

## Original Article

# Prognostic value of transducer of ErbB2.1 (TOB1) expression in patients with gastric cancer: tissue microarray analysis

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**Abstract:** Background: Transducer of ErbB2.1 (TOB1) protein plays an important role in cell cycle regulation, apoptosis induction, and metastasis inhibition. However, the prognostic value of TOB1 in the survival of gastric cancer (GC) patients has not been examined. Methods: In the present study, western blot and real-time qPCR were used to detect the protein and mRNA level of TOB1 in fresh samples from GC patients. A tissue microarray comprising 90 pairs of primary GC and adjacent normal tissues was analysed using immunohistochemistry. Western blot analysis of 16 samples from GC patients showed that 81% (13/16) of patients exhibited decreased expression of TOB1, and real-time qPCR analysis showed that GC patients had decreased expression of TOB1 mRNA. Results: Tissue microarrays showed that 72.2% of gastric cancer tissues exhibited down-regulated expression of TOB1, and the expression level was significantly lower in cases with poor differentiation and positive lymph node metastasis, compared with normal tissues. Moreover, the Kaplan-Meier analysis indicated that patients with low TOB1 expression had shorter survival time than those with high TOB1 expression (5-year survival rate, 26.2% vs. 56.0%,  $P=0.002$ ). In addition, multivariate analysis indicated that TOB1 was an independent prognostic factor for outcome in gastric cancer (HR, 0.256; 95% CI, 0.127-0.514;  $P=0.000$ ). Conclusions: Down-regulated TOB1 expression was found in gastric cancer. Furthermore, low TOB1 expression may be an independent indicator of poor prognosis in gastric cancer.

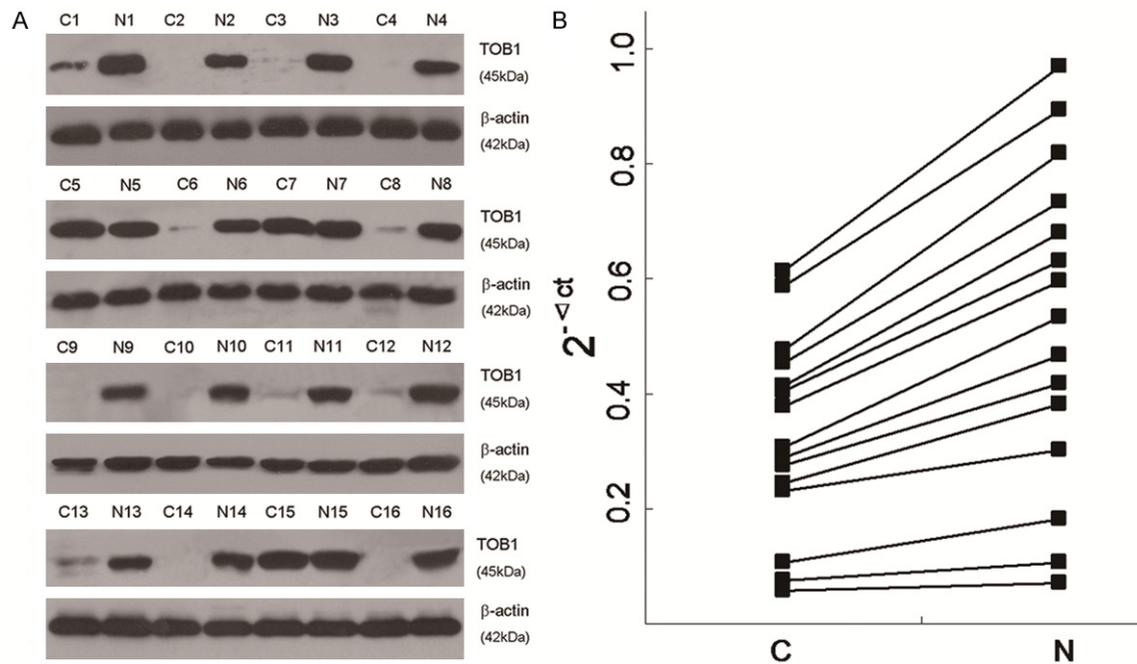
**Keywords:** Transducer of ErbB2.1, gastric cancer, prognosis

## Introduction

Gastric cancer (GC) ranks as the fourth most common malignancy and second leading cause of cancer mortality worldwide [1]. Incidence rates of this cancer vary regionally, and more than 70% of GC occurs in developing countries. GC in China constitutes approximately 33% of all cases worldwide [2-4]. The prognosis of this malignancy remains poor, with 25%-35% 5-year survival rate for loco-regional disease, despite its current treatment protocol incorporating chemotherapy or radiation into surgical resection [5, 6]. Thus, novel molecular markers that can be exploited to determine prognosis and identify targets should be explored and characterized for the development of novel therapies because of the high incidence and mortality of GC.

Transducer of ErbB2.1 (TOB1) is a member of the B-cell translocation gene/transducers of ErbB2 antiproliferative protein family, which was firstly discovered in 1996 [7, 8]. The antiproliferative activity of TOB1 is regulated through phosphorylation and nuclear translocation [9]. When activated, TOB is unphosphorylated, but phosphorylation of TOB1 keeps it inactivated [10-12]. Accumulated evidence confirms that TOB1 functions as a tumor suppressor in various human malignancies including lung and thyroid cancers [13, 14]. However, the prognostic significance of TOB1 in malignancies has not been extensively studied. We investigated the relevance of TOB1 protein expression with GC and its association with clinical outcome. Thus, we performed expression analysis on a patient cohort of 90 gastric can-

## Transducer of ErbB2.1 expression in gastric cancer



**Figure 1.** Expression of TOB1 in the GC samples. A. Western blot analysis of TOB1 protein expression in matched fresh tissues from GC patients. B. Real-Time PCR analysis of TOB1 mRNA expression.

cer cases with long-term follow-up and full clinical records.

### Patients and methods

#### Patients and specimens

Primary GC and adjacent normal tissues used for western blot and real-time qPCR were collected from 16 gastric cancer patients, including 11 males and 5 females in 2017. All samples were snap-frozen in liquid nitrogen after surgery and stored at  $-80^{\circ}\text{C}$  before the following RNA and protein extraction. Each case was reviewed by two experienced histopathologists who were blinded to the original diagnosis. The matched tissue samples used for tissue microarray were obtained from 90 GC patients. Surgery was performed from February 2008 to August 2008 at the Kunshan First People's Hospital affiliated with Jiangsu University, Kunshan, Jiangsu, China. No patient received chemotherapy or radiotherapy prior to the operation. The pathologic stages of all patients were evaluated according to the 6th edition of AJCC Cancer Staging Manual [15]. Surgical treatment comprised radical distal gastrectomy or total gastrectomy following the recommendations of the Japanese Research Society

for GC [16]. The patients were followed up for 6.5 years or until death. Survival time was measured as the time from the date of the initial surgery to the date of death. The materials to be analyzed were selected by a pathologist to ensure that the samples were macroscopically entirely cancerous and chosen from an area devoid of necrotic tissue. Written informed consent was obtained from each patient.

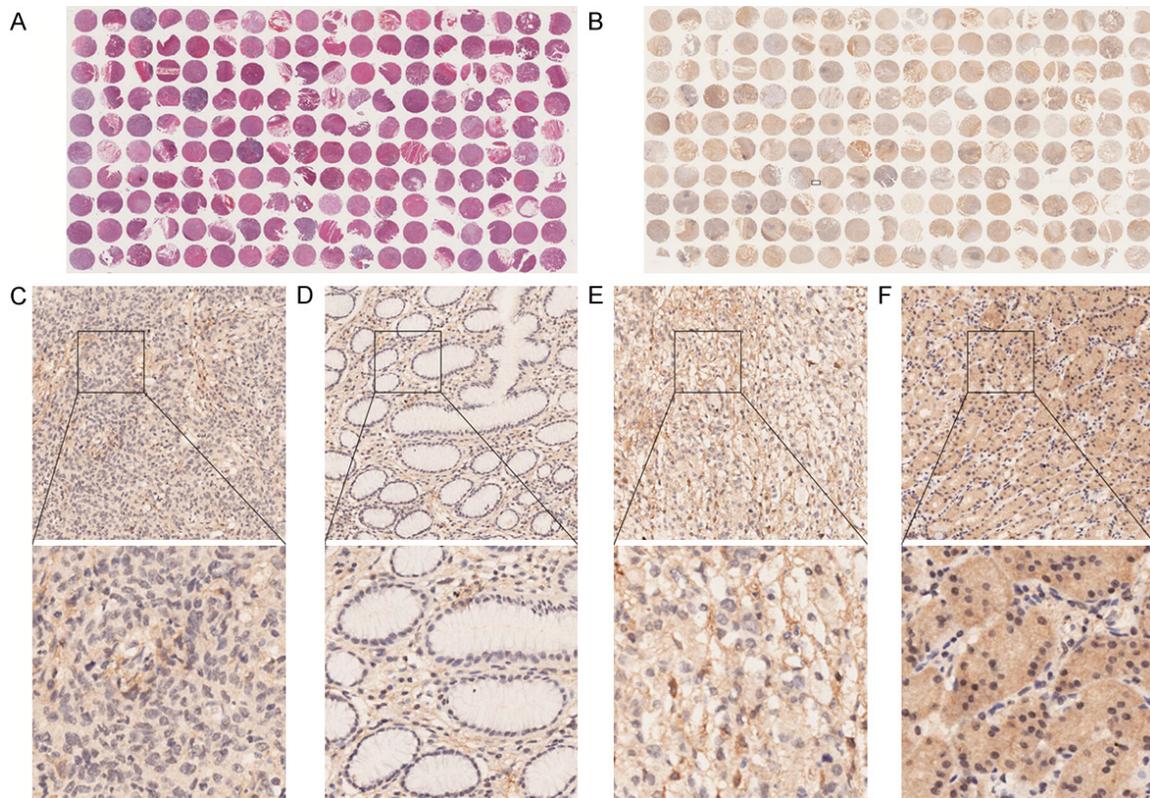
#### Ethics statement

This study was undertaken under the ethical committee of Jiangsu University, and written informed consents were obtained from all patients before enrolment. Collection of human gastric cancer resection specimens from patients conformed to the principles outlined in the Declaration of Helsinki.

#### Western blot analysis

Western blot was used to detect protein expression levels. Briefly, the tissues were sonically homogenized and lysed with RIPA buffer with protease inhibitor (cocktail, Roche). Protein was loaded at a concentration of 30  $\mu\text{g}$  per lane, separated on a 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel, and then transferred onto a nitrocellulose membrane. Next,

## Transducer of ErbB2.1 expression in gastric cancer



**Figure 2.** TOB1 expression in gastric cancer and adjacent normal tissues. (A) The gastric cancer tissues and adjacent normal tissues were confirmed by haematoxylin and eosin staining. (B) TOB1 expression was analysed by immunohistochemical staining. Immunohistochemical analysis revealed low cytoplasmic TOB1 expression in gastric cancers (C) ( $\times 100$ ), low cytoplasmic TOB1 expression in normal tissues (D) ( $\times 100$ ), high cytoplasmic TOB1 expression in gastric cancers (E) ( $\times 200$ ) and high cytoplasmic TOB1 expression in normal tissues (F) ( $\times 200$ ).

the membrane was blocked with 5% nonfat milk in PBS and then incubated with primary antibodies (Santa Cruz Biotech, Santa Cruz, CA, 1:500 dilution) TOB1 (H-18) and  $\beta$ -actin (C-4) at 1:1000 overnight at 4°C. The appropriate secondary antibodies horseradish peroxidase (HRP)-labeled goat anti-mouse (GAM-007) and goat anti-rabbit (SC-2004) IgG (Santa Cruz Biotech, Santa Cruz, CA, USA) were used at 1:2000. Positive antibody reactions were detected with the enhanced chemiluminescence system (Union Bioscience Corporation, Hangzhou, China) with prestained markers as molecular size standards.

### Real-time qPCR

Total RNA was isolated using an RNeasy Mini Kit (217184, Qiagen, Valencia, CA), according to the manufacturer's recommendations. After the purity and integrity of the obtained RNA was assessed, cDNA was synthesized using Superscript III platinum kit (R250-01,

Invitrogen) according to manufacturer's instructions. Real-Time PCR was performed using SYBR Green I (CS7561, Invitrogen) and run on an ABIPrism 7500 sequence detector (Applied Biosystems).  $\beta$ -actin was set as internal control. The sequences of the primers used were as follows: TOB1-F: 5'-TCACTCTGCTGCTGTAAGCC-3'; TOB1-R: 5'-GGGAGAAGTACGTGCAACCT-3';  $\beta$ -actin-F: 5'-CGACATGGAGAAAATCTGGCAC-3';  $\beta$ -actin-R: 5'-GATAGCACAGCCTGGATAGCAA-3'.

The cycle parameters were as follows: denaturing for 10 s at 95°C, annealing for 30 s at 60°C, and extending for 45 s at 70°C for a total of 40 cycles. Values were calculated using the comparative threshold cycle ( $C_t$ ) method after normalization to the control gene  $\beta$ -actin.

### Tissue microarrays (TMAs)

The TMA was prepared by Shanghai Superchip Co., Ltd, (Shanghai, China). All cases were histo-

## Transducer of ErbB2.1 expression in gastric cancer

**Table 1.** Correlation between expression of TOB1 and clinicopathologic factors

Characteristics	TOB1 expression		
	Low	High	P
Age			
<60	21	11	0.299
≥60	44	14	
Gender			
Male	46	16	0.534
Female	19	9	
Depth of invasion			
pT1	2	1	0.923
pT2	5	3	
pT3	41	15	
pT4	17	6	
Nodal status			
pN0	11	9	0.021*
pN1-3	54	16	
TNM stage			
I	5	1	0.558
II	19	10	
III	41	14	
Differentiation			
Grade I	0	3	0.044*
Grade II	16	10	
Grade III	49	12	

\*P<0.05.

logically reviewed using HE staining. The HE sections were examined by two independent pathologists under a light microscope. Duplicate 1-mm diameter cylinders from the tumor centre were selected from each case, together with precancerous nonmalignant gastric tissues as controls to ensure reproducibility and homogeneous staining of the slides. Single-core biopsy specimens were placed on a TMA mould with 180 pores and embedded with paraffin. Of all the 90 pairs of GC tissues and the compared normal tissue, 180 cores were applied per slide. The TMA blocks were prepared as 4- $\mu$ m thick sections and were stained with HE. The tissues were then examined to determine whether the appropriate tumor site had been selected. Pathological diagnosis, grade and stage were retrieved from the medical record.

### Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed using a standard streptavidin-biotin-

peroxidase complex method. TMA slides were incubated overnight at 4°C in a moist chamber with primary antibody specific for TOB1 (E-1) (Santa Cruz, CA, 1:1000). Staining with PBS, instead of primary antibody against TSP50, was used as negative control. TOB1 expression was determined by assessing semi-quantitatively the percentage of marked tumor cells and the staining intensity. The TOB1 expression level was determined by integrating the percentage of positive tumor cells and the intensity of positive staining. The intensity of staining was scored as follows: negative (score 0), weak (score 1) and strong (score 2). The staining extent was scored according to the percentage of positively stained tumor cells in the field, as follows: negative (score 0), 0%-50% (score 1) and 51%-100% (score 2). The product of the intensity and extent score was considered as the overall IHC score (values from 0 to 4). The staining was observed and assessed by two independent pathologists who were blind to the identity of the samples. Scores for percentage of positive cells and scores for expression intensities were multiplied to calculate the immunoreactive score (IRS). IRS values of 0 and 1 were judged as low expression, whilst IRS values of 2 to 4 were considered as high expression.

### Statistical comparisons

Statistical analysis was performed with the SPSS statistical software package (SPSS Standard version 17.0, SPSS Inc.). The  $\chi^2$ -test was performed to analyse the association between TOB1 protein expression and clinicopathological features. Kaplan-Meier analysis (log-rank test) was used for survival curves. The Cox proportional hazards model was used in the multivariate analysis of the factors that were determined to be significant for overall survival (OS) using univariate analysis. P<0.05 was considered a significant difference with a 95% confidence interval (CI).

### Results

#### Expression of TOB1 is decreased in GC tissues

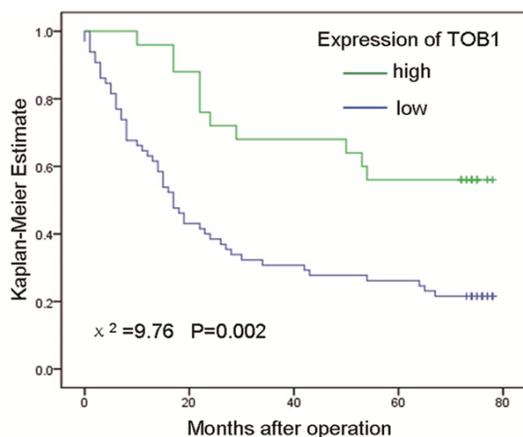
To assess whether TOB1 was involved in the pathogenesis of gastric cancer, we first detected the expression of TOB1 in 16 GC patients using western blot assay. Results showed that 81.3% of patients with GC (13/16) had significant reduction compared with the adjacent nor-

## Transducer of ErbB2.1 expression in gastric cancer

**Table 2.** Univariate and multivariate analysis of clinicopathologic factors affecting survival rate

Variables	5-year survival rate (%)	Univariate analysis P Value	Multivariate analysis	
			Relative risk (95% confidence interval)	P Value
Age				
<60	34.4	0.813	1.316 (0.728-2.381)	0.363
≥60	34.5			
Gender				
Male	38.7	0.415	1.129 (0.646-1.972)	0.671
Female	25.0			
Depth of invasion				
pT1	100	0.073	1.659 (1.118-2.461)	0.012*
pT2	62.5			
pT3	30.4			
pT4	26.1			
Nodal status				
pN0	76.0	0.000*	5.171 (1.431-18.682)	0.012*
pN1-3	18.5			
TNM stage				
I	100	0.000*	1.599 (0.563-4.540)	0.378
II	55.2			
III	16.4			
Differentiation				
Grade I	66.7	0.143	1.518 (0.820-2.809)	0.184
Grade II	45.2			
Grade III	26.8			
TOB1 expression				
Low	26.2	0.002*	0.256 (0.127-0.514)	0.000*
High	56.0			

\*P<0.05.



**Figure 3.** Survival curves of gastric cancer patients according to expression status of TOB1.

mal tissues (**Figure 1A**). To further investigate whether the aberrant expression of TOB1 oc-

curred in the transcriptional level, we adopted Real-Time PCR to determine its mRNA expression. Consistent with western blot, TOB1 mRNA expression in GC tissues was strongly decreased when compared with the adjacent normal tissues (**Figure 2B**). Together, these data demonstrate that the reduction of TOB1 expression in GC patients occurred both at protein and mRNA levels.

### Patient characteristics

The characteristics of the 90 patients are listed in **Table 1**. The samples included 62 (68.9%) male and 28 (31.1%) female patients with gastric cancer. A total of 32 (35.6%) patients were less than 60 years old, while the other patients were aged more than or equal to 60 years. Histological classification, grading and pathologic TNM staging of the tumors were based on the criteria defined by the 7th American Joint Committee on Cancer. Data showed that 56 (62.2%) patients had poorly differentiated tumors, whilst 34 (37.8%) patients had moderately or well

differentiated tumor. Six (6.7%) patients were stage I, 29 (32.2%) were stage II and 55 (61.1%) were stage III. A total of 65 (72.2%) patients had lymph node metastasis. TOB1 was highly expressed in 27.8% (25/90) of the 90 gastric cancer tissue samples. The corresponding rate in the 90 normal gastric mucosa samples was 87.8% (79/90). These results demonstrated that TOB1 expression in the cytoplasm was significantly down-regulated in cancerous tissues (P<0.05).

### Relationship between TOB1 protein expression and clinicopathological factors

All cases were histologically reviewed by HE staining (**Figure 2A**). TOB1 expression was analysed by IHC TMA (**Figure 2B**). Thus, representative examples for these significant molecules are shown in the TOB1 expression sample

(Figure 2C-F). Relationships between pathologic features and TOB1 are shown in Table 1. Reduced TOB1 expression was significantly associated with lymph node metastasis ( $P=0.021$ ) and differentiation grade ( $P=0.044$ ). No statistical connection was found between TOB1 expression and the other clinicopathologic factors, such as age, gender, and clinical stage (Table 1).

### *Association of TOB1 expression with survival of patients with gastric cancer*

Survival analyses using the Kaplan-Meier method in connection with the clinicopathological variables and the expression profiles of TOB1, VEGF and survivin are summarized in Table 2. Lymph node metastasis ( $P=0.012$ ) demonstrated significant correlation with OS. In terms of the three proteins analysed, patients with suppressed TOB1 expression were likely to exhibit significantly shorter OS compared with those with high TOB1 expression (5-year survival rate, 26.2% vs. 56.0%,  $P=0.002$ ) (Figure 3). Multivariate analysis using the Cox proportional hazards model was performed to evaluate the independent prognostic predictors. The results indicated that depth of invasion (HR: 1.659, 95% CI: 1.118-2.461,  $P=0.012$ ) and lymph node status (HR: 5.171, 95% CI: 1.431-18.682,  $P=0.012$ ) were significant predictors of cancer-specific survival. In addition, the suppressed expression of TOB1 (HR: 0.256; 95% CI, 0.127-0.514;  $P=0.000$ ) was found to be a statistically significant prognostic factor.

### **Discussion**

TOB1 is ubiquitously expressed in human adult tissues and was first identified by screening an expression library that detected protein-protein interactions with an ErbB2 probe [17]. TOB1 has been proposed as a putative tumor suppressor gene, because TOB1-knockout mice spontaneously formed tumors, and TOB1 expression was not observed in human lung and thyroid cancers [18]. However, TOB1 expression pattern has not yet been investigated in gastric cancer. In this study, we demonstrated that TOB1 expression was associated with the development and prognosis of gastric cancer. TOB1 expression level in the cancer tissue was significantly suppressed than in adjacent normal tissue. The expression level was significantly lower in poorly differentiated tissues and

positive lymph node metastasis. Furthermore, our study also indicated that TOB1 expression may be used as an independent prognostic factor for predicting patient OS, because low cytoplasmic expression is correlated with unfavourable outcome. Previous studies revealed that TOB1 proteins were distributed throughout the cytoplasm and nucleus [17]. Notably, subcellular distribution varied during cell cycle phases such that higher levels were detected in the cytoplasm during late S phase than during other phases of the cell cycle [11, 19]. In the current study, we identified that down-regulated TOB1 expression may play an important role in the carcinogenesis of gastric cancer. In addition, the down-regulated TOB1 expression correlated well with poor differentiation, lymph node metastasis and poor survival.

### **Conclusions**

The absence of TOB1 in gastric cancer may be an independent marker of a poor prognosis. Additional functional studies are needed to examine whether TOB1 activity has relevance in the treatment of HER2-positive GC with future therapies targeting ErbB2 signalling.

### **Acknowledgements**

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### **Disclosure of conflict of interest**

None.

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## Transducer of ErbB2.1 expression in gastric cancer

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