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Original Article

Prognostic significance of Notch1 expression in operable esophageal squamous cell carcinoma

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Abstract: *Objective:* To study the correlation between Notch1 expression and the prognosis of patients with esophageal squamous cell carcinoma. *Methods:* Notch1 expression was determined by immunohistochemistry and the correlation between Notch1 and patients' prognosis was evaluated. *Results:* A total of 228 patients with ESCC who had undergone curative resection in the Department of Thoracic Surgery, Yinzhou Hospital, were analyzed retrospectively. Notch1 expression was significantly associated with sex, age, tumor size, T stage, and N stage ($P < 0.05$). High expression of Notch1 was related with poor overall survival ($P < 0.05$). The Cox's proportional hazard model showed that patients with high expression of Notch1 had a 2.22-fold, 2.91-fold, and 1.86-fold increased relative risk of developing treatment failure, distant metastasis, and death respectively, compared with patients with low expression of Notch1. *Conclusions:* Expression of Notch1 is thus a predictive factor of distant metastasis and OS in operable ESCC patients.

Keywords: Notch1, esophageal squamous cell carcinoma, metastasis, prognosis

Introduction

Esophageal cancer is one of the most common cancers in China. The predominant histological type is esophageal squamous cell carcinoma (ESCC), which accounts for more than 80% of cases. Surgical resection is the standard treatment for early stage disease. However, most patients are newly diagnosed with ESCC at a locally advanced stage. Furthermore, even after radical surgery, ESCC patients still have very high risk of loco-regional recurrence and distant organ metastasis in a short time. Despite the progress of multidisciplinary therapy in past two decades, the prognosis for locally advanced or metastatic ESCC is still poor and unsatisfactory. Although the tumor-node-metastasis (TNM) stage after pathologic examination could provide the best prognostic information, patients with the same stages and histologic classification have remarkably different survival outcomes. Numerous prognostic factors, including molecular and biological biomarkers have been investigated to identify patients at high risk for recurrence or metastasis. However, the validity of these markers is still controversial and none of them has been proven effective in the clinical setting.

Notch signaling is a relatively conserved pathway that plays an important role in cell proliferation, tissue renewal, and cell-fate determination of progenitors [1]. Four Notch receptors (Notch1-4) and five ligands (JAG 1-2, DLL 1, 3 and 4) have been identified in mammals. Recently, Notch signaling has been found to be closely associated with development of several cancers. It can play as oncogene or tumor suppressor, depending on the tumor tissue or cancer cell types. Notch1 expression was found to be positively related with cervical lymph node metastasis [2], tumor invasion, and micro-vessel density [3]. Knockdown of Notch1 by siRNA transfection or inhibition of activated Notch by treating cells with γ-secretase inhibitor significantly reduced cell proliferation and decreased the ability of invasion [4]. In clinical studies, overexpression of Notch1 was related with short disease-specific survival time and overall survival time [5]. However, Notch1 was also

Table 1. Relationship between Notch1 expression and the clinicopathological characteristics in ESCC patients

Variables	Patients, n (%)	Notch1 expression		P
		Low, n (%)	High, n (%)	
Gender				
Male	205 (89.9)	125 (61.0)	80 (39.0)	0.041
Female	23 (10.1)	19 (82.6)	4 (17.4)	
Age (Year)				
≤ 65	201 (88.2)	122 (60.7)	79 (39.3)	0.036
> 65	27 (11.8)	22 (81.5)	5 (18.5)	
Smoking				
Never	41 (18.0)	29 (70.7)	12 (29.3)	0.267
Ever	187 (82.0)	115 (61.5)	72 (38.5)	
Alcohol				
Never	72 (31.6)	49 (68.1)	23 (31.9)	0.298
Ever	156 (68.4)	95 (60.9)	61 (39.1)	
Tumor location				
Upper	6 (2.6)	3 (50.0)	3 (50.0)	0.125
Middle	123 (54.0)	85 (69.1)	38 (30.9)	
Lower	99 (43.4)	56 (56.6)	43 (43.4)	
Tumor size				
≤ 5 cm	183 (80.3)	129 (70.5)	54 (29.5)	< 0.001
> 5 cm	45 (19.7)	15 (33.3)	30 (66.7)	
T stage				
T_{1-2}	85 (37.3)	71 (83.5)	14 (16.5)	< 0.001
T_{3-4}	143 (62.7)	73 (51.0)	70 (49.0)	
N stage				
N_0	127 (55.7)	89 (70.1)	38 (29.9)	0.015
N_{1-3}	101 (44.3)	55 (54.5)	46 (45.5)	

reported as tumor suppressor. Several reports investigated Notch1 expression between primary tumor tissues and corresponding normal tissue and found decreased Notch1 expression in tumor cells [6, 7]. Activation of Notch1 signaling pathway could lead to dramatic increase in p21 and p53 expression with decreases in Bcl-2 and β-catenin expression, which participate in the induction of apoptosis and cell cycle arrest [8].

In this study, the goal was to analyze the capability of Notch1 protein expression for predicting metastasis and survival in operable ESCC.

Patients and methods

Patients' selection

A total of 228 patients with ESCC who had undergone curative resection in the Department of Thoracic Surgery, Yinzhou Hospital, from January 2008 to December 2013 were retrospectively analyzed. All patients were newly confirmed to have esophageal squamous cell carcinoma and had not received treatment previously. Patients with other malignancies were excluded from this study. Each case was reassigned for TNM stage classification and clinical stage according to the American Joint Committee on Cancer (AJCC) 7th staging system. The following detailed clinical information was retrospectively collected and analyzed for each case: gender, age at treatment, smoking status, tumor location, and pathological TNM stage.

All surgical specimens were routinely fixed in 10% buffered formalin liquid and then embedded in paraffin. Each specimen was verified by two pathologists before inclusion in the present study.

Treatment and follow-up

All patients underwent total or subtotal transthoracic esophagectomy and regional lymphadenectomy with curative intent. All patients received standardized follow-up at a 3-month interval for the first 2 years after operation, a 6-month interval in the third year, and yearly thereafter. Evaluation comprised a physical examination, upper gastrointestinal endoscopy, complete blood count, chest and abdomen computed tomography.

Immunohistochemical analysis

Notch1 protein immunohistochemical staining was performed using EnVision™ detection kit (Dako Laboratories, CA, USA). First, 4 μm sections were prepared from paraffin-embedded tumor tissues. The sections were dewaxed and incubated for 30 min in 0.5% hydrogen peroxide (H_2O_2) in methanol. Thereafter, the slides were rinsed with distilled water, and incubated with a rabbit monoclonal anti-human Notch1 antibody (1:100 dilution, Abcam) for 1 hour at room temperature. HRP-linked anti-rabbit secondary antibody (1:1000 dilution, Cell signaling) was then applied for 30 minutes. The signal was visualized using avidin-peroxidase. Finally, the slides were counterstained with

Table 2. Relationship between clinicopathological characteristics, Notch1 expression, and three year survival rate in ESCC patients

Variables	3-year DFS rate	P	3-year DMFS rate	P	3-year OS rate	P
Gender						
Male	51.0	0.169	60.9	0.210	63.2	0.322
Female	59.0		71.8		75.5	
Age (Year)						
≤ 65	52.0	0.863	61.4	0.969	65.7	0.279
> 65	49.5		67.3		52.4	
Smoking						
Never	58.1	0.284	73.3	0.163	71.1	0.319
Ever	50.6		59.8		63.0	
Alcohol						
Never	53.8	0.393	68.0	0.367	73.9	0.193
Ever	50.9		59.3		60.1	
Tumor location						
Upper	66.7	0.655	66.7	0.458	27.8	0.013
Middle	53.2		62.8		68.1	
Lower	50.3		62.1		61.2	
Tumor size						
≤ 5 cm	53.9	0.316	66.2	0.072	67.4	0.016
> 5 cm	42.1		43.4		51.5	
T stage						
T_{1-2}	64.0	0.012	69.4	0.089	80.7	< 0.001
T_{3-4}	43.9		57.1		53.9	
N stage						
N_0	63.1	< 0.001	72.0	0.005	75.0	< 0.001
N_{1-3}	39.5		50.5		52.4	
Notch1						
High	61.6	< 0.001	73.6	< 0.001	73.4	< 0.001
Low	34.5		42.1		47.9	

Mayer's hematoxylin solution. Serial sections incubated with buffer alone instead of the primary antibody were used as negative controls.

Notch1 immunoreactivity was localized in the cell cytoplasm. The expression intensity of Notch1 was stratified into two categories scored as follows: tumor cells with positive staining in the cytoplasm ≥ 25% were defined as high expression, while < 25% were considered low expression.

Statistical analysis

Statistical analyses were performed by using SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL). The Chi-square test was performed to evaluate the association between the clinicopathological variables and Notch1 expression. Survival curves were estimated by the univariate Kaplan-Meier method. The log-rank test was applied to check the significant differences in the curves among groups. Furthermore, we used the Cox proportional hazards model with the backward selection method for multivariate analysis. Distant metastasis-free survival (DMFS) covered the date of definitive surgery to the date of distant metastasis was diagnosed. Disease-free survival (DFS) was defined as the time from surgery to any recurrence. Overall survival (OS) was calculated as the time from the date of surgery to death or censoring. Two-sided *P* values of < 0.05 were considered statistical significance.

Results

Patients' characteristics

The characteristics of the 228 ESCC patients are summarized in **Table 1**. The median age was 58 years (range: 37-72 years) and 89.9% of patients were males. The median length of the tumor was 4.0 cm (range: 0.5-11.0 cm). Tumors were located in the upper-thoracic esophagus in 2.6% of patients, while in the mid-thoracic esophagus in 54.0% of patients and in the lower-thoracic esophagus in 43.4% of patients. Based on staging system, 36, 49, 131, and 12 patients were classified as T_1, T_2, T_3, and T_4. According to lymph node metastasis in the N region, 127 patients were considered N_0, whereas 62 patients were classified as N_1, 28 patients were classified as N_2, and 11 patients were considered N_3.

Association between Notch1 expression and the clinicopathological variables of ESCC patients

High expression of Notch1 in tumor cells was observed in 84 of 228 (36.8%). Notch1 expression was significantly associated with sex, age,

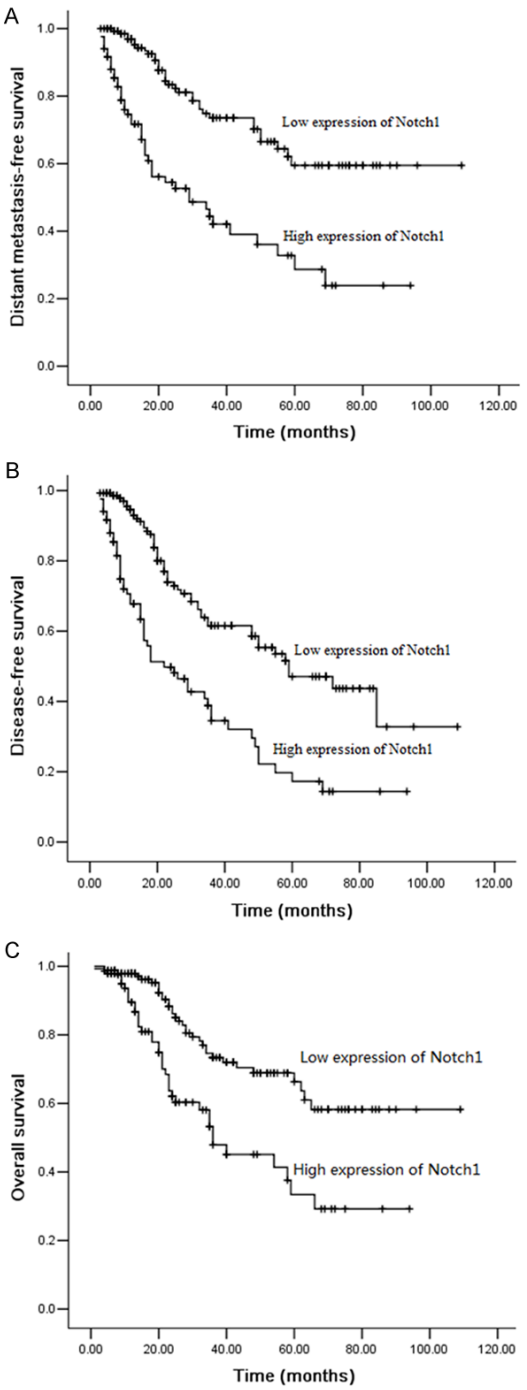

Figure 1. Notch1 expression and survival in ESCC patients. A: Kaplan-Meier plots of DMFS in patients with ESCC. B: Kaplan-Meier plots of DFS in patients with ESCC. C: Kaplan-Meier plots of OS in patients with ESCC.

tumor size, T stage, and N stage ($P < 0.05$). There were no significant differences in Notch1 expression and smoking status, alcohol behavior, and tumor location ($P > 0.05$). The percentage of high expression of Notch1 was significantly higher in patients with larger tumor size, advanced T stage and positive regional lymph node metastasis.

Correlation of Notch1 expression with survival in ESCC patients

After a median follow-up time of 40 months, 77 (33.8%) had distant metastasis, 103 (45.2%) had treatment failure and 70 (30.7%) died. The 3-year DMFS, DFS, and OS rates were 62.0%, 51.8%, and 64.2%, respectively. Kaplan-Meier survival analysis (**Table 2**) in 228 ESCC patients revealed that patients with high expression of Notch1 survived a significantly shorter time than patients with low expression of Notch1 ($P < 0.001$, **Figure 1A-C**). Univariate analysis for Notch1 expression was performed and other eight clinicopathological variables to find out the useful prognostic factors. Advanced T stage, lymph node metastasis, and high expression of Notch1 were significantly related with lower 3-year DFS rate ($P < 0.05$). Lymph node metastasis and high expression of Notch1 were also significantly associated with lower 3-year DMFS rate ($P < 0.01$). Furthermore, lower tumor location, larger tumor size, advanced T stage, lymph node metastasis, and high expression of Notch1 were related with poor overall survival ($P < 0.05$). After stratification by clinical stage, Notch1 expression remained of great prognostic value in patients with early stage (stage I-II, $P < 0.05$) and locally advanced stage (stage III, $P < 0.05$).

Cox's proportional hazard model (**Table 3**) showed that Notch1 expression was an independent prognostic factor in operable ESCC. With respect to DFS, DMFS, and OS, patients with high expression of Notch1 had a 2.22-fold, 2.91-fold, and 1.86-fold increased relative risk of developing treatment failure, distant metastasis and death compared with patients with low expression of Notch1.

Discussion

Esophageal cancer presents a highly aggressive characteristic with very poor prognosis. In this study, we performed immunohistochemistry to investigate Notch1 expression in ESCC tissues and evaluate the relationship between Notch1 expression and clinicopathological characteristics. Our results demonstrate that

Table 3. Correlation of Notch1 expression and patient survival analyzed by COX's proportional hazard model and adjusted for age, sex, T stage, and N stage

	Crude HR	95% CI	P	Adjusted HR	95% CI	P
DFS						
Low expression of Notch1	1			1		
High expression of Notch1	2.58	1.75-3.81	< 0.001	2.22	1.47-3.34	< 0.001
DMFS						
Low expression of Notch1	1			1		
High expression of Notch1	3.20	2.04-5.04	< 0.001	2.91	1.80-4.70	< 0.001
OS						
Low expression of Notch1	1			1		
High expression of Notch1	2.48	1.55-3.97	< 0.001	1.86	1.14-3.06	0.013

Notch1 is highly expressed in ESCC tumors and Notch1 expression is significantly related with tumor size, T stage, and N stage. Multivariate Cox regression revealed that Notch1 could act as an independent prognostic marker for ESCC patients. Patients with high expression of Notch1 had 2.91 times the risk of distant metastasis and 1.86 times the risk of death after radical surgery, compared to patients lacking Notch1 expression. Furthermore, after stratified by clinical stage, Notch1 expression remained of great prognostic value in patients with early stage (stage I-II) and locally advanced stage (stage III). Our data indicate that in clinical practice, if operable ESCC patients have high expression of Notch1, they could be at increased risk of distant organ metastasis and poor prognosis postoperatively. A close follow-up is needed or additional treatment, such as adjuvant chemotherapy or radiotherapy, could be recommended.

Previous studies revealed that high expression of Notch1 was correlated with histological grade of tumors, TNM stage, metastasis, as well as prognosis in patients with non-small cell lung cancer [9], breast cancer [10], gallbladder cancer [11], pancreatic cancer [12], and gastric cancer [13]. In these studies, high Notch1 expression was associated with advanced TNM stage, lymph node metastasis, rapid progression, early distant organ metastasis, and unfavorable prognosis. In our study, high expression of Notch1 was related to regional lymph node metastasis, tumor size, T stage, as well as poor prognosis. Ogawa et al. [14] reported similar result, of which they analyzed the expression of Notch1 in ESCC tumor tissues. The high expression of Notch1 was significantly related with low overall survival rate (P=0.0028). However, only 8 of 55 patients were found high Notch1 expression in this study and expression of Notch1 was not related with other prognostic factors, such as TNM stage, histological differentiation, lymphatic invasion, as well as blood vessel invasion. It is hard to explain how Notch1 influenced patients' prognosis. According to our study, high Notch1 was expressed in 36.8% (84/228) of ESCC patients, higher rate than that in above study. The discordance in these studies might be explained by the difference of ethnicity and epidemiology.

The mechanism of action of Notch1 in ESCC is not well understood. Recently, a number of studies have reported that Notch1 was not only described as oncogene but it also acted tumor suppressor [7]. In a recent study (written in Chinese), Yuan et al. [15] used immunohistochemistry to analyze the expression of Notch1 in 50 patients with ESCC, both in tumor tissues and paired adjacent normal tissues. The results showed that Notch1 expression was negatively correlated with lymph node metastasis and TNM staging (all $P < 0.01$). In this study, high Notch1 was found in 58.0% (29/50) of ESCC patients. The differences in definition of "high expression" between our study and this study might lead to different result. In our study, the Notch1 expression cutoff was defined as ≥ 25%, according to previous studies [11, 16]. In a report of Yuan et al., high expression of Notch1 was defined as ≥ 11%. Furthermore, the differences of immunohistochemistry method and antibody provider might also lead to various results. Finally, Yuan et al. did not provide the information that whether Notch1 expression was associated with prognosis in

ESCC patients. Thus, we devised this study and found that Notch1 expression in tumor tissues was not only associated with TNM stage but also with tumor metastasis and poor prognosis, indicating that Notch1 might act as tumor oncogene and reflect the patients' individual status rather than tumor burden and progression.

The major strength of our study is sufficient survival data for investigating. However, we acknowledge that our study has the following limitations. First, our study is a retrospective study with relative small sample size. Second, the patients enrolled in this study were treated in a single institution.

In conclusion, our findings demonstrate that high expression of Notch1 protein in ESCC is associated with cancer metastasis and survival and can serve as an independent prognostic factor. Targeting inhibition of Notch1 might be an option for developing new therapeutic agents.

Disclosure of conflict of interest

None.

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