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*Original Article*

# Expression of TMEM40 in bladder cancer and its correlation with clinicopathological parameters

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**Abstract:** Transmembrane protein 40 (TMEM40) is a 23-kDa protein in cell membrane. There is no report that TMEM40 is associated with cancer. However, our study found that TMEM40 was high expressed in bladder cancer tissues. Immunohistochemical analyses of TMEM40 expression were performed on a tissue microarray including 72 transitional cell carcinomas and 43 normal bladder tissues to investigate the expression and clinical significance of TMEM40 in bladder cancer. We adopted receiver operating characteristic (ROC) analysis to select the optimal cut-off score. TMEM40 expression was defined positive if above 62.5% of cells were stained, and below it was negative. Then, the expression of TMEM40 in bladder cancer cells was evaluated by quantitative real-time PCR and western blot analysis. A significantly high level of TMEM40 in bladder cancer cells was proved. On the basis of ROC curve analysis, TMEM40 expression was positive in 68.1% (n=49) and negative in 31.9% (n=23) of bladder cancer cases. TMEM40 staining was positive in 2.3% (n=1) and negative in 97.7% (n=42) of normal bladder tissues. It showed that TMEM40 was up-regulated in bladder cancer tissues compared to normal bladder tissues. Moreover, TMEM40 expression was significantly associated with histological grade ($P<0.05$), clinical stage ($P<0.05$), pT status ($P<0.05$), but not age. Our study demonstrates that high TMEM40 expression is associated with bladder cancer, and it could be a diagnostic biomarker for bladder cancer.

**Keywords:** TMEM40, bladder cancer, tissue microarray, diagnostic biomarker, clinicopathological parameters

## Introduction

Bladder cancer remains one of the most common cancers worldwide, especially in elderly men [1, 2]. Bladder cancer is classified as non-muscle invasive and muscle-invasive cancers. The majority of bladder cancers are non-muscle-invasive ones and limited to the mucosa or submucosa when they are diagnosed [3]. Although through the combined therapyapproaches, bladder canceris still high risk for its characteristics of high rate of recurrence and mortality [4]. Therefore, earlier diagnosis, aggressive radical surgery and adjuvant multimodal treatment are needed for survival improvement. Some parameters are used to predict the clinical outcome and treatment response, among which cystoscopy is an effective way to diagnose this cancer. However, these parameters are limited for a risk of some complications [5]. Recently, various molecular biomarkers have been discovered and several have high sensitivity, providing various targets for treatment. But there is hardly a biomarker applied for clinical purposes [6]. More relevant molecular biomarkers are desired for early detection, evaluation of prognosis and targeted therapies [7].

Transmembrane protein 40 (TMEM40) is a 233 amino acid protein encoded by a gene that located on chromosome 3p25.2. But few studies have explored the functions of this protein. A study shows that the level of TMEM40 is associated with damage of parietal lobule [8]. However, no reports have described the functions of TMEM40 in tumors. Here we demonstrated that TMEM40 was up-regulated in tumor cells of clinical bladder cancer samples and bladder cancer cells in vitro. TMEM40 expression was associated with the clinical stage, histological grade and pT status.

**Table 1.** The expression of TMEM40 in bladder tissues

| | TMEM40 staining | | |
|---|---|---|---|
| | All cases (%) | Negative expression (%) | Positive expression (%) |
| Transitional cell carcinoma | 72 | 23 (31.9%) | 49 (68.1%) |
| Normal bladder tissues | 43 | 42 (97.7%) | 1 (2.3%) |

**Table 2.** Relationship of TMEM40 expression between bladder normal tissues and bladder cancer tissues

| | TMEM40 staining | | | |
|---|---|---|---|---|
| | All cases (%) | Negative expression (%) | Positive expression (%) | *P* value* |
| Transitional cell carcinoma | 72 | 23 (31.9%) | 49 (68.1%) | 0.000 |
| Normal bladder tissues | 43 | 42 (97.7%) | 1 (2.3%) | |

**P* values are from Chi-square test.

**Table 3.** Relationship of TMEM40 expression and clinicopathological features in bladder cancers

| Variables | TMEM40 staining | | | |
|---|---|---|---|---|
| | Negative (%) | Positive (%) | Total | *P* value[b] |
| Sex | | | | |
| Male | 49 | 37 | 86 | 0.865 |
| Female | 16 | 23 | 29 | |
| Age (year) | | | | |
| ≤59.5[a] | 31 | 20 | 51 | 0.410 |
| >59.5 | 34 | 30 | 64 | |
| pT Status | | | | |
| T1 | 13 (54.2%) | 11 (45.8%) | 24 | 0.007 |
| T2 | 10 (24.4%) | 31 (75.6%) | 41 | |
| T3 | 0 (0.0%) | 7 (100.0%) | 7 | |
| Grade | | | | |
| 0 | 1 (100.0%) | 0 (0.0%) | 1 | 0.012 |
| 1 | 8 (53.3%) | 7 (46.7%) | 15 | |
| 2 | 14 (31.8%) | 30 (68.2%) | 44 | |
| 3 | 0 (0.0%) | 12 (100.0%) | 12 | |
| Stage | | | | |
| I | 13 (54.2%) | 11 (45.8%) | 24 | 0.029 |
| II | 10 (22.7%) | 34 (77.3%) | 44 | |
| III | 0 (0.0%) | 3 (100.0%) | 3 | |
| IV | 0 (0.0%) | 1 (100.0%) | 1 | |

[a]Mean age. [b]*P* values are from Chi-square test.

## Materials and methods

### *Cell lines and cultures*

T24, EJ, UMUC3, BIU87, 5637, and SVHUC1 cell lines were obtained from laboratory preservation. SVHUC1 cells were cultured in Kaighn's Modification of Ham's F-12 Medium (F-12K, Gibco, U.S.A.), and other cell lines were cultured in RPMI 1640 Medium (1640, Gibco, U.S.A.). All culture medium were supplemented with 10% fetal bovine serum (FBS, Hyclone, U.S.A), penicillin (100 units/mL), and streptomycin (100 units/mL) at 37°C in a humidified atmosphere of 5% $CO_2$.

### *Tissue samples*

We collected 115 paraffin-embedded bladder tissue samples in total from patients in the Nanfang Hospital of Southern Medical University between 2009 and 2016. These paraffin-embedded tissue cases included 43 bladder normal tissues and 72 transitional cell carcinoma without prior treatments. The information of tissue samples included sex, age, pTNM status, histology grade and clinical stage. The mean age was 59.5 and other details were presented in **Table 3**. Our study followed the protocol of the Ethic Committee of the Nanfang Hospital and all patients in our study had written informed consents.

### *RNA isolation, reverse transcription and quantitative real-time PCR*

We adopted quantitative real-time PCR to quantify and assess the relative mRNA expression of TMEM40 in bladder cancer cells. According to manufacturer's protocol, total RNAs were extracted from bladder cancer cells used RNAiso Plus (Takara, China) and dissolved in nuclease free water. In addition, biophotometer plus (Eppendorf, Germany) were used to measure the concentration and purity of RNA and made sure that the OD A260/A280 ratio was between 1.8 and 2.0. cDNA was reversibly transcribed using PrimeScript® RT reagent Kit (Takara, China) according to product manual. Then TMEM40 expression level was detected with the SYBR® Premix Ex TaqTM II Kit (Takara, China) in an ABI 7500 real-time PCR amplifier (Applied Biosystems, U.S.A.). The primers were as follows: TMEM40 forward (5'-CAGAG-

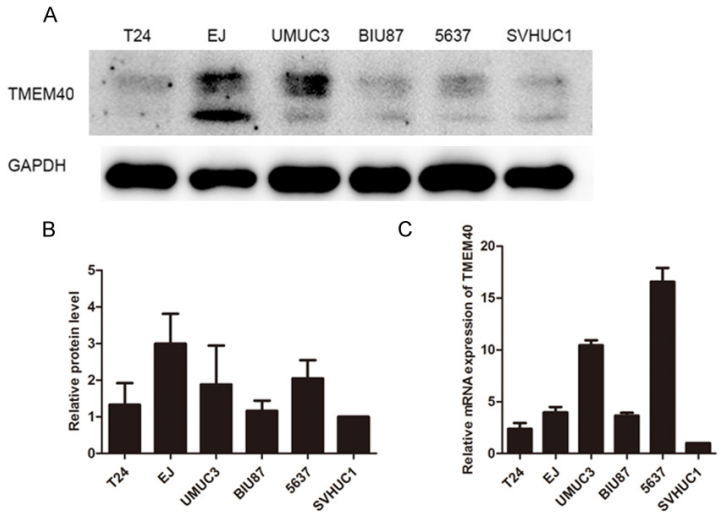


**Figure 1.** Real-time PCR and western blot analysis of TMEM40 expression in five bladder cancer cells and a normal immortalized human urothelial cell line. A. Significant differences of TMEM40 mRNA level between bladder cancer cells and normal cell line were analyzed statistically by $2^{-\Delta\Delta Ct}$ method. TMEM40 mRNA expressions were significantly higher in bladder cancer cells ($P<0.05$). GAPDH was used as internal control. B. Western blot indicated significant up-regulation in bladder cancer cells (T24, EJ, UMUC3, BIU87, 5637) in comparison with in the normal immortalized human urothelial cell (SVHUC1). GAPDH was used as internal control. C. Western blot was calculated as optical density value and expressed graphically. TMEM40 protein expressions were significantly higher in bladder cancer cells ($P<0.05$).

CAACCGGAAAACATCG-3') and reverse (5'-CTGGGCTACACTGAGCACC-3'), GAPDH forward (5'-AGAAGGCTGGGGCTCATTTG-3') and reverse (5'-AAGTGGTCGTTGAGGGCAATG-3'). Optimizing annealing temperatures for each pair of primers are as follows: 95°C for 10 min, 40 cycles of amplification at 95°C for 30 s and 60°C for 34 s, additional dissociation stage for detecting reaction specificity. Each PCR reaction was tested in triplicate for stable results. GADPH was selected as an endogenous control. The relative levels of TMEM40 were calculated using the Comparative Ct (ΔΔCt) method.

*Western blot*

Total proteins were isolated from bladder cell lines with Radio-Immunoprecipitation Assay (RIPA) buffer containing 1 mM PMSF (Beyotime, China) in the presence of proteinase inhibitor. Then, BCA Protein Assay Kit (Beyotime, China) was employed to determine protein concentration and using a standard BSA curve to normalize the values. The protein samples were dissolved in the loading buffer (Beyotime, China) and unstructured by boiling for 10 min. Eventually, equal amounts of protein (40-50 μg) were separated in 10% SDS-PAGE gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) by a standard wet-transfer device (Trans-blot SD, Bio-Rad, USA). Then, the membranes wereblocked using 5% non-fat milk diluted in Tris-Buffered Saline and Tween 20 (1×TBST) solution at room temperature. Two hours later, membranes were incubated overnight with TMEM40 primary antibody (Mouse monoclonal, 1:500, Santa Cruz Biotechnology, U.S.A.) and GAPDH primary antibody (Mouse mono-China) at 4°C. After washing with 1×TBST for 3 times, the membranes were incubated with secondary antibodies labeled with horseradish peroxidase-(HRP) (Go-at anti-Mouse IgG, 1:8000, MultiSciences, China; Goat anti-Mouse IgG, 1:8000, MultiSciences, China) at room temperature for 1 h. Subsequently, the membranes were washed again with 1×TBST for 3 times and then the enhanced chemiluminescence reagents (BeyoECL Plus Kit, Beyotime, China) were applied,figures were scanned and protein bands were observed by the SmartChemi610 (Beijing Sage Creation Science, China). The optical density value of theprotein bands were adopted quantified for evaluation.

*Tissue microarrays (TMA) construction and immunohistochemistry*

The TMA including 72 transitional cell carcinomas and 43 normal bladder tissues was constructed according to standard method [9]. Consecutive tissue sections of 5 μm were cut from TMA block for immunohistochemical analysis, and the staining was performed following standard procedures. Then sections were deparaffinized in xylene and rehydrated in graded ethanol. 0.3% $H_2O_2$ was applied to inhibit the activities of endogenous hydrogen peroxidase, following antigen retrieval heating with sodium citrate buffer (pH 6.0) for 20 min in an auto-

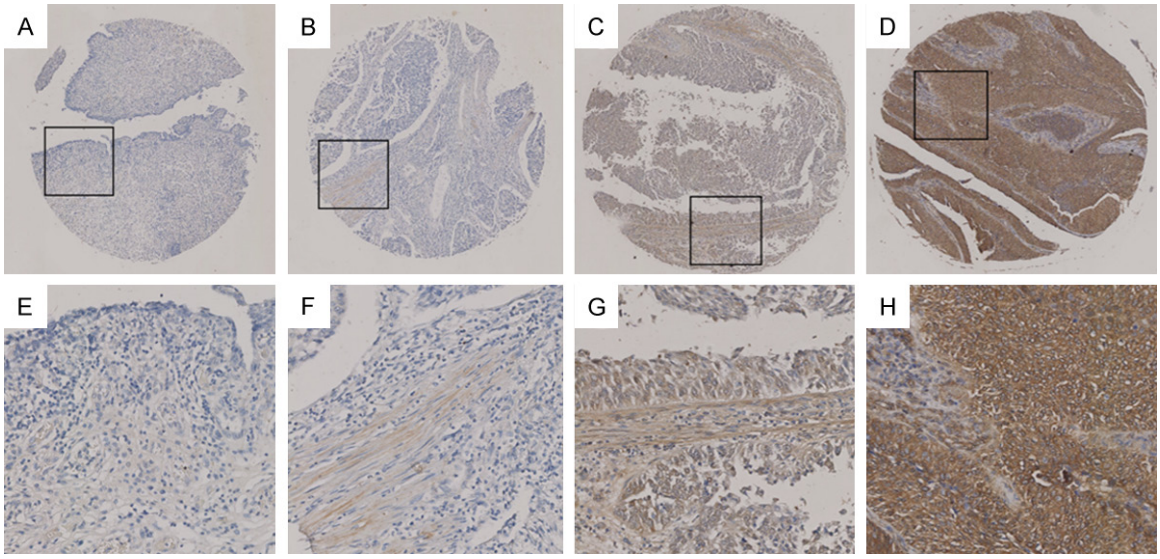


**Figure 2.** IHC results of the expression of TMEM40 in bladder normal tissues and bladder tumor tissues. (A) Negative expression of TMEM40 was detected in bladder normal tissue (case 82) (2.5×). (B) 30% positive expression of TMEM40 was detected in a transitional cell carcinoma (case 70) (2.5×). (C) 80% positive expression of TMEM40 was showed in a transitional cell carcinoma (case 19) (2.5×). (D) 95% positive expression of TMEM40 was showed in transitional cell carcinoma (case 43) (2.5×). (E-H) demonstrate the higher magnification (10×) from the area of black box in (A-D), respectively.

clave. To reduce nonspecific antibody binding, sections were subsequently incubated for 30min in 1×phosphate-buffered saline (PBS) containing 5% normal goat serumfor blocking. Sections were incubated with TMEM40 primary antibody (Mouse monoclonal, 1:200, Santa Cruz Biotechnology, U.S.A.) at 4°C overnight. After being washed in 1×PBS for 3 times, sections were incubated at room temperature for 30 min with HRP conjugated-secondary antibodies (MultiSciences, China). After additional wash with 1×PBS, the sections were stained by hematoxylin counter and dehydrated before mounting. Immunostaining was visualized by DAB Horseradish Peroxidase Color Development Kit (Beyotime, China). Phosphate-buffered saline rather than anti-TMEM40 antibody is a negative control.

*IHC evaluation*

Immunostaining results of TMEM40 were interpreted by two authors, who were unacquainted of the patients' clinicopathological characteristics in advance, in a semi-quantitative scoring method based on staining intensity and percentage. Positive immunostaining also contained cytoplasmic and membrane staining in tumor cells. Percentage scores were performed as 5% increments (0, 5%, 10%... 100%). The two authors ceased re-estimating only when differences settled.

*Selection of cut-off score*

Receiver operating characteristic (ROC) curve analysis was adopted to get a best cut-off score and the increasing expression of TMEM40 was used 0, 1-criterion. Clinicopathological parameters for ROC analysis were split into two groups as follows: histological grade (G0-G1, G2-G3), clinical stage (I-II, III-IV), pT stage (T1, T2-T3). Then the ROC curves were plotted on the basis of plotting the paired sensitivity and specificity of each TMEM40 score. The spot which was the nearest to point [0.0, 1.0] was regarded as the cut-off value. Therefore, when TMEM40 score was below the threshold, tumor was defined as TMEM40 "negative". Otherwise it would be TMEM40 "positive".

*Statistical analysis*

Statistical evaluation was completed using SPSS 20.0 software (SPSS, Chicago, IL, USA). The data was presented in the form of mean ± SD and at least three independent experiments. ROC was introduced to achieve an optimal cut-off score for TMEM40 positive. T test was performed to find out the expression differ-

ences between two groups. Chi-square test was used to assess the relationship between TMEM40 expression and the clinicopathological parameters of bladder cancer. Differences were considered statistically significant when the *P*-value was less than 0.05.

## Results

*TMEM40 expression in bladder cancer cells*

We examined the expression of TMEM40 by reverse transcription (RT)-polymerase chain reaction (PCR) (**Figure 1C**) and western blotting (**Figure 1A** and **1B**) in five human bladder cancer cell lines, T24, EJ, UMUC3, BIU87, 5637, and a normal immortalized human urothelial cell line, SVHUC1. The results suggested that the level of TMEM40 in the bladder cancer cells was significantly higher than that in the normal cell line.

*TMEM40 expression in tissue samples of bladder cancer*

In this study, we assessed the expression of TMEM40 in bladder cancers and normal bladder tissues by immunohistochemistry (IHC). Immunoreactivity was observed in the cytomembrane of tumor cells. Four representative samples of different levels of TMEM40 IHC staining were shown in **Figure 2**. Receiver operating characteristic (ROC) curve analysis was used to choose the optimal cut-off score. As shown in **Figure 3**, clinical stage and pT statuswere selected as the shortest distance to the point (0.0, 1.0) among the four clinical parameters. Thus, 62.5% was regarded as the optimal cut-off score and above it was defined as TMEM40 positive, and below it was negative. TMEM40 staining was positive in 68.1% (n=49) and negative in 31.9% (n=23) of a total of 72 bladder cancer tissues based on this cut-off score, and positive in 2.3% (n=1) and negative in 97.7% (n=42) of normal cases (**Table 1**). For further analysis, we used Chi square test to explore the relationship between TMEM40 expression and bladder tumor tissues and normal tissues. Results indicated that TMEM40 expression was significantly high in tumor tissues ($\chi^2$=47.331, *P*=0.000) (**Table 2**).

*Correlation between TMEM40 and clinicopathological parameters*

Furthermore, we analyzed the relationships between TMEM40 expression and clinicopathological parameters of bladder cancer respectively. As shown in **Table 3**, TMEM40 positive was correlated with clinical stage, histological grade and pT status (all *P*<0.05). However, there was no significant association between TMEM40 and age or sex.

## Discussion

Recently, the research of bladder cancer has made great progress. The Genome Atlas Research Network (the Cancer Genome Atlas, TCGA) revealed a study that contained outlined genome, transcriptome, mutational data and correlated many molecular events with specific stages and prognosis of patients in 131 urothelial tumors in 2014 [10]. Besides, studies have proved that familial mutations of the pRb-could increase the risk of bladder cancer [11, 12]. And the p53/pRb pathway is also often altered in bladder cancer [13, 14]. Additionally, evidences suggest that some individuals may be at a high risk of developing bladder cancer, such as smokers or smokers with genetically overactive CYP1A2 [15]. However, the etiology of bladder cancer is complicated, and further research of itsbiomarkers for diagnosis and prognosis is needed [16]. Little is known of the functions of TMEM40, and the role of human TMEM40 in tumorgenesis has not been reported. In our study, we found the differences of TMEM40 expression in bladder cancer cells and normal immortalized human urothelial cell line. We also studied the TMEM40 expression in bladder cancer tissues and normal bladder tissues to explore the clinicopathological significance of TMEM40 expression.

To investigate the expression of TMEM40 in bladder cancer cells, we detected it in five bladder cancer cell lines and one normal bladder immortalized human urothelial cell line by quantitative real-time PCR and western blot analysis. Bladder cancer cell lines are in vitro models which can represent the biological characteristics of different types of bladder cancers to some extent. Therefore, we found that TMEM40 levels in five bladder cell lines (T24, EJ, UMUC3, BIU87, and 5637) were significantly up-regulated compared with the normal bladder immortalized human urothelial cell line (SVHUC1) (*P*<0.05).

Furthermore, in order to assess the clinical value of TMEM40, we collected 72 bladder cancer tissues and 43 normal bladder tissues to

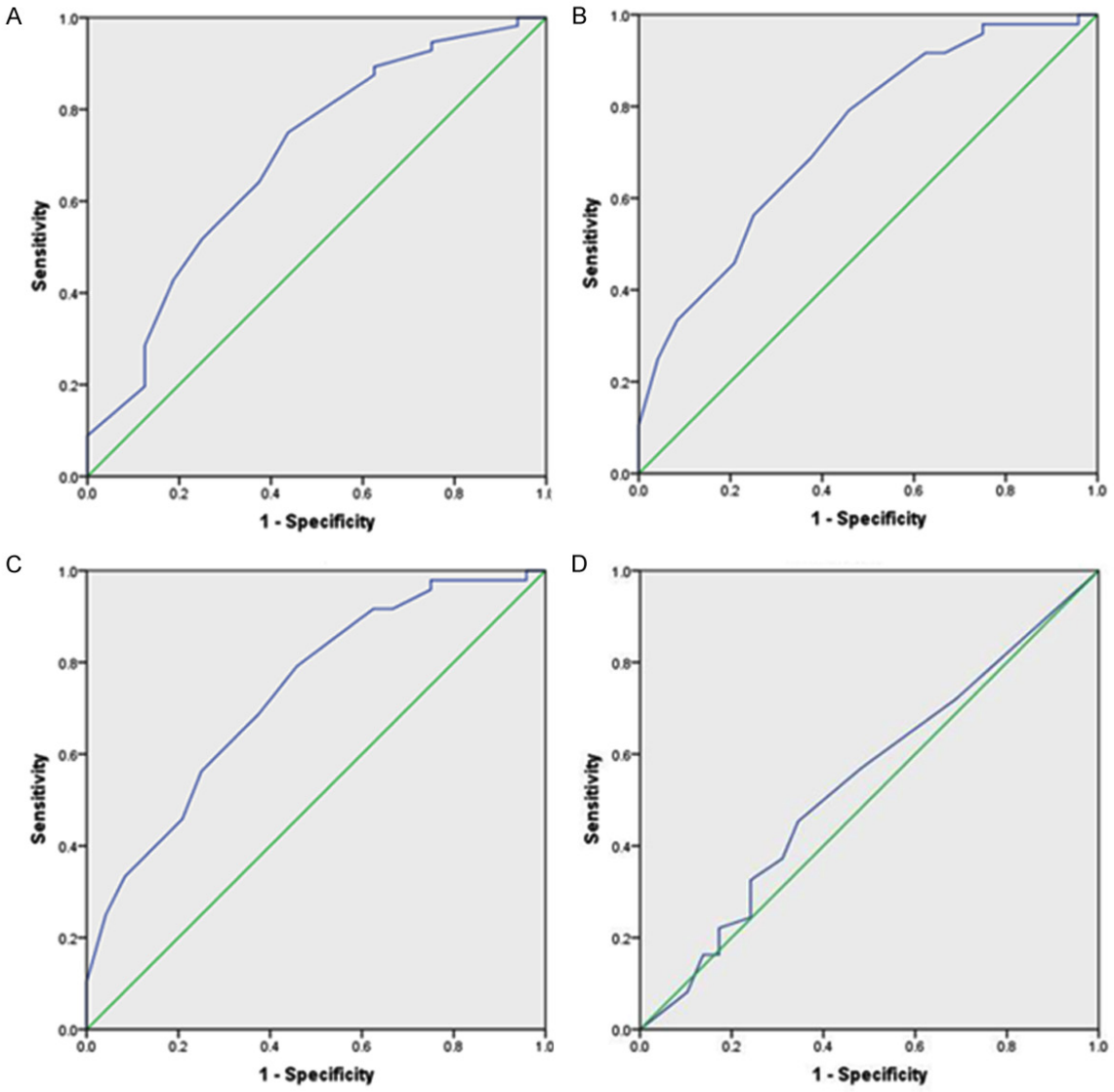


**Figure 3.** Receiver operating characteristic (ROC) were used to select the optimal cut-off score for positive expression of TMEM40: A. Histological grade. B. Clinical stage. C. pT stage. D. Sex.

construct a TMA stained with TMEM40. Then, we adopted ROC analysis to acquire the optimal cut-off score. Four ROC curves were created on the basis of clinicopathological parameters, including histological grade, clinical stage, pT stage and sex. Therefore, we obtained four cut-off scores of TMEM40 and selected 62.5% as the optimal cut-off value. TMEM40 positive expression was defined as staining over 62.5%. According to this cut-off value, the analysis results indicated that the expressions of TMEM40 in 72 cancer tissues with IHC were significantly higher than those in 43 normal bladder tissues ($\chi^2$=47.331, *P*=0.000). Otherwise, the statistical analysis indicated that high TMEM40 expression was significantly related to histological grade ($\chi^2$=10.920, *P*=0.012), clinical stage ($\chi^2$=9.049, *P*=0.029) and pT stage ($\chi^2$=9.814, *P*=0.007). However, there are some limitations in our present study. The number of samples is small and more studies of in vivo and in vitro are needed to explore its functions in bladder cancer.

In conclusion, our study found that TMEM40 was high expression in bladder cancer cells and tissues, and it was also associated with clinicopathological parameters of bladder cancer, which indicated that TMEM40 could be used as a diagnostic marker for bladder cancer.

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## Disclosure of conflict of interest

None.

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