of tumor cells (**Figure 1**). As shown in **Table 1**, WNT6 is over expressed in 50.7% (69/136) of ESCC patients. The relationship between WNT6 expression and age, gender, histopathological type, tumor stage was significant, except for the N status (P = 0.083). (**Table 2**).

Survival analysis is in ESCC patients

The median overall survival of the whole patients was 25 months. Kaplan-Meier analysis revealed that overall patients with over-expressionWNT6 and low levelWNT6 expression had significant difference in both OS and distant-metastasis survival (DMS) (**Figure 2A**, **2B**). We found that patients with positive WNT6 expression had signal better prognosis than the negative group. To determine the prognostic value of WNT6 expression, we carried out univariate and multivariate analysis using the Cox regression model. The result of univariate analysis implied that high level of WNT6 expression was a marker for poor survival [Hazard ratio (HR), 0.447; 95% CI, 0.301-0.663; P < 0.001 (**Table 3**)]. For DMS and OS, over expression of WNT6 remained the independent factor for worse prognosis (HR, 0.576; 95% CI, 0.372-0.894; P = 0.014 for DMS and HR, 0.464; 95% CI, 0.299-0.721; P = 0.001 for OS, respectively) (**Table 4**).

Discussion

WNT6 is an evolutionary conserved morphogenic factor promoting differentiation, survival and epithelial-to-mesenchymal transitions during embryonic organogenesis [17, 18]. The expression and function of WNT6 has been examined in several cancers such as gastric, bladder, breast, and colon cancer [19-22, 27], however, the role of WNT6 in ESCC is unclear. In this study we evaluated the expression and clinical-prognostic significance of WNT6 in ESCC for the first time.

The current data demonstrated that the overexpression of WNT6 in tumor tissue was correlated with poor prognosis and the recurrence in ESSC patients. For DMS and OS, over expression of WNT6 remained the independent factor for worse prognosis (hazard ratio (HR), 0.576; 95% CI, 0.372-0.894; P = 0.014 for DMS and HR, 0.464; 95% CI, 0.299-0.721; P = 0.001 for OS, respectively). These results implied that WNT6 might therefore play a role in the development and differentiation of ESCC, which could be a potential marker for recurrence and poor prognosis in ESCC patients. If in conjunction with other tumor markers, more data might allow clinicians to make better-informed therapeutic decisions for these ESSC patients.

EMT is one of the earliest steps of solid tumor progression, associated with tumor growth, invasion, and metastasis, and contributes to

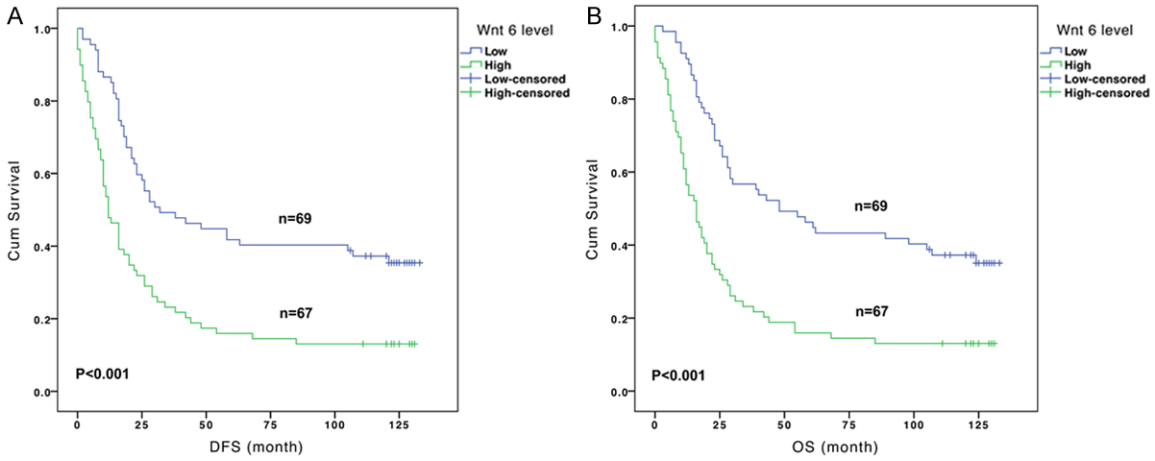

Figure 2. Kaplan-Meier survival analysis in patients with ESCC. A. Disease-free survival and overall survival curves for patients according to the low and high expression level of WNT6 in tumor cells. B. Disease-free survival and overall survival curves for patients according to the low and high expression level of WNT6.

Table 3. Univariate Cox Regression analyses of prognostic factors on DFS and OS of 136 patients with ESSC

	DFS			OS		
Variables[a]	HR	95% CI	*P*-value	HR	95% CI	*P*-value
WNT6 level (Low/High)	0.447	0.301-0.663	< 0.001*	0.412	0.271-0.613	< 0.001*

[a]Variables with *P* values greater than 0.05 in the univariate models were not included in the multivariate analysis. *Significant. DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

Table 4. Multivariate Cox Regression analyses of prognostic factors on DMS and OS of 136 patients with ESSC

	DMS			OS		
Variables[a]	HR	95% CI	*P*-value	HR	95% CI	*P*-value
WNT6 level (Low/High)	0.576	0.372-0.894	0.014*	0.464	0.299-0.721	0.001*
Gender (Male/Female)	1.430	0.766-2.668	0.262	1.677	0.912-3.081	0.096
Age (≤ 50/> 50)	1.251	0.807-1.939	0.316	1.218	0.791-1.877	0.371
WHO degree (1/2/3)	1.000		0.147	1.000		0.154
	0.772	0.438-1.360	0.371	0.780	0.444-1.372	0.389
	0.607	0.368-1.001	0.050	0.611	0.370-1.008	0.054
T status (T1/T2/T3/T4)	1.000		0.401	1.000		0.165
	1.616	0.173-15.144	0.674	1.910	0.207-17.650	0.568
	0.531	0.115-2.458	0.418	0.443	0.094-2.073	0.301
	0.461	0.109-1.943	0.291	0.364	0.084-1.583	0.178
N status (N0/N1)	1.225	0.162-9.261	0.844	1.294	0.167-10.031	0.805
M status (M0/M1)	1.400	0.332-5.896	0.647	1.207	0.284-5.125	0.798
Recurrence (No/Yes)	0.000	0.000-6.396E+100	0.909	0.000	0.0000-5.189E+101	0.909
Clinical stage (1/2/3/4)	1.000		0.822	1.000		0.820
	0.309	0.15-6.202	0.443	0.284	0.015-5.282	0.398
	0.587	0.073-4.706	0.616.	0.605	0.074-4.952	0.639
	1.168	0.382-3.575	0.785	0.074	0.362-3.274	0.879

[a]Variables with *P* values greater than 0.05 in the univariate models were not included in the multivariate analysis. *Significant. DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

the conversion of tumors from low-grade to high-grade malignancy [23, 24]. WNT signals received from the tumor microenvironment can initiate EMT [25]. WNT6 is one of the important components of the EMT initiation [26]. These findings suggest that WNT6 plays an important role in maintaining the stemness of ESCC. Moreover, over-expression of WNT6 potentiates as a target gene of caveolin-1 in gastric cancer [27], probably parallel to caveolin-1 expression and its function [28, 29]. Similar mechanisms may be at work in ESSC.

This is the first study to demonstrate the clinical significance of WNT6 expression in ESCC. Although based only on this retrospective study of patients with ESCC, we concluded that 1) WNT6 may play a role in tumor progression; 2) WNT6 overexpression inversely can be considered as a poor prognostic indicator of OS; and 3) WNT6 correlates as a predictor for recurrence.

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Disclosure of conflict of interest

None.

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