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Original Article

Expression of SOX18 is associated with poor prognosis in esophageal squamous cell carcinoma

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Abstract: SOX18 (Sex-determining region Y-box 18) is a transcription factor, which belong to the SOX (Sex-determining region on the Y chromosome related high mobility group box) family that involved in the regulation of embryonic development and in the determination of cell fate. This protein may act as a transcriptional regulator after forming a protein complex with other proteins. It plays a role in hair, blood vessel and lymphatic vessel development. It has been shown that SOX18 is closely related to the occurrence and development of tumor. However, the exact role of SOX18 in esophageal squamous cell carcinoma (ESCC) remains to be determined. Purpose: The present study was to explore clinicopathological significance and prognostic value of SOX18 expression in ESCC. Methods: We examined the expression of SOX18 in 100 cases of ESCC and their corresponding adjacent nonneoplastic esophageal tissues by immunohistochemical technique, and evaluated the association between SOX18 expression and clinicopathological parameters, prognosis in 100 ESCC patients. Results: The lever of SOX18 expression in ESCC was 78.0% (78/100), significantly higher than that in nonneoplastic esophageal tissues was 20.0% (16/80) (χ^2=59.923, *P*=0.000). The SOX18 protein expression was related to the degree of tumor cell differentiation, lymph node metastasis. In univariate analysis, we found that SOX18 expression, lymph node metastasis and AJCC clinical stage were independent risk factors for poor prognosis in ESCC patients. In multivariate analysis, SOX18 expression and AJCC clinical stage were independent risk factors. Furthermore, survival analysis revealed that the SOX18 negative expression group had significantly better survival rate than the positive group after curative surgery. Conclusion: SOX18 may play an important role in the pathogenesis and development of ESCC, high expression of SOX18 might be a novel valuable biomarker to predict poor prognosis of ESCC.

Keywords: Sex determining region Y box 18, esophageal squamous cell carcinoma, immunohistochemistry, tissue microarray, prognosis

Introduction

Esophageal cancer (EC) is the eighth most common malignancy in the world, with an estimated 456,000 new cases diagnosed per year, and the sixth most common cause of death from cancer with an estimated 400,000 deaths [1, 2]. More than 80% of the cases worldwide occur in less developed regions, especially in China. Esophageal squamous cell carcinoma (ESCC) is the predominant histologic subtype of EC, which characterized by high mortality rate in China [3]. In recent years, great advances in diagnosis and treatment has improved the prognosis of ESCC patients, but the survival time is not optimistic. Therefore, the discovery of novel predictive factors and therapeutic strategies are urgently needed.

The SOX (sex-determining region on the Y chromosome related high mobility group box) gene is a large family of transcription factors sharing a high-mobility group (HMG) domain [4, 5]. The SOX family is divided into ten groups (A-J) based on their amino acid homology, SOX18 is part of the F group (SOX F) of SOX proteins [6]. SOX18, is expressed in endothelial cells, has an important role in blood and lymphatic vessel development [7, 8], as well as in wound healing processes [9]. Recent findings revealed that SOX18 may take part in tumour genesis and development [10, 11]. It has been reported that the expression of SOX18 was increased in various malignancies such as invasive ductal breast cancer, gastric cancer, ovary carcinoma, cervical carcinoma and non-small cell lung cancer [12-16]. However, the function of SOX18

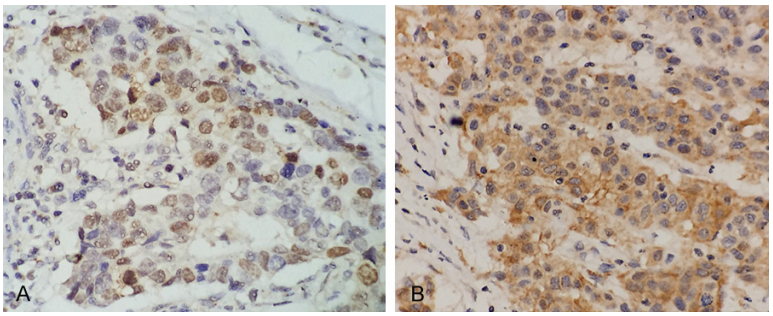

Figure 1. A: Nuclear SOX18 expression in ESCC. (SP×200) B: Cytoplasmic SOX18 expression in ESCC (SP×200).

expression in esophageal squamous cell carcinoma has not yet been determined. In this study, we detected the expression of SOX18 protein in ESCC and their corresponding adjacent nonneoplastic esophageal tissues, analyzed the relationship between SOX18 expression and clinicopathological parameters, survival rate and prognostic information so as to explore the significance of SOX18 expression in ESCC.

Materials and methods

Patients and tissue samples

Tumor tissue samples were obtained from 100 patients (74 female, 26 male; median age 59 years; range 36-81 years) who had undergone radical esophagectomy in the Heping Hospital affiliated Changzhi Medical College (Changzhi, Shanxi, China) from January 2006 to December 2008. All patients were selected at their first diagnosis and none had preoperative radiotherapy, chemotherapy and immunotherapy. Each tumor sample was matched with their corresponding adjacent nonneoplastic. All tumor tissues with a histopathologic diagnosis of ESCC were confirmed by two independent pathologists who were blinded to the original diagnosis, and we use the strict evaluation criteria to ascertain no metaplasia, dysplasia, and atypical hyperplasia in the nonneoplastic esophageal tissues. The patients' clinical stage were accorded to the American Joint Committee on Cancer (AJCC) staging system (2010, 7th edition) [18], and histologic grading were based on the WHO classification of tumors of the digestive system (2010, 4th edition) [19].

The follow-up was continuous after surgery until September 2014, which followed 5.8-7.8 years. The assessment of survival status was made every month during the first year after surgery, then trimonthly during the second year, and once half a year, thereafter. During this follow-up time, 67 patients were died of ESCC, with a median follow-up time of 32.0 months (1-78 months), 29 patients were still alive, 4 patients who died of other diseases or accidents were excluded from the study.

Construction of tissue microarray (TMA)

The tissue microarray was produced in collaboration with Shanghai Biochip Company (Shanghai, China). Tag all cases HE sliced lesions scope and design organization chip array arrangement, used tissue array instrument extraction wax block organization core and arranged according to the design on the blank receptor wax block. 180 TMA blocks tissues with formalin-fixed, paraffin-embedded (100 case of ESCC and 80 case of corresponding adjacent nonneoplastic esophageal tissues) were prepared.

Immunohistochemistry

TMA blocks were cut into 4 μm thick sections for the immunohistochemical reactions. Tissue sections were dewaxed and hydrated using xylenes, graded ethanols, immersed in 3% hydrogen peroxide solution for 10 min; High pressure heating repair in Ethylene diaminetetra acetic acid (EDTA) buffer (pH 9.0) at 100°C for 8 min. To block nonspecific binding sites, we incubated the sections with 10% normal goat serum at 37°C for 30 min. Then the sections were incubated with SOX18 rabbit monoclonal antibody (1:200, Abcam, Cambridge, UK, ab109194) overnight at 4°C. After washed three times with phosphate buffer solution (PBS) for 5 min, the sections were treated with corresponding streptavidin-peroxidase conjugated second antibody (Zhongshan Golden Bridge Corporation, Beijing, China) then color reaction by diaminobenzidine (DAB) reagent. Negative and positive controls were included for experiments. The positive gastric carcinoma sections were utilized as positive controls and PBS instead of primary antibody as negative controls.

Table 1. Correlation of SOX18 expression with clinicopathological parameters

Variable		n	SOX18 expression		χ^2	*P*-value
			+	-		
Overall frequency	Nonneoplastic	80	16 (20.0%)	64 (80.0%)	59.923	0.000*
	ESCC	100	78 (78.0%)	22 (22.0%)		
Gender	Male	74	59 (79.7%)	15 (20.3%)	0.496	0.481
	Female	26	19 (76.0%)	7 (24.0%)		
Age(yr) at surgery	≥60	68	57 (83.8%)	11 (16.2%)	4.200	0.400
	<60	32	21 (65.6%)	11 (34.4%)		
Tumor size(cm)	<4	24	17 (70.8%)	7 (29.2%)	0.945	0.623
	4-7	71	57 (80.3%)	14 (19.7%)		
	≥8	5	4 (80.0%)	1 (20.0%)		
Cell differentiation	High-grade	6	2 (33.3%)	4 (66.7%)	7.436	0.024*
	Middle-grade	69	56 (81.2%)	13 (18.8%)		
	Low-grade	25	20 (80.0%)	5 (20.0%)		
Depth of invasion	T1	5	3 (60.0%)	2 (40.0%)	2.169	0.538
	T2	13	9 (69.2%)	4 (30.8%)		
	T3	79	64 (81.1%)	15 (18.9%)		
	T4	3	2 (66.7%)	1 (33.3%)		
lymph node metastasis	(-)	46	31 (67.4%)	15 (32.6%)	5.587	0.018*
	(+)	54	47 (87.1%)	7 (12.9%)		
AJCC clinical stage	I+II	46	34 (73.9%)	12 (26.1%)	0.829	0.363
	III+IV	54	44 (81.5%)	10 (18.5%)		

*$P<0.05$.

Evaluation of immunohistochemical staining

SOX18 immunostaining were evaluated by a semiquantitative method based on a scale include the distribution and intensity of the staining. We considered yellow or brown particles in the cell nuclei and (or) cytoplasm as SOX18 positive specimen. Each section had 10 high power fields (×40) selected. Two pathologists who were blinded to the original histological diagnosis assessed the immunostaining. The staining intensity was scored as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The percentage of positive cell counts was scored as follows: 1 (0-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). The final score were obtained from two scores combined: negative (0-3), weakly positive (4-5), or strongly positive (6-7). Both weakly and strongly positive were considered as positive cases in our statistical analysis.

Statistical analysis

All analysis was performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, USA). The Chi-square test was used to analyze the correlation between SOX18 expression and clinicopathological parameters. Kaplan-Meier was used to draw survival curves. Univariate proportional hazards regression was used to estimate the dependence of survival on each variable. Multivariate survival analysis was based on cox proportional hazard model to test the variables selected by univariate analysis as having prognostic value. $P<0.05$ was considered statistically significant in all statistical analysis.

Results

The SOX18 expression in ESCC and the correlation between SOX18 expression and clinicopathological parameters

The result of Immunohistochemistry indicated that the SOX18 expression was in cell nuclei, as well as in cytoplasm (**Figure 1A**, **1B**). The SOX18 expression in ESCC was significantly higher than in nonneoplastic esophageal tissues. SOX18 expression was observed in 78 out of 100 (78.0%) cases of ESCC, whereas the positive rate of nonneoplastic esophageal tissues was 16/80 (20.0%). There was a significant up-regulation of SOX18 expression be-

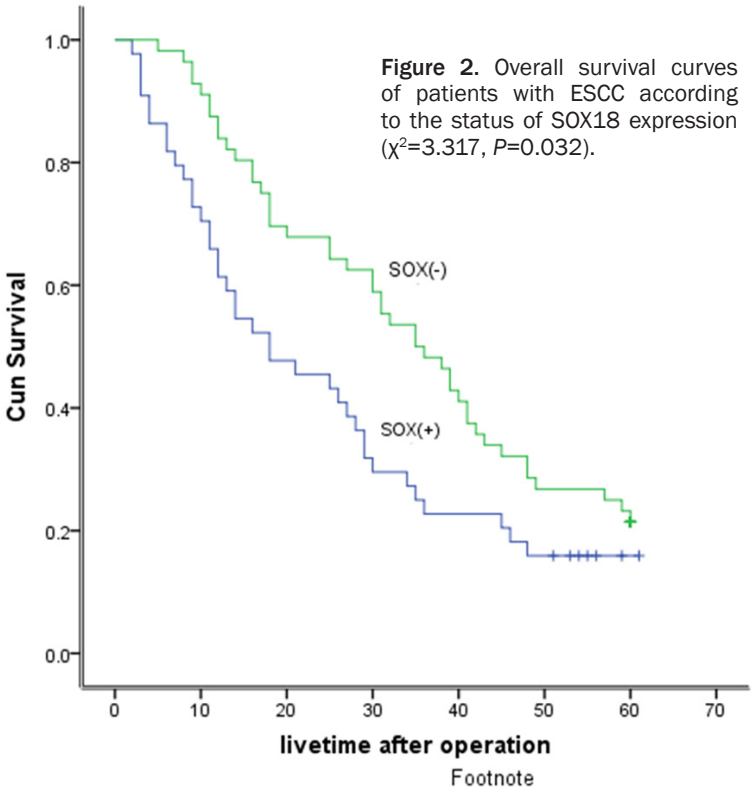

Figure 2. Overall survival curves of patients with ESCC according to the status of SOX18 expression (χ^2=3.317, *P*=0.032).

tween ESCC and the nonneoplastic esophageal tissue (χ^2=59.923, *P*=0.000). The statistical analyses also revealed the correlation between SOX18 expression and clinicopathological parameters. We found that an increasing trend of SOX18 expression from high grade to low grade, a significant difference was found among them (*P*=0.024). At the same time, the SOX18 positive expression percentage in the lymph node metastasis group was 47/54 (87.0%), whereas the group without lymph node metastasis was 31/46 (67.3%), a statistically significant was found between them (P=0.012). However, there were no significant difference between SOX18 expression and sex (*P*=0.481), age (*P*=0.400), tumor size (*P*=0.623), depth of invasion (*P*=0.538) and AJCC clinical stage (*P*=0.363) (**Table 1**).

Survival analysis

Kaplan-Meier survival curves for all 100 patients have demonstrated to determine whether SOX18 expression is a prognostic factor. The median survival time of SOX18 positive expression group was 18 (95% CI: 16.3.2-27.6) months and 32 (95% CI: 25.4-36.7) months of SOX18 negative expression group. The 1-year, 3-year and 5-year survival rate were 62.3%, 35.4% and 13.1% for patients with SOX18 positive expression (n=78) compared to 76.5%, 49.2% and 28.7% for patients with negative expression (n=22). A significantly poorer survival rate was found in the SOX18 positive expression group compare to the negative group (χ^2=3.317, *P*=0.032) (**Figure 2**).

In order to identify independent predictors for survival, univariate and multivariate cox-regression analyses have performed, all the clinicopathological parameters were entered into the analysis. Univariate survival analysis indicated that SOX18 expression, lymph node metastasis and AJCC clinical stage were poor prognostic factors for cancer-specific survival (P<0.05, **Table 2**). Multivariate survival analysis revealed that SOX18 expression and AJCC clinical stage were independent predictors for ESCC patients (P<0.05, **Table 3**).

Discussion

The SOX (Sex-determining region on the Y chromosome related high mobility group box) gene is a large family of diverse and well-conserved genes encoding transcription factors. All family members share a high-mobility group (HMG) domain, which specifically binds to the 5'-CA-AG-3' DNA sequence motif. Previously studies demonstrated that SOX genes have a wide range of roles in various developmental processes [4, 5].

The SOX family contains ten groups (A-J) on the basis of their amino acid homology, SOX18, together with SOX7 and SOX17, is part of the F group (SOX F) of SOX proteins [6]. It is reported that SOX18 has an important role in vascular development and postnatal neovascularization of different animal species. Murine SOX18 gene was expressed in endothelial cells of the newly formed blood vasculature in the pathological condition of wound healing or tu-

Table 2. Univariate analysis of predictive factors for survival

Prognostic factors	Relative risk (95% CI)	*P*-value
Univariate		
SOX18 (+) (-)	1.399 (1.216-1.973)	0.038*
Gender (Male) (Female)	0.875 (0.524-1.321)	0.351
Age (≥60 years) (<60 years)	0.965 (0.487-1.643)	0.384
Tumor size (<4 cm) (4-7 cm) (≥8 cm)	0.740 (0.354-1.732)	0.548
Cell differentiation (High-grade) (Middle-grade) (Low-grade)	1.413 (0.885-2.300)	0.437
Depth of invasion (T1) (T2) (T3) (T4)	0.610 (0.351-1.182)	0.085
Lymph node metastasis (+) (-)	1.831 (1.335-2.413)	0.046*
AJCC clinical stage (I+II) (III+IV)	1.761 (1.379-2.298)	0.040*

*$P<0.05$.

Table 3. Multivariate analysis of predictive factors for survival

Prognostic factors	Relative risk (95% CI)	*P*-value
SOX18 (+) (-)	1.796 (1.243-2.257)	0.029*
AJCC clinical stage (I+II) (III+IV)	1.811 (1.098-2.254)	0.032*

*$P<0.05$.

mor growth [9]. Furthermore, SOX18 have been shown to involve in endothelial cell proliferation, migration and vascular remodeling [10].

Recent findings indicate that SOX18 gene may play an important role in tumour growth. Pula B et al found that SOX18 expression increases in invasive ductal breast cancer (IDC) cells, SOX18 expression in the IDC samples correlated with malignancy grade and also associated with human epidermal growth factor receptor 2 (HER2) positivity [12]. In addition, Zhang et al found that the mRNA and protein expression levels of SOX18 were prevalently and significantly overexpressed in human breast cancer cell lines, knockdown of SOX18 significantly inhibited cell proliferation and invasion, but promoted apoptosis in breast cancer cells [17]. Eom et al revealed the expression of SOX18 was higher in gastric cancer compared to corresponding normal tissues, but SOX18 expression was exclusively noted in the stromal cells rather than in the cancer cells. The frequencies of both lymphovascular invasion and lymph node metastases were all significantly increased in the SOX18 positive group. Both the 5-year survival and the recurrence-free survival were shorter for SOX18 positive tumors [13]. Jethon et al demonstrated SOX18 mRNA expression was significantly lower in non-small cell lung cancer (NSCLC) than in non-malignant lung tissue. However, SOX18 protein expression levels were higher in NSCLC tissues and in the examined lung cancer cell lines. In paraffin sections, a positive correlation between the Ki-67 antigen and nuclear SOX18 expression was noted. In univariate survival analysis, cytoplasmic SOX18 expression correlated with poor patient outcome [16]. Nevertheless, the expression of SOX18 in ESCC has not yet been determined.

Over the years, the role of SOX18 in embryonic vascular and lymphatic development has been well investigated [20, 21]. Recently studies have revealed that high expression of SOX18 not only in blood and lymphatic vessels, but also in cancer cells. SOX18 might play an important role in promoting tumor angiogenesis and lymphangiogenesis. Now, the function of SOX18 gene is not just the development of embryonic vascular and lymphatic vessels, but the regulation of tumor angiogenesis and lymphangiogenesis [22, 23].

In our present study, we have examined SOX18 expression on the protein level in ESCC and nonneoplastic esophageal tissues, analyzed the clinicopathological data of the patients in order to determine its prognostic significance. Our research findings showed that the SOX18 expression in ESCC was significantly higher than in nonneoplastic esophageal tissues, SOX18 expression was significantly correlated with the differentiation degree of ESCC and the lymph node metastasis. Therefore, SOX18 may play an important role in the genesis and development of ESCC. Analysis of survival data revealed that positive expression of SOX18 was significantly associated with a poorer prognosis of ESCC. Furthermore, univariate survival analysis indicated that SOX18 expression, lymph node metastasis and AJCC

clinical stage were poor prognostic factors for cancer-specific survival. Multivariate survival analysis revealed that SOX18 expression and AJCC clinical stage were independent predictors for ESCC patients.

In summary, our primary research have demonstrated that SOX18 is expressed in cancer cells of ESCC for the first time, SOX18 expression could provide clinically useful prognostic information in cases of ESCC. SOX18 expression might be a prognostic tumor marker and a potential therapeutic target in ESCC. However, further investigation is still required to explore the molecular mechanisms of SOX18 during lymphangiogenesis and lymphatic invasion in ESCC.

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Disclosure of conflict of interest

None.

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